THE DIFFERENTIATION EFFECT OF ATORVASTATIN AND ROSUVASTATIN ON MITOCHONDRIAL FUNCTION IN L6 MUSCLE CELLS

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by

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DECLARATION

I hereby declare that this dissertation is the result of my own investigations, except where otherwise stated and duly acknowledged. I also declare that it has not been previously or concurrently submitted as a whole for any other degrees at Universiti Sains Malaysia or other institutions. I grant Universiti Sains Malaysia the right to use the dissertation for teaching, research and promotional purposes.

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Muhammad Azim Haikal Bin Ruslan

Date: 27/01/2025

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TABLE OF CONTENTS

LIST OF EQUATIONS

LIST OF FIGURES

LIST OF TABLES

LIST OF ACRONYMS, ABBREVIATIONS AND SYMBOLS

ATP	Adenosine Triphosphate
CHC	α-cyano-4-hydroxycinnamate
CI	Confidence Interval
CVDs	Cardiovascular Diseases
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
DM	Diabetes Mellitus
DMD	Duchenne muscular dystrophy (DMD)
DMEM	Dulbecco's Modified Eagle Medium
EDTA	Ethylenediaminetetraacetic Acid
ETC	Electron Transport Chain
FBS	Fetal Bovine Serum
HMG-CoA	3-hydroxy-3-methylglutaryl coenzyme A
IC_{50}	Half-Maximal Inhibitory Concentration
LDH	Lactate Dehydrogenase
MCT	Monocarboxylate Transporter
MCT1	Monocarboxylate Transporter 1
mtDNA	Mitochondrial DNA
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide
NO	Nitric Oxide
NRF	Nuclear Respiratory Factor
NT	Non-treated
OXPHOS	Oxidative Phosphorylation
PBS	Phosphate-Buffered Saline
PGC-1α	Peroxisome Proliferator-Activated Receptor Gamma Coactivator 1-Alpha
qPCR	Quantitative Polymerase Chain Reaction
RD	Rhabdomyosarcoma
ROS	Reactive Oxygen Species
SOD	Superoxide Dismutase
SRM	Statin-Related Myopathy
TCA	Tricarboxylic Acid
TFAM	Transcription Factor A, Mitochondrial

LIST OF APPENDICES

PERBEZAAN KESAN ATORVASTATIN DAN ROSUVASTATIN TERHADAP FUNGSI MITOKONDRIA DALAM SEL OTOT L6

ABSTRAK

Statin, yang banyak digunakan untuk menguruskan hiperlipidemia dan mengurangkan risiko kardiovaskular, dikaitkan dengan kesan buruk pada sel otot rangka, berkemungkinan disebabkan oleh disfungsi mitokondria. Kesan statin pada sel otot berbeza bergantung kepada sifat keterlarutannya. Kajian ini membandingkan kesan antara atorvastatin (statin lipofilik) dan rosuvastatin (statin hidrofilik) terhadap daya hidup (ujian MTT) dan fungsi mitokondria dalam sel otot rangka L6. Berdasarkan ujian MTT, atorvastatin menunjukkan sitotoksisiti yang lebih tinggi dengan nilai IC_{50} sebanyak 160.0 μM (95% CI: 136.6–201.7) berbanding rosuvastatin (IC_{50} > 200 μM). Ujian laktat dehidrogenase (LDH) menilai fungsi mitokondria, dengan sel yang dirawat dengan atorvastatin dan rosuvastatin menunjukkan kesan yang setanding pada pengekalan LDH dengan α-cyano-4-hydroxycinnamate (CHC), inhibitor MCT1 tidak spesifik. Aktiviti LDH berbanding sel yang tidak dirawat (NT) adalah jauh lebih tinggi dalam sel yang dirawat dengan CHC ($p < 0.0001$), atorvastatin ($p < 0.0001$), dan rosuvastatin ($p < 0.0001$). Aktiviti LDH juga dipengaruhi oleh bilangan peringkat sel di mana bilangan peringkat sel yang lebih tinggi menghasilkan perbezaan min yang signifikan dalam tahap LDH (masing-masing $p < 0.05$ dan $p < 0.01$). Penemuan ini mencadangkan bahawa statin lipofilik mempunyai kesan yang lebih ketara terhadap daya hidup sel L6 berbanding statin hidrofilik; namun, kedua-dua jenis statin mempengaruhi fungsi mitokondria pada tahap yang sama melalui modulasi MCT1. Kajian ini menyerlahkan akibat potensial kedua-dua statin memodulasi MCT1 dalam menyebabkan kesan terhadap fungsi mitokondria.

