Impact of Polyphenols on Lipid Accumulation Within Skeletal Muscle Cells

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Thesis submitted to The University of Nottingham for the degree of Doctor of Philosophy

March 2020

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Abstract

Obesity is characterised by a high-fat accumulation in many organs in the body and is associated with many diseases, including Type 2 diabetes mellitus (T2DM). As most T2DM patients, the major class of diabetes, are obese, researchers have started investigating the role of obesity in this disease. The skeletal muscles are the major organ in the disposal of blood sugar. Also, the accumulation of fats in non-adipocyte cells such as skeletal muscles can result in insulin resistance. The overall aims of this thesis were to establish a useful *in vitro* model to investigate intracellular lipids accumulation and the effects on insulin sensitivity. Besides, to examine whether dietary polyphenols have a beneficial effect on lipid accumulation and, subsequently, leads to amelioration in insulin sensitivity. C2C12 and L6 myotubes cells were exposed to 0.2mM of different fatty acids; palmitic acid (PA), oleic acid (OA), linoleic acid (LA), and a combination of these FAs (3xFA) total 0.2mM for 3 days, followed by Nile-Red staining. There was a consistently higher deposition of lipids in the LA treated cells after 3 days for both cell lines ($P<0.001$ and $P<0.0001$, respectively). C2C12 cells were treated with 0.2mM LA, which resulted in no significant increase in the mRNA of the peroxisome proliferator-activated receptor gamma-2 (PPARγ2) ($P>0.05$) and fatty acid-binding protein 4 (FABP4) (not detectable), which suggests that no adipocytes were occurring. L6 myotubes were treated with 0.2mM LA combined with different polyphenols (0.05µM, 0.1µM, and 1µM of resveratrol, quercetin, or epigallocatechin gallate (EGCG)) for 3 days. Only 1µM EGCG significantly inhibited the lipid deposition ($P<0.01$). Interestingly, LA treatment (0.2mM) of L6 myotubes for 3 days significantly decreased the insulin-stimulated glucose uptake ($P<0.05$), while this effect of LA was blocked after co-treatment with 1µM EGCG.

Acknowledgement

First of all, I would like to express my sincere gratitude to my lord, who helped me starting, progressing, and completing this thesis.

Next, I would like to say a huge thank you to my supervisors Prof. Tim Parr and Prof. John Brameld for their patience, motivation, the incredible support of my Ph.D. study. This thesis would not have been possible without their guidance and immense knowledge. Also, I'm grateful for their kindness when they accepted taking over my Ph.D. supervision when my first supervisor left the university. In parallel, I would like to thank Dr. Andrew Murton who started my Ph.D. supervision, which then led to the completion of this thesis. I wish all the best for him in his role in the USA.

Thank you for all the technicians in the division, Cathy Wells, Kirsty Jewell, Sarah Jones, and Dr. Zoe Daniel, who helped me by sharing their knowledge and expertise in molecular and cell biology techniques. Thank you for all the things you have done for me during the last 4 years. You can't imagine how you all made my studies less stressful with your sense of humor and endless support. Also, I would like to send my salutations to all the members of Nutritional Sciences faculty who have been very kind enough to me with their smiles, welcoming, and I do hereby acknowledge all of them.

I would also like to thank my fellow PhD colleagues for making such a fun, helpful, and friendly environment in the office, lab, and other places on the campus: Amanda, Kimberley, Zoe, Kerensa, Maddy, Burak. Also, I'm grateful to my friends at the campus: Waleed, Abdullah, Mohammad, and Ibrahim, and also to my neighbors who made my living like at home.

Last but not the least, a special and warm thank you to my family who always stands by me and asks for nothing in return. They were always present when I need them, especially my father Saad Alhodieb, my brothers Abdulrahman, Abdulaziz, Mohammad, Muath, Abdulmalik,

Muhannad; my sisters Nurah, Mona, Maha, Amjad, Sama, Saja for their priers and motivations. Especially, I greatly thank my wife Shrouq Alomri, for all your motivations, supports, and encouragement during my four years of Ph.D. study, and thank you for taking care of me. Finally, a warm appreciation to my son Saad who arrived at the life during my studies and having him increased my purposefulness, God bless him.

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List of Abbreviations

2-DG: 2-Deoxy-D-glucose

2DG6P: 2-deoxyglucose-6-phosphate

36B4: acidic ribosomal protein

4E-BP1: eukaryotic initiation factor 4E binding protein

ACC: acetyl-CoA carboxylase

ADA: American Diabetes Association

AGPAT1: 1-acyl-sn-glycerol-3-phosphate acyltransferase alpha

ANOVA: Analysis of variance

BSA: bovine serum albumin

cDNA: Complementary DNA Synthesis

CE: cholesterol esters

CK: Creatine kinase

DGAT: diacylglycerol acyltransferase

DGAT1: Diacylglycerol O-acyltransferase 1

DM: Diabetes mellitus

DMEM: Dulbecco's Modification of Eagle's Medium

DMSO: Dimethyl Sulfoxide

DNA: Deoxyribonucleic acid

dNTP: nucleoside triphosphate

ECs: Endocannabinoids

EGCG: epigallocatechin gallate

eIF4E: eukaryotic translation initiation factor- 4E

FABP4: Fatty Acid Binding Protein 4

FAs: fatty acids

FAS: fatty acid synthase

FAT/CD36: fatty acids translocate

FBS: Fetal Bovine Serum

FFAs: free fatty acids

G1P: glucose-1 phosphate

G6P: glucose 6-phosphate

G6PDH: glucose-6-phosphate dehydrogenase

GDM: gestational diabetes mellitus

GLUT 4: Glucose transport 4

GS: glycogen synthase

GSK-3: glycogen synthase kinase-3

H2O: Water

HBSS: Hank’s Balanced Salt Solution

IBMX: 3-isobutyl-1-methylxanthine

IL-6: interleukin-6

IR: insulin resistance

IRS 1: insulin receptor substrate 1

LA: linoleic acid

LDs: lipid droplets

mGPAT2: Glycerol-3-Phosphate Acyltransferase 2, Mitochondria

mRNA: Messenger RNA

mTOR1: mammalian target of rapamycin-1

NADP+: Nicotinamide adenine dinucleotide phosphate

NADPH: Reduced nicotinamide adenine dinucleotide phosphate

NEFA: non-esterified fatty acids

OA: oleic acid

P/S: Penicillin-Streptomycin

PA: Palmitic acid

PBS: phosphate buffer saline

PCR: Polymerase Chain Reaction

PDK1: P3- dependent kinase-1

PDK2: P3- dependent kinase-2

PI3K: phosphatidylinositol 3-kinase

PIP2: phosphatidylinositol (4,5)P2

PIP3: phosphatidylinositol (3,4,5)P3

PKB/Akt: protein kinase B

PPARg2: Peroxisome proliferator-activated receptor gamma-2

qPCR: Quantitative Polymerase Chain Reaction

quer: quercetin

resv: resveratrol

Rheb: Ras-homolog enriched in brain

RNA: Ribonucleic acid

S6K/P70S6: protein S6 kinase 1

SEM: standard error of the mean

SMs: skeletal muscles, 24

SREBP-1a: Sterol regulatory element-binding protein 1-a

SREBP-1c: Sterol regulatory element-binding protein 1-c

SREBP-2: Sterol regulatory element-binding protein 2

T1DM: type 1 diabetes mellitus

T2DM: type 2 diabetes mellitus

T3: Tri-iodothyronine

TAG: triacylglycerol

TBP: TATA Binding Protein

TNF- α: tumor necrosis factor α

TSC1: tuberous sclerosis complex 1

TSC2: tuberous sclerosis complex 2

UDP: uridine 5′-diphosphate

WHO: World Health Organisation

Chapter 1:

Introduction

1 Introduction

1.1 Diabetes Mellitus

Healthcare is one of the most costly issues in the world in terms of research and treatment. Although there are thousands of research centres globally, there are still diseases that have no cures to end the suffering of patients. Diabetes mellitus (DM) is one of the most common diseases in the world (Wei-Chih et al., 2012). The difficulty of maintaining a healthy diet and regular exercise has caused an increase in the number of cases of diabetics. The rapidly increased prevalence of DM worldwide is associated with urbanisation and lifestyle changes (Lei et al., 2011). Although a lot of research is being conducted, there is still no complete cure for DM (Williams et al., 2007). DM is one of the most harmful diseases, especially for adults over 60 years old (Williams et al., 2007). According to the Centres for Disease Control and Prevention (2011), diabetes is a leading cause of many diseases, including heart disease, blindness, and stroke. The expected number of DM patients by 2030 is approximately 439 million (Lei et al., 2011) (Figure 1.1). DM is a chronic disorder caused by a lack of insulin secretion by the pancreas, a defect in insulin action, or both (Salpeter et al., 2003). Even though many people are diagnosed with DM, the disease can be prevented in certain cases. The problem is that most people do not know how to prevent it or fail to act on the advice that they are given. Knowing the causes of DM will help people prevent this disease or, at least, decrease the risk of getting it. People should be educated on the risks and factors that cause DM.

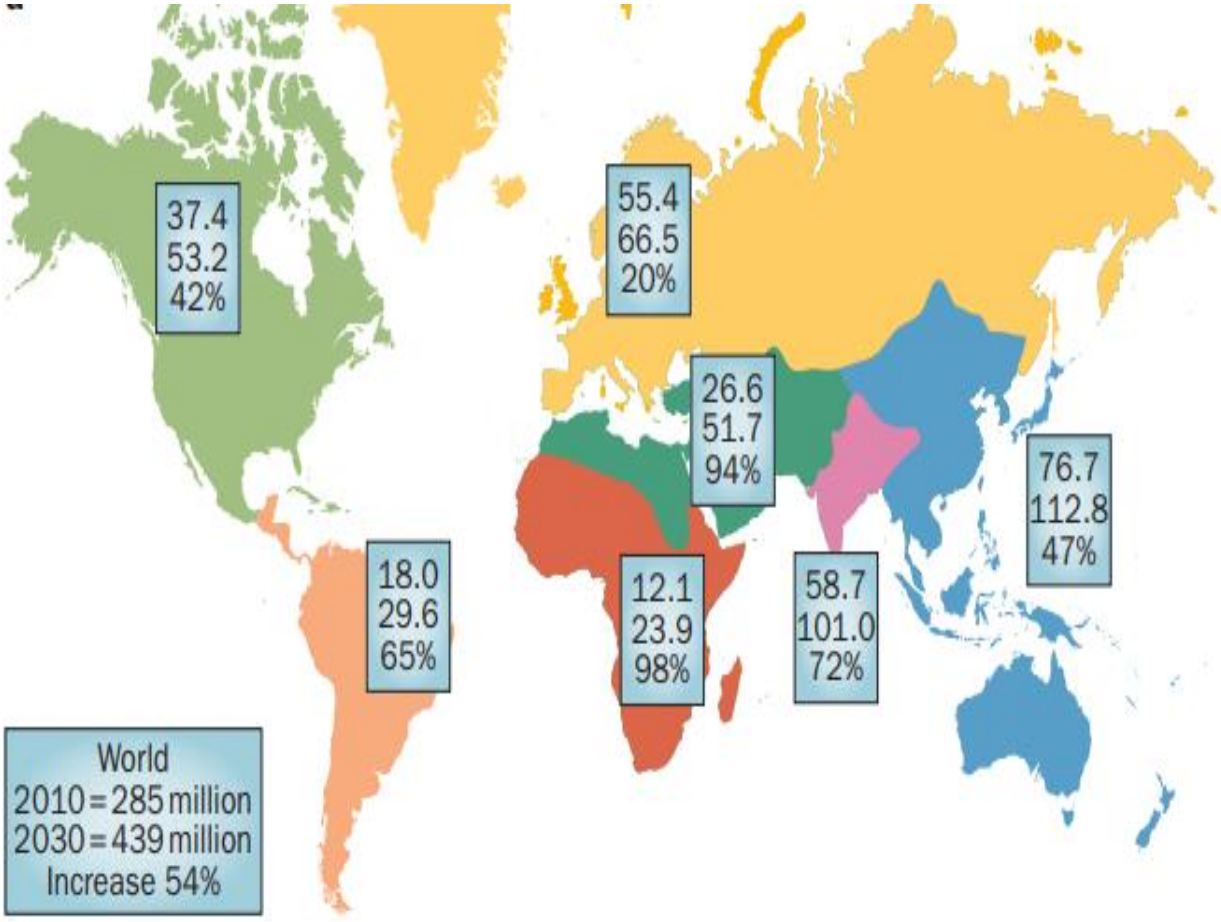


Figure 1-1 The prevalence of diabetes mellitus worldwide by 2030.

Obtain from (Lei et al., 2011).

1.1.1 Classification of Diabetes

According to the American Diabetes Association (ADA, 2018), DM can be classified into (i) type 1 diabetes mellitus (T1DM), which is a result of autoimmune β-cell destruction, (ii) type 2 diabetes mellitus (T2DM), which is a result of prolonged deficiency of insulin secretion or inactivity of insulin, or (iii) gestational diabetes mellitus (GDM), which is diagnosed during pregnancy and may develop into T2DM. T2DM is the major class of diabetes, and the number of patients is rapidly increasing throughout the world. A variety of environmental and genetic factors, as well as obesity, can cause T2DM. Inactivity of the insulin in the major organs, such as skeletal muscles (SMs), are known to be the markers of T2DM (Lillioja et al., 1993). T2DM is clearly associated with hypertension and dyslipidaemia; however, T2DM can be prevented by maintaining a healthy diet and performing regular exercise (Williams et al., 2007). Teams of scientific researchers, as well as other related researchers, are still investigating a cure for DM. They have expanded their investigations to other relevant topics such as obesity, lipids, and insulin resistance (IR) due to their clear association with T2DM.

1.1.2 Glucose Homeostasis

The pancreas, which is located behind the stomach, is a vital organ that possesses key roles in the secretion of important hormones in order to maintain metabolism and energy homeostasis (Röder et al., 2016). The pancreas consists of exocrine and endocrine cells, each of which has unique functions (Slack, 1995). The exocrine cells produce the enzymes required for digestion in the gastrointestinal tract, while the endocrine cells produce the plasma hormones, such as insulin and glucagon

(Catherine et al., 2004). Insulin is a hormone produced by β-cells in Langerhans islets of the pancreas (Wilcox, 2005), while glucagon is secreted by α-cells in the same islets, where insulin reduces the glucose levels and glucagon increases them (Röder et al., 2016). This reaction is referred to by the term glucose homeostasis (see Figure 1.2).

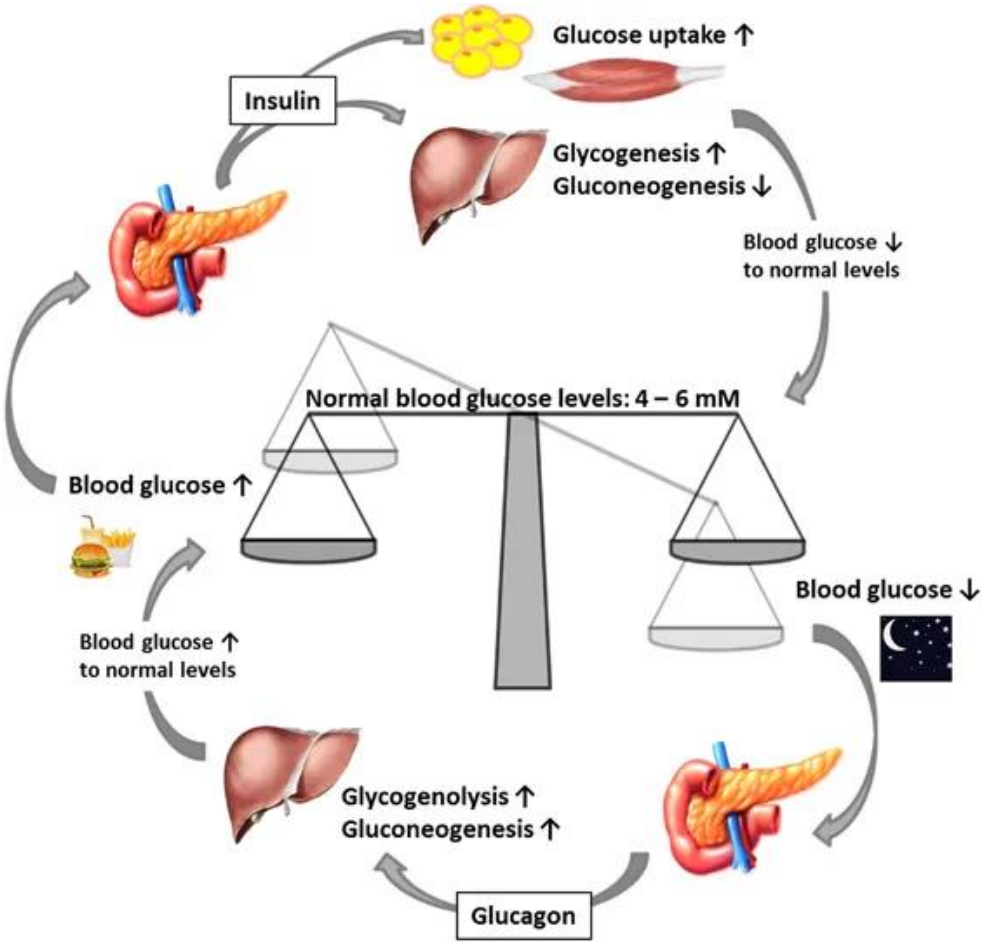


Figure 1-2 Glucose homeostasis regulation by insulin and glucagon. Obtained from (Röder et al., 2016).

Insulin stimulates the uptake of glucose into SMs and adipose tissues when the glucose levels in the blood are high (i.e. after a meal). The glucose will then be stored as glycogen via glycogenesis. Glucagon increases the glucose levels in the blood when the glucose levels are low (i.e. during sleep or between meals) through stimulation of hepatic glycogenolysis and gluconeogenesis.

To maintain blood glucose levels, insulin facilitates several cellular events such as glucose uptake and carbohydrate regulation (Wilcox, 2005). The secretion of insulin is in response to elevated glucose levels in the blood after a meal; as a result, the blood glucose level is rapidly returned to the normal level of 4–6mM (see Figure 1.3) (Röder et al., 2016). In contrast, during periods of fasting, the glucose levels in the blood go down; as a result, α-cells release glucagon to encourage hepatic glycogenolysis to increase the glucose levels (Aronoff et al., 2004) and promote liver and kidney gluconeogenesis in response to prolonged fasting (Röder et al., 2016).

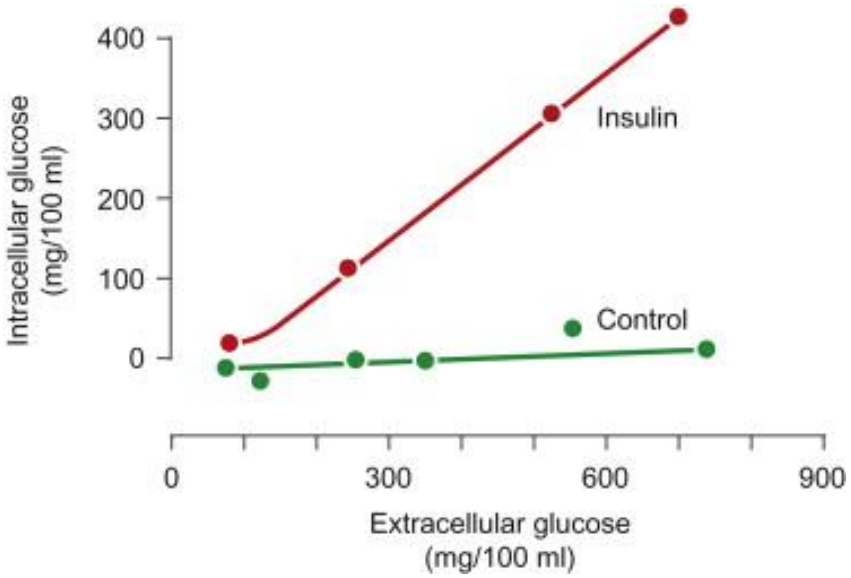


Figure 1-3 Insulin stimulates glucose uptake to SMs. Obtained from (Baura Gail, 2012).

In the absence of insulin (control), the intracellular glucose level is around zero. However, when insulin is present, the level of intracellular glucose increases by insulin stimulation of glucose uptake into muscle cells.

1.1.3 Mechanisms of Action of Insulin

Insulin starts its stimulation of glucose uptake by activating the insulin receptor. The insulin receptor consists of two subunits: (i) alpha-subunit, which is an extracellular unit, and (ii) beta-subunit, which is a transmembrane unit (Guo, 2014). After insulin binds to its receptor (at alpha-subunit), it catalyses one of the two beta sub-unit tyrosine kinase activity resulting in a fast receptor auto-phosphorylation (Ryder et al., 2001). This activation of tyrosine kinase is a crucial step in the insulin action on glucose metabolism (Abdul-Ghani and DeFronzo, 2010). Afterwards, tyrosine kinase activation promotes the phosphorylation of insulin receptor substrate (IRS), especially IRS-1 (Ryder et al., 2001), leading to the activation of phosphatidylinositol 3-kinase (PI3K) (Huang and Czech, 2007). Subsequently, PI3K stimulates the phosphorylation of phosphatidylinositol (4,5)P2 (PIP2) at the membrane to phosphatidylinositol (3,4,5)P3 (PIP3) (Guo, 2014; Huang and Czech, 2007; Ruth et al., 2019). As a result, PIP3 recruits the P3- dependent kinase-1 and 2 (PDK1 – PDK2) which then activates protein kinase B (PKB, also known Akt) (Sabatini David and Sarbassov Dos, 2011). The activation of the Akt leads to multiple biological events include (i) inactivation (phosphorylates) of glycogen synthase kinase-3 (GSK-3), which in turn activates (dephosphorylation) of glycogen synthase (GS), which is the rate-limiting enzyme in glycogen synthesis (glucose storage) (Vaag et al., 1992) (ii) stimulation of GLUT 4 translocation from intracellular storage vesicles to the membrane, therefore, allowing the glucose transport into the cells (see Figure 1.4) (Guo, 2014; Ruth et al., 2019). This is the part of the insulin pathway which is responsible for glucose uptake was the focus of the thesis.

However, the activation of Akt regulates other components of the insulin pathway which, ultimately, stimulates protein synthesis (James et al., 2017).

Akt activates the mammalian target of rapamycin-1 (mTOR1), which a master regulator for cell growth and protein synthesis, through inhibiting (phosphorylation) tuberous sclerosis complex 1 and 2 (TSC1 and TSC2) (Laplante and Sabatini, 2009). The inhibition of TSC 1 and 2 allows the small GTPase Rheb (Ras-homolog enriched in brain) to stimulate mTOR1 because the Rheb is an essential regulator of mTOR1 (Neuman and Henske, 2011). During the active state, TSC 1 and 2 convert GTPase Rheb from the active form to the inactive form (GDP-bound) as they act as Rheb GAP (GTPase activation protein) (Kim, 2009; Laplante and Sabatini, 2009). The activation of mTOR1 leads to the phosphorylation of ribosomal protein S6 kinase 1 (S6K/P70S6) and eukaryotic initiation factor 4E binding protein (4E-BP1) (Laplante and Sabatini, 2012). 4E-BP1 in the un-phosphorylated form binds to eukaryotic translation initiation factor- 4E (eIF4E), which prevents the mRNA translation (Jossé et al., 2016). Thus, mTOR1 inhibits the 4E-BP1 activity and stimulates S6K; therefore, promoting the cap-dependent translation of eIF4E and S6K (see Figure 1.4) (Jossé et al., 2016; Kim, 2009; Laplante and Sabatini, 2009).

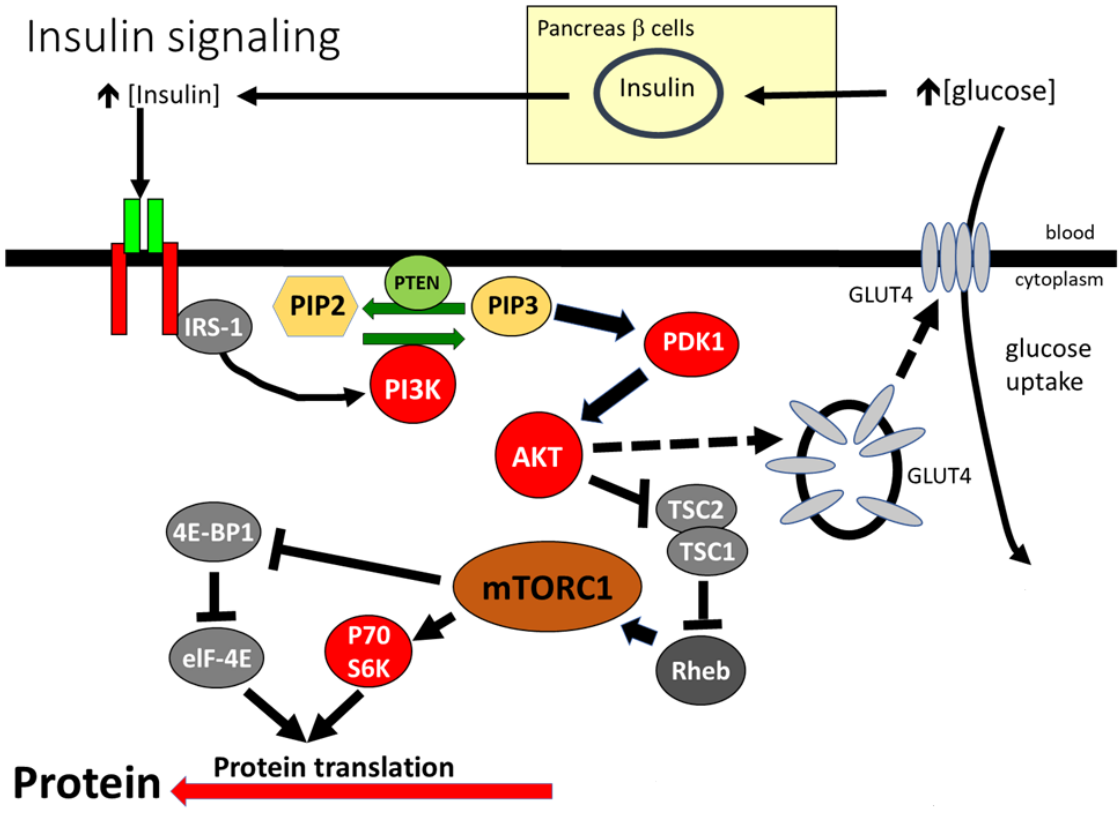


Figure 1-4 Insulin stimulation of GLUT 4 translocation and protein synthesis.

Obtained from (Parr, T., Lecture materials., 2020)

Insulin binds to its receptor which in turn recruits IRS1, stimulating Akt through several actions involving in the phosphorylation of PI3-K, PIP2 to PIP3 conversion, and recruitment PDK1. Activation of Akt stimulates the glucose transporter 4 to translocate to the cell membrane, ultimately, permits glucose transportation into the cell. In the other way, Insulin stimulates mTOR1 via inhibition of TSC1 and 2 leading to the activation of Rheb. mTOR1 phosphorylate S6K and 4E-BP1, preventing the attachment of 4E-BP1 to eIF-4E, promoting the translation of protein. **IRS:** Insulin receptor substrate**. PIP2:** phosphatidylinositol (4,5)P2. **PIP3:** phosphatidylinositol (3,4,5)P3. **PI3-K:** phosphatidylinositol 3-kinase **PDK1:** P3- dependent kinase-1 **Akt:** protein kinase B. **TSC1-2**: tuberous sclerosis complex 1 and 2. **Rheb:** Ras-homolog enriched in brain **mTOR1:** mammalian target of rapamycin-1 **S6K:** protein S6 kinase 1. **4E-BP1**: eukaryotic initiation factor 4E binding protein. **eIF4E:** eukaryotic translation initiation factor- 4E

1.1.4 Glucose Metabolism in Muscle Cells

Once the glucose is inside the muscle cell, it undergoes the glycolysis process, where hexokinase is the rate-limiting enzyme in the process. Subsequently, if the glucose is not used for energy, the hexokinase phosphorylates the glucose to generate glucose 6-phosphate (G6P) (Bouskila et al., 2008; Novikova, 1980). The conversion of glucose into G6P is an important step in glucose homeostasis because the conversion of glucose prevents the glucose from reverting to plasma (Chen et al., 1988). Following the conversion of glucose to G6P, the enzyme phosphoglucomutase-1 converts the G6P to glucose-1 phosphate (G1P). Afterwards, the G1P incorporated into UDP-glucose in a process catalysed by uridine 5′-diphosphate (UDP)-glucose pyrophosphorylase (Adeva-Andany et al., 2016). Hence, the glycogen synthesis is initiated by glycogen in, which generates a short chain of glucose moieties, followed by the protraction of the chain by glycogen synthase, by the addition of glycosyl moiety from UDP-glucose (see Figure 1.5) (Adeva-Andany et al., 2016; Kanungo et al., 2018).

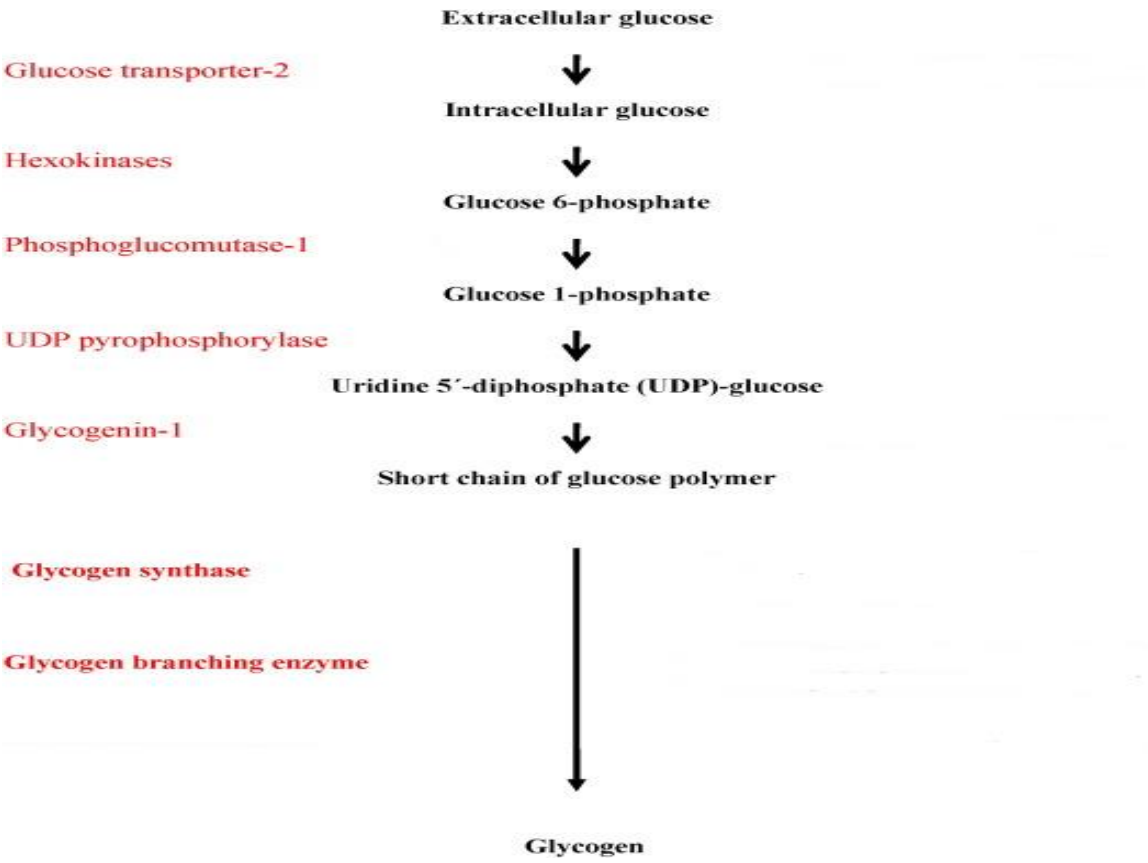


Figure 1-5 Regulation of glucose to glycogen. Obtained from (Adeva-Andany et al., 2016).

1.1.5 Insulin Resistance

The first step in response to insulin is the activation of the insulin receptor tyrosine kinase (Morris et al., 1985). However, studies reported that the activity of tyrosine kinase was decreased in T2DM patients (Pirola et al., 2004). Moreover, in the SMs of T2DM patients, insulin binding to its receptor and receptor phosphorylation is reduced. Even though the expression of GLUT4 was found to be normal in the SMs of T2DM patients, it was suggested that the impairment of glucose transportation resulted from a defect in GLUT4 translocation or fusion (Kahn and Flier, 2000).