THE DIFFERENTIATION EFFECT OF ATORVASTATIN AND ROSUVASTATIN ON MITOCHONDRIAL FUNCTION IN L6 MUSCLE CELLS

ABSTRACT

Statins, widely prescribed for managing hyperlipidemia and reducing cardiovascular risks, are associated with adverse effects on skeletal muscle cells, potentially due to mitochondrial dysfunction. The effects of statins on muscle cells vary due to their solubility properties. This study compares the differential effects of atorvastatin (lipophilic statin) and rosuvastatin (hydrophilic statin) on the viability (MTT assay) and the mitochondrial function in L6 skeletal muscle cells. Using MTT assays, atorvastatin exhibited greater cytotoxicity with an IC_{50} value of 160.0 μM (95% CI: 136.6–201.7) compared to rosuvastatin (IC_{50} > 200 μM). Lactate dehydrogenase (LDH) assays assessed mitochondrial function, with cells treated with atorvastatin and rosuvastatin exhibiting a comparable effect on LDH retention with α-cyano-4-hydroxycinnamate (CHC), a non-specific MCT1 inhibitor. Specifically, the LDH activity compared to non-treated (NT) cells was significantly higher in CHC-treated cells ($p < 0.0001$), atorvastatin-treated cells ($p < 0.0001$), and rosuvastatin-treated cells ($p < 0.0001$). LDH activity was also found to be affected by cells passage number resulting in significant mean differences in the LDH levels ($p < 0.05$ and $p < 0.01$, respectively). These findings suggest that lipophilic statin exerts a more pronounced effect on L6 cells viability than hydrophilic statin; however, both types of statins affect mitochondrial function to a similar degree with MCT1 modulation. The study highlights the potential consequences of both statins modulating MCT1 in causing an effect on mitochondrial function.

CHAPTER 1

INTRODUCTION

1.1 Research Background

Statins are widely prescribed medications that lower blood cholesterol levels by inhibiting 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, a key enzyme in cholesterol biosynthesis (Pereira et al., 2016). They are categorized into two types based on their solubility: lipophilic statins (e.g., atorvastatin, simvastatin) and hydrophilic statins (e.g., rosuvastatin, pravastatin). While their primary function is to reduce cardiovascular risk, statins can induce side effects, particularly on skeletal muscle cells. These effects, including muscle pain and weakness (myopathy), are attributed to mitochondrial dysfunction (Turner & Pirmohamed, 2019). Peroxisome Proliferator-Activated Receptor Gamma Coactivator 1-Alpha (PGC-1α), a master regulator of mitochondrial biogenesis (Scarpulla, 2011), and ATP5g1(Turner & Pirmohamed, 2019), a key component of ATP synthase for ATP synthesis are significantly affected by statin treatment.

Previous study from our group has demonstrated that lipophilic statins, compared to hydrophilic statins, inhibited the functional expression of monocarboxylate transporter 1 (MCT1), in the L6 rat skeletal muscle model (Bakar et al., 2016). It remains unclear whether the inhibition of statin-related MCT1 function contributes to alterations in mitochondrial pathways, gene regulation, and cellular metabolism in skeletal muscle. Bridging this knowledge gap is essential for optimizing statin therapy to reduce side effects while preserving its therapeutic benefits.

1.2 Research Problem

Although statins are highly effective in reducing cardiovascular risks, their adverse effects on skeletal muscle cells, particularly mitochondrial dysfunction, pose significant challenges. The differential effects of lipophilic and hydrophilic statins on mitochondrial pathways and gene expression are not fully understood. This lack of knowledge prevents the development of strategies to mitigate side effects and optimize the therapeutic potential of statins.

1.3 Research Questions

1. How does treatment with atorvastatin and rosuvastatin affect the viability of L6 muscle myotube cells?
2. What are the effects of statin treatment on mitochondrial function, including LDH production, in L6 muscle myotube cells?
3. How do other parameters, such as statin concentration and cell passage number, affect LDH activity?
4. How does statin treatment affect LDH activity in the presence of CHC, a non-specific MCT1 modulator, in L6 skeletal muscle cells?

1.4 Research Objectives

General Objective

This study aims to investigate the impact of atorvastatin and rosuvastatin on mitochondrial function in L6 muscle myotube cells.

Specific Objectives

1. To assess the impact of statin treatment (atorvastatin and rosuvastatin) on cell viability of L6 muscle myotube cells.
2. To evaluate the impact of statin treatment on mitochondrial function including LDH production in L6 muscle myotube cells.
3. To determine the effects of other parameters (statin concentration and passage number) on the LDH activity in L6 cells.
4. To compare the effects of statin treatment on LDH activity with CHC, a non-specific MCT1 modulator.

1.5 Research Hypotheses

It is hypothesized that atorvastatin will have a higher IC50 value compared to rosuvastatin and will induce greater LDH activity in L6 myotubes, indicating a more pronounced impact on mitochondrial function

Null Hypothesis (H0): There is no significant difference between atorvastatin (lipophilic) and rosuvastatin (hydrophilic) in their effects on cell viability and LDH activity of L6 myotubes cell.

Alternate Hypothesis (H1): Atorvastatin (lipophilic) has a significantly greater impact compared to rosuvastatin (hydrophilic) in their effects on cell viability and LDH activity of L6 myotubes cell.

CHAPTER 2

LITERATURE REVIEW

2.1 Statin

Statins are a class of lipid-lowering drugs widely prescribed to reduce cholesterol levels and prevent cardiovascular diseases (CVDs). Mevastatin (ML-236B), the first agent discovered from *Penicillium citrinum*, was never marketed due to negative side effects. The first licensed statin, lovastatin, was isolated from *Aspergillus terreus* and approved in 1987. Statins are categorized into three generations based on their potency and efficacy: first-generation (lovastatin, pravastatin, fluvastatin), second-generation (simvastatin, atorvastatin), and third-generation (rosuvastatin, pitavastatin) (Climent et al., 2021).