It has been reported that the imperfection of insulin signalling cascade in target tissues, such as SMs, develops IR. The IR in SMs predicts the development of T2DM. SMs require a higher concentration of insulin in order to permit the glucose to enter the cells in response to elevated glucose (Inzucchi, 2002; Lillioja et al., 1993). IR is usually defined as a decreased disposal of a glucose load by the body in response to a given amount of insulin (see Figure 1.6) (Turcotte and Fisher, 2008).

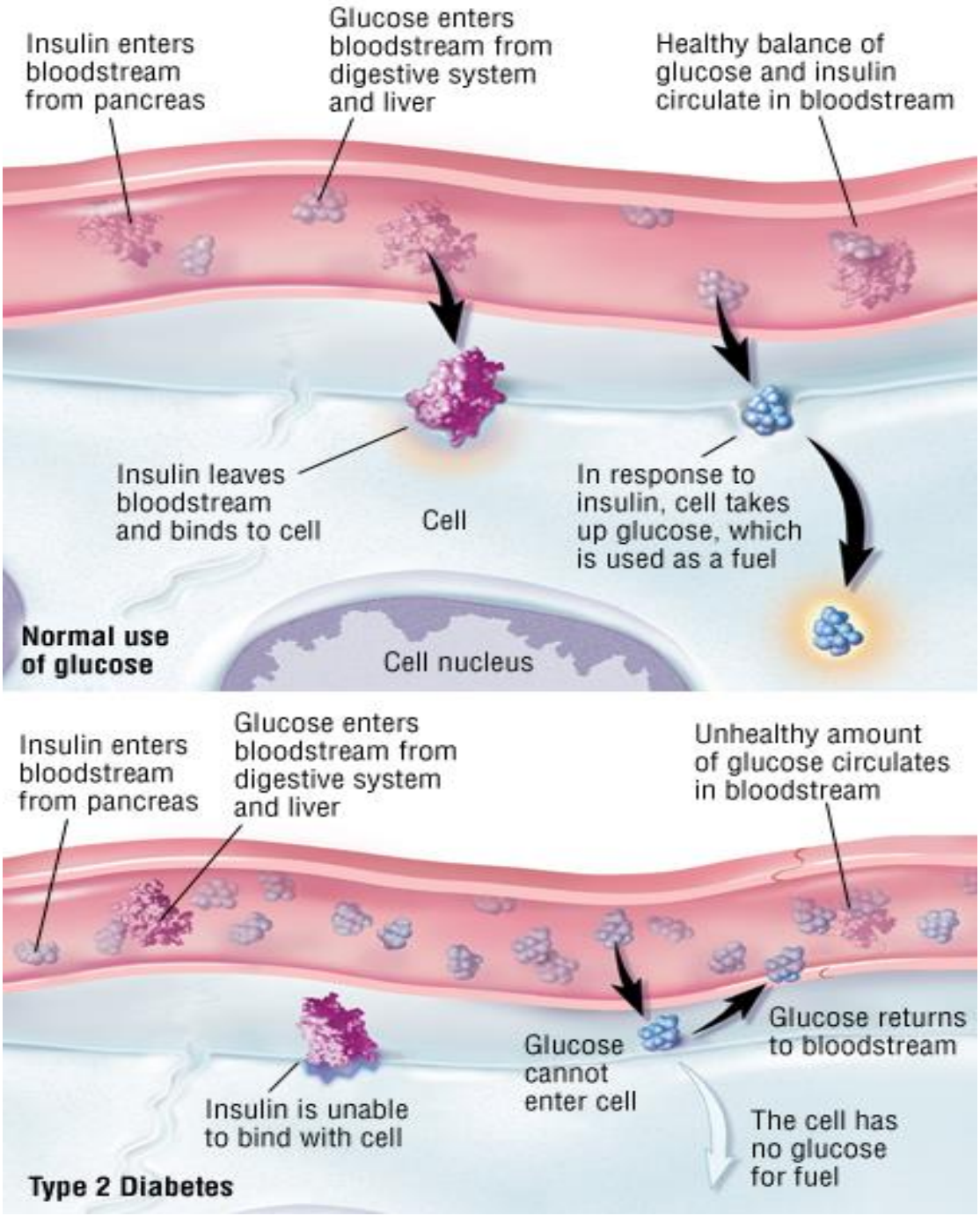


Figure 1-6 Schematic diagram illustrates the insulin resistance theory.

Obtained from (Harvard Health Publishing, 2018).

1.2 Skeletal Muscles and Obesity

1.2.1 Overview of Lipid Storage and Metabolism in the Body

The principal organ in lipid storage is the adipose tissue. The liver and SMs also store a small number of lipids. More than 95% of the lipids stored in adipose tissue are found as triacylglycerol (TAG) (Coppack et al., 1994). Lipolysis is a process through which fatty acids (FAs) and glycerol are generated by the hydrolysis of TAG within the adipocyte. The adipocyte releases the FAs into plasma circulation while bound to albumin as free fatty acids (FFAs). The liver and SMs then oxidise the FFAs for energy or the FFAs are re-esterified into TAG by the adipocyte (Large et al., 2004). Lipolysis is a critical process to ensure the body has enough lipids to fuel the body's tissues (Terjung and Kaciuba-Usciłko, 1986).

1.2.2 Obesity and Insulin Resistance

According to the World Health Organisation (WHO) 2017, obesity is characterised by excessive accumulation of fat in the body (WHO, 2017; fact sheet 2017) and by increased intracellular TAG accumulation in different tissues (Sitnick et al., 2013). Besides, its consequences include heart disease and diabetes (WHO, 2017; fact sheet 2017). Obesity has been reported to affect whole-body metabolism, as it is characterised not only by lipid accumulation but also in hypertension, insulin resistance, and low HDL-cholesterol (Amanda Oliva et al., 2014). Metabolic syndrome, which one of major obesity's consequences, has been raising the world wildly. Metabolic syndrome along with obesity are associated and highly linked to many mortality and morbidity (Wahba and Mak, 2007). Moreover, it was suggested that abdominal (central) obesity is the main component of metabolic syndrome, which is linked to many organ dysfunctions and other chronic diseases such as

dyslipidaemia, glucose intolerance (T2DM), and high risk of renal failure (Eckel et al., 2005). Also, the same author indicates that metabolic syndrome is associated with an increased risk of cardiovascular disease and predicts after time DM.

Notably, visceral obesity is associated with chronic inflammation that leads to IR in the liver too. The liver regulates whole-body energy homeostasis and its functions include Gluconeogenesis, glycogenolysis, lipogenesis, and plasma protein synthesis (Leclercq et al., 2007). Additionally, Insulin is one of the hormones that regulate these functions of the liver. Therefore, in the liver's IR state, normal functions of the liver are disrupted and lead to several complications such as hepatic very-low-density lipoprotein production and increased supply of FFA into the liver by adipose tissues (Meshkani and Adeli, 2009).

1.2.3 Lipids Accumulation in Skeletal Muscles

High fat in the body results in the storage of the fats as lipid droplets (LDs) in non-adipocyte cell types, such as SMs (Greenberg et al., 2011). LDs are comprised mainly of the deposition of cholesterol esters (CE) and TAG and are surrounded by phospholipid monolayer (see Figure 1.7) (Martin and Parton, 2006; Zamani et al., 2017). The mechanism for storing the fats in LDs involves fats entering the body as neutral lipids, such as TAG and CE. The adipocytes store the lipids in LDs to fuel the body with energy. Moreover, in obese people, the storage of TAG in the adipocytes is elevated (Greenberg et al., 2011). Hence, the adipocytes store their fat as TAG in LDs. Cells protect themselves against lipotoxicity by storing the FAs as TAG in LDs. Furthermore, the rate of lipolysis in adipocytes appears elevated in most obese people, resulting in an increase in FFAs in the circulation, which later may be stored as TAG

in LDs within the liver and SMs (Greenberg et al., 2011). An elevated intramyocellular TAG has been found to be associated with IR (Shulman, 2000).

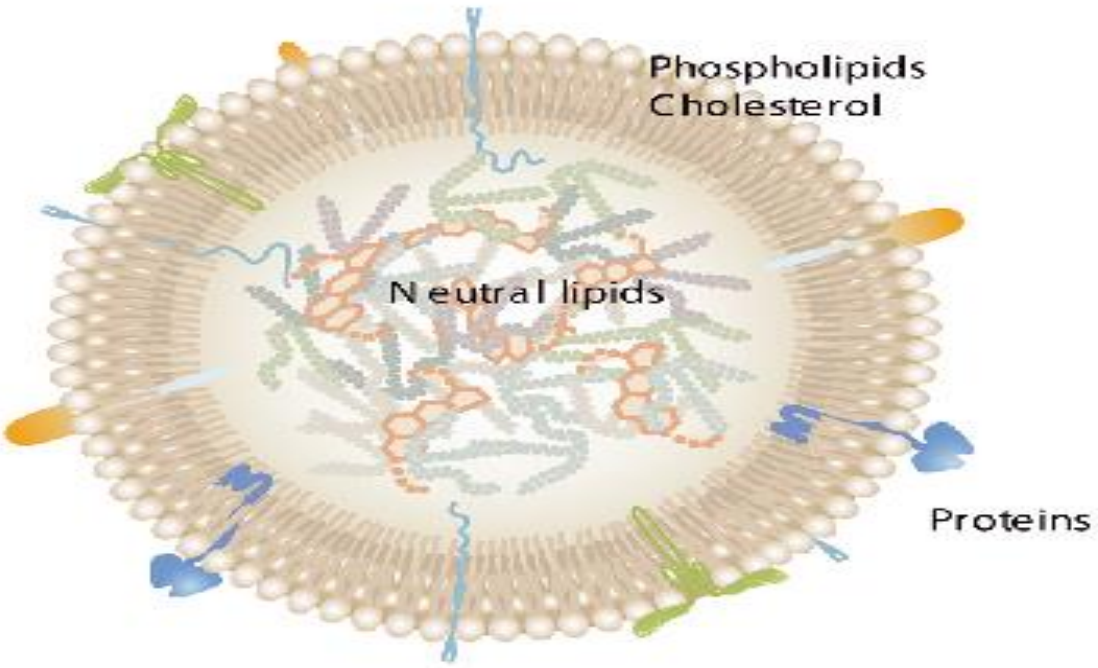


Figure 1-7 Lipid droplet structure. Obtained from (Borén et al., 2013)

Neutral lipids (CEs and TAGs) are being stored within the LD and surrounded by phospholipids monolayer and associated proteins.

1.2.4 The Linkage between Lipids and Insulin Resistance in SMs

For a long time, it was believed that IR, which is a predictor of T2DM (Shulman, 2000), was a downstream complication of obesity (Samuel et al., 2010). It was suggested that the mechanism by which the lipids induce IR was a defect in the insulin signalling cascade, accumulation of lipid intermediates, and inflammation (Høeg et al., 2011). It was hypothesised that intramyocellular lipid accumulation in

obese individuals initiates cellular events that lead to the development of IR. Hence, intramyocellular TAG has been found to be associated with IR (Shulman, 2000).

Many studies have demonstrated that the level of intramyocellular lipids is high in obese T2DM patients when compared to lean individuals (Bosma et al., 2012). The most important tissue in insulin-stimulated glucose disposal is the SMs (Defronzo and Tripathy, 2009; Turcotte and Fisher, 2008). Thus, the IR seen is thought to be mostly the result of changes in the SMs. It was proposed that the mechanisms by which the TAG influences the insulin action resulted from the SMs' TAG lipolysis' product called non-esterified fatty acids (NEFA). This product of TAG lipolysis suppresses glucose transportation by either immediate inhibition or substrate competition (Forouhi et al., 1999; Petersen and Shulman, 2006) or reduces glucose phosphorylation within SMs (Perseghin et al., 1999). Moreover, elevated intramyocellular lipids within SMs tend to negatively affect the SMs' oxidation capacity (Goodpaster et al., 2001) and result in lipotoxicity (Laurens and Moro, 2016). Two decades ago, it was thought that lipid accumulation in muscle negatively affects the insulin stimulation of glucose uptake. However, later, it was found that increased levels of lipids in muscle also impairs insulin signalling, which ultimately leads to the occurrence of IR (Samuel et al., 2010; Samuel and Shulman, 2012).

Besides, adipose tissues act not only as fat storage but also as an endocrine organ that produces adipokines and cytokines such as tumor necrosis factor α (TNF-α) and interleukin-6 (IL6), leptin, and adiponectin (Heilbronn and Campbell, 2008; Ouchi et al., 2003). Adipose tissue comprises several types of cells including adipocytes and immune cells (Huh et al., 2014). Adipose tissues release the FFA and adipocytokines to the circulation to communicate with the body (Sell et al., 2006).

Macrophages, which are types of innate immune cells in adipose tissue, are involved in the regulation of insulin sensitivity as they act as anti-inflammatory immune cells in lean adipose tissues. However, on the obesity state, the adipose tissues cannot respond to the high demand for fat storage, which then leads to an increase in their size and numbers (Suganami et al., 2012). It was reported that numbers of macrophages cells in visceral adipose tissues were more than double in obese rodents and human models (Heilbronn and Campbell, 2008; Huh et al., 2014). Also, macrophages infiltration in visceral adipose tissues of obesity state has been found to increase the production of pro-inflammatory adipocytokines such TNF-α, IL6, leptin (Heilbronn and Campbell, 2008; Suganami et al., 2012), and decrease the anti-inflammatory adipocytokines adiponectin (Suganami et al., 2012). As a result, this mismatch secretion of the pro- and anti-inflammatory adipocytokines observed in visceral adipose tissue is implicated in the metabolic syndrome and IR development (Suganami et al., 2012).

Endocannabinoids (ECs), which are lipids-derived metabolites and regulate energy homeostasis (Abdulnour et al., 2014), have been demonstrated to be elevated in peripheral tissues such as SMs in obese and D2TM patients (Taube et al., 2009). Moreover, IR has been linked to increased levels of pro-inflammatory adipocytokines (Pradhan et al., 2001; Taube et al., 2009), ECs (Abdulnour et al., 2014; Pagano et al., 2008; Taube et al., 2009), FFAs (Delarue and Magnan, 2007; Griffin et al., 1999) and reduction of adiponectin (Nicholson et al., 2018). The causes of IR within SMs are not yet understood (Amati et al., 2011), but the accumulation of lipids is clearly involved (Samuel and Shulman, 2012). Figure 1.8 illustrates the relationship between excess fat storage in the body and insulin sensitivity.

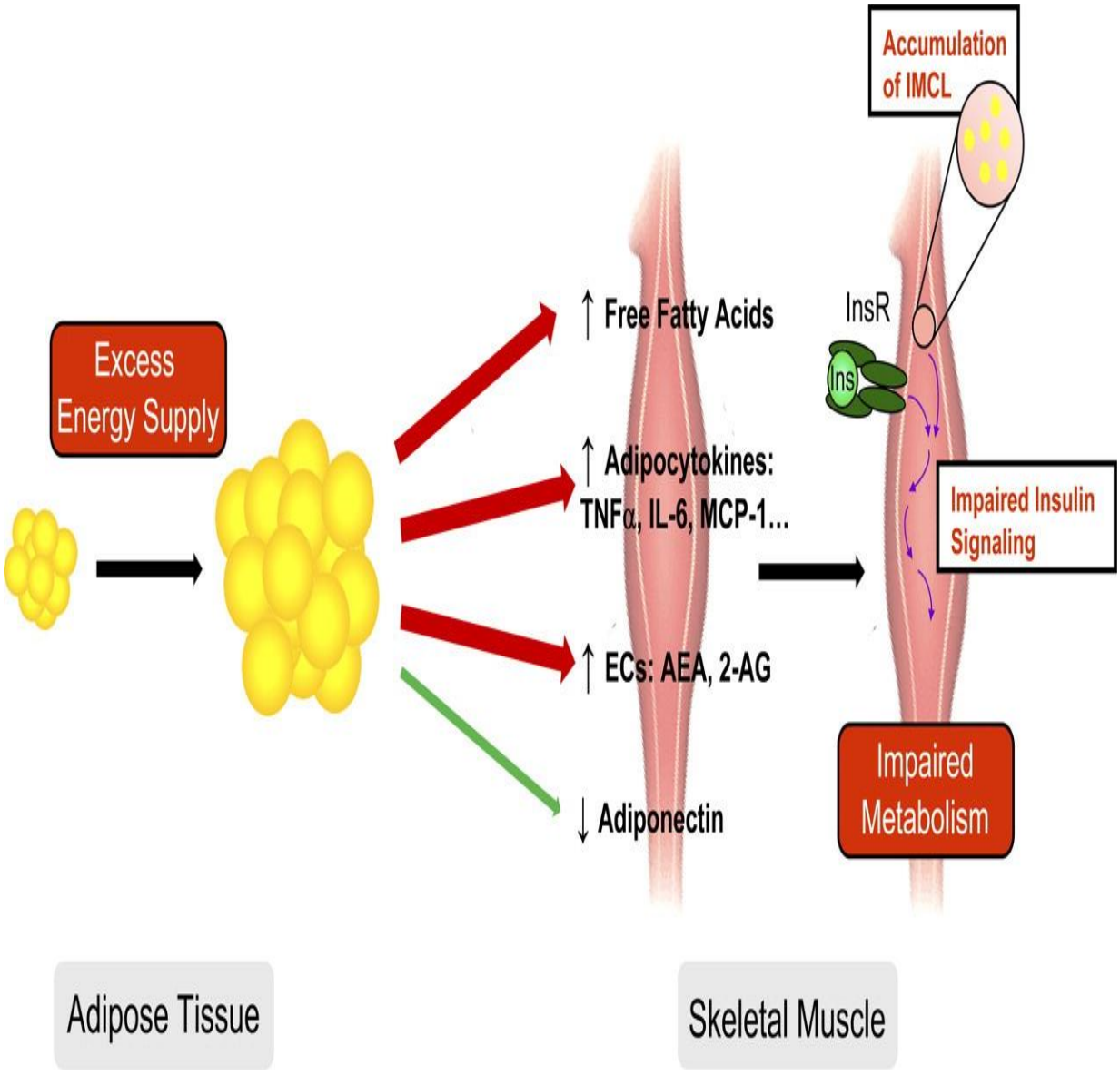


Figure 1-8 The relationship between fat accumulation and insulin sensitivity. Obtained from (Taube et al., 2009).

The relationship between a high-fat diet and the occurrence of IR has been reported in both humans (Bachmann et al., 2001) and animals (Goodpaster and Wolf, 2004; Matsuzawa-Nagata et al., 2008). It was demonstrated that high dietary fat consumption is implicated in weight gain and IR in SM. This association was due to

increased intracellular ceramides, damage insulin signalling pathway, and decrease glucose uptake in humans, rodents, and in *vitro* myotubes (Lark et al., 2012). Recently, a high-fat diet exhibited excessive glucose intolerance in normal human subjects using glucose tolerance tests (Czech, 2020). Additionally, It was reported that after 3 days of mice fed a high-fat diet induces inflammations in adipose tissues and after several weeks in SM and liver. These inflammations were associated with IR in parallel with increased proinflammatory macrophages in adipose tissue (Lee et al., 2011). (Lee et al., 2006) indicated that the type of FA is crucial in terms of IR. The main types of FAs found in diets are saturated FA (e.g. palmitic acid), monounsaturated FA (e.g. oleic acid), and polyunsaturated FA (e.g. linoleic acid) (see Figure 1.9) (Tvrzicka, 2011). It was demonstrated that saturated and polyunsaturated FAs are associated with a reduction in insulin sensitivity (Frangioudakis et al., 2010). In parallel, (Lovejoy, 2002) stated that monounsaturated FAs are also implicated in the development of IR. Thus, one of each FA type will be used in this thesis in order to compare their effects on lipid accumulation in SM cells *in vitro*.

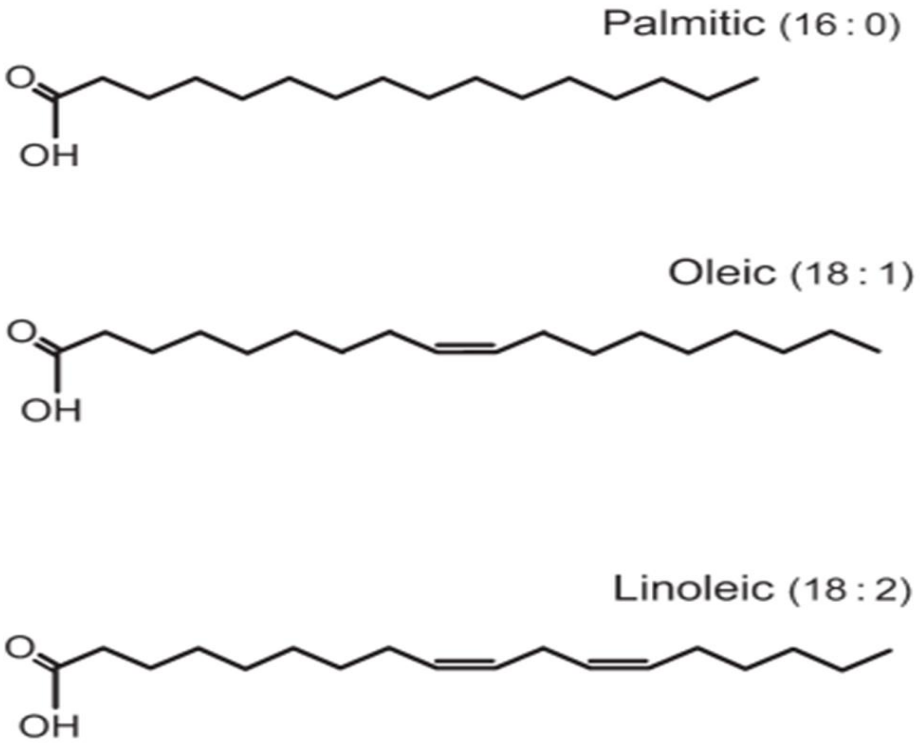


Figure 1-9 Structure of fatty acids used in this thesis.

Obtained from (Vanhercke et al., 2013).

1.2.5 In Vitro Muscle Cells Models

As yet, several muscle systems have been applied to investigate the muscle-related studies such as muscle function, differentiation, and glucose uptake. L6 and C2C12 cells are most cell lines that have been used to study muscles' glucose metabolism in SM (Owens et al., 2013; Yap et al., 2007). However, L6 cells, derived from rat skeletal muscle, are the most frequent cells model used to study glucose uptake by insulin in both types myoblast and myotubes. They have been reported to have the ability to respond to insulin stimulates GLUT4 translocation to the membrane. In parallel, mouse skeletal muscle-derived cells also have been used to study insulin stimulation of glucose uptake into muscle, despite that, they were limited to the study of muscle differentiation, proliferation development (Nedachi and

Kanzaki, 2006). C2C12 cells have been reported to display a smaller extent in the insulin stimulated-glucose uptake when compared to the L6 cell line, as C2C12 cells were suggested to express insufficient GLUT4 proteins even after differentiation (Sarabia et al., 1990; Yap et al., 2007).

Despite that, cell lines usually are immortalized in the laboratories and incubated for a long time in an artificial environment, they are most likely to swerve from normal functions and behaviours (Owens et al., 2013). Human primary cells are the most valuable in *vitro* model to investigate tissue-related events as they are directly isolated from tissue origin and maintain their function and morphological characteristics (Pastor et al., 2010). However, the primary cells are reported to have short life-span, which makes them less suitable for the use of repeated experiments. Even though using multi-extracting primary cells can be an option, myogenic heterogeneity during each extracting may result in different results between experiments (Muses et al., 2011).

1.3 The Polyphenols

1.3.1 What are the Polyphenols?

Polyphenols exist in plant-derived foods, and there are more than 8000 compounds (Bahadoran, et al, 2013). They are natural phytochemical compounds and are found in many daily foods, including tea, cocoa, fruits, vegetables, and whole grains. Most antioxidants that are found in diets are derived from polyphenols (Scalbert et al., 2005). Polyphenols have many biological functions, such as acting as antioxidants and anti-inflammatory agents (Bahadoran, et al, 2013). The estimated average daily intake of polyphenols is 1 g/d (Cook and Samman, 1996; Scalbert et al., 2005). Polyphenols have been shown to have a positive role in human health

(Jakobek, 2015). Currently, the literature confirms the potential effect of polyphenols on the prevention of many diseases, including cardiovascular diseases, cancers, and diabetes (Corrêa and Rogero, 2019). The most biological action of polyphenols is the antioxidant feature, which previous experimental data has reported (Hongxiang et al., 2007; Petti and Scully, 2009). Nevertheless, polyphenols contribute to many sensory properties of food, such as colour and flavour.

An estimated 5–10% of the body's total intake of polyphenols are absorbed in the small intestine (Corrêa and Rogero, 2019). Once absorbed, the polyphenols undergo coupling in the liver and/or small intestine. The polyphenols reach the colon, where they are metabolised by gut microbiota, ultimately, they reach plasma and tissues in an amalgamation form (Corrêa and Rogero, 2019; Manach et al., 2005; Milenkovic et al., 2013). It is difficult to estimate the intake of polyphenols that produces the most benefit; the reasons for this include the diversity of polyphenols' structures and the content variations of particular foodstuffs (Corrêa and Rogero, 2019; Petti and Scully, 2009; Scalbert and Williamson, 2000).

1.3.2 The Polyphenols Classifications

Phenolic acids, flavonoids, stilbenes, lignans, and curcuminoids are polyphenol compounds that have been found to be abundant in whole plant foods (Bahadoran et al., 2013). Polyphenols have been classified in several ways, including original source, biochemical features, and chemical structure. Polyphenols are comprised of phenolic structural features (see Figure 1.10) and, as such, they are called polyphenols. The polyphenols are highly diverse groups of phenolic structural features and structurally, they are classified into four groups: (i) phenolic acids such as benzoic acid, (ii) flavonoids such as quercetin and epigallocatechin gallate

(EGCG), (iii) polyphenolic amides such as avenanthramides, and (iv) non-flavonoids (stilbenes) such as resveratrol (Rong, 2010).

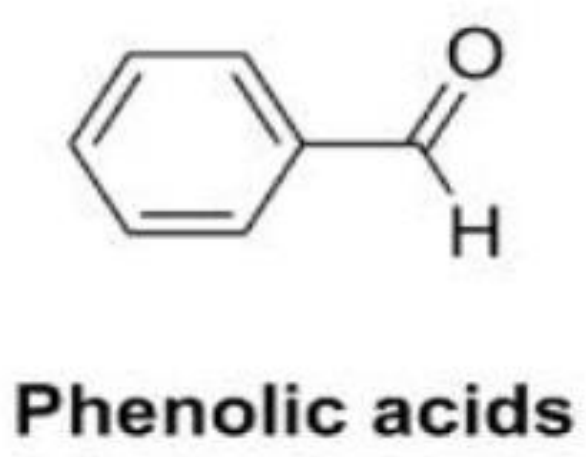


Figure 1-10 Diagram of the chemical structure of phenol, the structure on which the polyphenol family is based.

Obtained from (Baselga-Escudero et al., 2014).

Some of the most studied polyphenols include quercetin, EGCG, and resveratrol (which are used in this thesis). Each of these polyphenols has a different and unique phenolic structure (see Figure 1.11). Also, each comes from the same or different sources of foods or beverages; Table 1.1 shows the different sources of each polyphenol.

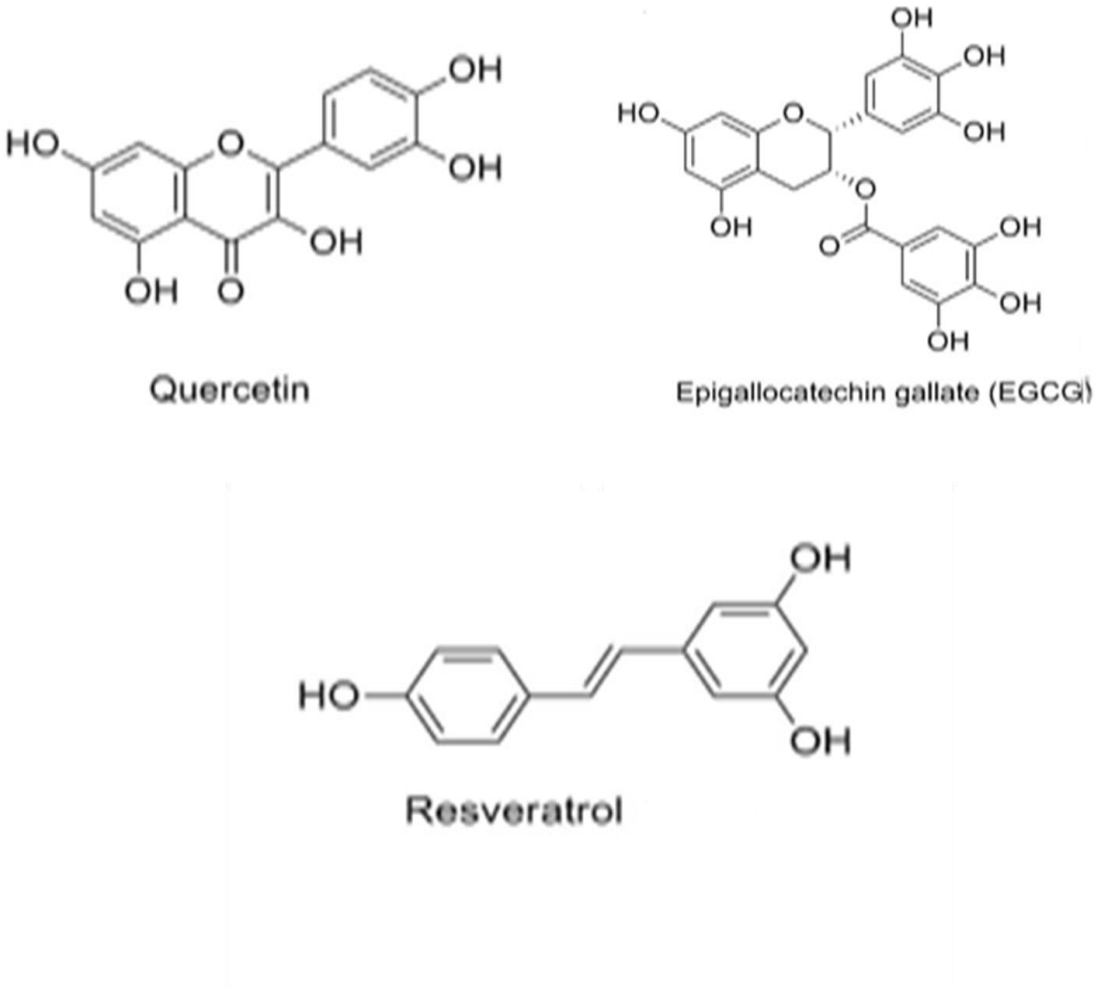


Figure 1-11 Structures of the resveratrol, quercetin, and EGCG polyphenols.

Obtained from (Baselga-Escudero et al., 2014).

Table 1-1 Sources of dietary polyphenols.

Obtained from (Hongxiang et al., 2007).

Group	Polyphenol	Source
flavonoid	Quercetin	Vegetables: capers, chives, onions, red onions, dock leaves, fennel, hot peppers, cherry tomatoes, spinach, sweet potato leaves, lettuce, broccoli, and beans(green/yellow) Fruits: apples, apricots, grapes, cherries, and black currant juice. Others: red wine, tea (green, black), cocoa powder, turnip (green), endive, and leek.
catechin	EGCG	Fruits: apples, apricots, grapes, peaches, nectarines, pears,plums, raisins, cherries, berries (black, blue, rasp, cran, and elder). Others: red and white wines, tea (green, black), chocolate (dark, milk), and cocoa
Stilbenes	Resveratrol	Mainly found in Red wine and Grapes

1.3.3 The Advantageous Impact of Polyphenols on Human Health

Polyphenols have been shown to have beneficial effects on the health of humans. Besides, these compounds can be used to treat or prevent numerous illnesses (see Figure 1.12) (Aguirre et al., 2014). Evidence shows that polyphenols ameliorate dyslipidaemia (Chen et al., 2011) and IR (Rivera et al., 2008). Moreover, the consumption of polyphenols provides enhanced protection against chronic diseases, including cardiovascular disease, cancer, aging, and diabetes (Ganesan and Xu, 2017). For thousands of years, Eastern medicine has been therapeutically using plants and spices that contain the major subgroup of polyphenols: flavonoids (Middleton et al., 2000). Previously, it had been thought that polyphenols only possessed antioxidative capacity; however, after extensive research on polyphenols, it has become clear that the ability of polyphenols exceeds that (Mudgal et al., 2010). Stevenson and Hurtst (2007) have reviewed different beneficial effects of polyphenols on human health. They concluded that polyphenols have the ability to maintain intracellular signalling cascades, which are implicated in cell growth's regulation, inflammation, and other processes (Stevenson and Hurst, 2007).