Statin effectiveness and safety have established them as the first-line therapy for managing hyperlipidemia and associated conditions. Statins primarily act by inhibiting HMG-CoA reductase, the rate-limiting enzyme in cholesterol biosynthesis (Turner & Pirmohamed, 2019). However, their effects extend beyond cholesterol reduction, offering additional cardiovascular and systemic benefits known as pleiotropic effects.

2.1.1 Types of Statins

Statins are classified based on their solubility into lipophilic and hydrophilic statins. Lipophilic statins, such as atorvastatin, simvastatin, and lovastatin, easily penetrate lipid membranes, leading to broader tissue distribution, including skeletal muscle and the central nervous system. However, this extensive tissue penetration is associated with an increased risk of muscle-related side effects (Turner & Pirmohamed, 2019). In contrast, hydrophilic statins, such as rosuvastatin and pravastatin, primarily act in the liver due to their selectivity for hepatocytes. Their limited distribution in non-

hepatic tissues makes them less likely to cause muscle-related adverse effects (Bouitbir et al., 2020)

2.1.2 Clinical Uses of Statins

Statins are prescribed primarily for their cholesterol-lowering effects, but their clinical applications extend beyond lipid reduction, making them essential in preventing CVDs and other health conditions. Statins are the first-line therapy for managing hypercholesterolemia and atherosclerosis, as they effectively reduce low-density lipoprotein cholesterol (LDL-C) levels, a major contributor to plaque formation and progression of cardiovascular diseases(Pereira et al., 2016).

In addition to their lipid-lowering effects, statins exert pleiotropic effects, which are beneficial in reducing cardiovascular events beyond merely lowering cholesterol levels. They improve endothelial function by enhancing nitric oxide (NO) production in endothelial cells, leading to better vasodilation and improved blood flow, which helps prevent endothelial dysfunction, a key early event in atherosclerosis (Hargreaves & Spriet, 2020). Additionally, statins reduce inflammation by lowering C-reactive protein (CRP) levels and decreasing the activity of pro-inflammatory cytokines. This anti-inflammatory effect plays a crucial role in slowing the progression of atherosclerosis and stabilizing plaques, reducing the risk of rupture and thrombosis (Turner & Pirmohamed, 2019). Furthermore, statins exert antioxidant effects by increasing the activity of antioxidant enzymes while reducing the production of reactive oxygen species (ROS), which are implicated in vascular damage and atherosclerosis (Bouitbir et al., 2020).

2.1.3 Mechanisms of Action of Statins

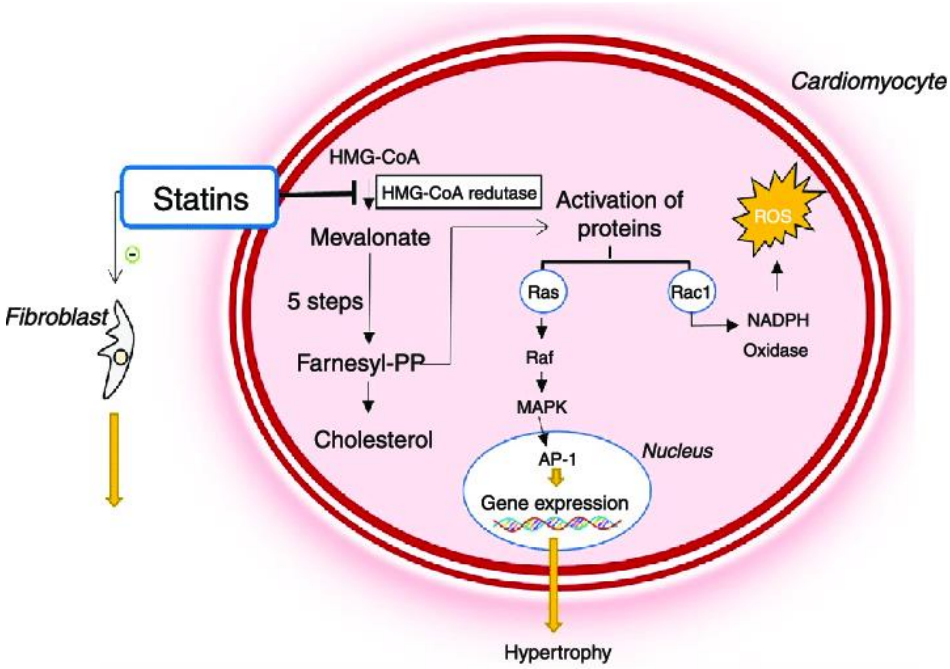

Figure 2.1 Mechanism of action of statins

Statins work primarily by inhibiting HMG-CoA reductase, an enzyme crucial for the synthesis of cholesterol in the liver. This enzyme catalyzes the conversion of HMG-CoA to mevalonate, an early step in the biosynthesis of cholesterol. By blocking this enzyme, statins effectively lower the production of cholesterol within the liver. The decrease in intracellular cholesterol levels stimulates an increase in LDL receptors on hepatocytes, leading to enhanced clearance of LDL cholesterol (LDL-C) from the bloodstream (Pereira et al., 2016).

2.1.4 Potential Side Effects of Statins

Statins, while widely used and effective in lowering cholesterol and reducing cardiovascular risks, are associated with several potential side effects, particularly with long-term use. One of the most common adverse effects is statin-associated myopathy (SAM), which is characterized by muscle pain, weakness, and, in severe cases, rhabdomyolysis. Lipophilic statins, such as atorvastatin, are more likely to cause

myopathy due to their ability to penetrate muscle cells, increasing the risk of mitochondrial dysfunction and energy deficits in muscle tissue (Turner & Pirmohamed, 2019).

Additionally, hepatotoxicity can occur in rare cases, leading to elevated liver enzymes. While severe liver damage is uncommon, it is still advisable to monitor liver function regularly during statin therapy (Bouitbir et al., 2020). Statins have also been linked to an increased risk of type 2 diabetes, particularly in individuals who are already at high risk for the condition. This effect is thought to arise from changes in glucose metabolism, although the exact mechanisms remain unclear (Pereira et al., 2016). Moreover, some patients may experience neurological effects, such as memory loss or cognitive decline, though evidence linking statins to cognitive impairment remains inconclusive. To minimize these risks, healthcare providers often adjust dosages, switch to hydrophilic statins, or explore alternative treatments when necessary. Understanding and managing these side effects is crucial to improving patient adherence and ensuring the overall safety and effectiveness of statin therapy.

2.2 Mitochondrial

Mitochondria are critical organelles in muscle cells, supporting cellular energy production, signaling, and homeostasis. They play a pivotal role in maintaining muscle functionality and adaptability to metabolic demands, especially under stress or pathological conditions (Hood et al., 2020). This section delves into the key aspects of mitochondrial structure, energy production, biogenesis, dynamics, and roles in apoptosis, oxidative stress, and muscle-related disorders.

2.2.1 Structure and Function of Mitochondria

Mitochondria are double-membrane-bound organelles that harbor a unique structure enabling them to perform energy-intensive cellular functions. The outer membrane contains porins, which regulate the transport of molecules, while the inner membrane forms cristae to house the electron transport chain (ETC) and ATP synthase complexes. The mitochondrial matrix harbors enzymes essential for the tricarboxylic acid (TCA) cycle, where substrates are oxidized to generate reducing equivalents, namely NADH and $FADH_2$. These intermediates fuel the ETC, making mitochondria the powerhouse of the cell (Hood et al., 2019).

2.2.1.1 Mitochondrial Role in Cellular Energy Production

Mitochondria are the powerhouses of the cell, producing adenosine triphosphate (ATP) through oxidative phosphorylation. This process occurs within the inner mitochondrial membrane, where electrons from NADH and $FADH_2$ traverse the electron transport chain, culminating in ATP synthesis. This efficient energy production mechanism is essential for muscle function, particularly during sustained or intense activity. Impairments in this process can lead to muscle fatigue and dysfunction, as seen in various mitochondrial dysfuntion (Brooks, 2009; Zhou et al., 2016).

Beyond ATP production, mitochondria play a crucial role in regulating the balance between energy demand and supply. During exercise, mitochondrial activity dynamically increases to meet the heightened energy requirements of skeletal muscles. This is achieved through the upregulation of oxidative phosphorylation and substrate oxidation pathways. Additionally, mitochondria serve as metabolic hubs, integrating signals from nutrients and hormones to optimize cellular energy production and sustain physiological function (Brand et al., 2013). Dysregulation of this system has been implicated in

metabolic diseases, including diabetes and obesity, which are often associated with mitochondrial dysfunction.

2.2.1.2 Mitochondrial Biogenesis and Dynamics

Mitochondrial biogenesis refers to the synthesis of new mitochondria, a process regulated by peroxisome proliferator-activated receptor-gamma coactivator 1-alpha (PGC-1α). PGC-1α activates nuclear respiratory factors (NRF1 and NRF2), which enhance the expression of mitochondrial transcription factor A (TFAM) and the replication of mitochondrial DNA (mtDNA) (Scarpulla, 2011). Dynamic processes like fusion and fission maintain mitochondrial quality. Fusion, mediated by mitofusins (MFN1/2) and optic atrophy 1 (OPA1), ensures the exchange of mitochondrial content, while fission, regulated by dynamin-related protein 1 (DRP1), facilitates the segregation of damaged mitochondria for autophagic degradation (Hood et al., 2019).

The coordination between biogenesis and dynamics is vital for cellular adaptation to metabolic stress. For instance, during endurance exercise, increased PGC-1α expression drives mitochondrial biogenesis, enhancing the oxidative capacity of muscle fibers. Simultaneously, mitochondrial dynamics ensure the removal of dysfunctional mitochondria through mitophagy, preserving cellular homeostasis (Youle & Van Der Bliek, 2012). Aberrations in these processes are associated with aging and diseases like neurodegeneration and cardiomyopathies.

2.2.1.3 Mitochondrial Role in Apoptosis and Cell Survival

Mitochondria play a central role in regulating apoptosis and cell survival through the release of pro-apoptotic factors such as cytochrome c, which triggers the caspase cascade. Additionally, mitochondrial dysfunction often leads to dysregulated cell death, contributing to the pathology of various muscle disorders. Calcium overload within the

mitochondria is a critical mediator of this process, influencing mitochondrial permeability transition and promoting apoptosis (Zhou et al., 2016).