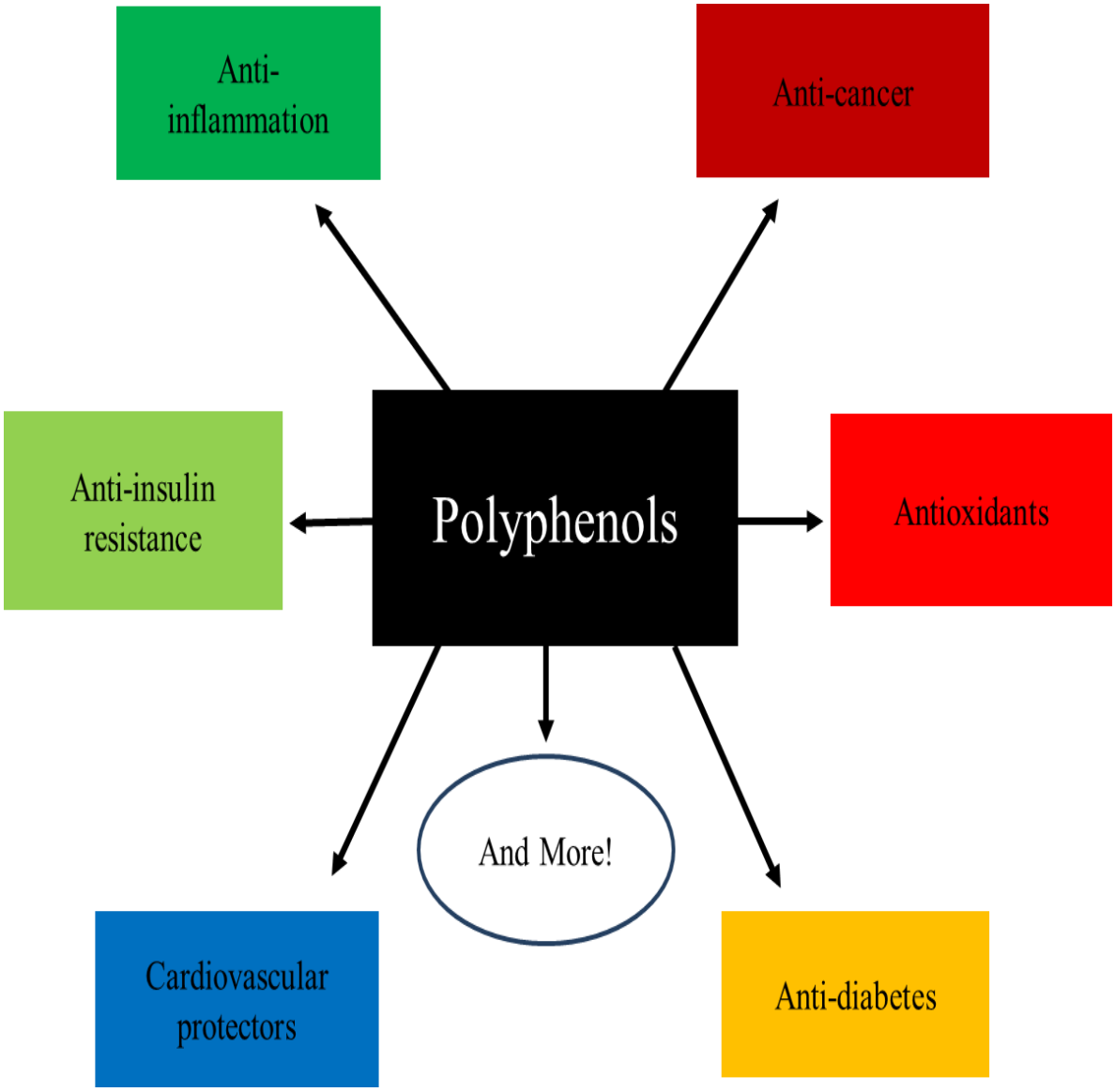


Figure 1-12 Diagram illustrates the beneficial effects of polyphenols on human health.

1.3.4 Polyphenols and Type 2 Diabetes

Evidence shows that natural polyphenols can minimise the risk of diabetes and diabetes complications because these polyphenols are natural products and, unlike other drugs, less likely to have potential side effects (Xiao and Högger, 2015). Polyphenol-rich foods and beverages have been shown to reduce hyperglycaemia (both fasting and postprandial) and improve insulin sensitivity in humans and animals (Aryaeian et al., 2017). Additionally, the same author stated that the positive role of the polyphenols on glucose homeostasis is well-recognised and reported in human trials, animal models, and *in vitro*. The possible mechanisms of polyphenols' action towards T2DM include activation of insulin receptors and glucose uptake in insulin target tissues, modulation of intracellular signalling pathways, energising of insulin secretion from the β cells of the pancreas, and suppression of the digestion of carbohydrates (Amiot et al., 2016).

Epidemiological studies, despite being inconsistent (Cao et al., 2017), suggested that anthocyanidins and flavonoids showed a significant effect on lowering the risk of T2DM (Xiao and Högger, 2015). Polyphenols had been demonstrated to boost the uptake of glucose into SMs and adipocytes and improve liver gluconeogenesis. Moreover, polyphenols, including resveratrol, quercetin, and EGCG, have been reported to protect against inflammatory conditions, which are linked to obesity-induced T2DM (Anhê et al., 2013). Oxidative stress, inflammation, and glycaemia biomarkers of obese T2DM patients have all been improved by flavonoids from grape extracts (Cao et al., 2017). Table 1.2 illustrates some of the studies conducted on polyphenols and their effects on obesity and T2DM.

TabTable 1-2 Overview of studies conducted on different polyphenols' effects related to lipids and DM.

Subjects/models	Treatment	Effect	Reference
10 T2DM patients	10mg Resv/day for 4 weeks	Decrease the IR	(Timmers et al., 2011)
10 healthy subjects	Polygonum cuspidatum extract (40mg Resv)/day for 6 weeks	Suppressed the plasma TNF-α and IL-6	(Ghanim et al., 2010)
23 T2DM patients	583 mg catechin/day for 12 weeks	Increased adiponectin and insulin levels	(Nagao et al., 2009)
T2DM patients	1 g/day Resv for 45 days	Reduction of fasting plasma glucose and increased insulin levels	(Movahed et al., 2013)
32 obese T2DM	Grape-seed extract (Flavonoid)	Improved inflammation's biomarker, glycemia, and oxidative stress	(Sun et al., 2020)
HepG2 cells and mouse primary hepatocytes	0.1-10uM EGCG cotreated with high glucose (30mM)	Increased AKT and GSK phosphorylation compared to high glucose treatment in a dose-dependent manner.	(Ma et al., 2017)
cultured C2C12 cells	0.1-1mM Curcumin	Increased glucose uptake and GLUT4 proteins levels at membrane	(Cheng et al., 2009)

1.4 Hypothesis and Objectives

1.4.1 Hypothesis

It was realised previously that most T2DM patients are obese. This has become more obvious after confirming that obesity is a major cause of T2DM. Moreover, T2DM is characterised by the inability of insulin to dispose of elevated blood glucose into major glucose metabolism tissues such as SMs. At present, IR has become a major target in the investigation of the etiology of T2DM. IR has been clearly linked to the accumulation of lipids. In addition to the many contemporary studies in the literature looking for the relationship between IR and lipids, none of them have specifically looked into the association between lipid accumulation and IR within SMs *in vitro*. Moreover, many studies have reported the beneficial effects of polyphenol compounds on insulin sensitivity. Again, none of these studies elucidated whether dietary polyphenols have a direct impact on the accumulation of lipids within muscle cells, which therefore ameliorates IR *in vitro*.

Hence, the overall aim of this thesis was to establish an *in vitro* model that enables us to determine whether the accumulation of lipids within muscle fibres is implicated in IR and to investigate the potential effects of polyphenols on that. The hypothesis of this thesis is that lipid accumulation within cultured SM myotubes cells will cause an alteration of insulin stimulation of glucose uptake, and dietary polyphenols will improve insulin sensitivity via decreasing the lipid deposition.

1.4.2 Objectives

1. To determine whether exposing muscle cells (C2C12 and L6) to different types of fatty acids for a period of time will result in an increase in lipid accumulation within muscle myotubes.

2. To determine the effects of certain polyphenols on the formation of lipid droplets in cultured muscle cells.

3. To investigate the effect of the accumulation of lipid droplets within myotubes on the capacity of insulin to stimulate glucose uptake.

4. To examine whether polyphenols have a positive influence on insulin sensitivity.

Chapter 2:

General Materials and Methods

2 General Materials and Methods

2.1 Cell Culture

In this thesis, two different cultured cell lines were used (C2C12 mouse muscle cells and L6 rat muscle cells). Notably, all techniques, methods, and materials were the same for both cell lines. However, the only difference between the cells is that the process of C2C12 differentiation into myotubes takes 4-5 days, whereas L6 takes a longer time – about 6-7 days. The materials described in this thesis were provided from different suppliers; however, the specific details and codes are included either within this chapter or within specific chapters. In addition, Class II microbiological safety cabinets were used for all cell culture work complying with procedures relating to manufacturing safety. Corning Filtration Systems (250ml; 431096 or 500ml; 431097) were used to filter media before being added to the cells in order to minimize contamination. Reagents and media in small quantities were filtered using 0.2µm filters (Sarstedt) attached to a syringe. All non-sterilized equipment was autoclaved before being used at 121°C for 15 min (MP24 Control System, Rodwell Scientific Instruments). All methods, reagents, and equipment used for cell culture were performed as the standard operating procedure from our laboratory in the division.

2.1.1 Cells Proliferation

For regular proliferation and expansion of myoblast cells, they were grown in 75 cm2 culture flasks (430641U, Corning) in a growth medium that comprised of Dulbecco's Modification of Eagle's Medium (DMEM; D5796, Sigma, USA) supplemented with 10% Fetal Bovine Serum (FBS, Sigma; F7524), and 1% Penicillin-Streptomycin (P/S; P4333, Sigma, USA) inside a humidified incubator at 37°C with 5% CO2. After reaching 60-80% confluence, these cells were then

transferred to a new flask (passaged) in order to maintain the cells in an optimal growth condition.

2.1.2 Passaging cells

The passage process is perfumed to keep the cells in an optimal proliferative condition. It commences when the cells attain 60-80 % confluence in the flask. Thereafter, the supplemented medium was removed and the cells washed using 10 ml sterile phosphate buffer saline (PBS, phosphate buffer saline tablets, Sigma-Aldrich; P4417). Subsequently, 2 ml of warm trypsin-EDTA solution (0.25% trypsin-EDTA, Sigma-Aldrich; T3924) was added in order to allow the cells to detach from the surface. The cells were then incubated for 4-5 minutes in an incubator at a temperature of 37 °C, followed by shaking the flask and adding 8 ml of growth media into the flask. Additionally, the suspension in the flask was transferred to a universal tube to be centrifuged for 5 minutes at 1000rpm (approx.: 168xg) (Eppendorf Centrifuge 5702R). Then, the supernatant was removed so that it could be re-suspended with a 10ml fresh growth medium; the desired number of cells from the resultant cell suspension was eventually transferred to a new flask.

2.1.3 Counting cells

Haemocytometer counting equipment was used to count the myoblast cells in suspension. Counting cells is essential for laboratory experiments, it ensures the same number of cells are used and reduces the variability across experiments. The counting process begins by taking 10ul from the suspension and place it between the counting chamber and the glass cover on the haemocytometer. Then, calculate the average of 3 squares of 4 squares in the counting chamber and multiply the average by 10^4 (See

Figure 2.1). The result is the number of cells per ml. Using the following formula allows you to know the total cells in the suspension.

Total cell number= *total cells per ml 𝑥 total volume of cell suspension*

To seed the cells at the appropriate density for an experiment, each culture plate size needs a specific density of seeding. Table 2.1 shows the appropriate seeding density for each specific plate size. The following formula was used to determine the volume needed from the cell suspension to seed a desired number of cells into a culture plate:

Required volume = *(Desired cell number/total cell number) X total volume of cell suspension*

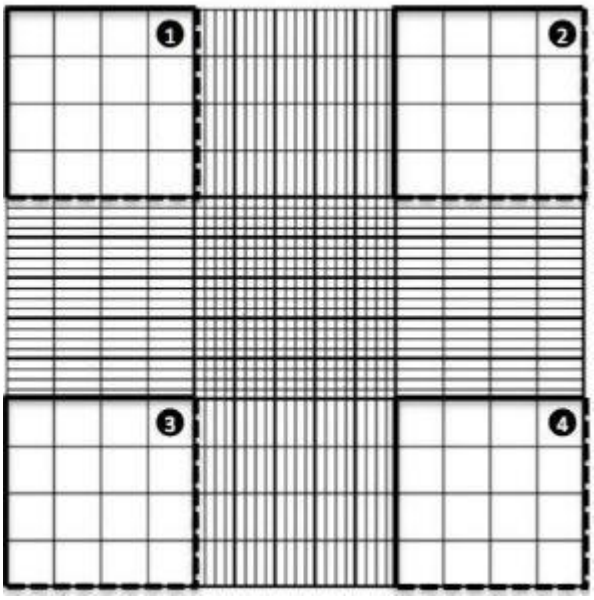


Figure 2-1 haemocytometer gridlines.

Haemocytometer big squares contain 16 small squares. Taking the number of cells in three of the four big squares and average them before multiply by 10^4 gives the total number of cells in 1 mL.

Table 2-1 Cell culture seeding density different culture plates. (Obtained from Thermo fisher scientific, 2015)

Culture Plates	Seeding density	Growth medium (ml)
6-well plate	0.3×10^6	2
12-well plate	0.1×10^6	1
24-well plate	0.05×10^6	0.5

2.1.4 Freezing Cells

Myoblast cells were frozen in liquid nitrogen in order to create a storage of cells for later experiments. To freeze cells, the passage process, which was explained before, was followed until the cells pelleted. Cells were then re-suspended in a freezing medium of FBS with 10% Dimethyl Sulfoxide (DMSO, D/4120/PB08, Fisher Scientific). In the end, the cells were transferred to vials and slowly frozen in a freezing container in 100% isopropanol (p/7500, Fisher Scientific) at -80 for 48 hours before being transferred to liquid nitrogen.

2.1.5 Thawing Cells

Cells were frozen in liquid nitrogen to be used when needed. To thaw the desired cells, one vial was taken from the liquid nitrogen and placed in a water bath at 37º for 2–3 minutes. When the cells were thawed, they were immediately transferred into a new flask that contained a 13 ml growth medium. The flask was then incubated at 37º for 1–2 days.

2.1.6 Cell Differentiation

2.1.6.1 Myogenic Medium

The myoblasts cells were encouraged to differentiate into myotubes when they reached 50–60% confluence by replacing the growth medium with a differentiation medium, consisting of DMEM with 2% horse serum (Gibco, New Zealand) and 1% P/S. Myoblasts were expected to start fusing to form multinuclear myotubes from day 3 to day 5 for C2C12 cells and from day 5 to day 7 for L6 cells after applying the differentiation media. The differentiation medium was changed every 24–48 hours.

2.1.6.2 Adipogenic Medium

To allow the cells to differentiate into adipocyte cells, myoblast C2C12 cells were treated with the adipogenic medium consisting of two different media. Firstly, after reaching 80-90% confluence, the cells were treated with induction medium (DMEM, 10% FBS, 1% P/S, 50mM 3-isobutyl-1-methylxanthine (IBMX), 2.5mM Dexamethasone, 170mM Insulin, and 1µM Tri-iodothyronine T3) for 48 hours. Secondly, after 48 hours, the induction medium was replaced with differentiation medium (DMEM, 10% FBS, 1% P/S, 170 mM Insulin, and 1µM T3). The medium was changed every 24–48 hours for 8 days and then the cells were used for further analyses.

2.2 Fatty Acid Treatments

The FAs described and examined in this thesis are Palmitic acid (PA) 16:0 in solid form, oleic acid (OA) 18:1 dissolved in ethanol, and linoleic acid (LA) 18:2 dissolved in ethanol. The FA were purchased from Sigma Aldrich and the initial protocol for the FA assessment experiment was derived from Jové and colleagues (2006) who showed that saturated FA at the dose of 0.75mM induced inflammation and glucose suppression (Jové et al., 2006).

2.2.1 Preparation of Fatty Acids

The dissolved FAs were delivered in lidless glass tubes. The tubes were broken and contents were transferred to 1.5ml tubes and then multiple aliquots were made. The FA were kept in a freezer at -20 ^{o}C until used. The palmitic acid, which was received as a solid, was stored at room temperature, as guided by manufacture. Initially, the treatments were prepared to apply to the cells as described previously (Chavez et al., 2003; Jové et al., 2006; Schmitz-Peiffer et al., 1999). The treatments for the initial experiment were as follows:

a) 2% bovine serum albumin (BSA) in DMEM – (control)

b) 0.75 mM PA in 2% BSA in DMEM (PA)

c) 0.75 mM OA in 2% BSA in DMEM (OA)

d) 0.75 mM LA in 2% BSA in DMEM (LA)

As the OA and LA were already dissolved in ethanol, but the PA was not, 100 µl ethanol was added to the PA to be dissolved. Moreover, to negate any effect that the ethanol may have on the cells, ethanol was added to the control treatment.

However, the concentrations described above was found to be high and might be harmful to the cells. Additionally, to increase the power of the experiment, a time course was needed. Also, the high concentration of FA could kill the cells when incubated for a longer time. The typical FFA level found in the human fasting plasma is 0.3mM and can be ranged from 0.1 to 0.7mM according to gender, nutritional status, and smoking (Frayn et al., 1996). This concentration can be raised in obese individuals on average of 70uM (Karpe et al., 2011). Zoe Huggett, a Ph.D. student in

the lab, was conducting a similar experiment with similar FAs. She found that a lower concentration (0.2mM) could be used to induce lipid deposition in liver cells. Therefore, the same concentration was used for all subsequent experiments. The treatments were prepared as follows:

a) 2% BSA in DMEM (Control)

b) 0.2 mM PA in 2% BSA in DMEM- (PA)

c) 0.2 mM OA in 2% BSA in DMEM- (OA)

d) 0.2 mM LA in 2% BSA in DMEM- (LA)

e) 0.2 mM total fat at the ratio 4:4:2 (80uM PA, 80uM OA, 40uM LA) (3x FA)

Again, because all FAs were dissolved in ethanol, ethanol was added to the control treatment as well. The prepared treatments were stored in the fridge for no more than 3 days. The ratio of 3xFA was chosen to be close to the typical distribution of FAs found in the plasma which is reported to be approximately 45 OA;30 PA; and 25 LA % (Nikolaou et al., 2016).

2.2.2 Application of Fatty Acids

After the treatments were prepared, they were then stored in the fridge no more than 3 days before use in the experiment. For the initial protocol derived from Jové et al. (2006), the cells at a differentiated state (4 days for C2C12) were subjected to the FA treatments after the removal of the differentiation medium. Each treatment

was applied to 3 wells of a 12 well plate (n=3). The treatments were then administered for 16 hours, after which they were removed and the cells were washed with PBS. They were then stained with Oil red O to localise the LDs. According to Jové et al. (2006), PA incubation for 16 hours, with the concentration of 0.75mM, elevates TNFa mRNA expression. Additionally, PA under these conditions perturbs the metabolism of the carbohydrates, which is illustrated by reduced mRNA expression of GLUT4.

For the second FA protocol, the C2C12 cells were differentiated for 4-5 days and then subjected to the FA treatment. The cells were then incubated at 37ºC for 1 day, 2 days, or 3 days before they were stained with Nile red (in 12 or 24 well plates) or harvested for subsequent analyses (in 6 well plates).

2.3 Polyphenol Treatments

All the polyphenols described in this thesis were purchased from Sigma - Aldrich (St Louis, MO, USA). The polyphenols resveratrol (Resv; R5010), Epigallocatechin Gallate (EGCG; E4143), and Quercetin (Quer; Q-0125) were received in powder form, and then a stock of 100mM in DMSO was made for each polyphenol and stored at -20 ºC.

2.3.1 Polyphenol preparations

The working concentrations were obtained from (Nicholson et al., 2010) and the concentrations were as follows: 1µM, 0.1µM, 0.05µM, 0.01µM, 0.005µM, and 0.001µM. Notably, the maximum concentration of polyphenols in plasma is ranged between 0.1-1 µM (Nicholson et al., 2010). The 100mM stock of each polyphenol was diluted further in DMSO to 1mM, 0.1mM, 0.05mM, 0.01mM, 0.005mM, and

0.001mM, and stored in aliquots at -20 ºC. DMSO must be no more than 0.1%(V/V) of the total volume, otherwise, it can affect the proliferation and differentiation of muscle cells. Therefore, when adding these concentrations to the media, they were diluted 1000 times (see Table 2-2).

Table 2-2 Example of working solution preparation

Stock Concentration	**Medium volume**	**Added volume**	**Final concentration**
1 mM	10 ML	10 µL	1 µM

2.3.2 Polyphenol application

After the preparation of LA, as described in 2.2.1, 5-10 ml of the LA treatment was transferred to multiple universal tubes. Next, 5-10 µl of each stock of polyphenols concentration was added to the related tube (see Table 2-3). The treatments were applied to the cells in 24 well plates and incubated at 37ºC for up to 3 days. Additionally, the cells were either stained using Nile Red on day1, day2, or day3 or used for Glucose Uptake Assay (see section 2.6). The lipid stained with Nile red in the cells was then measured using a fluorescent plate reader (Fluostar Optima, BMG Labtech).

Table 2-3 Working treatment contents used for the polyphenols experiments

Treatment	Treatment contents
Control	DMEM + 2% BSA + ethanol (100% pure) + DMSO
LA	DMEM + 2% BSA + 0.2mM LA + DMSO
Polyphenols	DMEM + 2% BSA + 0.2mM LA + Polyphenols

2.4 Cell Staining

2.4.1 Oil Red-O Staining Method

To identify lipid accumulation in the cells, the oil red O (Sigma) staining method was used (for 0.75mM fatty acids). The staining procedure was followed as described previously (Ryan et al., 2013). To stain the cells with oil red O, the treatment was removed and cells were washed twice with PBS. Next, 4% paraformaldehyde was added for 30 mins, to fix the cells, and then the solvent was removed, before staining with 0.4% (w/v) oil red O in 60% (v/v) isopropanol for 30 mins. Afterwards, the cells were washed with PBS, and images were captured using an Olympus SZH10 microscope (Olympus, UK) and analysed using Image-Pro (version 5.1, USA).

2.4.2 Nile Red Staining Method

A better staining method to localise intracellular lipid droplets (used for 0.2mM fatty acids) is the dye Nile Red (9-diethylamino-5H-benzo[alpha]phenoxazine-5-one), which can be imaged using fluorescence microscopy. The preparation and application of Nile Red staining were followed as instructed by Zoe Hugget, a PhD student in our lab.

2.4.2.1 Preparation of Nile Red

To stain cells with Nile Red dye, a stock of 1mM Nile Red solution (N-3013, Sigma) in DMSO, with 1% Pluronic F-127 (Polyethylene-polypropylene Glycol; P-2443, Sigma) was made and stored at -20 ºC in aliquots. Just before staining the cells, the stock was diluted to 30uM in Hank's Balanced Salt Solution (H6648, HBSS, Sigma, USA) to make a working solution. The working solution consists of a 0.4 ml Nile Red stock solution in12.6ml HBSS (see Table 2-4).

Table 2-4 Nile Red working solution for cell staining

HBSS	Nile Red stock solution	Final volume
12.6 ML	0.4 ML	13 L for 24 well plate

2.4.2.2 Application of Nile Red

Cells were washed twice with HBSS before being stained with Nile Red. The stained cells were incubated for 12-15 mins in the dark, at room temperature. The cells were washed once with HBSS and 100ul HBSS was added to each well before reading the plate in a fluorescence plate reader (Fluostar, Omega) at excitation 485, emission 590.

2.5 DNA Assay

DNA assay was used to quantify the number of cells in an individual well. The assay quantifies the content of the DNA of cultured cells; therefore, it can be used as a marker of cell number. The DNA assay was performed using Hoechst dye following a standard operating procedure from the division. This method was used once to normalise the polyphenols results in chapter 4 section 4.3.2.

2.5.1 Sample collection from cell culture

After staining the cells with Nile Red and measuring the lipid deposition (lipids and polyphenols), the plate containing the cells was frozen and thawed 3 times to allow cells lyse. Next, 400µl of distilled water was added to each well, and cells were harvested and transferred into 1.5ml Eppendorf tubes and stored at -20 ^{o}C.

2.5.2 Detection reagent preparation

To prepare the detection solution, 10x TNE buffer ((100mM Tris, 1M NaCl, 10mM EDTA; pH 7.4 in distilled water) was diluted to 2x TNE by adding 3ml of 10xTNE to 12ml RNase-free water to make 15 ml stock. Just before the assay, 30µl of 1mg/ml Hoechst dye was added to the 15ml 2x TNE and covered by foil (see Table 2-5).

Table 2-5 Composition of detection reagent

Reagent	Volume
10x TNE	3ML
RNase- free water	12ML
Hoechst dye	30µL

2.5.3 The assay procedure

40µl (contains 40µg DNA) of calf thymus DNA was added to 960µl, then 500µl was transferred into 500 RNase-free water (1:2 dilution; therefore, 20µg/ml). Dilution was repeated to 0.3125µg/ml to make a standard curve of serial dilutions (from 40µg/ml to 0.3125µg/ml). Besides, 20-60µl of samples was added and topped up to 100µl with RNase-free water. Finally, 100µl of detection solution was added to each well and quickly scanned using a fluorescent plate reader (excitation at 350 nm and emission at 460 nm; Fluostar Optima, BMG Labtech). All reactions were run in triplicate on a 96 well plate.

2.6 Glucose uptake assay

2.6.1 The assay protocol standard

Glucose Uptake-Glo™ Assay kit (J1343 50ml and 500ml, Promega) was used to determine the glucose uptake by L6 cells and the response to insulin. According to Glucose Uptake-Glo™ Assay Instructions for Use of Products (2017, Promega), the

assay measures glucose uptake in cultured mammalian cells, predicated on the detection of 2-deoxyglucose-6-phosphate (2DG6P) (Glucose Uptake-Glo™ Assay Instructions for Use of Products, 2017).

The basis of the glucose assay depends on the 2-Deoxy-D-glucose (2-DG). 2DG structure is relatively close to that of glucose. Therefore, insulin can stimulate the uptake of 2DG into the cells (Wick et al., 1957). Additionally, in the presence of 2DG, the insulin-stimulates 2DG uptake into the cell and being phosphorylated quickly to form 2DG6P, but the 2DG6P cannot be metabolised by the cell's enzymes that metabolise glucose-6-phosphate (G6P). Therefore, the 2DG6P accumulates in cells and it can be detected using a detection solution. The detection solution consists of glucose-6-phosphate dehydrogenase (G6PDH), NADP+, Reductase, Ultra-Glo™ Recombinant Luciferase, and proluciferin substrate. Following the addition of the detection solution, G6PDH oxidises 2DG6P to 6-phosphodeoxygluconate, concurrent with reducing NADP+ to NADPH. This is followed by the conversion of proluciferin to luciferin, which then produces a luminescent signal by combining with the Ultra-Glo™ Recombinant Luciferase reagent (see Figure 2.2) (Glucose Uptake-Glo™ Assay Instructions for Use of Products, 2017).

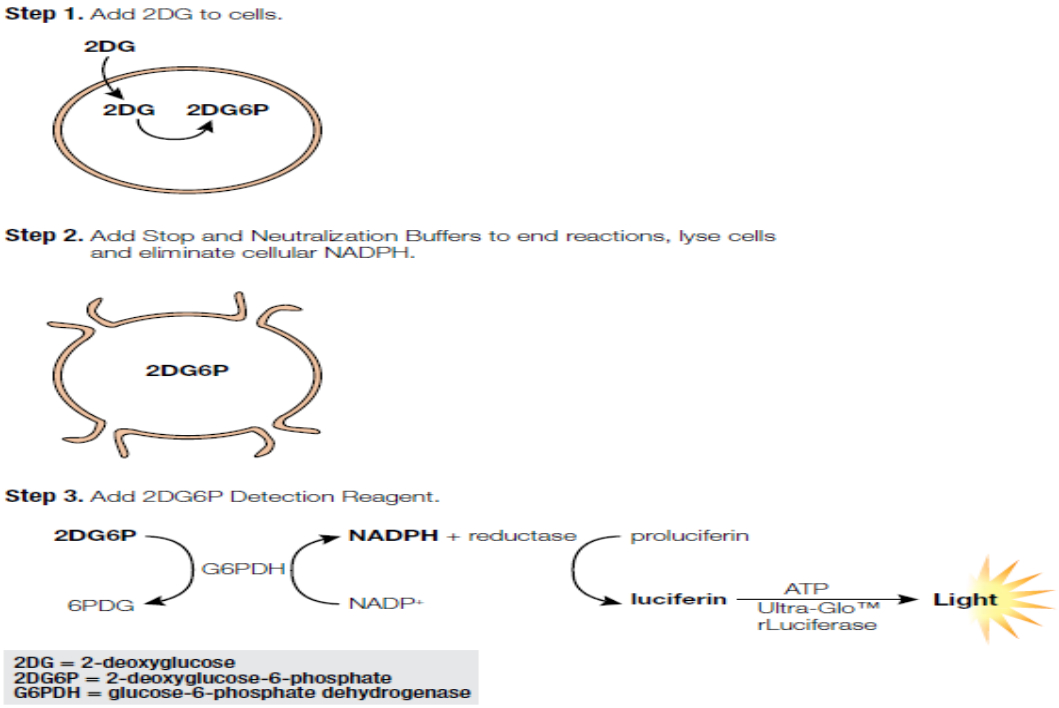


Figure 2-2 The basis of the glucose uptake assay. Obtained from Glucose Uptake-Glo™ Assay Instructions for Use of Products, 2017.

2.6.2 Sample preparation

Myoblast L6 cells were differentiated into myotubes (DMEM, 2% horse serum, 1% P/S) for 6-7 days in 24-well plates (Costar®, 3524). Then, the treatments were added to the cells, then the cells were switched to normal DMEM (no serum) for 24hrs to allow cells to enter starvation mode.

2.6.3 Detection reagents preparation

The assay reagents were thawed from -80^{o}C to room temperature. All components were slowly thawed on ice except the Stop Buffer, Luciferase Reagent, and Neutralization Buffer, which were kept at room temperature. After the detection reagents were thawed, they were mixed, according to Table 2-6, in a universal tube an hour before use, to minimize assay background.

2.6.4 Assay Procedure

The procedure was followed as manufacturer's instructions after treating cells with DMEM with no serum for 24hrs, the cells were incubated at 37ºC in 5% CO2 for 1h in the presence or absence of 1mM insulin in DMEM. Next, the DMEM containing the insulin was removed and 0.1mM 2DG in PBS was added after the cells were washed once with PBS. Following 30 minutes of incubation at 25ºC in 5% CO2 with 2DG, the uptake was stopped by adding stop buffer for 12-15 minutes and mixed thoroughly. The assay is designed for 96 well plate, therefore, 75µl from each well was transferred to 96 well plate (Costar®, 3904). The cells were then neutralized by adding a 25µl neutralization buffer and were then shaken. Eventually, each sample was topped up with 100µl of the detection reagent mixture and incubated at 25ºC in 5% CO2 for 1h before the measurement by a luminometer (Fluostar, Omega).

Table 2-6 Volumes required from each reagent for detection mixture

Reagent	**Each reaction requires**
Luciferase Reagent	100µl
NADP+	1µl
G6PDH	2.5µl
Reductase	0.5µl
Reductase Substrate	0.0625µl

2.7 Real-Time Quantitative Polymerase Chain Reaction (qPCR)

2.7.1 Total RNA Extraction

2.7.1.1 Sample preparation from cell culture

After the cells were treated with the required treatment in 6 well plates, the treatments were removed and 200ul ice-cold RNase-free PBS was added. Cells were harvested using a cell scraper to detach the cells from the surface. The harvested cells were then stored at -80 ^{o}C for further analysis.

2.7.1.2 Extraction procedure

The total RNA was extracted using the High Pure RNA Isolation Kit (Roche, Mannheim, Germany) as the manufacturer's guide. The frozen cells were lysed by adding 400µl of the lysis-binding solution followed by 15 seconds vortex. The tube containing the 600µl sample was centrifuged through a spin column at 8000xg for 15 seconds at room temperature. Afterwards, the flow-through was discarded and 90µl of DNase-incubation buffer containing 10µl DNase I was added and then incubated at room temperature for 15 minutes. The precipitated RNA in the spin column's membrane was washed with 500µl of wash solutions I and II, via centrifugation at 8000xg, for 15 seconds for each wash solution. Additionally, to ensure all contaminants were removed from the RNA, the RNA was washed again with 200µl of wash solution II, via centrifugation, at 13000xg for 2 minutes. Finally, to elute the RNA into a fresh 1.5 tube, 50µl of RNase-free H2O was added and centrifuged at 8000xg for 1 minute and stored at -80°C.