Furthermore, the crosstalk between mitochondria and the endoplasmic reticulum (ER) is integral to maintaining calcium homeostasis. Disruption in this communication leads to excessive calcium influx into mitochondria, triggering permeability transition pore opening and subsequent cell death. This mechanism is particularly relevant in ischemia-reperfusion injury, where mitochondrial calcium overload exacerbates tissue damage. Therapeutic strategies targeting mitochondrial calcium regulation hold promise for mitigating apoptosis in pathological conditions (Giorgi et al., 2018).

2.2.1.4 Impact of Oxidative Stress on Mitochondria

Oxidative stress arises from an imbalance between the production of reactive oxygen species (ROS) and the cell's ability to detoxify these reactive intermediates. Mitochondria are particularly susceptible to oxidative damage due to their central role in energy production and high ROS generation rates during oxidative phosphorylation. Excessive ROS can impair mitochondrial membranes, proteins, and DNA, leading to dysfunction and apoptosis. In skeletal muscle, this contributes to reduced functionality, a hallmark in disorders such as sarcopenia and myopathies (Chen et al., 2022).

The accumulation of oxidative damage over time is a key factor in the aging process and age-related decline in muscle function. Mitochondria employ several antioxidant defense mechanisms, including superoxide dismutase (SOD) and glutathione peroxidase (GPx), to mitigate ROS damage. Enhancing these antioxidant pathways through dietary or pharmacological interventions has shown potential in preserving mitochondrial function and improving muscle health in aging populations (Pham-Huy et al., 2008). Additionally, interventions like caloric restriction and exercise are known to

attenuate oxidative stress and promote mitochondrial resilience, highlighting their role in maintaining cellular health.

2.2.2 Mitochondrial Dysfunction in Muscle Disorders

Mitochondrial dysfunction is implicated in a variety of muscle disorders, particularly those that manifest as muscle weakness, atrophy, and fatigue. Mitochondrial myopathies, caused by mutations in mitochondrial DNA (mtDNA) or nuclear genes that affect mitochondrial function, result in impaired oxidative phosphorylation, leading to insufficient ATP production. This dysfunction is exacerbated by abnormal calcium handling, where excessive calcium within mitochondria triggers the opening of the mitochondrial permeability transition pore, promoting cell death. Additionally, dysregulated mitochondrial dynamics, including impaired fusion and excessive fragmentation, contribute to the pathogenesis of disorders like Duchenne muscular dystrophy (DMD) and age-related sarcopenia. The inability of damaged mitochondria to undergo proper mitophagy also exacerbates oxidative stress, leading to further muscle degeneration and reduced muscle regenerative capacity (Chen et al., 2022).

2.3 Effects of Statins on Mitochondrial Function

Statins, as inhibitors of HMG-CoA reductase, primarily reduce cholesterol synthesis, but their effects extend beyond lipid regulation. Emerging evidence shows significant interactions between statins and mitochondrial function, potentially explaining their myotoxic side effects. Mitochondrial dysfunction is implicated in statin-induced myopathy, a common adverse effect, characterized by reduced cellular energy, oxidative stress, and altered enzyme activity.

Furthermore, research suggests that the lipophilicity of statins plays a critical role in their impact on mitochondria. Lipophilic statins, such as atorvastatin, are more readily taken up by non-hepatic cells, including skeletal muscle cells, where they may impair mitochondrial respiration by inhibiting complexes of the electron transport chain (ETC) (Bouitbir et al., 2012). This can lead to decreased ATP production and increased reactive oxygen species (ROS), further contributing to oxidative stress and cellular damage (Schirris et al., 2015). Hydrophilic statins, like rosuvastatin, are less likely to cross cell membranes, potentially resulting in a lower risk of mitochondrial dysfunction. These differences underscore the importance of statin selection and dosing in minimizing adverse effects while maintaining therapeutic efficacy.

2.3.1 Mitochondrial Respiration

Statins disrupt mitochondrial respiration by inhibiting electron transport chain (ETC) complexes. Notably, atorvastatin reduces PGC-1α expression, affecting biogenesis (Bouitbir et al., 2012). Similarly, Schirris et al. (2015) found that statin lactones inhibit mitochondrial complex III, reducing ATP synthesis and increasing ROS production.

One of the most notable findings is the inhibition of mitochondrial complex III (CIII) by statins, particularly the lactone forms, which inhibit CIII activity by up to 84%

in muscle cells (Schirris et al., 2015). This inhibition of CIII, a critical enzyme in the mitochondrial ETC, impairs ATP production and contributes to the muscular toxicity observed in statin-treated patients. The reduced CIII activity in statin-treated skeletal muscle has been confirmed in muscle biopsies from patients suffering from statin-induced myopathy, where a reduction of 18% in CIII activity was documented.

2.3.2 ATP Production

Reduced mitochondrial respiration directly affects ATP synthesis. Bouitbir et al. (2012) linked atorvastatin-induced mitochondrial dysfunction with decreased ATP production in skeletal muscle, correlating this with impaired oxidative phosphorylation capacity.