2.7.1.3 Quality check

To check the quality and quantity of an extracted RNA, the RNA was measured by spectrophotometry (Nanodrop -2000, Thermo Scientific, USA). The ratio of 260/280 ~2.0 is an indicator of pure RNA.

2.7.1.4 Integrity test

To ensure that the RNA was intact and not degraded, the 18S and 28S bands of ribosomal RNA were inspected by gel electrophoresis (figure 23).

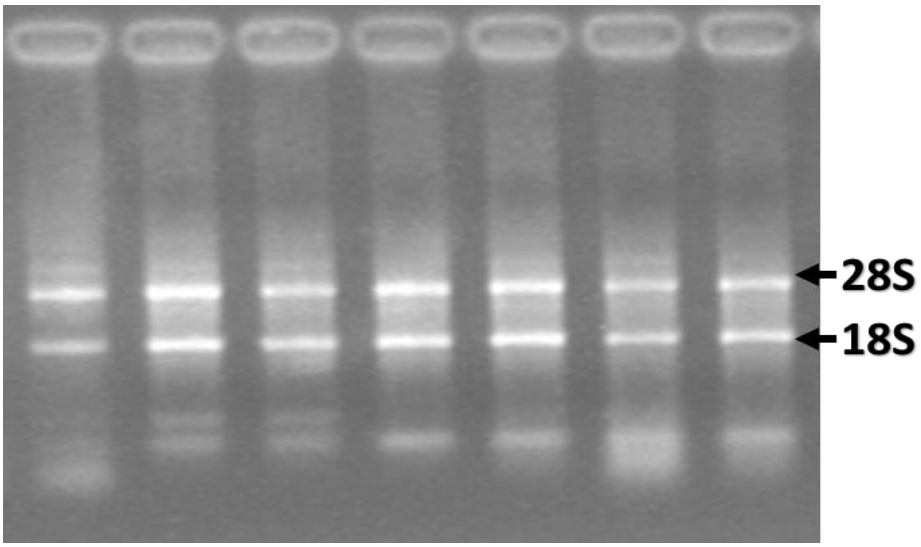


Figure 2-3 An example of an RNA integrity check by gel electrophoresis.

2.7.2 Complementary DNA Synthesis (cDNA)

RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, Lithuania) was used to synthesise single-strand cDNA as guided by manufacturers. The synthesis of cDNA was performed as directed by the manufacturer. For each reaction, 5 µl of 100 ng/ µl of RNA sample, 6 µl RNase-free water, and 1 random hexamer primers (12ul total volume) were mixed in 200 µl clear PCR tube and incubated at 65 ºC for 5 mins. Meanwhile, 4µl of 5x reaction buffer, 1µl Ribolock RNase inhibitor (20U/µl), 2µl Deoxynucleotide mix (10mM dNTP) and 1µl Revert aid (200U/µl) (8µl master mix) were mixed in the PCR tube and placed on ice. Immediately, the samples were taken from the incubator and placed on ice and 8 ul of the master mix was added to the PCR tube (see Table 2-7). Afterwards, the final volume of 20 µl was incubated at

25 ºC for 5 mins, followed by 60 mins at 42 ºC, then the reaction was terminated by being incubated at 70ºC for 5 mins. Eventually, the samples were cooled on ice and stored at -20 ºC for further analysis.

Table 2-7 The cDNA synthesis reagents

Reagent	**The volume required for 1 reaction**
Total RNA sample (100ng/µl)	5 µl
5x reaction buffer	4 µl
Ribolock RNase inhibitor (20U/µl)	1 µl
dNTP (10mM)	2 µl
RevertAid transcriptase (200U/µl)	1 µl
Random hexamer primers	1 µl
RNase-free water	6 µl
Total volume	20 µl

2.7.3 Real-Time Quantitative Polymerase Chain Reaction (qPCR):

The Lightcycler 480 system (Roche, USA) was used to perform the qPCR as a standard operating procedure from the division. The working solution was as follows:

5µl cDNA sample (diluted to 1:8), 7.5µl SYBR green I master mix (04707516001, Roche), 0.45 µl of each forward and reverse primers of each gene, and 1.6µl RNase free H2O (15µl in each well) (see Table 2-8). The reaction was performed in 384 well optical plate and each sample run in triplicate. TATA Binding Protein (TBP) and acidic ribosomal protein (36B4) were used as housekeeping/ reference genes since their expression was shown to be unaffected by the treatments (P=0.255 Figure 2-3 and P=0.187 Figure 2-4, respectively). The qPCR was performed as follows: 1 cycle for pre-incubation for 5 min at 95ºC; 45 cycles for amplification for 40 seconds each (denaturation: 95°C for 10 seconds; annealing: 60°C for 15 seconds; elongation: 72°C for 15 seconds); 1 cycle for melting curve at 95ºC; then one cooling cycle at 40ºC. cDNA samples were run in the qPCR after making a pool of cDNA and made series of dilutions: 1:2, 1:4, 1:8, 1:16, 1:32, 1:64, 1:128, 1:248 and one non-templet (control) sample to assess the efficiency of the PCR (see Figure 2-5). These samples were performed in triplicate in the Lightcycler 480.

Table 2-8 qPCR solution components using SYBER Green I

Component	**Volume**
cDNA sample (1:8 dilution)	5 µl
SYBER Green I master mix	7.5 µl
Forward primer	0.45 µl
Reverse primer	0.45 µl
RNase-free water	1.6 µl
Total volume	15 µl

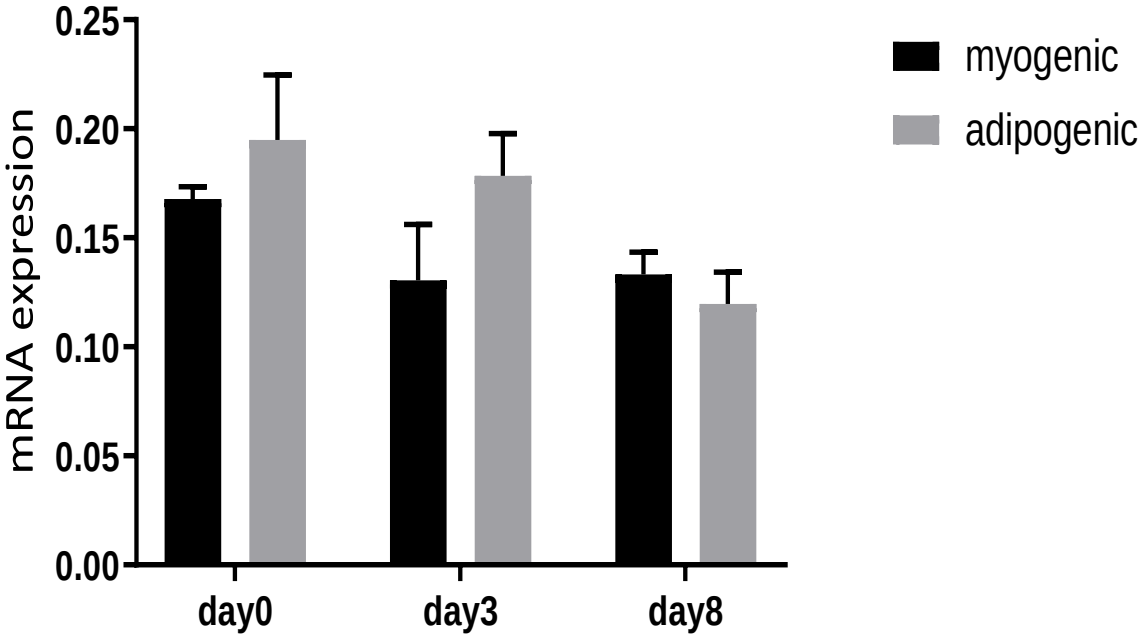


Figure 2-4 Effect of Adipogenic and Myogenic media for time course on TBP gene expression of C2C12 cells.

C2C12 cells were treated with either myogenic or adipogenic medium and harvested in 3-time points: day 0, day 3, and day 8. Two-way ANOVA showed no significant difference in the expression of TBP during the experiment $P>0.05$. Results are mean ± standard error of the mean (SEM) for n=3.

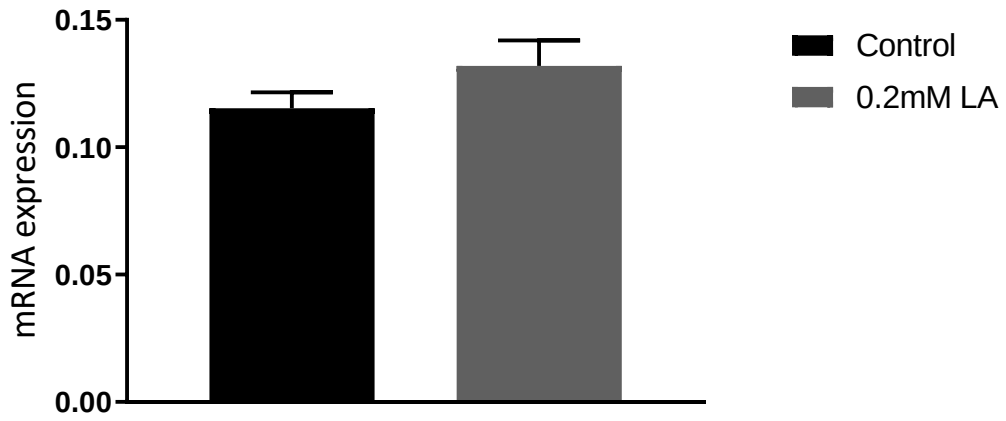
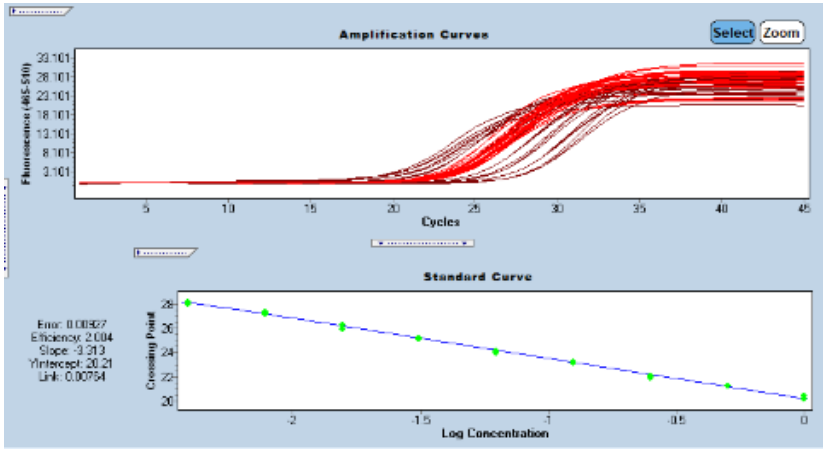


Figure 2-5 Effect of treatments on the expression of housekeeping gene 36B4 of C2C12 cells.

Differentiated C2C12 cells to myotubes for 5 days were treated with or without 0.2mM LA for 3 days. T-test showed no significant difference between treatments $P>0.05$. Results are mean ± standard error of the mean (SEM) for n=3.

A



B

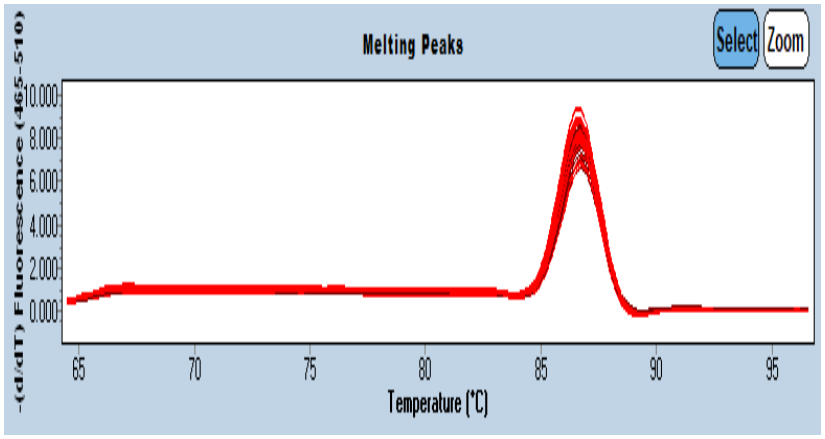


Figure 2-6 Images from the LightCycler 480 program illustrate the standard curve method.

A. A serial dilution of pooled cDNA samples (1:8 dilution) was used to generate the standard curve (brown lines). The measurement of the slope is to check the efficiency of the reaction, and the optimal slope is 3.3. Moreover, the optimal efficiency is 2.0.

B. The melting curve analysis, this to check the reaction specificity and primer-dimers. Only one melt peak was produced during the reaction, which confirms a single amplicon was generated by the PCR.

2.7.4 Quantification of total cDNA using Oligreen

Sometimes the housekeeping genes were affected by treatments. Therefore, the Oligreen (Invitrogen, Paisley, UK), which specifically binds with single-stranded DNA molecules, can be used to quantify the total cDNA concentrations in a sample. The cDNA quantification was measured by Lightcycler 480® ((Roche, Burgess Hill, UK), using samples derived from FA treatment of C2C12 following a standard operating procedure from the division. To quantify the amount of the cDNA in the samples, a working solution of Oligreen was made by adding 5µl Oligreen to 995µl 1x TE buffer as previously described (Rhinn et al., 2008). Each reaction contains 5µl Oligreen working solution and 5µl cDNA sample (1:8 dilution) and performed in 384 well plate in triplicate. The results of the Oligreen were then used to normalize the 36B4 housekeeping gene (see Figure 2-6).

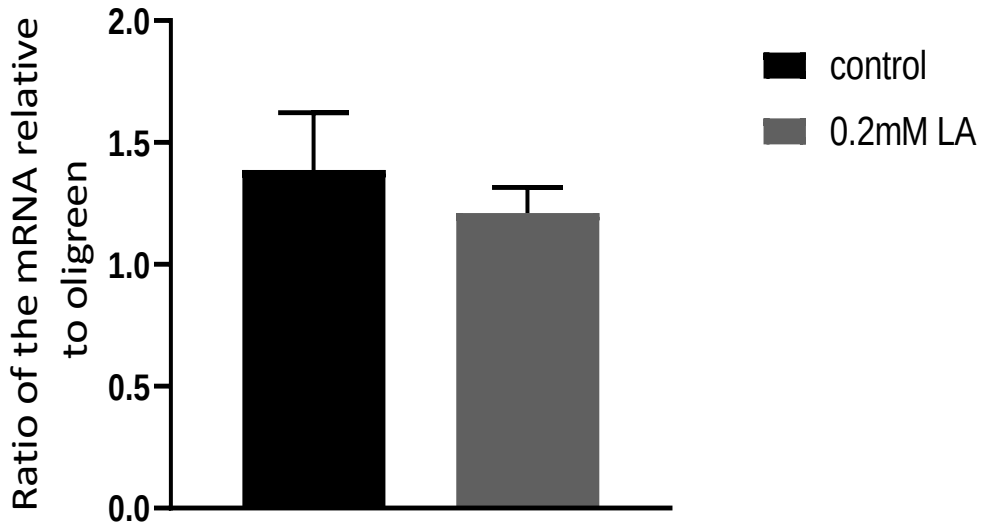


Figure 2-7 Effect of treatments on the expression of housekeeping gene 36B4 of C2C12 cells.

Differentiated C2C12 cells for 5 days were treated with or without, 0.2mM LA for 3 days. The T-test showed no significant difference between treatments P>0.05. The gene was normalised to Oligreen and results are mean ± standard error of the mean (SEM) for n=3.

2.7.5 Primer design

The primer sets of the oligonucleotide, for the genes used in the experiments, were designed using Primer Express v3 software. The mRNA of TBP, or 36B4, were used as reference genes for target gene normalisation. Fatty Acid Binding Protein 4 (FABP4), Peroxisome proliferator-activated receptor gamma-2 (PPARg2), Myogenin, Creatine kinase (CK), Diacylglycerol O-acyltransferase 1 (DGAT1), 1-acyl-sn-glycerol-3-phosphate acyltransferase alpha (AGPAT1), Glycerol-3-Phosphate Acyltransferase 2, Mitochondria (mGPAT2), Sterol regulatory element-binding protein 1 (A and C) and 2 (SREBP-1a, SREBP-1c, and SREBP-2) are the genes that were measured. The primer sequences used for these genes are displayed in Table 2-9.

Table 2-9 The sequences of gene-specific primers of all genes used for quantitative real-time PCR analysis.

Primer's Name	Sequence
1- FABP4	Forward: 5'- AAGTGGGAGTGGGCTTTGC-3'
	Reverse: 5'-TGGTGACCAAATCCCATTT-3'
2- PPARg2	Forward: 5'- GCATGGTGCCTTCGCTGA-3'
	Reverse: 5'- TGGCATCTCTGTGTCAACCATG -3'
3- Myogenin	Forward: 5'- CCCATGGTGCCCAGTGAA-3'
	Reverse: 5'- GCAGATTGTGGGCGTCTGTA-3'
4- Creatine Kinase	Forward: 5'- GCACTGGCCGCAGCAT-3'
	Reverse:5' GAGGGTAGTACTTGCCCTTGCCCTTGAACTC-3'
5- DGAT1	Forward: 5'- CTCCAGTGGGTTCCGTGTTT-3'
	Reverse: 5'-GGTTGACATCCCGGTAGGAAT-3'
6- AGPAT1	Forward: 5'- CAGGATGTGAGAGTCTGGGTTTT-3'
	Reverse: 5'- GAAGGCGCCTCGTTTGAAG-3'
7- mGPAT2	Forward: 5'- GCTGCAACCGTAGAGTCTGTG-3'
	Reverse: 5'- CCTGCTATCCCAGCAACTTCTAG-3
8- SREBP-1a	Forward: 5'- AGGCGGCTCTGGAAACAGA-3'
	Reverse: 5'- ATGTCGTTCAAACCGCTGTGT-3
9- SREBP-1c	Forward: 5'- ATCGGCGCGGAAGCTGTCGGGGTAGCGTC-3'
	Reverse: 5'- ACTGTCTTGGTTGTTGATGAGCTGGAGCAT-3
10- SREBP-2	Forward: 5'- CAAGTCTGGCGTTCTGAGGAA-3'
	Reverse: 5'- ATGTTCTCCTGGCGCAGCT-3

2.8 Data Analysis

The mean ± standard error of the mean (SEM) was used when displaying the results. GraphPad Prism versions 7 and 8 and were used to analyse the data. One-way ANOVA, two-way ANOVA, or a T-test were used, as appropriate. To consider that the results are statistically significant, the P-value must be less than 0.05 ($P<0.05$).

Chapter 3:

Effect of Fatty Acids on Lipid Deposition Within Skeletal Muscle Cells

3 Effect of Fatty Acids on Lipid Deposition Within Skeletal Muscle Cells

3.1 Introduction

High body fat results in the storage of fats as lipid droplets (LDs) in non-adipocyte cell types such as SMs (Andrew et al., 2011). Elevated intramyocellular lipids within SMs tend to negatively affect the SMs' oxidation capacity (Goodpaster et al., 2001) and result in lipotoxicity because the excess lipids result in inappropriate storage of lipids out of adipocytes (Laurens & Moro, 2016).

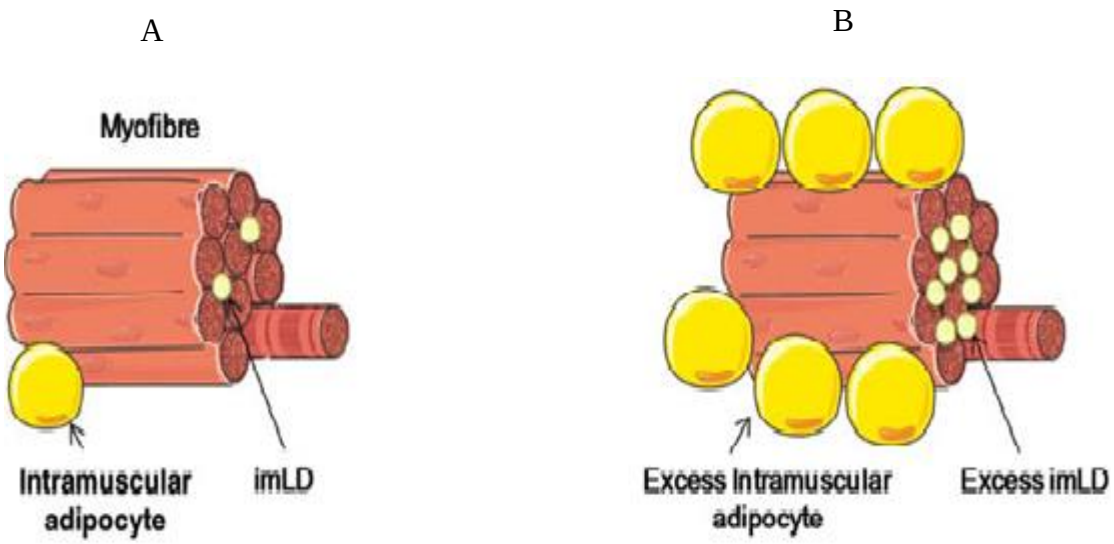


Figure 3-1 Representation of the lipid distribution in Lean Individuals (A) or Obese Individuals (B). Obtained from (Laurens & Moro, 2016).

Lipid droplets (LDs) are present in lean people, which reflects their vital activity. However, in obese people, there are more intramyocellular lipid droplets (IMLDs) within muscle fibres.

Figure 3-1 shows two different types of lipids accumulation in muscle cells. In B, the lipids accumulation around the muscle fibres, which are adipocytes, has been reported previously within the laboratory (Ryan et al., 2013). They found that the muscle cells (C2C12) can transdifferentiate into adipocytes when incubated with adipogenic medium (see Figure 3.2). However, this chapter hypothesizes that incubation of muscle cells with different FAs (palmitic acid PA (saturated), oleic acid OA (monounsaturated), and linoleic acid LA (polyunsaturated) (see Figure 3.3)) will induce lipid accumulation within muscle fibres. This would be another model to test the effect of lipids on SM's metabolism. Thus, a study that compares myogenic and adipogenic media has been done. This is to check if the accumulation of lipids around the muscle cells (in adipocytes) is reproducible within the lab to be used as a positive control. Moreover, if the intended model (lipid deposition within muscle fibers) did not get the expected results, the previous model (accumulation of lipids around muscle cells) can be used instead. Thus, this experiment (adipogenic and myogenic media comparison) was then followed by an experiment examining the different FAs. This is to determine which fatty acid results in the highest fat deposition in cultured C2C12 mouse muscle cells and L6 rat muscle cells myotubes.

Besides, Gene expression was measured to determine whether fat deposition was associated with the muscles cells accumulating fat or adipocytes were appearing by measuring genes related to adipogenesis (Peroxisome proliferator-activated receptor gamma-2 (PPARg2) and Fatty Acid Binding Protein 4 (FABP4)) and myogenesis (Creatine kinase (CK) and myogenin). Also, as the accumulated fats have been suggested to be stored as TAG and CE, genes related to TAG synthesis, including Diacylglycerol O-acyltransferase 1 (DGAT1), Glycerol-3-Phosphate Acyltransferase 2, Mitochondria (mGPAT2) and 1-acyl-sn-glycerol-3-phosphate

acyltransferase alpha (AGPAT1), as well as genes related to cholesterol, including sterol regulatory element-binding proteins -1a, -1c and -2 (SREBP-1a, SREBP-1c and SREBP-2), were measured to verify this hypothesis. Each gene isoform of SREBPs family has a specific target; SREBP-2 has been showing to promote enzymes involved in cholesterol synthesis, while SREBP-1c is involved in fatty acid synthesis. Moreover, SREBP-1a has been reported to be involved in both pathways (Malhotra et al., 2016).

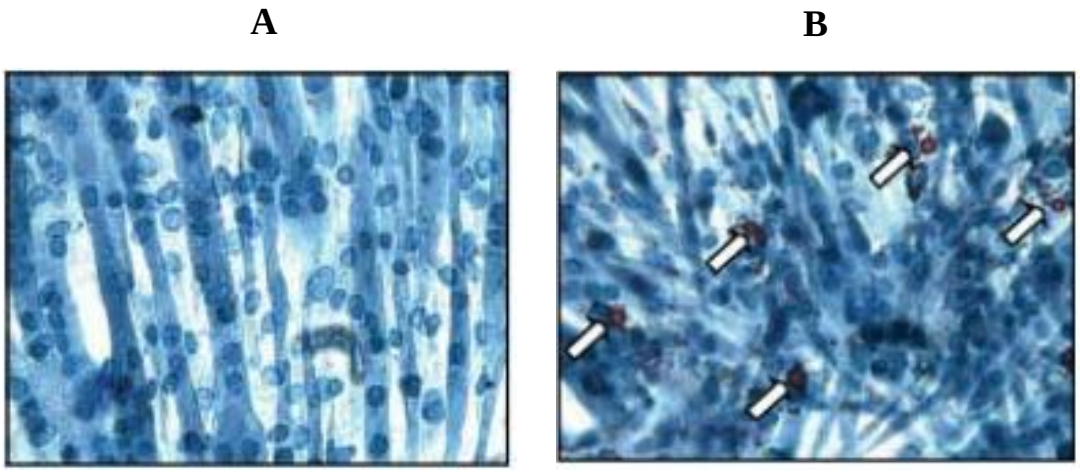


Figure 3-2 Representative Image of C2C12 Cells Incubated for 6 Days in Myogenic or Adipogenic Medium.

C2C12 cells were cultured for 6 days in myogenic (A) or adipogenic (B) media then stained with Oil Red-O (as indicated by white arrows) and counterstained with haematoxylin to indicate lipid droplet and nuclei/myofibre structures respectively. Obtained from (Ryan et al., 2013).

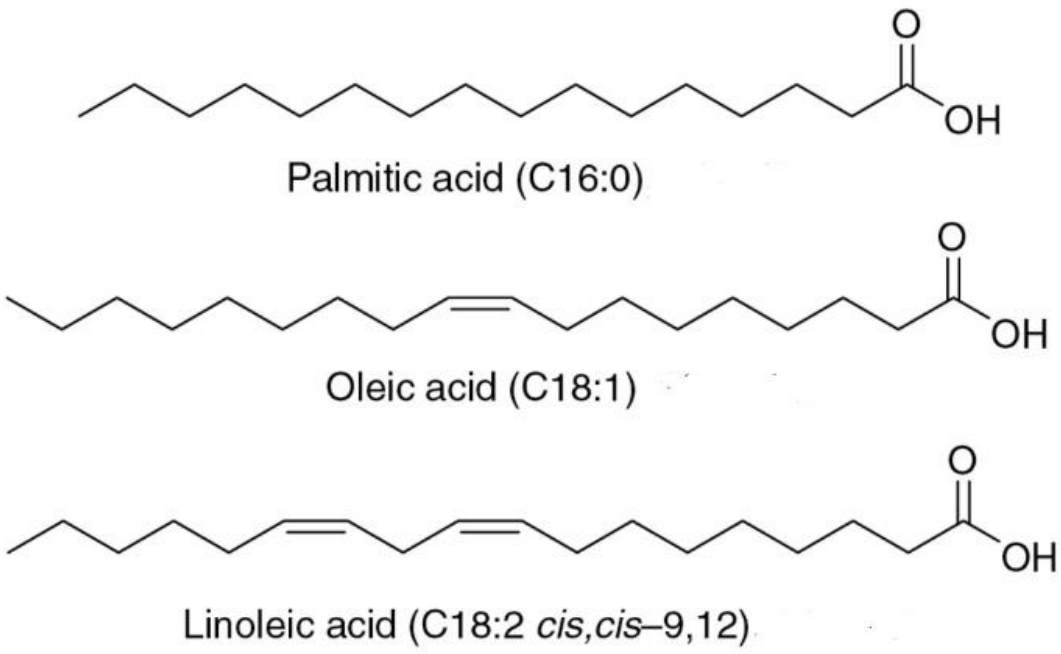


Figure 3-3 Structure of the Fatty Acids Used in This Study.

Obtained from (Armylisas et al., 2017).

3.2 Materials and Methods

3.2.1 Cell Culture

C2C12 and L6 cell lines were used in this section of the research. For detailed methods, please refer to section 2.1. The treatments and time period for the study are explained in detail in this chapter.

3.2.2 Adipogenic and Myogenic Media Treatments of C2C12 Cells

C2C12 cells were seeded in six 6-well plates ($1x10^5$ density), at a point when they reached 70-80% confluence they were then treated with either adipogenic or myogenic media (three plates each). The cells were then harvested at three different points of time: day zero (just before adding the treatments), day three, and day eight. Cell scrapers were used to harvest the cells into 1.5ml Tubes and then the cells were stored at -80^{o}C for subsequent analysis. For full details of media contents please see section 2.1.6.

3.2.3 Staining of the C2C12 Cells with Oil Red O

C2C12 cells were differentiated into myotubes by incubating the cells with the myogenic medium for 4-5 days in 12 well plates. This staining method was used after incubation of the cells for 16 hours with 0.75mM of different FAs (see Table 3.1). Please see section 2.4.1 for details of the staining procedure.

3.2.4 Staining of C2C12 and L6 Cells with Nile Red

C2C12 or L6 cells at a differentiated stage (4-5 days post confluence) were treated with 0.2mM of different FAs (see Table 3.1), after which the cells were stained with this method at different time points (day1, 2, and 3). Details of the procedure were explained in section 2.4.2.

3.2.5 Fatty Acids Treatment

PA, OA, and LA were examined in two different concentrations (0.75mM and 0.2mM) in order to find out which FA gives the highest lipid deposition within differentiated muscle cells. The first concentration (0.75mM) was derived from the work of (Jové et al., 2006) and the second concentration (0.2mM) was derived from the work of Huggett, who is a Ph.D. student in this laboratory (see section 2.2). These treatments are described in detail in Table 3-1.

Table 3-1 The Fatty Acids Treatments Contents

Treatment	**Content**
0.75 mM FAs	Derived from (Jové et al., 2006)
Control	2% bovine serum albumin (BSA) in DMEM
PA	0.75 mM PA in 2% BSA in DMEM
OA	0.75 mM OA in 2% BSA in DMEM
LA	0.75 mM LA in 2% BSA in DMEM
0.2 mM FAs	Derived from Zoe Huggett (A PhD student in the same laboratory)
Control	2% bovine serum albumin (BSA) in DMEM
PA	0.2 mM PA in 2% BSA in DMEM
OA	0.2 mM PA in 2% BSA in DMEM
LA	0.2 mM PA in 2% BSA in DMEM
3xFA	0.2 mM total fat at the ratio 4:4:2 (80uM PA, 80uM OA, 40uM LA)

3.2.6 Quantitative-RT-PCR

The procedure followed was described in section 2.7. C2C12 cells were seeded in 6-well plates ($1x10^5$ density) (three plates for each treatment) and then encouraged to proliferate until they reach 80% confluence. Cells were then treated with either adipogenic or myogenic media, after which the media was removed. Cells were harvested on day zero (just before adding treatments), day three, and day eight. This experiment was done to examine the differences between adipogenic and myogenic media. To determine the lipid deposition within the myotubes, other differentiated C2C12 cells (by incubating with the myogenic medium) were treated with 0.2mM LA or the control (no FA, plus Bovine Serum Albumin (BSA)) in 6-well plates (two plates) and incubated for three days at 37^oC. Once the cells were harvested and the total RNA was extracted, the cDNA was synthesised and the mRNA expression was analysed. Final results were normalised to housekeeping genes (see section 2.7.3).

3.2.7 Statistical Analysis

All analyses were carried out on GraphPad Prism and student T-tests and one or two ANOVA tests were used when appropriate. Statistical significance was accepted when the P-value was less than 0.05 ($P<0.05$) and the ± standard error of the mean (SEM) was used when displaying the results.

3.3 Results

3.3.1 A Comparison Between Adipogenic and Myogenic Media

As mentioned before, C2C12 cells have been reported previously to transdifferentiate into adipocytes when incubated with adipogenic medium (Ryan et al., 2013). Therefore, this experiment was done to ensure that the results were reproducible and whether the fat deposition was associated with the muscle cells accumulating fat or there were adipocytes appearing. There was no significant difference in the expression of TATA Binding Protein (TBP) mRNA so this was used to normalize QPCR data. The adipocyte associated mRNAs Peroxisome proliferator-activated receptor gamma-2 (PPARg2) and Fatty Acid Binding Protein 4 (FABP4) were assessed to determine whether adipocytes were present. Likewise, the myotube associated mRNAs Creatine kinase (CK) and Myogenin were used as measures of myogenic differentiation. Two-way ANOVA showed that there was a significant interaction between both media and time for the expression of all mRNAs ($P<0.01$ for PPARg2 and $P<0.001$ all others). Additionally, a Dunnett's test was performed and showed that in adipogenic medium samples, there was a significant increase ($P<0.001$) in mRNA expression of all genes by day eight compared to day 0, whilst at day three there was a significant increase in FABP4 ($P<0.001$), PPARg2 ($P<0.01$) and myogenin ($P<0.05$). In the myogenic medium, there was a significant increase in the mRNA of CK and Myogenin by day three and day eight compared to day 0 ($P<0.001$), but there was no effect on PPARg2 ($P=0.244$ day three; $P=0.202$ day eight) and no expression detected for FABP (see Figure 3-4).

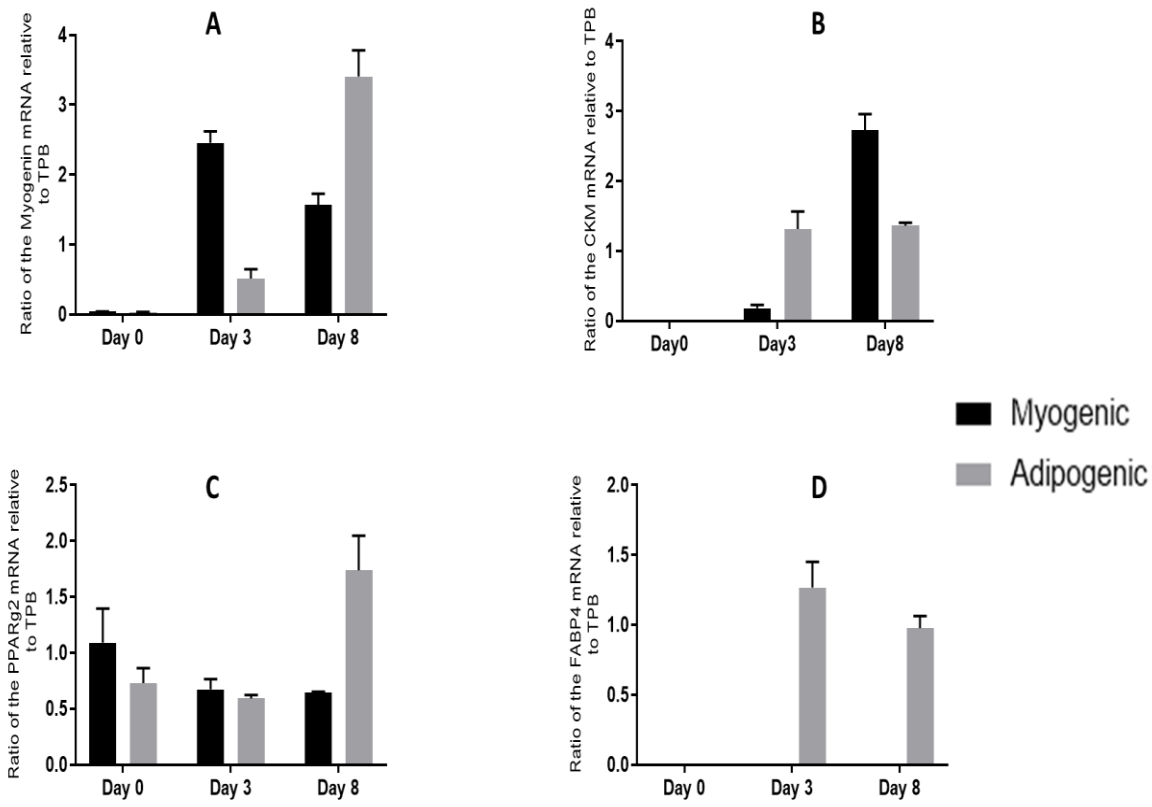


Figure 3-4 The Effect of Myogenic or Adipogenic Media on the Expression of Myogenin, CKM, PPARg2, and FABP4 Over time in C2C12 during Differentiation.

C2C12 cells (60-70% confluent) were treated with either adipogenic or myogenic media and harvested at three-time points (day zero, day three, and day eight). Two-way ANOVA showed that there was a significant interaction in all mRNA expressions of myogenin, CKM, PPARg2 $P< 0.01$, and FABP4 $P<0.001$. All genes were normalised to TBP and the data are presented as means ± SEM; n=6 wells per treatment group.

3.3.2 Effect of Different Fatty Acids (0.75mM) on the Accumulation of Lipids of C2C12 Cells over 16 Hours

C2C12 cells were seeded into 12-well plates and differentiated into myotubes by incubating with the myogenic medium for 4-5 days, then 0.75mM of each FA and the control (no FA) were added to three wells of 12-well plates. The cells were exposed to the treatments for 16 hrs at 37ºC. Afterwards, the cells were stained with

Oil Red O and observed under the microscope (all at the same magnification). LDs had formed on all treatments including the control (see Figure 3-5). This shows that LDs form naturally, both with and without the presence of FAs. However, the Oil-Red O staining method is not the best method as it was not clear from the images exactly where the LDs had formed. An alternative staining method using Nile red is a much better method for staining cells to localise the lipids, due to it being a fluorescent stain and therefore has a high sensitivity. Moreover, the protocol was changed in order to incubate with the FA for a longer time and different time points (3days). The original concentration used (0.75mM) was designed for incubation of 16 hours but not for a longer period. Hence, the concentration was reduced to avoid possible toxicity of such a high level of FAs and possible effects on myotube formation as demonstrated previously (Hurley et al., 2006).

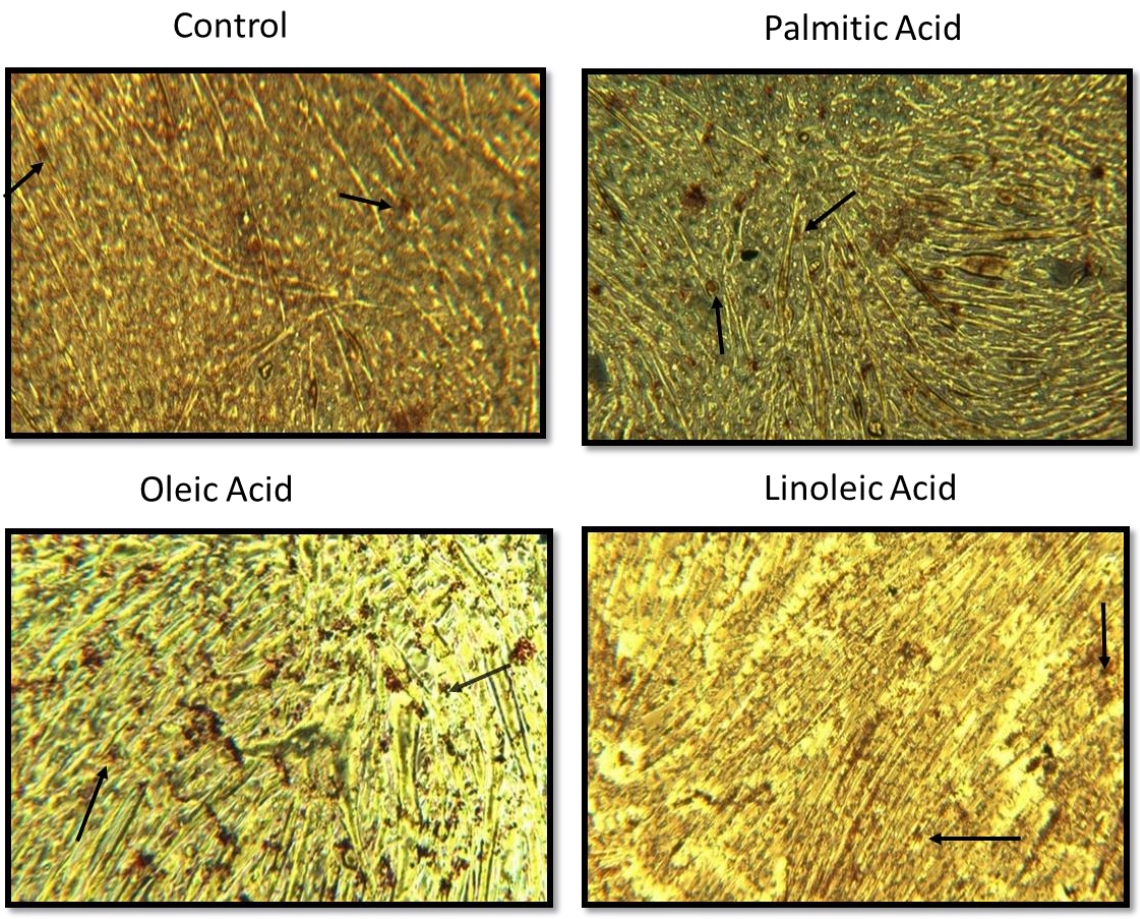


Figure 3-5 Effect of 16-hour exposure to Different FAs on Lipid Deposition in C2C12 Muscle Cells.

C2C12 cells that had been differentiated for four or five days were treated with 0.75mM of PA, OA, LA, or the control (no FAs) for 16 hours. The cells were stained with Oil Red O to localise the LDs. 6 images per well per treatment.

3.3.3 Effect of Different Fatty Acids (0.2mM) on the Accumulation of Lipids in C2C12 Cells

As mentioned in the last experiment, the concentration had to be reduced to 0.2mM to allow for longer time points to be used. Therefore, the effects of 0.2mM of different FAs (PA, OA, LA) on lipid deposition at different time points (3 days) were examined and the Nile red staining method used. The objective was to establish a suitable model and protocol for future experiments. The FAs used are representing

the three main types of FAs found in the body and they are saturated to different levels. As mentioned in the 2.2 section, the 0.2mM concentration was chosen based on a study performed by Zoe Huggett, another PhD student in the lab, who found that this concentration of the PA, OA and LA resulted in LD accumulation in HepG2 cells. C2C12 cells were allowed to differentiate into myotubes by incubating the cells with the myogenic medium for 5 days (after reaching 60-70% confluence), then exposed to different FAs (PA, OA, LA, and combination of all 3xFA) for up to 3 days with staining with Nile red carried out on each day. The fatty acid LA consistently gave the highest lipid deposition within myotubes and this can be seen in the images (see Figure 3-7). However, the two way ANOVA result was not significant ($P>0.05$) (see Figure 3.8).

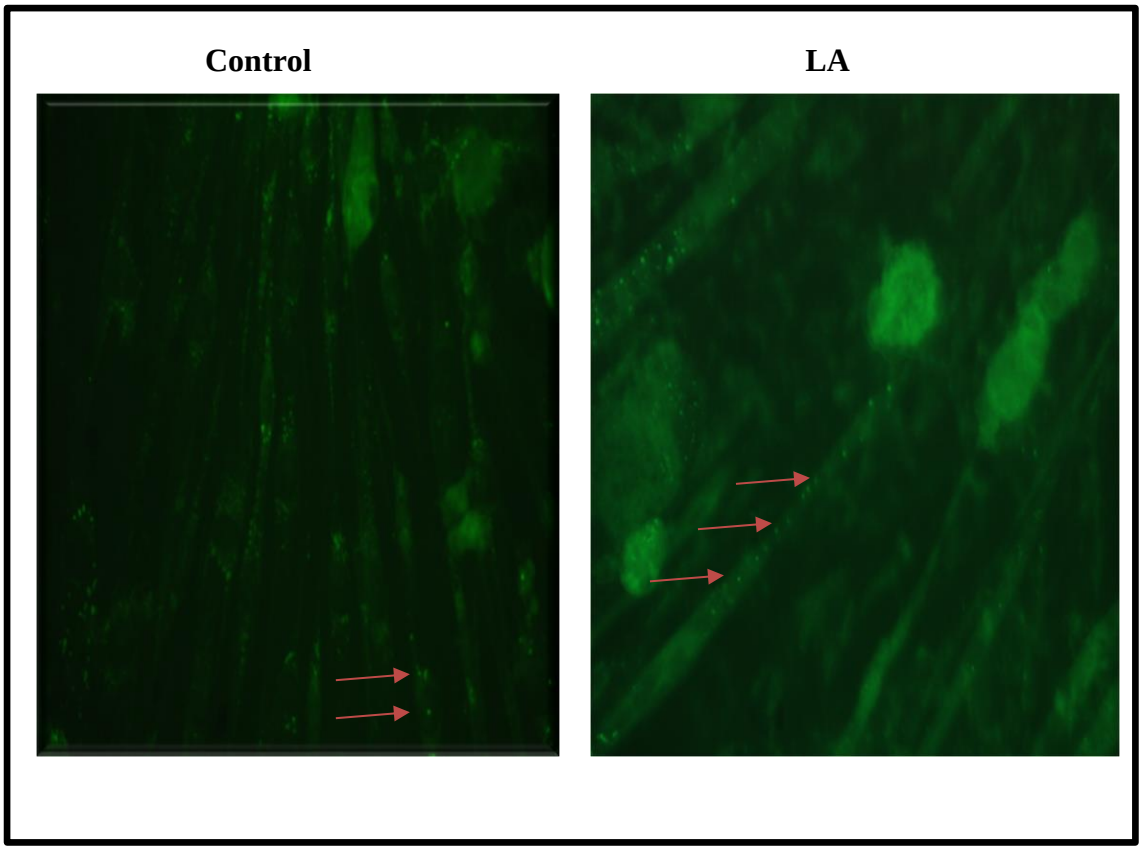


Figure 3-7 Accumulation of Lipid Droplets Within C2C12 Myotubes Following the Incubation of Different FAs.

Cultured C2C12 cells were encouraged to differentiate into myotubes for five days and then were treated with 0.2mM LA or control (no FA) for three days. Then, the cells were stained with Nile red and imaged using the blue filter at x40 magnification. The arrows indicate the lipids deposition within the myotubes. 8 images per well per treatment.

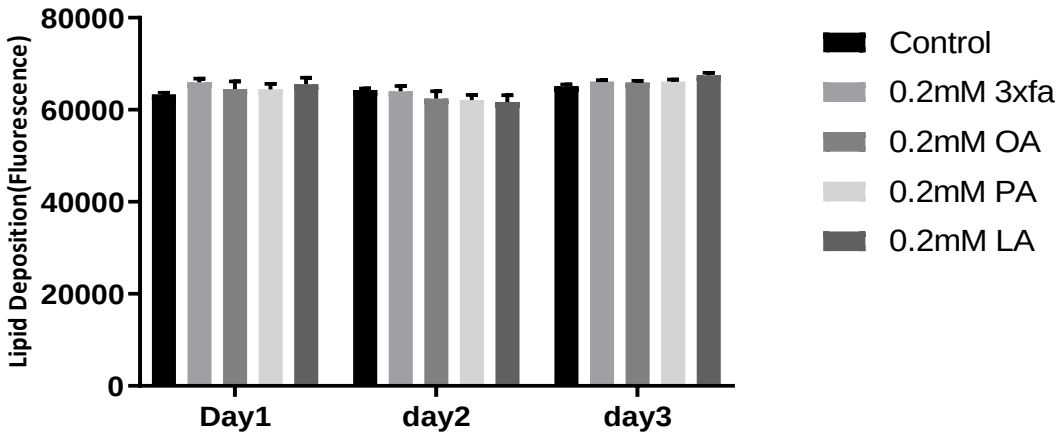


Figure 3-8 The Effect of Different FAs on C2C12 Cells Over Time.

C2C12 cells that had been differentiated for five days were treated with 0.2mM of OA, PA, LA, 3xFA, or the control (no FAs). The cells were stained with Nile red at three points (day one, day two, and day three). Two-way ANOVA showed no interaction ($P>0.5$). Dunnett's test showed a significant increase in LA on day three ($P<0.05$) compared to the control sample. Data are presented as means ± SEM; n=3 wells per treatment group.

3.3.4 Effect of Different Fatty Acids on C2C12 Muscle Cells for Three Days.

The previous experiments suggest that the lipid deposition typically occurs after three days of exposure to FA. To confirm this, a single experiment with only a one-time point (three days) was done using 0.2mM FAs either individually or combined (3xFAs). Again, images were taken and they showed that the LDs were within the myotubes (see Figure 3-10). There was a significant effect of FA treatment ($P<0.001$). Post-hoc analysis (Dunnett's test) indicated there was a significant increase with LA alone ($P<0.001$), PA alone ($P<0.01$) or when OA, PA, and LA were combined (3xFAs) ($P<0.05$) when compared to the control (see Figure 3-11).

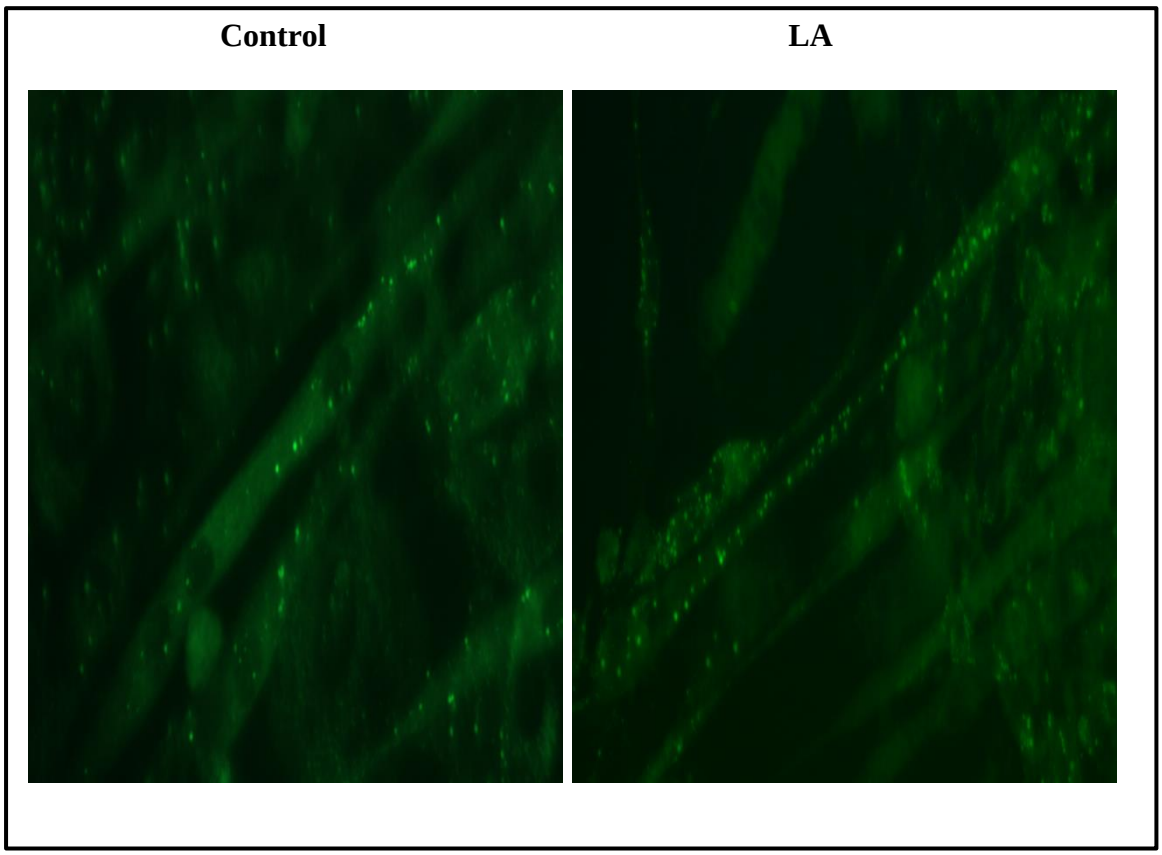


Figure 3-6 Microscopy Images of C2C12 Cell Myotubes.

C2C12 cells that had been differentiated for five days were treated with 0.2mM LA or the control (no FAs). The cells were stained with Nile red on day three and images of x40 magnification show the difference between the LA and control myotubes. 8 images per well per treatment.

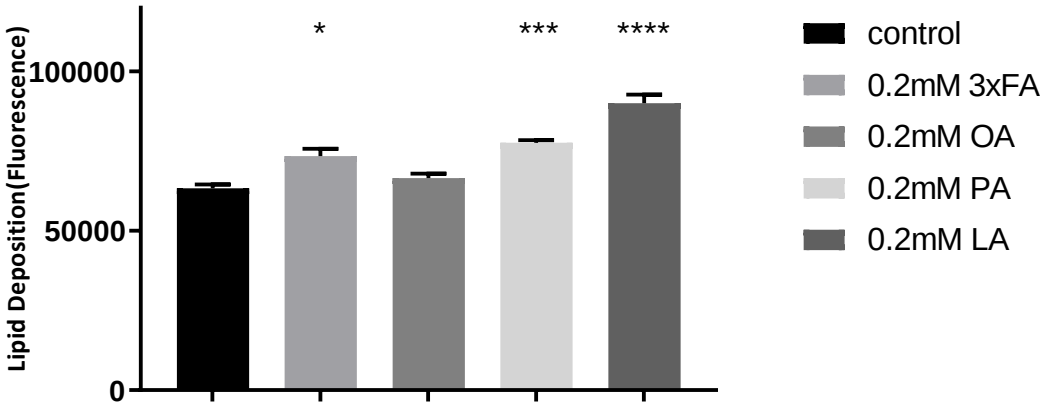


Figure 3-7 The Effect of Different FAs on C2C12 Cells Over Three Days.

C2C12 cells that had been differentiated for five days were treated with 0.2mM of OA, PA, LA, 0.2mM 3xFA (ratio of 4:4:2 respectively), or the control (no FAs). The cells were stained with Nile red on day three. A one-way ANOVA test showed a significant difference ($P<0.0001$). Stars are representative of post-hoc Dunnett's test. Data are presented as means ± SEM; n=3 wells per treatment group.

As the 0.2mM LA treatment was consistently providing the highest lipid deposition within myotubes compared to the other FAs, 0.2mM LA would be used in future experiments.

3.3.5 Impact of Linoleic Acid treatment of C2C12 Cells on Expression of Genes Related to Adipogenesis, Myogenesis, and Lipogenesis

Genes associated with adipogenesis (PPARg2 and FABP4) and myogenesis (CK and myogenin) were measured to determine whether the exposure to FAs caused transdifferentiation and the appearance of adipocytes which then would presumably be responsible for lipid accumulation. Treatment had no effect on the mRNA expression of PPARg2 ($P>0.05$) and FABP4 was not detectable (data are not shown). In contrast, there was a significant decrease in CK ($P<0.01$) and myogenin ($P<0.05$) caused by the treatment with LA (Figure 3-12).

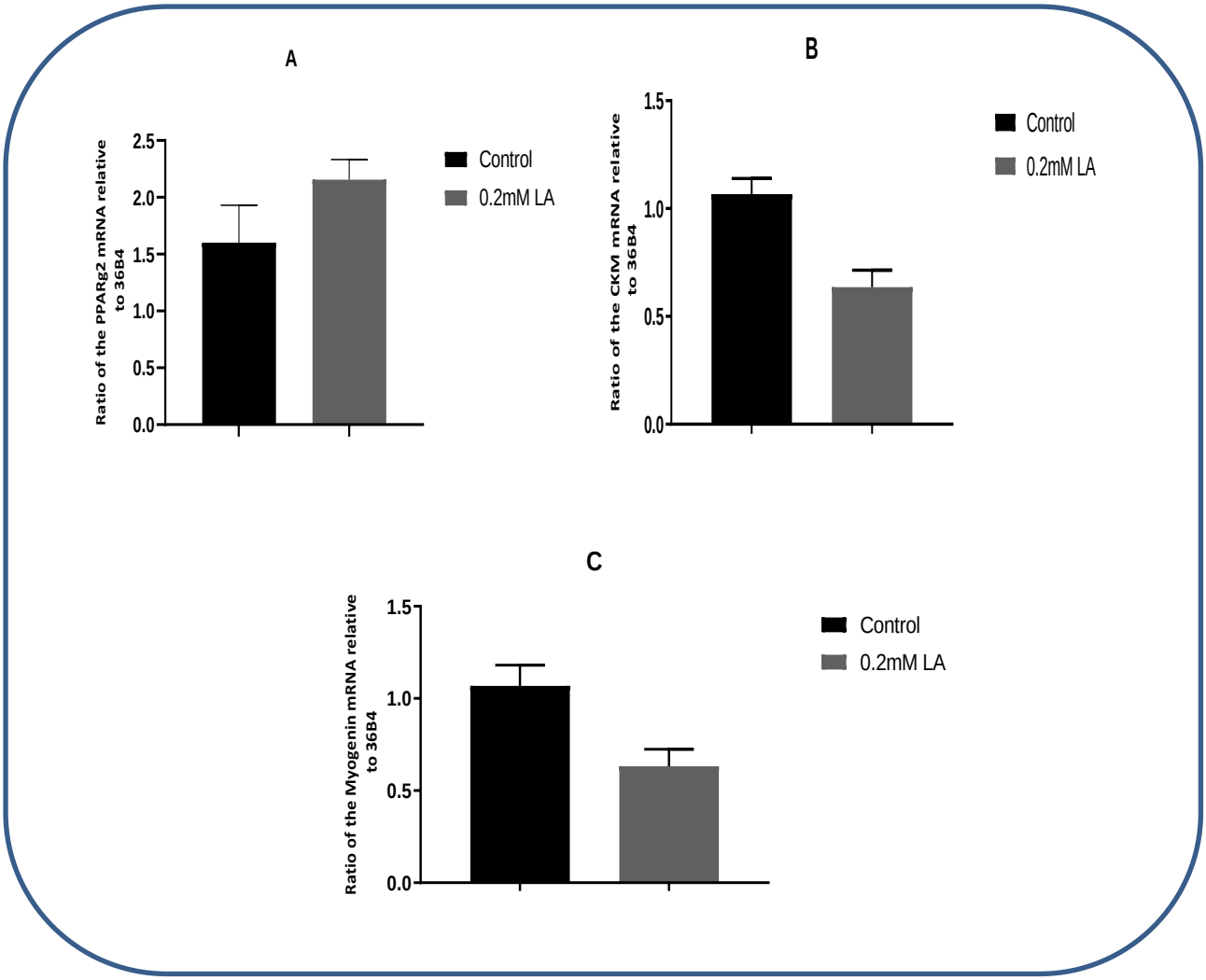


Figure 3-8 The Effect of LA on mRNA Expression of PPARg2, CKM, and Myogenin of C2C12.

C2C12 cells were differentiated for five days and then treated with or without 0.2mM LA for three days. Total RNA was extracted and cDNA synthesised. The T-test showed no significant effect in PPARg2 $P>0.05$ and a significant decrease in CKM ($P<0.1$) and myogenin ($P<0.05$). All genes were normalised to 36B4 and data are presented as means ± SEM; n= 6 wells per treatment group.

Three genes that relate to TAG synthesis were measured: Diacylglycerol O-acyltransferase 1 DGAT1, Glycerol-3-Phosphate Acyltransferase 2, Mitochondria mGPAT2, and 1-acyl-sn-glycerol-3-phosphate acyltransferase alpha AGPAT1. There was no significant change in mRNA expression of any of the genes compared to the control (P>0.05) (see Figure 3-13). All genes were normalised to the reference gene B364 and were run in triplicate.

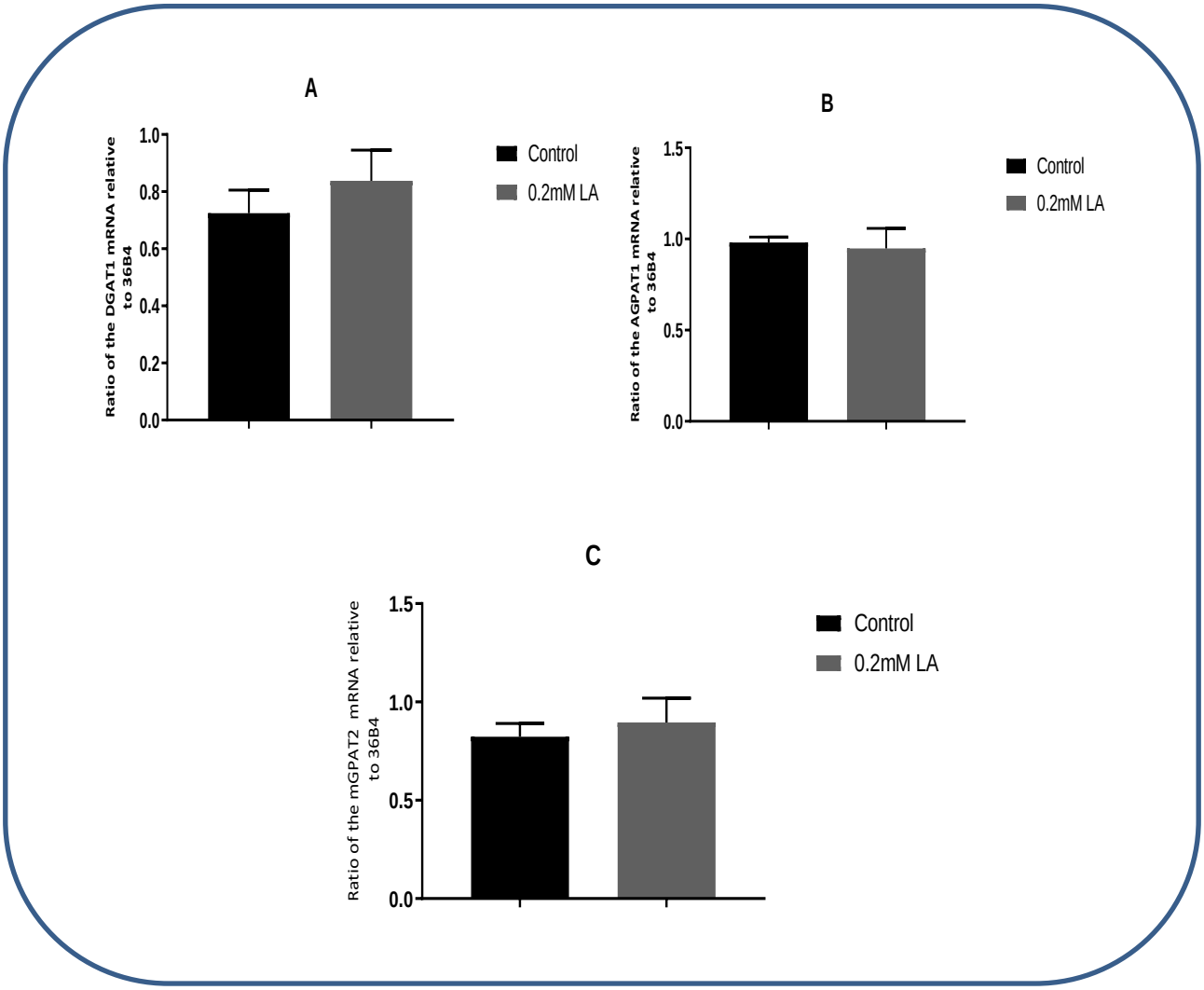


Figure 3-9 The Effect of LA on mRNA Expression of DGAT1, AGPAT1, and mGPAT2 of C2C12 Cells.

C2C12 cells were differentiated into myotubes for five days, then treated with or without 0.2mM LA for three days. The total RNA was extracted and cDNA synthesised. The T-test showed no significant difference in expression of DGAT1, AGPAT1, or mGPAT2 (P>0.05). All genes were normalised to 36B4 and data are presented as means ± SEM; n=6 wells per treatment group.

Unfortunately, cDNA samples were not large enough for all genes. Consequently, another cDNA set was made from the RNA samples and housekeeping 36B4 was tested in the qPCR. It is unlikely that the mRNA expression of the 36B4 was altered significantly by the treatment (see Figure 3-14).

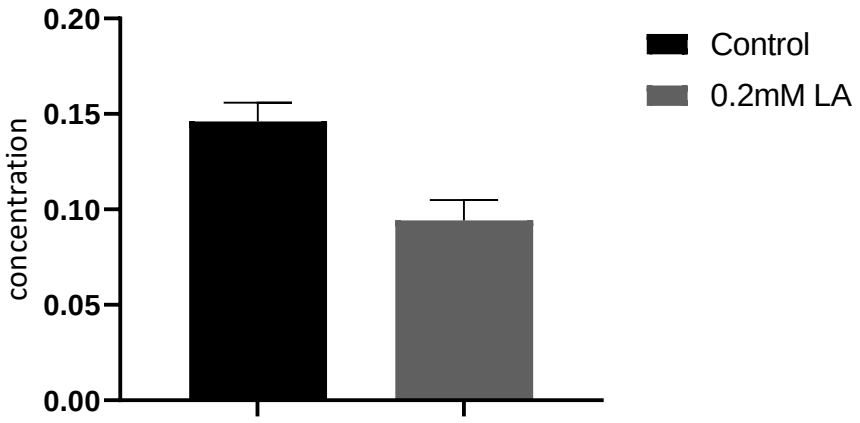


Figure 3-10 Effect of Treatments on Housekeeping Gene 36B4 and Gene Expression of C2C12 Cells.