Similarly, Larsen et al. (2013) found that statin treatment reduced ATP content in skeletal muscle. This reduction is attributed to the reduction in oxidative phosphorylation (OXPHOS) capacity in skeletal muscle. In particular, long-term treatment with simvastatin results in a decrease in the content of Coenzyme Q10 (CoQ10), an essential component of the electron transport chain (ETC), which directly impacts ATP synthesis (Larsen et al., 2013)

Despite the reduction in mitochondrial function, mitochondrial content within the skeletal muscle does not significantly change, indicating that the observed decrease in ATP production is not due to a reduction in the number of mitochondria, but rather to a decrease in their functional efficiency. This reduction in ATP synthesis leads to a compromised energetic state within the muscle cells, contributing to myalgia (muscle pain) and exercise intolerance, which are common side effects of statin therapy (Larsen et al., 2013)

2.3.3 Statins and Reactive Oxygen Species (ROS)

Statins, while beneficial for reducing cholesterol levels, have been shown to induce an increase in reactive oxygen species (ROS), particularly in muscle cells. The elevation in ROS production is thought to be associated with the impaired mitochondrial function that results from statin treatment. Specifically, simvastatin has been found to reduce the efficiency of mitochondrial respiration and oxidative phosphorylation (OXPHOS), which leads to an increase in ROS production as a byproduct of incomplete mitochondrial respiration (Larsen et al., 2013).

This increased ROS generation may contribute to the oxidative stress observed in statin-treated patients, leading to cellular damage and potentially exacerbating side effects such as muscle pain (myalgia) and exercise intolerance. ROS can damage mitochondrial proteins, lipids, and DNA, further impairing mitochondrial function and leading to a vicious cycle of mitochondrial dysfunction (Bouitbir et al., 2012).

2.3.4 Influence of Statins on Mitochondrial Enzyme Activities

Statins have been implicated in altering mitochondrial enzyme activities, with significant effects on the function of the electron transport chain (ETC) and related antioxidant systems. This decrease in enzyme activity disrupts mitochondrial function, leading to an overall reduction in oxidative phosphorylation capacity. Additionally, statins appear to trigger mitochondrial calcium leak and impair sarcoplasmic reticulum calcium cycling, further compromising cellular energy homeostasis and contributing to the onset of muscle damage (Schirris et al., 2015).

Statins also affect antioxidant enzymes involved in maintaining mitochondrial integrity. In cardiac muscle, statin treatment has been shown to increase the expression of SOD1 and SOD2, key antioxidant enzymes that help protect against reactive oxygen

species (ROS). However, in skeletal muscle, excessive ROS production induced by statins may overwhelm the antioxidant defenses, leading to oxidative stress, mitochondrial dysfunction, and muscle cell apoptosis (Bouitbir et al., 2012).

Additionally, Sirvent et al. (2008) reviewed the mechanisms underlying statin-induced myopathies, noting that the inhibition of mitochondrial enzymes, such as citrate synthase, may be implicated in statin-associated side effects (Sirvent et al., 2008).

2.4 Monocarboxylate Transporters

Monocarboxylate transporters (MCTs) are a group of transmembrane proteins belonging to the SLC16 family, responsible for the proton-linked transport of monocarboxylates such as lactate, pyruvate, and ketone bodies across cellular membranes. Of the 14 identified MCT isoforms, only MCT1–4 have been confirmed to exhibit proton-dependent activity (Halestrap, 2012). These transporters play a pivotal role in maintaining pH balance, facilitating metabolic flux, and supporting intercellular communication, particularly in tissues with high metabolic activity.

The tissue-specific expression of MCT isoforms reflects their specialized functions. MCT1 is ubiquitously expressed and supports the uptake of lactate in oxidative tissues such as the heart and red skeletal muscle, enabling lactate utilization in the tricarboxylic acid cycle. Conversely, MCT4 is predominantly expressed in glycolytic tissues, including white skeletal muscle fibers and astrocytes, where it mediates lactate efflux to prevent intracellular acidosis and ensure sustained glycolysis (Halestrap, 2012). The functionality of these transporters depends on their association with glycosylated ancillary proteins, such as basigin (CD147) or embigin, which are essential for their plasma membrane localization and activity (Halestrap, 2012).

2.4.1 Function of Monocarboxylate Transporters

MCTs serve as key regulators of intracellular and extracellular lactate levels, ensuring efficient energy metabolism and cellular homeostasis. MCT1 facilitates the uptake of lactate into oxidative cells, where it is converted to pyruvate and enters the tricarboxylic acid cycle for ATP production. This process underscores the importance of MCT1 in oxidative tissues, which rely on lactate as a significant metabolic substrate (Halestrap, 2012).

On the other hand, MCT4 is adapted for lactate efflux in cells with high glycolytic activity, such as white muscle fibers. Its lower affinity for lactate compared to MCT1 is well-suited to environments with elevated lactate concentrations, enabling efficient transport and preventing intracellular acidification. This lactate export supports glycolytic metabolism and pH balance under hypoxic or anaerobic conditions (Halestrap, 2012). The ability of MCT isoforms to function in tandem across different tissues forms the basis of the "lactate shuttle," a mechanism that allows lactate generated in glycolytic cells to be utilized as a fuel in oxidative cells (Halestrap, 2012).

2.4.2 Association with Mitochondrial Function

Monocarboxylate transporters contribute indirectly to mitochondrial function by regulating the availability of pyruvate and lactate, which are critical substrates for mitochondrial oxidative phosphorylation. MCT1 plays a central role in this process by importing lactate into cells that rely on oxidative metabolism. Once inside the cell, lactate is converted to pyruvate, which is transported into mitochondria for energy production via the tricarboxylic acid cycle (Halestrap, 2012).

This metabolic coupling between glycolytic and oxidative pathways, mediated by MCTs, supports energy efficiency and flexibility, especially under conditions of

fluctuating oxygen availability. The activity of MCTs ensures a continuous supply of metabolic intermediates to mitochondria, optimizing ATP production and reducing the accumulation of metabolic byproducts such as lactate.

2.4.3 Monocarboxylate Transporter 1

Monocarboxylate transporter 1 (MCT1) is a member of the solute carrier (SLC) family of transporters, specifically SLC16A1. MCT1 is responsible for the bidirectional transport of monocarboxylates, such as lactate, pyruvate, and ketone bodies, across cellular membranes. This transporter plays a crucial role in cellular metabolism, particularly in maintaining the balance of lactate production and utilization.

MCT1 is highly expressed in tissues with high metabolic rates, including skeletal muscle, cardiac muscle, and the brain. In skeletal muscle, MCT1 facilitates lactate uptake, which can be oxidized by mitochondria to produce ATP. By shuttling lactate between cells, MCT1 supports the Cori cycle and ensures efficient energy production during intense physical activity (Halestrap, 2012).

MCT1 function is closely linked to mitochondrial activity. Increased lactate transport by MCT1 can enhance mitochondrial respiration, particularly during aerobic metabolism. Conversely, impairments in MCT1 expression or function can disrupt lactate homeostasis, contributing to metabolic dysfunction. Recent evidence also suggests that MCT1 expression may be regulated by statins, which can alter mitochondrial metabolism and lactate transport. Statin-induced myopathy, characterized by muscle pain and fatigue, may involve disruptions in MCT1 function, further highlighting its importance in maintaining mitochondrial health and energy homeostasis (Halestrap, 2012).

2.4.4 Statin Effects on Monocarboxylate Transporters

Statins have been shown to influence MCT activity, particularly that of MCT4. Lipophilic statins, including atorvastatin and simvastatin, inhibit MCT4-mediated lactate transport in a dose-dependent manner, which can disrupt muscle homeostasis by impairing lactate efflux. This inhibition has been implicated in the development of statin-induced myopathy, a known adverse effect of statin therapy (Kikutani et al., 2016).

In vitro studies using Rhabdomyosarcoma (RD) cells, a model of skeletal muscle, demonstrated that atorvastatin significantly reduced cell viability and simultaneously upregulated MCT4 expression. This suggests a compensatory mechanism aimed at maintaining lactate transport despite the inhibitory effects of statins. Furthermore, knockdown of MCT4 in these cells reduced statin-induced cytotoxicity, confirming the role of MCT4 in mediating these effects. This interaction highlights the dual role of MCTs in maintaining metabolic balance and contributing to drug-induced side effects (Kikutani et al., 2016).

2.5 Genes Involve in The Mitochondrial Pathways

Understanding how statins target mitochondrial pathways can help in developing strategies to mitigate their side effects. Mitochondrial function is intricately regulated by various genes that influence energy metabolism, ROS production, and overall cellular homeostasis. Key among these are UCP2, PGC-1α, and ATP5G1

2.5.1 Uncoupling Protein 2

UCP2 is a mitochondrial protein that plays a role in regulating energy efficiency and ROS production. Ucp2 dissipates the proton gradient across the mitochondrial membrane, reducing ATP synthesis efficiency and limiting the production of ROS. This

protective mechanism helps prevent oxidative stress-induced damage in mitochondria (Bouillaud et al., 2016).

In addition to its role in mitigating oxidative stress, UCP2 has been implicated in regulating energy metabolism, particularly during conditions of high metabolic demand or oxidative stress. UCP2 expression is upregulated in various tissues in response to stimuli like high-fat diets, hypoxia, and increased ROS levels. This adaptive response helps maintain cellular redox balance and protect against damage from oxidative stress. Moreover, UCP2 is thought to influence mitochondrial calcium homeostasis and apoptotic pathways, making it critical for maintaining mitochondrial integrity (Kumar et al., 2022).

Emerging research suggests that UCP2 also plays a role in metabolic disorders, including diabetes and obesity, by modulating glucose and lipid metabolism. Its dysregulation has been associated with increased mitochondrial dysfunction and chronic metabolic diseases. These findings underscore the importance of UCP2 as a therapeutic target for conditions involving mitochondrial dysfunction and oxidative stress (de Oliveira Bristot et al., 2019).

2.5.2 Peroxisome Proliferator-Activated Receptor Gamma Coactivator 1-Alpha

Peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) is a key regulator of mitochondrial biogenesis and energy metabolism. PGC-1α interacts with various nuclear transcription factors, such as NRF1 and NRF2, to activate the expression of genes involved in oxidative phosphorylation and mitochondrial DNA replication. This coactivator also stimulates the production of enzymes that combat oxidative stress, thereby enhancing mitochondrial function and cellular resilience (Liang & Ward, 2006).

In skeletal muscle, PGC-1α expression increases in response to exercise, fasting, and other energy-demanding conditions, emphasizing its role in adapting to metabolic stress. By regulating mitochondrial biogenesis, PGC-1α enhances ATP production and supports endurance capacity in muscle tissue. Beyond its role in energy metabolism, PGC-1α also modulates other cellular processes, including thermogenesis in brown adipose tissue, lipid metabolism, and ROS detoxification (de Oliveira Bristot et al., 2019).