C2C12 cells that had been differentiated for five days were treated with or without 0.2mM LA for three days. The total RNA was extracted and the cDNA synthesised. The T-test showed a significant difference for treatments where ($P<0.01$). Data are presented as means ± SEM; n=6 wells per treatment group.

As a result, the OliGreen method was used to check the cDNA samples. The OliGreen method, which binds to double-strand DNA, is a useful method to determine how much cDNA in the sample. The T-test showed a significant difference between the treatments $P<0.05$ (see Figure 3-15). The control samples were found to be higher than the LA treatments, which suggests that there was a mistake in the dilution as the control samples have higher cDNA concentrations than the LA treatment samples. Thus, the housekeeping 36B4 results were normalised to the OliGreen and a T-test

showed that there was no significant difference between the treatments after the normalisation with the OliGreen P>0.05 (see Figure 3-16). Therefore it was appropriate to normalise the other genes using either Oligreen or 36B4, but 36B4 was used for consistency.

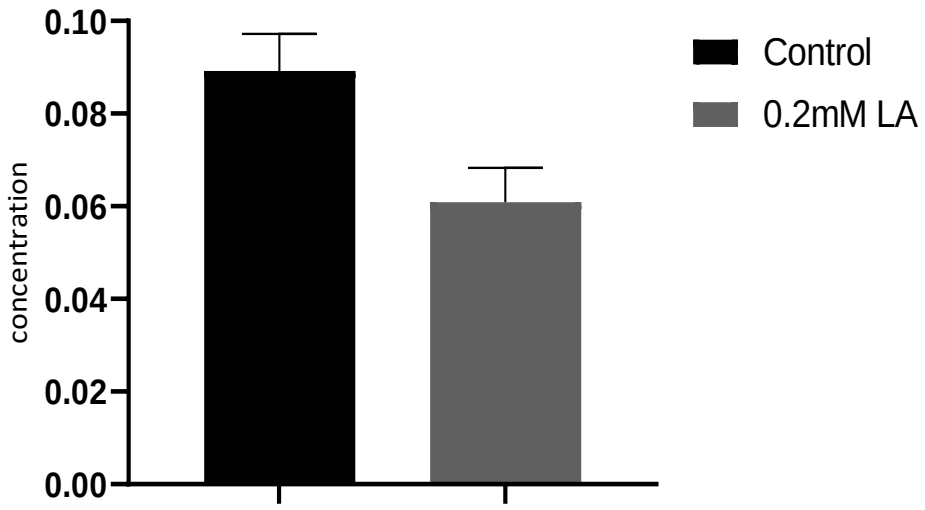


Figure 3-11 The OliGreen Data from Control and LA cDNA Samples of C2C12 Cells.

The samples from control and LA cDNA were run using the OliGreen method to detect how much cDNA was in the sample. The T-test showed a significant difference between treatments where P<0.05. Data are presented as means ± SEM; n=6 wells per treatment group.

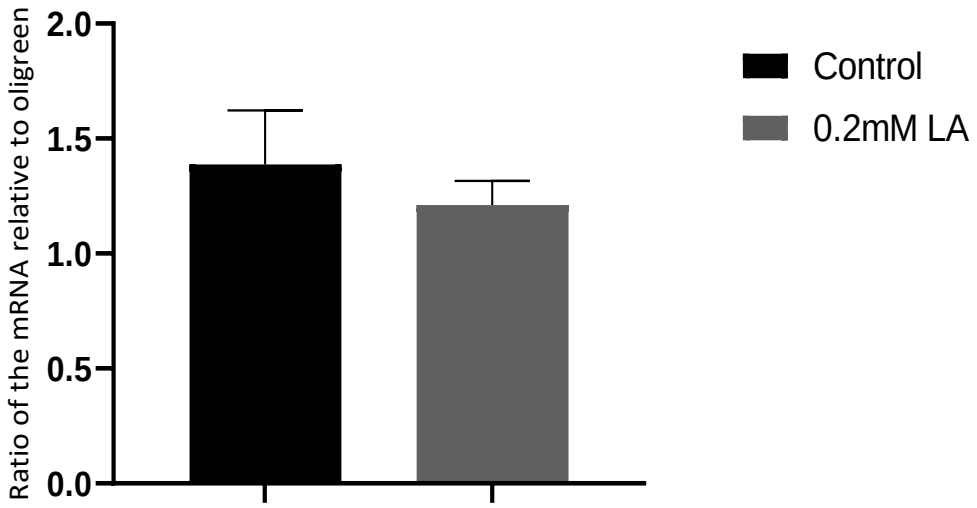


Figure 3-12 Effect of Control and LA Treatments on the mRNA Expression of the Housekeeping Gene 36B4 of C2C12 Cells.

C2C12 cells that had been differentiated for five days were treated with or without 0.2mM LA for three days. The total RNA was extracted and the cDNA synthesised. The T-test showed no significant difference between treatments where $P>0.05$. Genes normalised using the OliGreen method and the resulting data are presented as means ± SEM; n=6 wells per treatment group.

Ultimately, sterol regulatory element-binding proteins -1a, -1c and -2 (SREBP-1a, SREBP-1c and SREBP-2) were measured. There was a significant increase in the mRNA expression of SREBP-1a ($P<0.01$) and SREBP-2 ($P<0.05$), but no significant effect on mRNA expression of SREBP-1c (See Figure 3-17). It has been reported that SREBPs-1a and 2 are predominant in most cell lines in *vitro*, but not 1c. Moreover, SREBP-1a activates the other two isoforms SREBP-1c and 2 who mediates fatty acids, TAG synthesis, and cholesterol biosynthesis, respectively (Horton et al., 2002). The SREBP-1a is implicated in the synthesis of TAG and cholesterol, and SREBP-2 is restricted to the cholesterol biosynthesis (Xu et al.,

2013). The results indicate that genes SREBP-1a and 2 are significantly expressed in the LA treatment which suggests that genes involved in cholesterol and lipogenesis metabolism were expressed by LA treatment. However, further examination is needed to check the protein change in the TAG, fatty acids, and cholesterol synthesis.

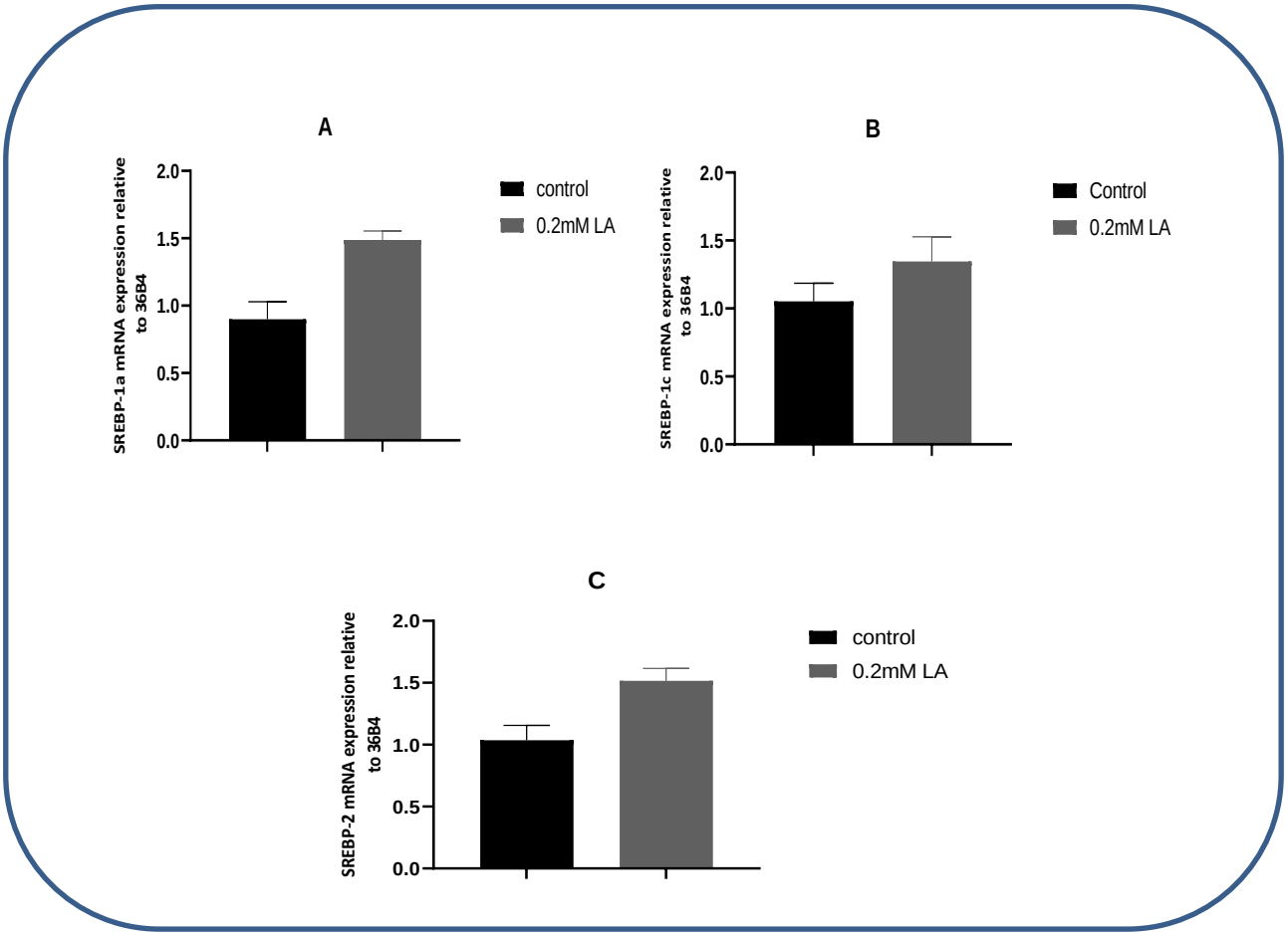


Figure 3-13 Effect of LA on mRNA Expression of the SREBP-2 Gene of C2C12 Cells.

C2C12 cells were encouraged to differentiate into myotubes for five days, then treated with or without 0.2mM LA for three days. The T-test showed a significant difference in SREBP-1a ($P<0.01$) and SREBP-2 ($P<0.05$) and no significant difference of SREBP-1c ($P>0.05$). All genes were normalised to the 36B4 gene and the data are presented as means ± SEM; n=6 wells per treatment group.

3.3.6 Determination of Different Fatty Acids' Ability to Deposit Lipids Within Muscle Cells of L6 Cells

Even though C2C12 cells have previously been examined for insulin-stimulation of glucose uptake with positive results, it has been reported that L6 cells exhibit larger glucose uptake than C2C12 cells (Nedachi and Kanzaki, 2006; Sarabia et al., 1990; Yap et al., 2007). Moreover, C2C12 cells are predominately used to examine muscle cell development and differentiation (Nedachi and Kanzaki, 2006). Even after differentiation, C2C12 cells' responsiveness to insulin has been determined to be minimal (Kotliar and Pilch, 1992). In addition, Qiong Yin (a Ph.D. student in this lab) tested both C2C12 and L6 muscle cells in terms of glucose uptake in response to insulin. She found a twofold increase in glucose uptake in response to the insulin in L6 cells, but no response in C2C12 cells. As a result, L6 cells were considered a better model to examine insulin sensitivity in the future. Therefore, the L6 cells were tested to examine whether they deposited lipids within myotubes, as was observed with C2C12 cells. The same protocol as that used for testing the effect of the different FAs on lipid deposition in C2C12 myotubes was used (see sections 2.1, 2.2, and 3.3.3). The images show that the LDs were accumulated within myotubes (see Figure 3.18). There was a significant interaction between FA used and the incubation time ($P<0.0001$). As with the C2C12 cells, 0.2mM LA treatment resulted in the highest lipid deposition on day three (see Figure 3-19). The results for the L6 cells agreed with those for the C2C12 cells.

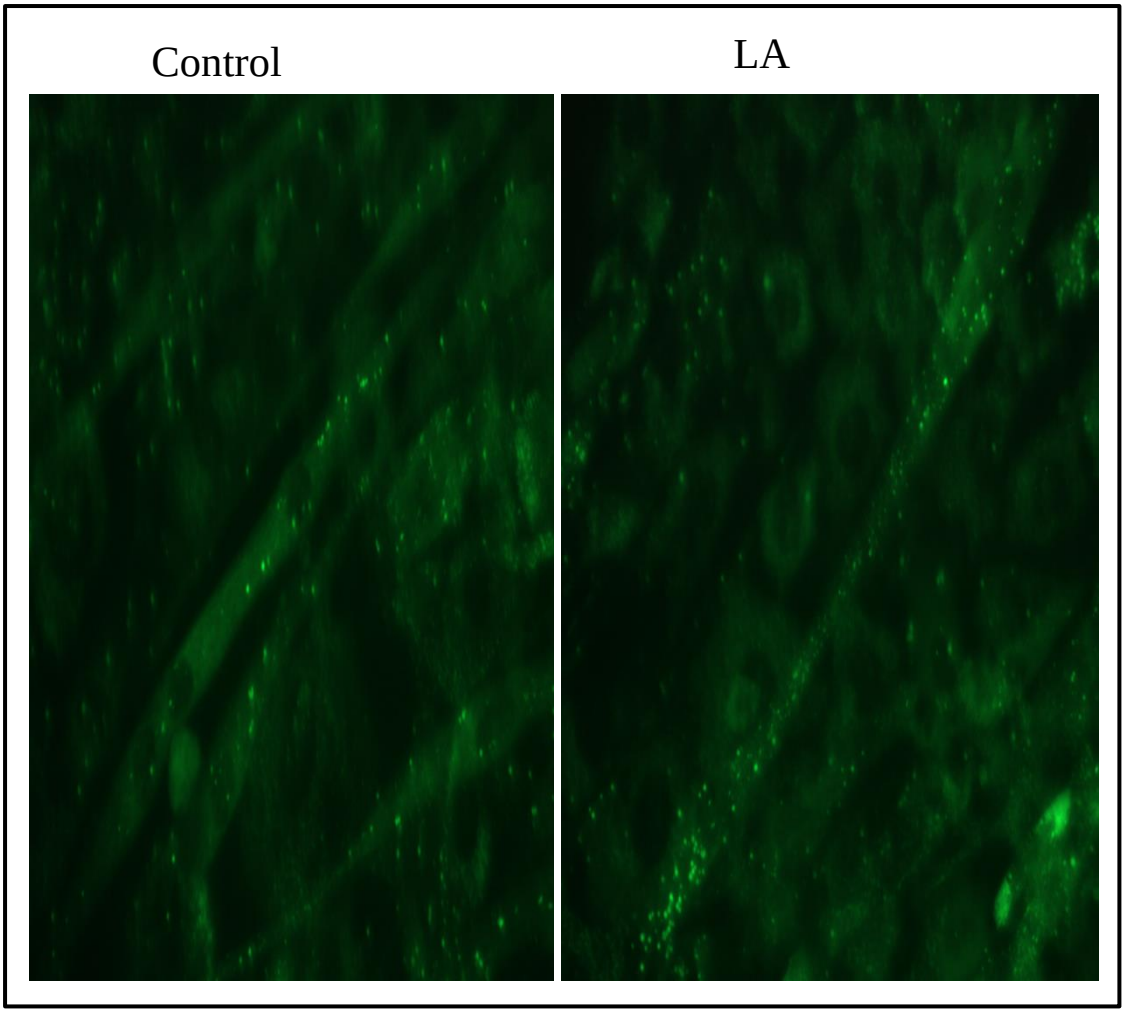


Figure 3-14 Microscopy Images of L6 Cells Myotubes.

C2C12 cells that had been differentiated for five days were treated with 0.2mM LA or the control (no FAs). The cells were stained with Nile red on day three and x40 magnification images show the difference between LA and control myotubes. 4 images per well per treatment.

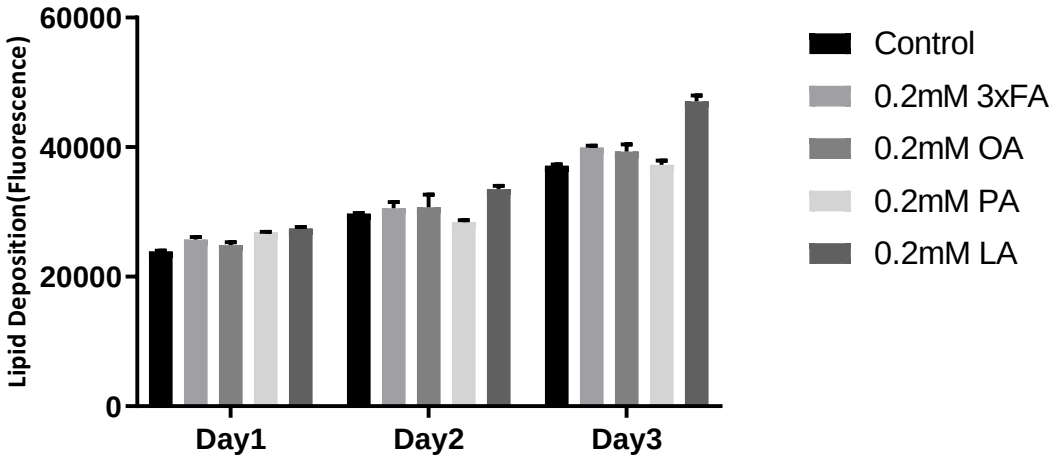


Figure 3-15 Impact of Different Fatty Acids on Lipid Deposition in L6 Cells Over the Time Course.

L6 cells were differentiated into myotubes for six days and then treated with 0.2mM of OA, PA, LA, 3xFA, or the control (no fats). The cells were stained with Nile red on day one, day two, and day three. Two-way ANOVA showed an interaction of $P<0.0001$. Data are presented as means ± SEM; n=3 wells per treatment group.

3.4 Discussion

For the first time, this study shows that differentiated muscle cells have the capacity to accumulate lipids within differentiated myotubes. The results obtained from the two-media experiments on the C2C12 cell line showed that treatment with the myogenic medium resulted in the generation of myotubes resembling muscle fibres. Meanwhile, treatment using the adipogenic medium resulted in adipogenesis, which means that the accumulation of fats occurs in mononuclear adipocytes (data not shown), as previously shown (Ryan et al., 2013; Sanna et al., 2009). In addition, no fat accumulation occurred when the cells were treated with myogenic media, which is in contrast with the results of using the adipogenic medium. Hence, the myogenic medium is a good model to determine fat accumulation within myotubes.

According to the results of the lipid accumulation assessments using C2C12 cells, treatment with 0.2mM LA for three days resulted in increased lipid deposition. High consumption of soybean oil, rich in LA, has been linked to the increasing rate of obesity in the United States; possibly by increasing the activity of the endocannabinoid system (Alvheim et al., 2012; Simopoulos Artemis, 2020). The endocannabinoid system is implicated in energy balance regulation and consists of cannabinoid receptors (CB1 and CB2) and related enzymes. The arachidonic acid from LA derived endocannabinoid which has been demonstrated as pro-inflammatory (Simopoulos Artemis, 2020). However, there was an increase in lipid deposition with other fatty acids (3xFA and PA). Nevertheless, the purpose of this assessment was to establish a model for future work. These experiments found that treatment with LA for three days consistently resulted in the highest level of lipid deposition within myotubes.

The findings relating to the mRNA expression showed that treatment with 0.2mM LA had no significant effect on adipogenesis related genes. This confirms that LDs were not deposited in adipocytes but within myotubes, which proves this study's hypothesis. However, 0.2mM LA had a significant decrease in myogenin and CKM mRNA expression, confirming that the LA is negatively correlated with muscle cell differentiation, as has been reported previously by (Hurley et al., 2006). Also, this study showed there were no significant changes in the mRNA expression of TAG-related genes DGAT1, AGPAT1, and mGPAT2, but there were significant increases in the mRNA expression of SREBP-1a and SREBP-2. This finding is not in accordance with the results of (Carro et al., 2013). They stated that the manner in which the treatment with the polyunsaturated fatty acid LA (0.1mM) converts into the TAG is to protect the cells from lipotoxicity that is induced by FAs. By converting FAs into neutral lipids in lipid droplet storage, this results in the prevention of apoptosis (Laura et al., 2003).

The two cell lines used in this study, C2C12 and L6, are the most used *in vitro* model systems in skeletal muscle studies. However, the results obtained from these cells are not always consistent and often differ from study to study. For example, the C2C12 cells used here show no response in glucose uptake to insulin (Qiong Yin's Ph.D. thesis). This may be due to the restricted expression of sufficient GLUT4 protein levels (Kotliar and Pilch, 1992). As a result, the decision to switch from C2C12 to L6 cells was crucial as the overall purpose of the project was to investigate the impact of intra-myocellular LDs on insulin-stimulated glucose uptake.

Importantly, L6 cells were shown to exhibit the same lipid deposition in myotubes as C2C12 cells. The ability of L6 to accumulate lipids has been reported

previously by (Watt et al., 2006). Again, LA was shown to be the FA to result in a consistent increase in the lipid deposition, the highest being on day three. Hence, as the L6 cells responded to the fatty acids and accumulated lipids within myotubes and they have been reported to respond to insulin-stimulation of glucose uptake, they will be used as a model in all subsequent *in vitro* experiments.

Chapter 4:

Investigation of novel polyphenols influence on lipids accumulation within the muscle fibres of L6 cells

4 Investigation of novel polyphenols influence on lipids accumulation within the muscle fibres of L6 cells

4.1 Introduction

The previous study (chapter 3) had exhibited, for the first time, that 0.2mM linoleic acid (LA) increased the lipid deposition within C2C12 or L6 muscle myotubes cells. Also, the study demonstrated that lipid deposition was within myotubes without the apparent formation of adipocytes. Thus, using this model this chapter will investigate whether dietary polyphenols will interfere or inhibit the lipid deposition in myotubes.

Polyphenols, which are natural phytochemical compounds exist daily foods including tea, fruits, and vegetables (Bahadoran et al., 2013). Antioxidants and anti-inflammatory are some of the properties that have been linked to the polyphenols (Bahadoran et al., 2013). Polyphenols have shown a potential interaction with other nutrients such as carbohydrates and lipids. Besides, the interaction between polyphenols and lipids in the gastrointestinal system results in the reduction of fat absorption along with a decrease in lipid peroxidation (Jakobek, 2015).

Green tea polyphenols, which epigallocatechin gallate (EGCG) is the major content, have been reported to lower plasma lipids. Moreover, it has been suggested that these polyphenols can inhibit the absorption of lipids in the intestine in rats (Matsumoto et al., 1998). Grapes' polyphenols, which include resveratrol (Resv), have been found to lower the triacylglycerol (TAG) levels in the plasma (Zern and Fernandez, 2005). Kim et al, 2013 have reported that polyphenols include EGCG and Resv have effects on reducing lipolysis (Kim et al., 2013). Besides, quercetin (Quer), which is one of the most abundant polyphenols in fruits, has shown interesting effects such as anti-obesity and reduction of lipid accumulation (Jung et al., 2013).

Lipid-associated oxidative stress, which is a result of an imbalance between the oxidants and antioxidants, occurs in normal conditions under a certain amount (Pandey and Rizvi, 2009). However, elevated levels of oxidative stress are associated with numerous diseases including obesity and implicated in diabetes mellitus' pathogenesis (Sies et al., 2005). Also, oxidative stress occurs due to high levels of polyunsaturated fatty acids (Altan et al., 2003) and increased Fatty Acids (FAs) oxidation has been linked to insulin resistance (IR) (Koves et al., 2008). It is being recommended that the consumption of high antioxidant food can be beneficial in the prevention of diseases (Sies et al., 2005). It has been reported that polyphenols play a role in the prevention of lipid peroxidation since they act as free-radical scavengers, and electron or hydrogen donors. Some of the most studied polyphenols are Quer, Resv, and EGCG which are all reported to have anti-oxidant and anti-inflammation properties (Anastasiadi et al., 2010). In addition, Quer has been known to promote anti-diabetic activity, (Pandey and Rizvi, 2009) and EGCG has been reported to have potential health effects, including reducing oxidative stress (Jin et al., 2015).

Many studies have been conducted on polyphenols and their impact on lipids. Those studies have measured the effects of polyphenols on lipids in different organs and tissues *in vitro*, including the liver (Freise et al., 2013)), vascular tissues (Kim et al., 2013) and adipocytes (Freise et al., 2010). All of these studies showed significant positive effects of different polyphenol compounds on lipid accumulation. However, none of them had measured the effect of polyphenols on lipid accumulation in SMs. Therefore, this chapter hypothesis was that the polyphenols (Resv, Quer, or EGCG) would decrease the lipid deposition induced by 0.2mM LA in L6 myotubes.

4.2 Material and methods

4.2.1 Cell culture

L6 cells were used in this study and the detailed methods are described in section 2.1.

4.2.2 Fatty acid treatment

L6 myotubes were prepared as per section 2.1.6, followed by the addition of 0.2mM LA to induce lipid deposition (see section 2.2 for full details). The measurement of lipid deposition was performed using Nile Red staining and a fluorescence plate reader (see section 2.4.2 for full details).

4.2.3 Polyphenols preparation and application

Full details of polyphenols preparation and application, including the concentrations used are described in section 2.3.

4.2.4 DNA assay

DNA assay was used as a marker of cell numbers and was used to normalise for the variability that occurred in the staining due to differences in cell numbers (results section 4.3.2). The procedure of the assay was performed as per section 2.5.

4.2.5 Statistical Analysis

All analyses were carried out on GraphPad Prism using one or two ANOVA when appropriate. When appropriate, one-way ANOVA was followed by post-hoc Tukey's test, while two-way ANOVA was followed by post-hoc Bonferroni's test. Statistical significance was accepted when the P-value was less than 0.05 ($P<0.05$) and the ± standard error of the mean (SEM) was used when displaying the results.

4.3 Results

4.3.1 Effect of DMSO on Lipid Deposition Within Muscle Fibres.

As the polyphenols were all dissolved in DMSO, 0.1% (v/v) DMSO was added to all other treatments including the controls and the incubation of the differentiated cells with just LA. Thus, it was thought that DMSO may affect the capacity of the myotubes to deposit lipids within cells. To confirm, L6 cells were differentiated into myotubes for seven days then treated with 0.2mM LA, 0.2mM LA + DMSO, control (no lipids, no DMSO), and DMSO (no lipids + DMSO) for three days, followed by Nile Red staining. Two-way ANOVA showed no significant interaction between DMSO and LA ($P>0.05$), but there was a significant increase with LA treatment ($P<0.0001$) and no effect of DMSO ($P>0.05$, Figure 4.1). This confirms that the addition of DMSO did not affect lipid deposition when incubated along with LA.

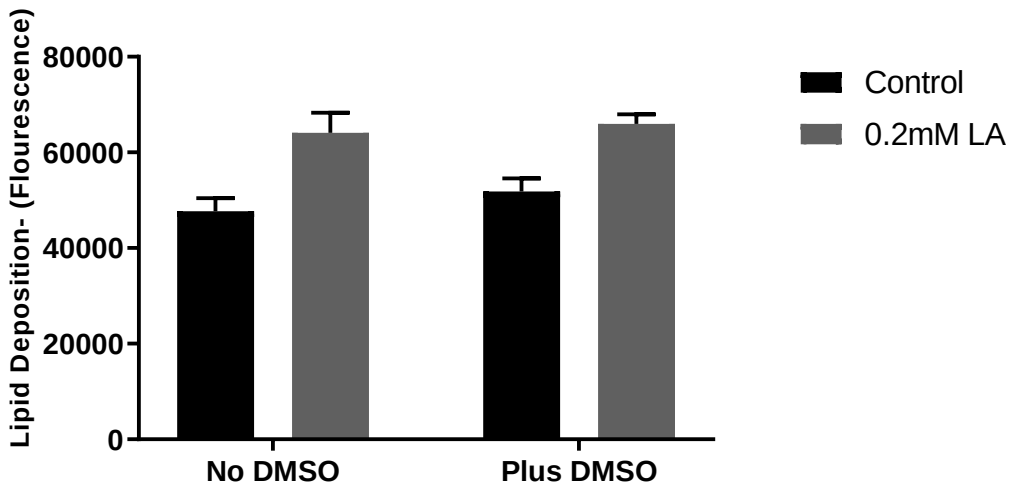


Figure 4-1 The effect of DMSO on lipid deposition of L6 cells in the presence of 0.2mM Linoleic acid (LA).

L6 cells were differentiated into myotubes for six days, then they were treated with or without DMSO and 0.2mM LA. The cells were incubated for three days, followed by staining with Nile Red. Two-way ANOVA showed no interaction between DMSO and LA ($P > 0.05$), but there was a significant increase with the 0.2mM LA treatment ($P < 0.0001$), with no effect of DMSO ($P > 0.05$). Results are mean ± standard error of the mean (SEM) for n=6 wells per treatment group.

4.3.2 Assessment of The Polyphenols Effect on Lipid Deposition and Normalisation to The DNA Assay for 3 Days.

Three different concentrations (0.05, 0.1, and 1 µM) of each polyphenol type (Resv, EGCG, and Quer) were tested initially to see what concentration of polyphenol would show an effect on lipid deposition. All the concentrations and polyphenol types were obtained from (Nicholson et al., 2010). They reported that these polyphenols (out of twelve polyphenols tested) have shown a positive effect on genes related to blood pressure in cultured vascular endothelial cells (HUVEC). They used different concentrations of different polyphenols and the maximum concentration was 1 µM, as the highest concentration of polyphenols in plasma is around 1 µM. Moreover, as

demonstrated in chapter 3, only after three days of incubation with 0.2mM LA was there sufficient lipid deposition to allow polyphenol treatment's effect to be determined. Thus, 0.05 µM, 0.1 µM, and 1µM were examined for three days. L6 cells were differentiated into myotubes by incubating the cells with the myogenic medium for 6-7 days. After the cells fully differentiated, they were treated with 0.2 mM LA, 0.2mM LA + polyphenols, or control (no LA no polyphenol; plus DMSO and BSA instead) for 3 days in serum-free DMEM before they were stained with Nile red. The Nile Red staining results were normalised to the DNA assay to account for variability in cell numbers. One-way ANOVA indicated significant differences between treatments in the Quer and EGCG experiments ($P<0.01$), but not the Resv experiment ($P>0.05$). Post-hoc Tukey's tests indicated there were significant increases with LA compared to the control in both the EGCG and Quer experiments ($P<0.01$ and $P<0.05$ respectively). Moreover, 1µM EGCG significantly decreased lipid deposition relative to LA alone ($P<0.05$ for Tukey's), but no significant effect of Quer concentrations relative to LA ($P<0.05$ for Tukey's). Notably, while ANOVA indicates there is no significant difference in Resv treatment, the LA treatment always greater than the control across the experiments (see Figure 4.2).

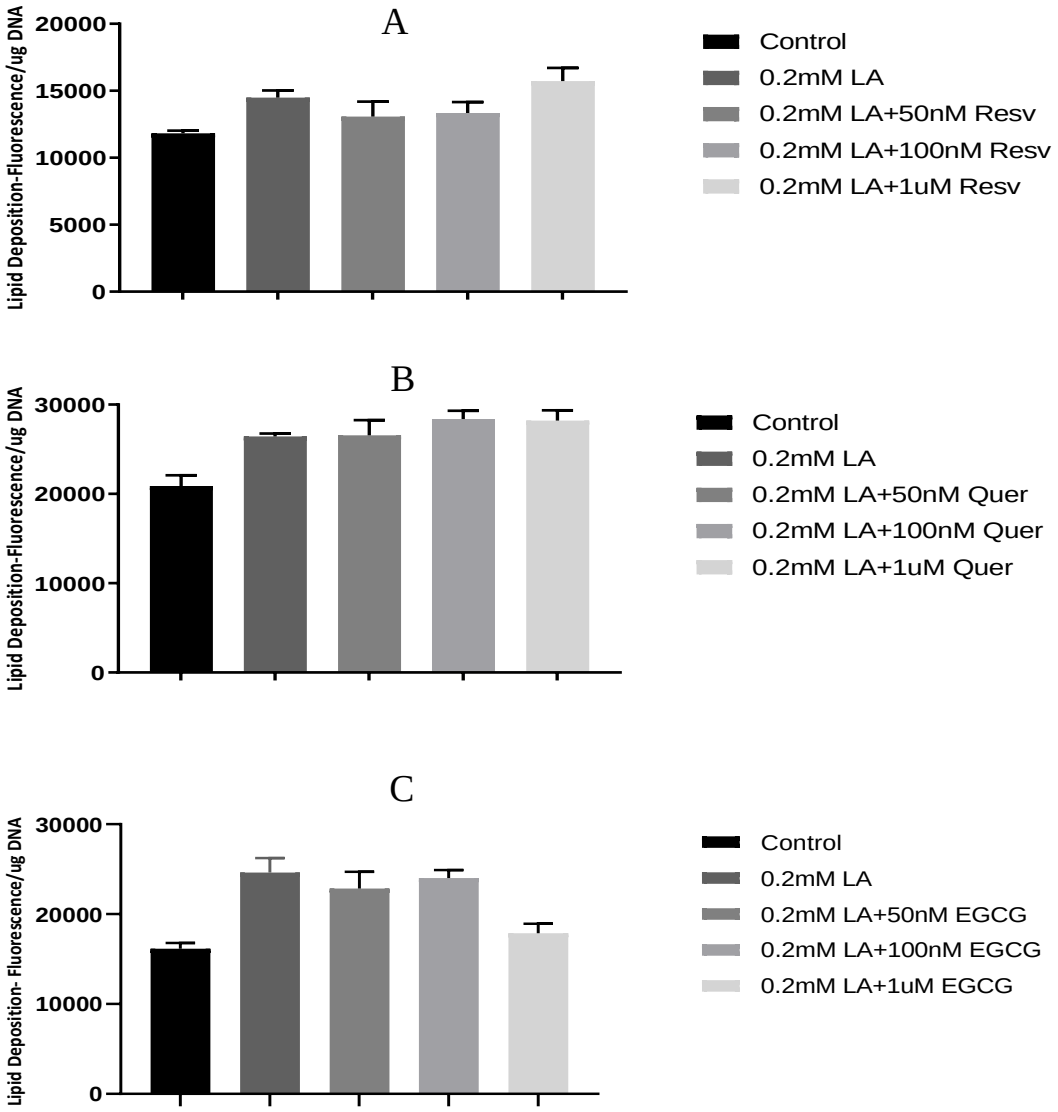


Figure 4-2 The effect of different polyphenols on lipid deposition of L6 cells in the presence of 0.2mM Linoleic acid (LA).

L6 cells were differentiated to myotubes for six days, then exposed for three days to 0.2mM LA in the presence of different concentrations of each of the polyphenols A. Resveratrol (Resv); B. Quercetin (Quer); C. EGCG. The cells were stained with Nile Red and fluorescence normalised to the DNA. There was a significant effect of EGCG and Quer treatments $P<0.01$, but no effect of Resv $P>0.05$, post-hoc showed a significant increase of LA compared to control in treatments of EGCG $P<0.01$ and Quer $P<0.05$ (for Tukey's). Moreover, 1µM EGCG showed a significant decrease of lipid deposition relative to LA $P<0.05$ (Tukey's), but no significant effect of Quer concentrations relative to LA $P>0.05$ (for Tukey's). Although ANOVA indicated there were no significant differences for Resv, consistently across the experiments the LA always greater than the control. Results are mean ± standard error of the mean (SEM) for n=3 wells per treatment group.

To confirm the previous results and ensure that the findings were consistent, a single experiment was performed. Differentiated L6 myotubes were incubated with LA in the presence or absence of 1µM of Resv, Quer, or EGCG for three days and lipid deposition measured. Images were taken which show the lipid deposition and differences between the treatments (see Figure 4.3). One-way ANOVA showed a significant effect of treatment ($P<0.001$, Figure 4-4) and post-hoc Tukey's tests showed a significant increase with LA ($P<0.01$), LA+ Quer ($P<0.05$) and LA+Resv ($P<0.05$) compared to control, and a significant decrease in the LA+EGCG treatment relative to LA alone ($P<0.01$).

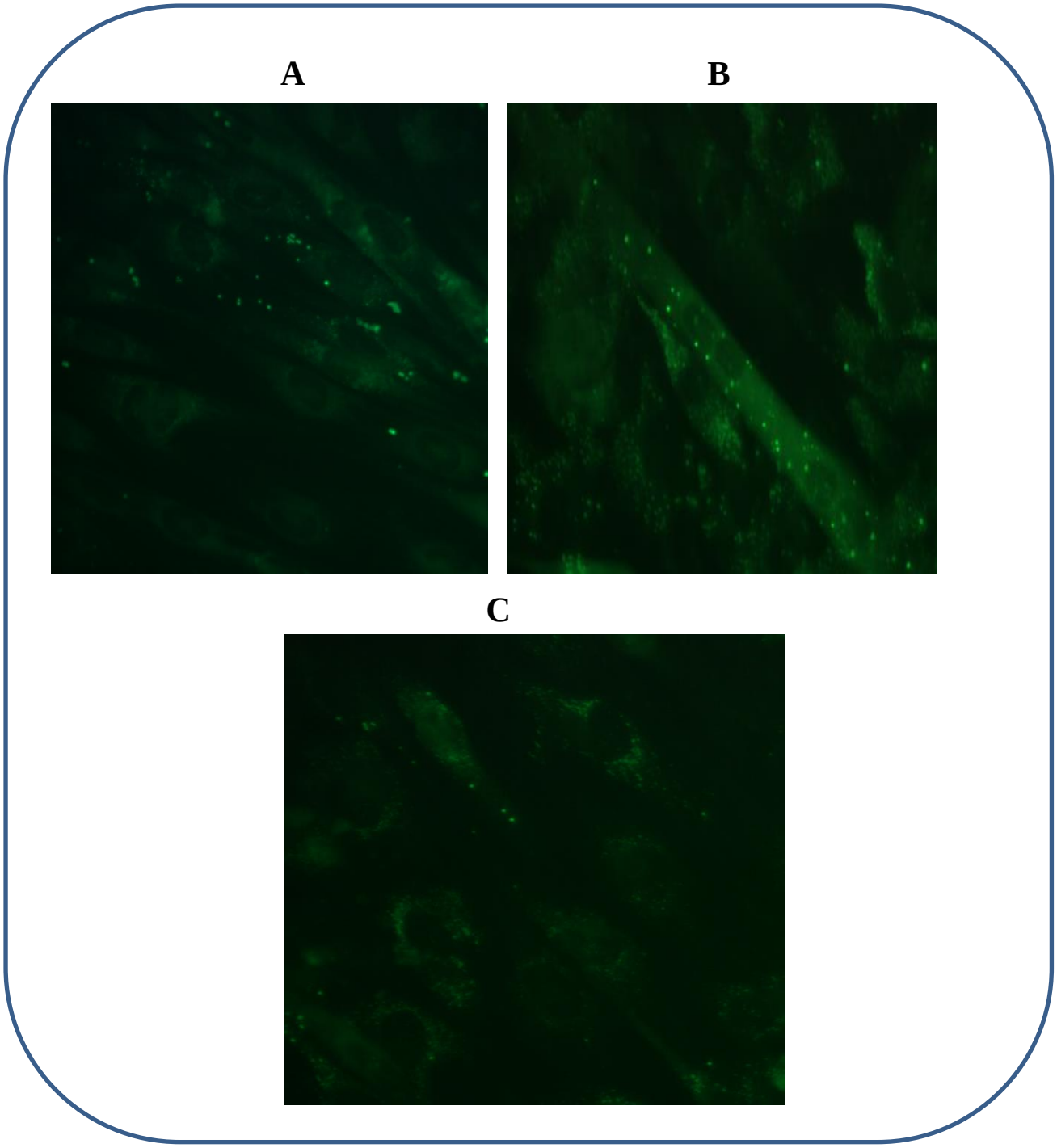


Figure 4-3 Effect of 1µM EGCG on lipid deposition of L6 cells for 3 days.

L6 cells were differentiated into myotubes for six days then treated with A: control (no lipids, no EGCG), B: 0.2mM LA, and C: 0.2mM LA + 1µM EGCG, for three days. The cells were then stained with Nile Red and images were taken at 40x magnification. 4 images per well per treatment.

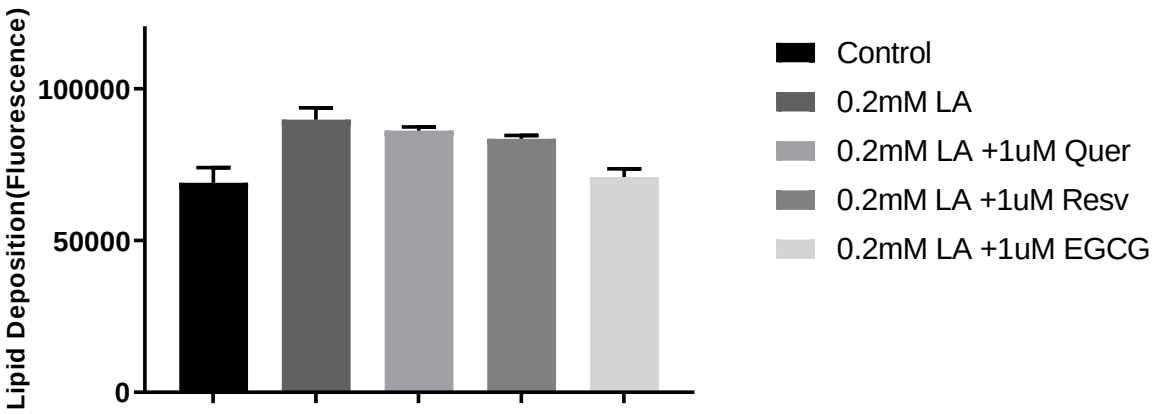


Figure 4-4 Effect of 1µM resveratrol, quercetin, and EGCG on lipid deposition of L6 cells for 3 days in the presence of 0.2mM Linoleic acid (LA).

Differentiated L6 cells were treated for six days with 0.2mM LA, LA+1µM Quer, LA+1µM Resv, LA+1µM EGCG, or control (no LA, or polyphenols) for three days before staining with Nile Red. One-way ANOVA showed a significant difference ($P<0.001$), and Tukey's tests showed a significant increase with LA ($P<0.01$), LA+Quer and LA+Resv ($P<0.05$) compared to the control, and a significant decrease with EGCG treatment on the lipid deposition relative to LA ($P<0.01$). Results are mean ± standard error of the mean (SEM) for n=4 wells per treatment group.

4.4 Discussion

The outcomes of this study were promising and ended in agreement with the study's hypothesis that the polyphenols can reduce the accumulation of lipids within muscle cells. The current study showed that the polyphenols, Resv and Quer, had no effect on lipid deposition within muscle myotube cells. However, EGCG showed a significant decrease in lipid deposition. This effect was seen after incubating L6 myotubes with 1µM of EGCG and LA for three days. Notably, the concentrations used in this study were chosen to not exceed those physiologically found in the blood. The polyphenols' extreme concentrations after consumption of rich-polyphenol foods were around 0.1–1µM (Nicholson et al., 2010). Most published studies tend to use higher concentrations of polyphenols (>100 µM), which are not physiologically relevant (Nagle et al., 2006).

Herbal extracts and nutrients such as polyphenols have been widely studied, gaining a lot of attention (Liu et al., 2017). Polyphenols have been reported to promote potential positive effects on human health (de Camargo et al., 2014; Kong et al., 2018). A study on black tea polyphenols (Uchiyama et al., 2011) and another study on apple polyphenols (Sugiyama et al., 2007) have shown that these polyphenols inhibited intestinal lipid absorption by inhibiting the lipase activity in vitro and in vivo. Also, they found that the levels of TAG elevation and absorption in rat plasma were suppressed. It has been reported that the reduction of lipid absorption in the intestinal resulted in an improvement of insulin sensitivity of mice fed a high-fat diet (Tsuchida et al., 2012)

EGCG is the most abundant polyphenol found in green tea (the component consists of more than 50%). The remaining green tea components, which are

classified as Catechins, are epicatechin-3-gallate (ECG), epigallocatechin (EGC), and epicatechin (EC) (Nagle et al., 2006). EGCG has been reported to have potential health effects in that it possesses antioxidant (Jin et al., 2015), anticancer (Pan et al., 2019), anti-inflammation, and anti-obesity properties (Wolfram et al., 2006).

Due to the lack of literature on the relationship between polyphenols and lipids in SMs, it is hard to decide the exact mechanism of which EGCG inhibited or reduced the lipid accumulation within muscle cells. However, it was demonstrated previously that consumption of green tea for a long time promotes positive effects on lipid *in vivo*; it was reported that green tea catechins reduce lipid absorption in the intestine of rats fed a high-fat diet (Raederstorff et al., 2003), plasma TAG (Crespy and Williamson, 2004; Kao et al., 2000a); also *in vitro* (Watanabe et al., 1998), and catalyse energy expenditure and the oxidation of fats (Huang et al., 2014; Murase et al., 2002). It was suggested previously that green tea catechins interfere with the proteins responsible for lipid uptake by enterocytes (Koo and Noh, 2007). In *vitro*, EGCG downregulates multiple enzymes involved in lipid metabolism including acetyl-CoA carboxylase (ACC) and fatty acid synthase (FAS) (Dulloo et al., 1999; Hodgson et al., 2013; Lee et al., 2009; Moon et al., 2007). ACC facilitates the first step in the biosynthesis of FAs by which acetyl-CoA being converted to malonyl-CoA (Kao et al., 2000b; Zheng et al., 2004). Also, FAS catalyses all the subsequent steps in the production of palmitate using acetyl-CoA and malonyl-CoA (Lin and Lin-Shiau, 2006). (Brusselmans et al., 2005) demonstrated that EGCG inhibited the activity of FAS in prostate cancer cells. EGCG at 10 μM inhibited FAS gene transcription in breast cancer cells and suppressed ACC in rat liver at 5 μM (Babu and Liu, 2008). Moreover, it was suggested that the reduction of FAS results in the reduction of liver TAG synthesis in rats fed catechins (Ikeda et al., 2005). It was demonstrated

previously that decreased production of malonyl CoA by inhibition of ACC leads to increased FAs oxidation (Harwood et al., 2003) and decreased TAG synthesis (Tan et al., 2017).

Therefore, taking all these together suggest that the mechanisms of which EGCG has reduced or inhibited the lipid deposition may be due to (i) inhibition of enzymes implicated in the uptake of fats into SMs, such as fatty acids translocate (FAT; also known as CD36), (ii) enhancing the turnover of FAs (maintains the rate between catabolism and anabolism), or (iii) inhibiting TAG synthesis by downregulating enzymes such as diacylglycerol acyltransferase (DGAT), which catalyzes the final step in TAG synthesis. In support of these hypotheses, it was reported recently that hepatic mRNA expression of DGAT and FAT were suppressed by green tea extract in mice fed a high-fat diet (Li et al., 2016). Moreover, catechins were demonstrated to upregulate FAs β-oxidation enzyme activities (Murase et al., 2002; Sugiura et al., 2012) and inhibit enzymes implicated in FAs synthesis (Sugiura et al., 2012). Green tea extract induced a significant decrease in the mRNA expression of DGAT in adipose tissue of obese rats (Rocha et al., 2016).

Interestingly, when myotubes were co-treated with LA and 1µM EGCG, the level of deposited lipids was returned to the same as the control level. Therefore, this suggests that EGCG might be blocking the LA uptake into the SMs. 1µM EGCG treatment over the time course indicated (3 days) brought the lipids to the same level of control, however, it might drop below that over time. This could be determined if there was a control experiment that compares the EGCG with the control without the presence of LA. Additionally, the Nile red staining method, which used to pinpoint the intracellular lipids in this study, is a vital method to detect intracellular lipid

droplets (mainly TAGs) (Greenspan et al., 1985). The study described in this thesis suggests also that the EGCG reduced TAG accumulation compared to the LA treatment. When the FAs were restrained from permeating into the SMs or the TAG synthesis inhibited, the fewer TAGs would be detected by the fluorescence measurement. However, to test these theories, a further investigation of FAs uptake related enzymes such as FAT and TAG related enzymes such as DGAT are needed. Additionally, a good control experiment would explain whether EGCG has further reduced the basal lipid deposition or no effect.

As each polyphenol used in this study has a different configuration, the results suggest that each polyphenol has specific activity depending on its structure (Fraga et al., 2010). For example, it has been suggested that Resv possesses a potential anti-obesity effect (Corrêa and Rogero, 2019) as it reduces lipid accumulation in adipocytes (Rayalam et al., 2008) whereas Quer has been reported to decrease lipid accumulation in hepatocytes (Wang et al., 2013). Additionally, it has been demonstrated that the polyphenols' mechanism of action depends on the deactivation or activation of transcription factors implicated in fat accumulation and lipid metabolism (Ali et al., 2014). Moreover, the type of cell might play a critical role in the polyphenols' capacity to have beneficial health properties (Kim et al., 2014). Therefore, this might explain the reason why only EGCG has shown an effect on lipid accumulation in L6 myotubes. Indeed, the mechanism of which EGCG has decreased lipid deposition in muscle fibres needs to be examined for further understanding.

Chapter 5:

The Impact of Polyphenols on Insulin Stimulated Glucose Uptake in L6 Muscle Cells

5 The Impact of Polyphenols on Insulin Stimulated Glucose Uptake in L6 Muscle Cells

5.1 Introduction

Polyphenols, especially green tea catechins, have many demonstrated to have biological functions and health benefits, such as reducing body weight, reducing blood glucose levels (Fukino et al., 2005), improving insulin resistance (Brown et al., 2008). Besides, drinking green tea can protect the pancreas from oxidative damage in rat fed green tea extract (Kim et al., 2003). Epigallocatechin gallate (EGCG) can increase the expression of glucose transporter 4 (GLUT4) in SMs and it has been suggested that EGCG is capable of improving energy metabolism, leading to the prevention of weight gain and the enhancement of insulin sensitivity (Jang et al., 2013).

Insulin resistance (IR) is usually defined as the decreased disposal of a glucose load by the body in response to a given amount of insulin (Turcotte & Fisher, 2008). Elevated intramyocellular lipids within skeletal muscles (SMs) tend to cause a defect in the SMs' oxidation capacity (Goodpaster et al., 2001) and stimulate lipotoxicity (Laurens & Moro, 2016). For a long time, it was believed that IR, which is a predictor of T2DM (Shulman, 2000), was a downstream complication of obesity (Samuel et al., 2010). Two decades ago, it was thought that lipid accumulation in muscle negatively affected the insulin-stimulated uptake of glucose. Later, it was found that increased levels of fat in muscles also impairs insulin signalling, which ultimately leads to IR (Samuel et al., 2010; Randle et al., 1963; Samuel & Shulman, 2012). However, the causes of IR within SMs are not yet understood (Amati et al., 2011) but the accumulation of lipids is clearly involved (Samuel & Shulman, 2012).

The outcomes from the last two chapters showed, firstly, that L6 muscle cells treated with 0.2mM linoleic acid (LA) over three days significantly increased lipid deposition within muscle myotubes. It was hypothesised that this elevation of intramyocellular lipids would be associated with reduced insulin sensitivity. Secondly, combining 1µM of EGCG with 0.2mM LA for three days significantly decreased the lipid deposition within the muscle myotubes of L6 cells. If the hypothesis (lipid deposition within muscle cells is associated with reduced insulin sensitivity) is correct, the effect of EGCG treatment on lipid deposition should result in no change in insulin sensitivity compared to controls.

This study aimed to (i) assesses the effects of intramyocellular lipid deposition, caused by incubation of differentiated L6 myotubes with 0.2mM LA, on insulin-stimulated glucose uptake. and (ii) whether treatment with 1µM EGCG would reverse any effects of lipid deposition on insulin sensitivity.

5.2 Materials and methods

5.2.1 Cell cultures

L6 cells were again used in this study, with the detailed methods described in section 2.1.

5.2.2 Fatty acid treatment

0.2mM LA was supplemented in DMEM containing 2% BSA and added to L6 cells, which had been differentiated into myotubes by incubating the cells with myogenic medium (DMEM containing 2% horse serum) for six to seven days. The cells were then incubated for a further three or four days, then either stained with Nile red followed by fluorescence plate reading (see section 2.4.2 for full details of staining) or used for glucose uptake assay (see section 2.6 for glucose uptake assay details).

5.2.3 Polyphenol treatment

1µM EGCG (dissolved in DMSO) was added to a prepared 0.2mM LA treatment (see section 5.2.2) and then applied to the L6 cells, which had been differentiated into myotubes by incubating cells with a myogenic medium (DMEM containing 2% horse serum) for six to seven days. The cells were then incubated for a further three days. The EGCG treatment was removed and DMEM with no serum added to the cells for 24 hours. The resulting cells were either stained or used for glucose uptake assay. 1µM resveratrol (Resv), quercetin (Quer), or EGCG were added to DMEM with no serum for 24 hours before the treatments were removed and a glucose uptake assay was performed. As the polyphenols were dissolved in DMSO, all other treatments, including the control, were supplemented with 0.1% (v/v) DMSO. For further details on polyphenols and preparation please see section 2.3.

5.2.4 Glucose uptake assay

The glucose uptake assay was performed as per section 2.6 and the insulin was incubated either per the manufacturer's protocol or along with the polyphenols.

5.2.5 Statistical analyses

All analyses were carried out in GraphPad Prism versions 7 and 8. One- or two-way ANOVA were used when appropriate. When appropriate, one-way ANOVA was followed by post-hoc Tukey's test, while two-way ANOVA was followed by post-hoc Bonferroni's test. Statistical significance was accepted when the P-value was less than 0.05 ($P<0.05$) and the mean ± standard error of the mean (SEM) was used when displaying the results.

5.3 Results

5.3.1 The effect of 0.2mM LA on lipid deposition in L6 cells over three and four days

The glucose uptake assay protocol requires incubation of the cells with DMEM containing no serum for 24 hours before the assay. Therefore, treatments needed to be removed 24 hours before the assay begins, which might have an effect on the lipid deposition. To ensure that the lipid deposition would remain within the L6 myotube cells, 0.2mM LA was incubated for three and four days, then stained with Nile red. This was done to examine whether the lipid deposition increased, decreased, or remained the same. A two-way ANOVA showed a significant interaction ($P<0.0001$) and the lipid deposition increased in the 0.2mM LA at day four, compared to day three ($P<0.0001$ post-hoc Bonferroni's test; see Figure 5.1).

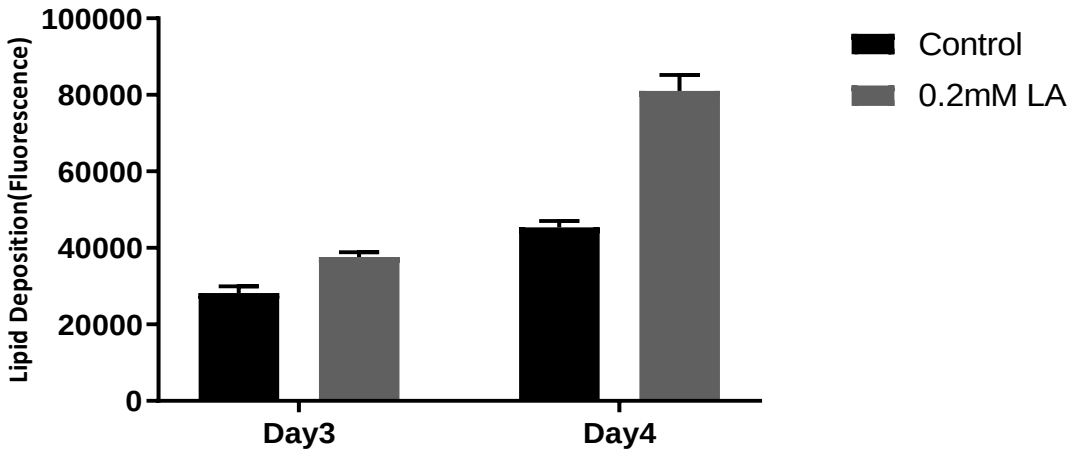


Figure 5-1 Effect of fatty acids on lipid deposition in L6 cells over time

L6 cells were differentiated into myotubes for six days and then treated with 0.2mM LA or control (no fatty acids, plus BSA). Then, the cells were stained with Nile red on days three and four. A two-way ANOVA showed a significant time x treatment interaction ($P<0.0001$) and a post-hoc showed that the lipids had increased with the 0.2mM LA at day four compared to day three ($P<0.0001$; for Bonferroni's). Results are mean ± standard error of the mean (SEM) for n=6 wells per treatment group.

5.3.2 The effect of removal of LA for 24 hours on the lipid deposition in L6 cells

Following the previous experiment, which confirmed the increase of lipid deposition after three days, a second experiment was conducted. This was to confirm that the removal of the 0.2mM LA and serum 24 hours before the glucose uptake assay would not affect the lipid deposition. Two plates were treated with 0.2mM LA; one plate was stained after three days of incubation and the second plate was switched to DMEM with no serum or LA, for 24 hours and then stained with Nile red. A two-way ANOVA showed no significant interaction ($P>0.05$) and the lipid deposition was

not affected by the removal of the serum or LA as there was a significant increase in LA treatment (P<0.0001; post-hoc Bonferroni's; see Figure 5.2).

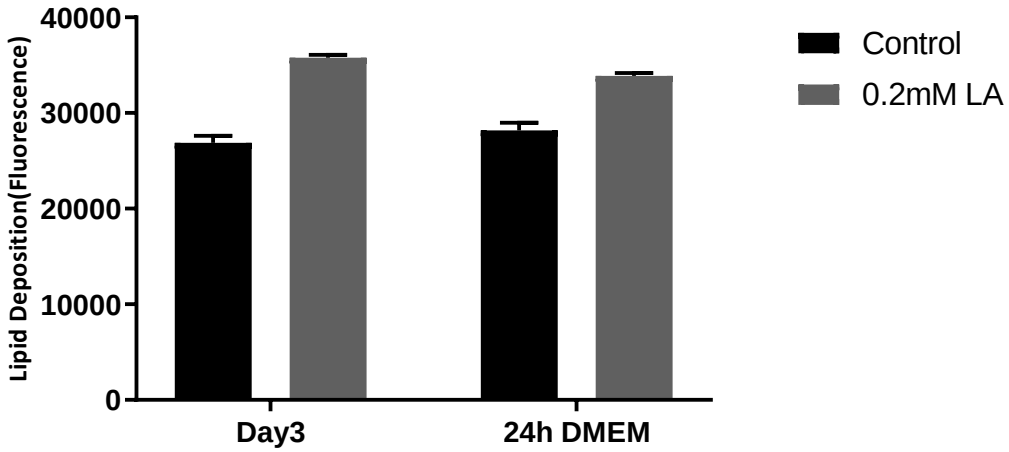


Figure 5-2 Effect of DMEM treatment on lipid deposition in L6 cells over 24 hours

L6 cells were differentiated into myotubes for six days, then treated with or without 0.2mM LA for three days. Cells were then stained with Nile red before and after removing the LA and/or serum for 24 hours. A two-way ANOVA showed no significant interaction (P>0.05), but there was still a significant increase with LA treatment P<0.0001(for Bonferroni's). Results are mean ± standard error of the mean (SEM) for n=6 wells per treatment group.

5.3.3 The effect of intramyocellular lipid deposition on the insulin-stimulated glucose uptake of L6 myotube cells

The previous study in chapter 3 showed that treatment with 0.2mM LA for three days increased the lipid accumulation within muscle fibres. Therefore, this study was conducted to investigate whether this accumulation of lipids was associated with reduced insulin-stimulated glucose uptake. Three experiments were combined and analysed to increase the replicate numbers and ensure consistent results. The results showed there was a significant insulin x LA interaction (P<0.05; see Figure 5.3). In the absence of lipids (i.e. LA), insulin increased the glucose uptake compared to the

control treatment (P<0.01; for Bonferroni's). However, in the presence of lipids, there was no effect of insulin on glucose uptake (P>0.05; for Bonferroni's).

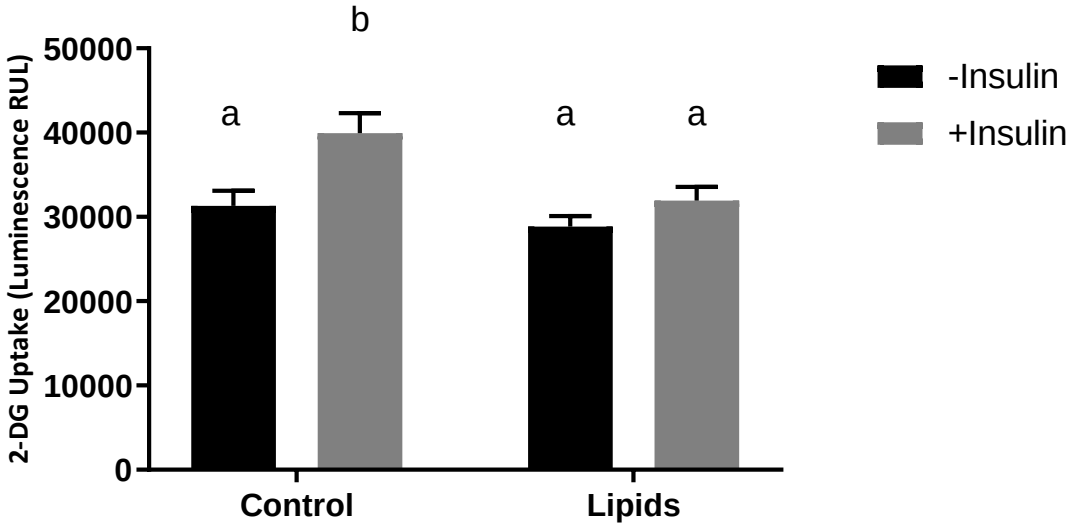


Figure 5-3 Effect of intramuscular lipids on the insulin-stimulated glucose uptake of L6 cells

Differentiated L6 cells were treated with or without 0.2mM LA for three days, then treated ± 1mM insulin for one hour before the measurement of 2-DG uptake. A two-way ANOVA showed a significant insulin x lipids interaction (P<0.05). Results are mean ± standard error of the mean (SEM) for n= 19 wells per treatment group.

5.3.4 The effect of 24 hours of serum, LA, and EGCG removal on the EGCG influence on lipid deposition.

As mentioned previously, serum and other treatments had to be removed 24 hours before the glucose uptake assay. This means there is a chance that the treatment effects could differ or be lost. Therefore, this study examined the effects of removing the EGCG and serum for 24 hours. Images were taken to check that there were still lipids within the myotubes (see Figure 5.4). The results confirmed that there was no alteration in the EGCG effect after removal for 24 hours (see Figure 5.5). A one-way ANOVA showed a significant effect of treatment $P<0.0001$. In the absence of EGCG, there was an increase of LA ($P<0.0001$; post-hoc Tukey's) relative to the control, while in the presence of EGCG the lipid deposition within muscle cells was suppressed ($P<0.0001$; post-hoc Tukey's) relative to LA even after the removal of treatments for 24 hours.

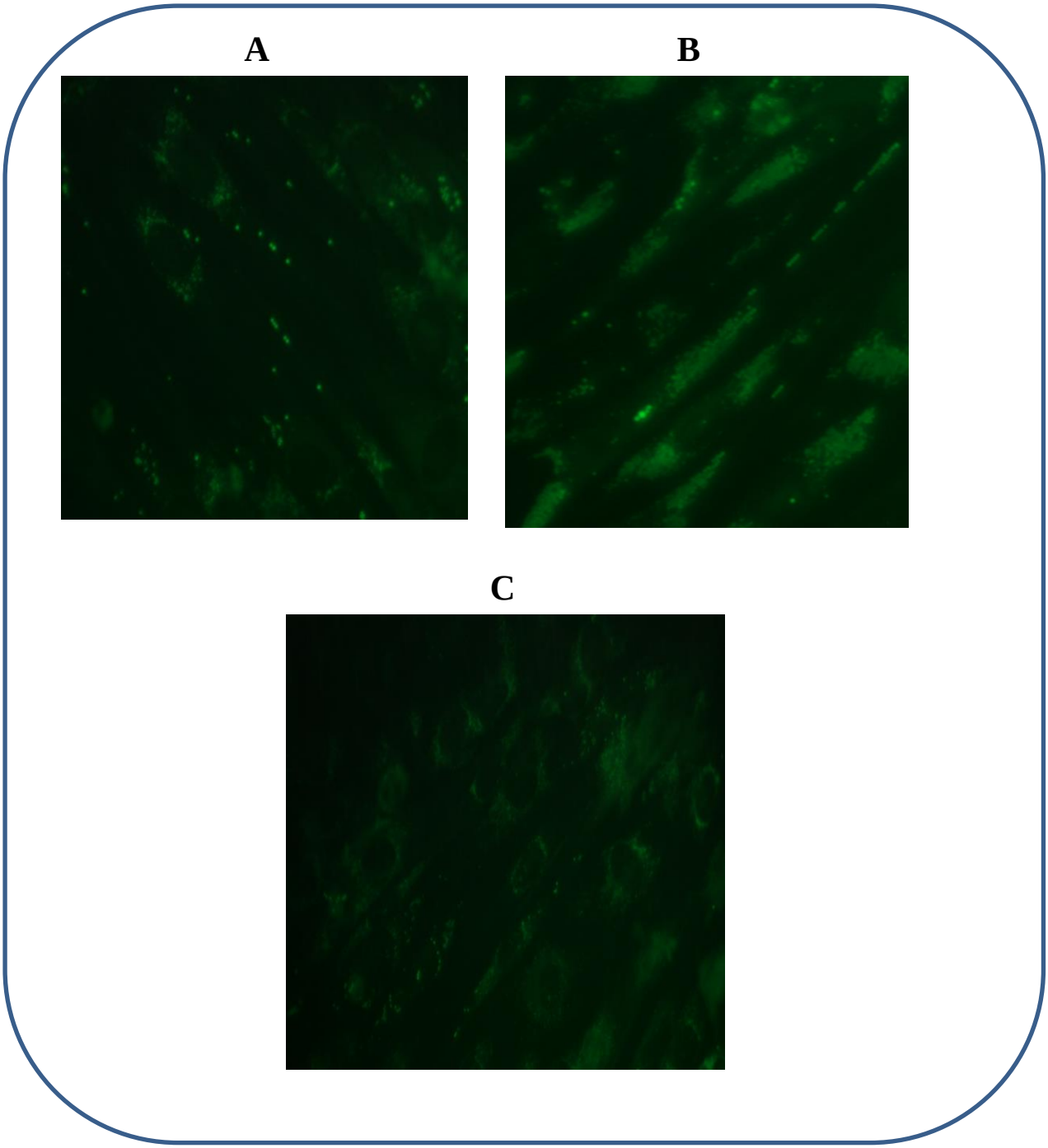


Figure 5-4 Effect of 1 µM EGCG treatment on lipid deposition after 24 hours of DMEM treatment

L6 cells were differentiated into myotubes for six days and then treated with A. control (no lipids or EGCG); B. 0.2mM LA; C. 0.2mM LA + 1µM EGCG. After three days, the treatments were removed and DMEM, no serum, was applied to the cells for 24 hours before the cells were stained with Nile red. Images were taken at 40x magnification. 4 images per well per treatment.

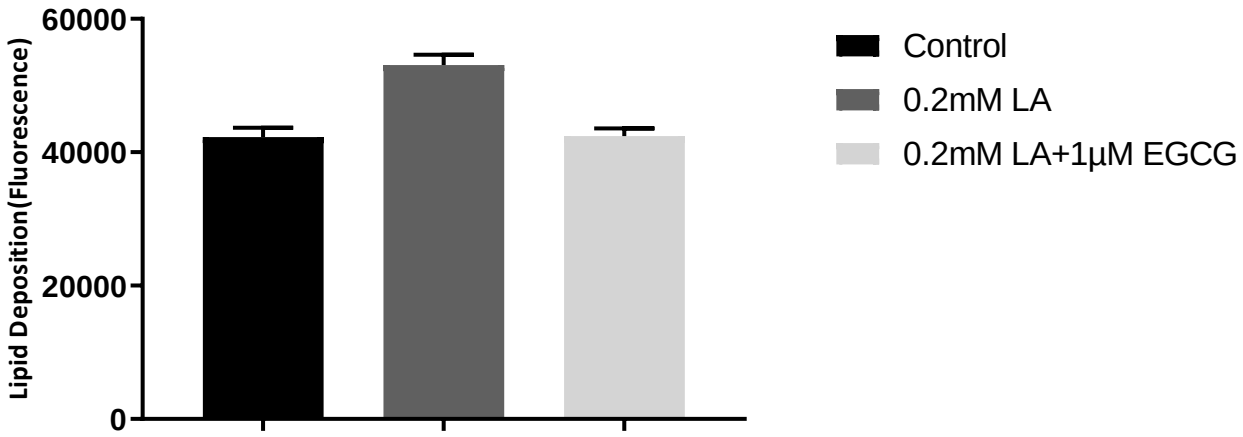


Figure 5-5 Effect of 1µM EGCG on lipid deposition after 24 hours of removal of treatments

Differentiated L6 cells were treated with 0.2mM LA, 0.2mM LA + 1µM EGCG, or control (no LA or ECGC) for three days. Then, the treatments were removed and DMEM with no serum added to the cells for 24 hours before they were stained with Nile red. A one-way ANOVA showed a significant difference ($P<0.0001$). The LA was increased relative to the control in the absence of EGCG ($P<0.0001$; for Tukey's), but when the EGCG presence the lipid deposition was decreased relative to the LA ($P<0.0001$; for Tukey's). Results are mean ± standard error of the mean (SEM) for n= 8 wells per treatment group.

5.3.5 The effect of 1µM EGCG on insulin-stimulated glucose uptake via decreasing the deposition of lipids

This study aimed to examine the subsequent effects of the 1µM EGCG treatment on insulin-stimulated glucose uptake (via decreasing lipid deposition). The previous study showed that there was no change in EGCG's influence on lipid deposition after treatment with DMEM with no serum for 24 hours before the glucose uptake assay. The results showed a significant EGCG x insulin interaction ($P<0.01$; see Figure 5.6). Once again, there was a clear insulin-stimulated increase in glucose uptake in the controls and lipid accumulation induced by LA treatment inhibited the

response to insulin. Importantly, there was a clear insulin-stimulated increase in glucose uptake following LA + EGCG treatment, presumably due to EGCG inhibiting the lipid accumulation.

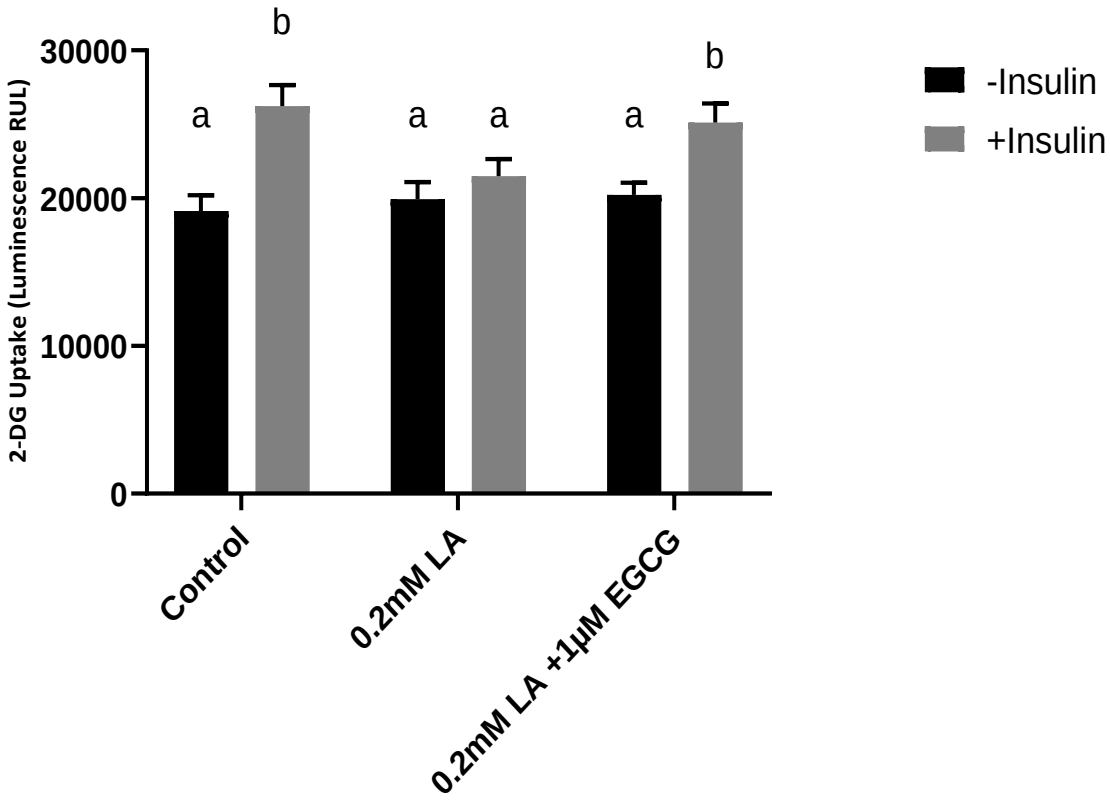


Figure 5-6 Effect of 0.2mM LA+ 1µM EGCG on the insulin-stimulated glucose uptake of L6 cells

L6 cells were differentiated for six days, then treated with 0.2mM LA, 0.2mM LA + 1µM EGCG, or control (no LA no EGCG) for three days before 1mM insulin treatment. This was followed by 2-DG uptake measurement. A two-way ANOVA demonstrated a significant interaction for EGCG x insulin ($P<0.01$). [a,b] Means with different letters are significantly different from one another ($P<0.05$ post-hoc Bonferroni) whereas those with the same letter are not significantly different ($P>0.05$ post-hoc Bonferroni). Results are mean ± standard error of the mean (SEM) for n= 9 wells per treatment group.

5.3.6 The effect of 1µM of different polyphenols on insulin sensitivity

To investigate whether polyphenols have a direct effect on insulin sensitivity, L6 myotubes were treated with 1µM of Resv, Quer, or EGCG in the presence or absence of insulin before measurement of glucose uptake. The polyphenols were added along with DMEM with no serum for 24 hours and then polyphenols were included with or without 1mM insulin for one hour before 2DG uptake measurement. None of the polyphenols had any effect on the basal or insulin-stimulated glucose uptake ($P>0.05$ for polyphenol x insulin interaction and polyphenol alone), but there was a significant increase with insulin ($P<0.001$; for Bonferroni's; see Figure 5.7).

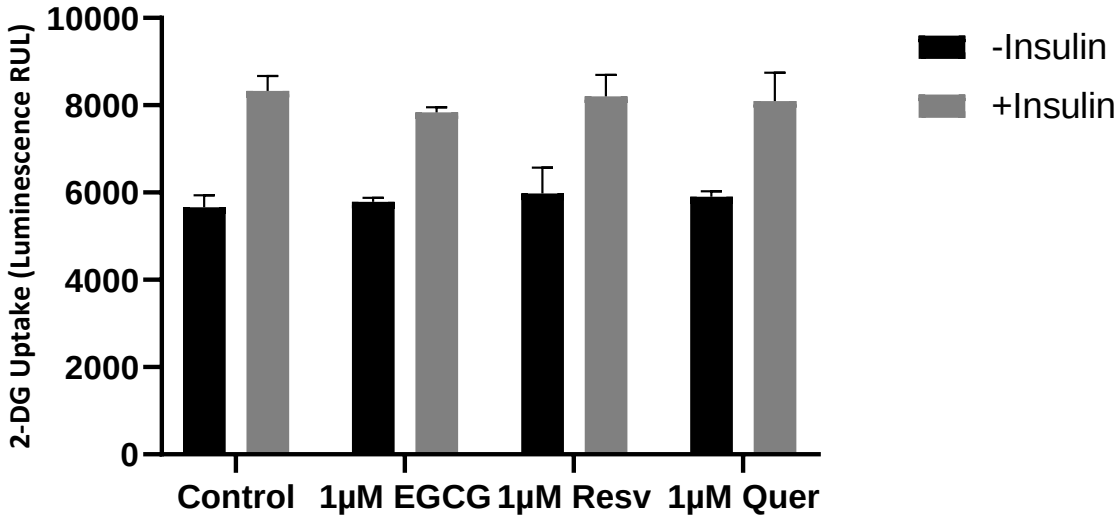


Figure 5-7 Effect of different polyphenols on the insulin-stimulated glucose uptake of L6 cells

L6 cells were differentiated for six days, then treated with 1µM of EGCG, Resv, Quer, or control (no polyphenols) for 24 hours before one hour +/- 1mM insulin +/- polyphenols treatment. This was followed by 2-DG uptake measurement. A two-way ANOVA showed no interaction ($P>0.05$) and no effect of polyphenol treatments, but a significant effect of insulin ($P<0.001$; for Bonferroni's). Results are mean ± standard error of the mean (SEM) for n=3 wells per group.

5.4 Discussion.

The study results confirmed that the insulin-stimulated glucose uptake was significantly suppressed in the presence of the intramyocellular lipids. Interestingly, when 0.2mM LA was combined with 1µM EGCG for three days, the insulin-stimulated glucose uptake was similar to controls. However, the polyphenols (EGCG, Resv, and Quer; 1µM each) had no direct impact on the basal or insulin-stimulated glucose uptake. Notably, lipid deposition within L6 muscle myotubes was not affected by the removal of serum or treatments for 24 hours. Also, the effects of the 1µM EGCG did not change when the treatment was removed and replaced with DMEM with no serum for 24 hours before the glucose uptake assay.

The main tissue for glucose disposal in humans is SMs. The inability of this tissue to stimulate glucose uptake in response to a given amount of insulin indicates the IR most frequently found in SMs (Hulver and Lynis Dohm, 2004). Many efforts were made to establish an efficient model for an in-depth understanding of the IR in muscle cells. At present, one of the most useful muscle models in *vitro* for the glucose uptake investigation is the L6 rat muscle cells (Yap et al., 2007). Besides, it was confirmed previously in chapter 3 that L6 myotubes cells could accumulate intramuscular lipids. Therefore, this chapter attempted to investigate the relationship between intramuscular lipids and IR.

IR is associated with lipid accumulation in SMs and leads to imperfections in FA metabolism, resulting in amendments in the muscle uptake of FAs and FA oxidation (Turcotte and Fisher, 2008). Specifically, intramyocellular triacylglycerol (TAG) accumulation is associated with IR in SMs (Hulver and Lynis Dohm, 2004; McGarry, 2002; Pan et al., 1997). However, Hulver and Lynis Dohm, (2004)

suggested that TAG itself is not responsible for the defects in insulin activities in SMs. But, TAGs are dormant signs of other lipid intermediates, such as diacylglycerol (DAG) and ceramide, which are associated with IR and can, directly and indirectly, alter insulin signalling. To our knowledge, this is the first time to measure the influence of lipid accumulation on insulin sensitivity using this model (L6 myotubes cells) in *vitro,* as all the previous studies in the literature have demonstrated the effects of lipid accumulation within SMs by using animal or human subjects (*in vivo*). Consequently, the hypothesis, that the accumulation of lipids, such as TAG, within SMs, is associated with insulin sensitivity alteration *in vitro,* was established.

Decades ago, it was suggested that increased lipid accumulation in SMs is associated with T2DM (Goodpaster et al., 2001; Kelley et al., 2002; Shulman, 2000). The characteristics of IR in SMs include reduced oxidative capacity, fatty acid oxidation by muscles, and impaired muscle oxidation abilities (Goodpaster et al., 2001). In the SMs of obese insulin-resistant people, the phosphorylation of the insulin receptor and insulin receptor substrate 1 (IRS 1) was found to be diminished (Goodyear et al., 1995; Griffin et al., 1999; Hulver and Lynis Dohm, 2004). Additionally, intramyocellular lipids are associated with inhibition of GLUT 4 translocation to the membrane (Chavez et al., 2003). GLUT 4 is a highly connected and responsive transporter of insulin and is expressed in SMs (Samuel et al., 2010). (Hegarty et al., 2003) reported that the GLUT 4 expression in SMs does not change in IR patients; however, the translocation of GLUT 4 to the membrane decreases in the SMs of patients with T2DM (Goodyear et al., 1995; Samuel et al., 2010). This defect of GLUT 4 is associated with a reduction of the insulin signalling pathway components, such as IRS 1 (Goodyear et al., 1995). The mechanisms by which the accumulation of lipids within SMs reduce insulin-stimulated glucose uptake could

include the defects in GLUT 4 translocation from inner stores to the plasma membrane, the interaction of TAG synthesis intermediates (such as DAG and ceramide) with insulin signalling and/or the impairment of glucose phosphorylation. Further investigation is highly necessary for understanding the mechanisms involved in IR caused by the accumulation of lipids within SMs.

It has been demonstrated that lowering intramuscular lipid levels is associated with the improvement of insulin sensitivity (Oakes et al., 1997). As reported in chapter 4, treatment with EGCG suppressed the elevation of lipid deposition within the SMs of L6 cells. Interestingly, the reduction of FFA and TAG is one of the mechanisms of action of antidiabetic drugs used to improve insulin sensitivity, such as thiazolidinedione (Kendall, 2006).

Consumption of green tea, a major component of which is EGCG, is associated with the delay of T1DM (Yang et al., 2014) and reduce the risk of T2DM (Mackenzie et al., 2007; Preedy and ProQuest, 2013). 4 cup consumption of green tea has shown to reduce the risk of developing diabetes by 30% in a prospective cross-sectional study of 38,018 women age 45 years with no history of diabetes (Sarker et al, 2000). EGCG consumption, as a food supplement, was demonstrated to improve the tolerance of glucose in rodents with diabetes and enhance the metabolism of lipids and glucose in hepatocytes (Zhang et al., 2010). In the present study, the co-treatment of 1μM EGCG with 0.2mM LA showed both a reduction in LA-induced lipid accumulation and an improvement in insulin-stimulated glucose uptake. This finding is in support of (Zhang et al., 2010), who reported that treating L6 myotubes with pre-insulin resistance, induced by exposer to 1μM dexamethasone, with 20-40μM of EGCG for 24 hours markedly improved insulin-stimulated glucose uptake and

significantly stimulated the translocation of GLUT 4 to the membrane. However, the concentrations used in that study were not physiologically relevant, as they exceeded ~1µM.

Comparably, these findings are in contrast with investigations on the effect of lipid accumulation on GLUT 4 translocation, which showed an inhibitory effect of intramyocellular lipids on the translocation of GLUT 4 and insulin-stimulated glucose uptake (Alkhateeb et al., 2007; Chavez et al., 2003). As mentioned in chapter 4, the potential mechanisms by which EGCG reduces/inhibits lipid accumulation within SMs includes blocking FA uptake into SMs, increasing lipid oxidation, and/or suppressing the synthesis of TAG. In the present study, insulin sensitivity was altered by lipid accumulation, then the co-treatment with EGCG improved the insulin-stimulated glucose uptake. Therefore, the potential mechanisms of the beneficial effect of EGCG on insulin sensitivity due to inhibiting lipid accumulation, thereby, improving the insulin signalling cascades which in turn stimulate GLUT 4 translocation. This inhibition of lipids accumulation could be via decreasing FA uptake/increasing FA oxidation or decreasing TAG/FA synthesis. However, further investigations should aim to determine the exact mechanisms of the effect of lipid accumulation on IR and the impact of green tea catechins, especially EGCG, on lipid accumulation and associated insulin sensitivity.

Chapter 6:

General Discussion and Future Work

6 General Discussion and Future Work

6.1 General Discussion

6.1.1 Conclusion

Obesity has been increasing worldwide over recent decades and has reached an epidemic level. T2DM is mostly seen in obese individuals. Many studies have indicated that the storage of TAG in non-adipocyte cells results in a defect of several metabolism pathways. The accumulation of lipids as TAG was reported to affect the insulin signalling within SMs. However, the exact mechanisms of the relationship between intracellular lipid accumulation and IR are not fully understood yet. Therefore, the overall aims of these studies were to (i) establish a novel *in vitro* model, (ii) examine the dietary polyphenols beneficial effect on the intracellular lipids accumulation, (iii) determine the effects of intracellular lipid and accumulation on insulin-stimulated glucose uptake and (iv) determine the effects of polyphenols on insulin sensitivity via effects on lipid accumulation. Rather than using human or animal subjects, which are very expensive, not always controllable, and have ethical issues, an *in vitro* model was developed to examine the role of intracellular lipids in IR and the potential mechanisms, which is better as it costs less.

C2C12 and L6 cells are the most used *in vitro* models for muscle cell-related research (Yap et al., 2007). However, not all C2C12 cells respond to insulin in terms of glucose uptake (Nedachi and Kanzaki, 2006). This was confirmed recently within the lab by Qiong Yin, a PhD student, who showed that insulin did not affect glucose uptake in the C2C12 cells used in the lab, while L6 cells showed a two-fold increase in glucose uptake in response to insulin. Therefore, this led to the switch from C2C12 cells to L6 cells for the current work after confirming that C2C12 cells are capable to

deposit lipids. This deposition of lipids was seen after incubation of C2C12 myotubes cells with 0.2mM LA for three days. Additionally, adipocyte biomarkers were not expressed during the LA treatment, which suggests that the cells were not transdifferentiated into adipocytes as was seen in the adipogenic medium study. L6 cells also have shown such results and, again, the LA showed the highest lipid deposition after three days, which is the same as C2C12 cells had. Therefore, L6 cells were used as a model in subsequent studies.

LA was reported previously to induce lipid accumulation in 3T3-L1 cells at physiological concentrations 0.3-0.6 mM for 4-8days of treatment (Kokta et al., 2008). Moreover, elevated LA level in Znt7 KO mice' skeletal muscles was linked to oxidative stress (Huang et al., 2018). It is well documented that essential fatty acids such as LA (n-6) and alpha-linolenic acid (ALA; n-3) are capable to promote positive effects on human health. Arachidonic acid, docosahexaenoic acid (DHA), and eicosapentaenoic (EPA) are the biologically active forms of LA and ALA (National Research Council . Committee on Nutrition and ProQuest, 2011). However, it was demonstrated that eicosanoids synthesised from LA are pro-inflammatory, while those from ALA are anti-inflammatory, thus increased consumption of LA results in high risk of chronic disease developments (Jeyapal et al., 2018). The normal ratio of n-6/n-3 is ~1:1, however, the present western diet tends to have a higher n-6/n-3 ratio (~20:1) (Kang, 2005). In the present study, the LA treatment has shown a consistent increase of lipid droplets compared to other FAs treatments of L6 muscle cells. On the other hand, Kokta et al (2008) have reported that OA induces higher lipid accumulation compared to LA treatment using 3T3-L1 cells (Kokta et al., 2008). They used a closely similar concentration to the present study (0.3 µM) and incubation period of 4 and 8 days. Therefore, the results from the present study

suggest that LA induces lipid deposition in skeletal muscle cells more than other tissues cells.

Notably, dietary polyphenols were reported in the literature to maintain body weight and reduce lipid accumulation (Mohsen and Syeda, 2010). Therefore, the potential effect of Resv, Quer, and EGCG, which are mostly used in lipid and IR studies, on the lipid deposition of L6 myotubes cells were examined. 1µM EGCG showed significant inhibition of the lipid deposition (co-treated with 0.2mM LA for 3 days). While other polyphenols did not affect the lipid accumulation in all concentrations used (0.05, 0.1, 1 µM). It was reported that EGCG suppressed adipogenic differentiation of 3T3-L1 cells and also EGCG at 10-200 µM for 4-6 days reduced lipid deposition of bovine mesenchymal stem cells (BMSCs) (Jeong et al., 2015). Interestingly, after co-treating L6 myotubes cells with 0.2mM LA and 1µM EGCG, the lipid deposition was the same as the control treatment (no FA added). This suggests that the EGCG was blocking the LA mediated lipid deposition, possibly via effects on FA uptake. However, more investigations are required to elucidate how EGCG inhibited lipid accumulation.

The accumulation of lipids within myotubes of L6 cells (induced by 0.2mM LA for 3 days) showed an inhibitory effect on the insulin-stimulate glucose uptake. This inhibitory action could be associated with a defect in the insulin pathway that mediated GLUT4 translocation. (Lara-Castro and Garvey, 2008), have demonstrated that intramyocellular lipids accumulation of L6 cells is associated with the degree of IR. Moreover, it was suggested previously that a reduction in the insulin receptor activity is associated with a decrease in the GLUT 4 translocation. However, the exact mechanism involved in the suppression of insulin-stimulate glucose uptake caused by

intramuscular lipid accumulation is not clear yet. As the hypothesis of the thesis indicated that polyphenols would improve IR induced by lipid accumulation, co-treatment of EGCG and LA was tested in terms of insulin-stimulate glucose uptake. The results showed that after the co-treatment of 0.2mM LA with 1µM EGCG, the insulin-stimulate glucose uptake was improved and returned to that seen in the control (no LA). This improvement might be due to enhancing the translocation of GLUT 4 to the cells membrane by a reduction of TAG synthesis. Moreover, this stimulation of insulin-stimulate glucose uptake may also a result of EGCG increased lipid oxidation, therefore, improve in the insulin signalling cascade. Polyphenols' potential direct effect on insulin sensitivity was also examined. The results indicate that the polyphenols (Resv, Quer, and EGCG) have no direct impact on the insulin-stimulate glucose uptake at the concentrations and time used (1µM for 24 hours, and along with the insulin (1hour)). Further, advanced inspection is needed to understand the mechanism behind LA mediated lipid accumulation within L6 myotubes and the inhibitory effect on the insulin-stimulate glucose uptake and how the EGCG resulted in an improvement in the IR via reducing the intramuscular lipids accumulation.

Importantly, the novel *in vitro* model established in this thesis (Figure 6.1) not only allows us to investigate the impact of intramuscular lipid accumulation on insulin sensitivity in terms of glucose uptake but also allows us to investigate other components of the insulin signalling cascade. Insulin signalling cascade influences biological processes that are connected to each other, particularly the deposition of macronutrients for example, it influences fat and glycogen deposition as well as protein synthesis. Additionally, the *in vitro* model developed in this thesis will allow not only examine the influence of fat deposition on SMs, but also the relationship between fat deposition and the development IR, as well as the associated potential

changes on whole insulin signalling and the impact on the macronutrient metabolism. A very recent study by (Yin et al., 2020) has demonstrated that L6 myotubes cells are capable to respond to insulin in terms of glucose uptake and signalling pathway. A previous study has shown that SMs cells (C2C12 cells) can accumulate lipids, but that was seen as the cells were transdifferentiated to fat cells (adipocytes), not intramuscular lipids (Ryan et al., 2013). Five decades ago, (Denton and Randle, 1967) had found that lipids deposition exists in muscle. Since that, many studies have demonstrated the relationship between lipid deposition and insulin resistance (Goodpaster et al., 2000), obesity, and T2DM (Goodpaster and Wolf, 2004) in vivo. Thus, *in vitro* model allows looking deeper into the relation of LDs accumulation within SMs myotubes cells and IR, therefore, understand the mechanism of IR occurrence.

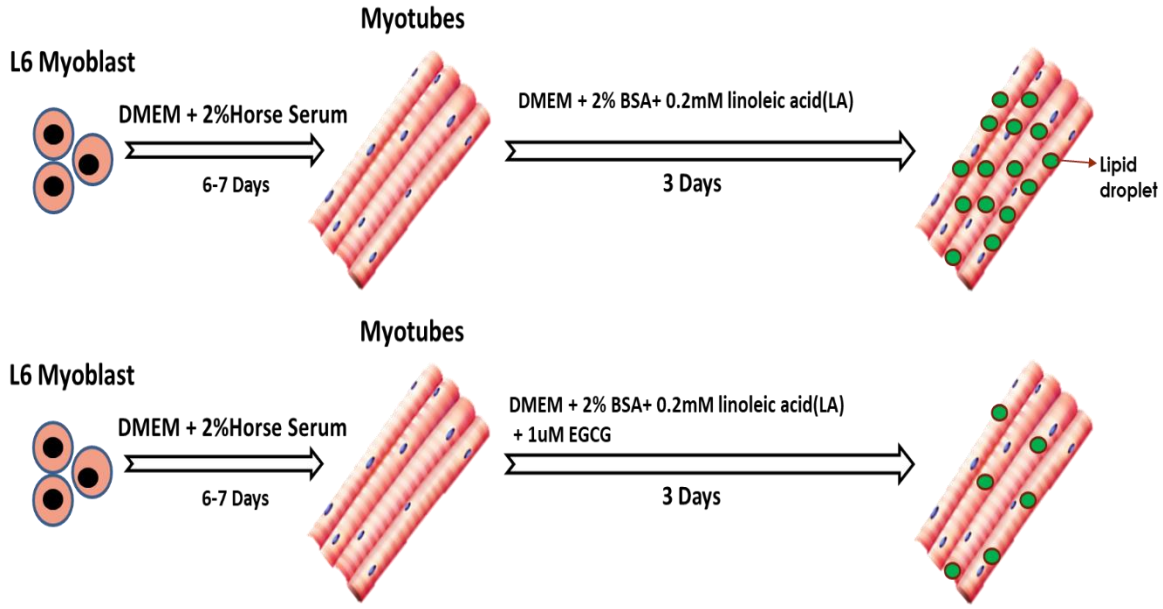


Figure 6-1 A diagram illustrates the novel model established in this study.

6.1.2 Final Summary of The Novel Findings of This Thesis

In conclusion, this body of work has shown, for the first time, the following:

1. C2C12 and L6 myotubes can accumulate intramuscular lipids by treating them with 0.2mM LA for three days, and that the intracellular lipids were deposited within muscle fibres and no adipocytes generated.

2. 0.2mM PA and 3xFAs for three days also showed a significant increase in lipid deposition of C2C12 myotubes cells.

3. Different concentrations of resveratrol and quercetin have no beneficial effect on the lipids accumulation within myotubes of L6 cells when co-treated with 0.2mM LA for three days. However, 1µM EGCG (which is a physiologically relevant concentration) has shown a significant decrease in the lipid accumulation in the same period of time used for other polyphenols.

4. The increased intramuscular lipids accumulation has shown alteration of insulin-stimulate glucose uptake leading to the occurrence of IR.

5. As a result of the reduction of intramuscular lipids by co-treating the LA with EGCG, the insulin stimulation of glucose uptake was improved compared to the LA treatment group.

6. 1µM of resveratrol, quercetin, and EGCG had no direct effect on insulin-stimulate glucose uptake after incubating with the L6 myotubes cells for 24 hours.

7. L6 myotubes cells are capable to deposit lipids within myotubes (treating with 0.2mM LA for three days) and also respond to insulin in term of glucose

uptake, therefore, it makes the L6 rat muscle cells is the first *in vitro* model that enables to investigate the relationship between intramuscular lipid accumulation in insulin sensitivity.

6.2 Limitations and Future Work

6.2.1 Limitations

Firstly, all work in this thesis was conducted using physiologically relevant concentrations and environments, however, the insulin concentration used in the insulin-stimulated glucose uptake (1mM) was much higher than physiological concentrations, which should not exceed (~0.01µM). The use of the 1mM insulin was as recommended by the manufacturer protocol in the glucose uptake assay. Because of the short time (1hour) required by the protocol to incubate the cells with the insulin, the high insulin concentration was required to enable the assay to work. For future works, I would use a physiological concentration of insulin. Secondly, due to the time of PhDs studies in the UK (3 years), there was not enough time to further investigate the mechanisms involved in the lipids induced IR and subsequent treatments of polyphenols (EGCG). Third, it should be noticed that this study was conducted in a controlled artificial environment (include individual FA or polyphenol) which is not equal to the normal environment found in human beings, therefore, it is good to check this study using living subjects (humans or animals) in their natural environment to confirm the results. Lastly, the difference between human and rodents muscle fibre and metabolism is should be considered as the result might be different when repeated using human subject (in vivo)

6.2.2 Future Work

In this thesis, the accumulation of lipids within myotubes was confirmed but still, some questions arise. Why LA induces more lipid deposition than other FAs, tests could be tried such as expression of enzymes involved in FA uptake include FATP4 and CD36. Moreover, the lipids deposited within myotubes were suggested as TAG or cholesterol esters. This hypothesis needs to be confirmed and specific assays can be used such as TAG assay or the use of western blotting to determine the amount of protein. As the lipid deposition by LA was confirmed, it is not clear whether this accumulation of lipids within muscle fibers is implicated in an alteration of muscle metabolism. Measure the cells' rate of oxygen consumption by (real-time Seahorse Analyser) to indicate the normal cell function.

The mechanism involved in the insulin-stimulation of glucose uptake alteration in the LA treatment is not understood yet. Thus, further examination of the specific mechanism is highly recommended and should be a focus of future work. A mechanistic study could be done on the insulin pathway that related to the GLUT4 translocation such as PI3K phosphorylation. PI3K is a principal protein implicated in GLUT4 translocation regulated by insulin (Kanai et al., 1993). Furthermore, if the insulin pathway such as the phosphorylation of PI3K was affected by the LA treatment, this would confirm that the lipids are implicated in the reduction of insulin-stimulated glucose uptake.

The results of this thesis showed that the polyphenols EGCG had significantly decreased the lipid deposition and also improved the insulin-stimulation glucose uptake compared to the LA treatment. Again, the exact mechanism involved is not clear and further examination is required and should be a direct objective of future

work. As mentioned that the mechanism of LA increased lipid deposition within myotubes might be as a result increased FAs uptake by muscle cells. Thus, a study comparing the enzymes involved in FA uptake such as FATP could be tried to determine whether the EGCG implicated in blocking the LA mediate FA uptake. Additionally, This thesis indicated that EGCG inhibited the lipid deposition within myotubes when co-treated with LA compared to the LA alone. However, it is not clear whether EGCG can reduce lipids that already accumulated within muscle fibres. In vivo animal study with LA/high-fat diets +/- EGCG then muscle biopsies taking to investigate the effect of EGCG on deposited lipids. Understanding the exact mechanism opens new chances to maintain or decrease the occurrence of the IR which therefore leads to the improvement of T2DM patients.

Chapter 7:

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7 References

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