Mutations or downregulation of PGC-1α are associated with metabolic syndromes, neurodegenerative diseases, and mitochondrial myopathies. Statins, which are known to disrupt mitochondrial function, have been reported to affect PGC-1α levels, further linking this coactivator to mitochondrial health and its regulation in pharmacological contexts.

2.5.3 ATP5G1

ATP synthase membrane subunit c locus 1 (ATP5G1) is a critical subunit of the mitochondrial ATP synthase complex (Complex V) that is responsible for ATP production. ATP synthase couples the proton gradient generated by the electron transport chain (ETC) with ATP synthesis, making it a central player in cellular energy metabolism. ATP5G1 forms part of the proton-conducting F0 region of ATP synthase, essential for the rotation mechanism that drives ATP synthesis (de Oliveira Bristot et al., 2019).

Proper functioning of ATP5G1 is vital for maintaining cellular energy levels and overall mitochondrial health. Dysregulation of ATP5G1 can impair ATP production, leading to energy deficits and increased susceptibility to mitochondrial dysfunction. This has been implicated in various pathologies, including neurodegenerative diseases, cancer, and metabolic disorders.

Statins can impair ATP5g1 expression by disrupting mitochondrial electron transport chain function and reducing coenzyme Q10 levels. This downregulation leads to decreased ATP production, which can impair muscle cell contraction and endurance (Turner & Pirmohamed, 2019). The impact of statins on ATP5g1 may be a key factor in understanding their adverse effects on mitochondrial energy metabolism in L6 muscle cells.

2.5.4 Statin Effects on Key Mitochondrial Genes

Studies have shown that statins can downregulate the expression of PGC-1α, a master regulator of mitochondrial biogenesis, thereby impairing mitochondrial replication and function in skeletal muscle cells (de Oliveira Bristot et al., 2019). Reduced PGC-1α activity has been associated with diminished oxidative capacity and increased susceptibility to muscle fatigue. Similarly, statins have been found to affect ATP5G1, a subunit of ATP synthase, potentially disrupting ATP production and contributing to energy deficits in muscle cells (Bouitbir et al., 2012).

The role of UCP2, which regulates ROS production and mitochondrial efficiency, is also impacted by statins. Increased oxidative stress due to altered UCP2 expression may exacerbate mitochondrial dysfunction and muscle damage (Yang et al., 2018). These findings underscore the multifaceted effects of statins on mitochondrial gene regulation, providing insights into their potential mechanisms of toxicity in skeletal muscle cells.

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

Table 3.1 List of chemical and reagents.

Chemical and Reagents	Manufacturer
Atorvastatin	Glentham life sciences
Dimethyl Sulfoxide (DMSO)	HmbG
Distilled Water	-
Double Distilled Water	-
Ethanol	HmbG
Fetal Bovine Serum (FBS)	HyClone Laboratories Inc.
Horse Serum	Gibco,
L6 Rat Muscle Cell Line	American Type Culture Collection (ATCC)
LDH assay kit	Elabscience Biotechnology Inc.
Low-Glucose Dulbecco's Modified Eagle Medium (DMEM)	Pricella Biotechnology
Methanol	HmbG
MTT Powder	Sigma-Aldrich
Penicillin-Streptomycin (PenStrep) Antibiotics	HyClone Laboratories Inc.
Phosphate-Buffered Saline (PBS)	Vivantis Technologies Sdn Bhd
Rosuvastatin	Glentham life sciences
Trypan blue	Cytiva
Trypsin	Cytiva

Table 3.2 List of consumables.

Consumables	Manufacturer
0.22 µm Syringe Filter	Biologix
12-Well Plates	Biologix
96-Well Plates	Biologix
Aluminium Foil	-
Countess cell counting chamber slide	Fisher Scientific Inc.
Falcon tubes (15 mL, 50 mL)	Biologix
Microcentrifuge Tubes	SPL Life sciences
Parafilm	Bemis
Pipette Tips (10 µL, 200 µL,1000 µL)	Biologix
Syringe (10 ml, 50 ml)	Ciringe
T25 Cell Culture Flask	Biologix
Tissue Paper	-

Table 3.3 List of laboratory apparatus and equipment.

Laboratory apparatus and equipment	Manufacturer
Analytical Balance	Shimadzu
Biosafety Cabinet Level II	ESCO
Centrifuge	Hettich
Centrifuge (Refrigerated)	Hettich
CO2 Incubator	ESCO
Countess Automated Cell Counter	Thermo Fisher Scientific Inc.
Deep Freezer -80°C	Ilshin
Inverted Microscope	Leica
Microcentrifuge tubes	Hettich
Micropipette (10 µL, 200 µL,1000 µL)	Gilson
Microplate Reader	Tecan
Multichannel Pipette (200 µL)	IKA
Orbital Shaker	-
Sonicator	Thermo Fisher scientific Inc.

Table 3.4 List of computer application, and software.

Programs/software	Manufacturer
GraphPad Prism 10	GraphPad Software Inc.
Microsoft office	Microsoft Corp.

3.2 Cell Culture and Differentiation of L6 Rat Muscle Cells

The L6 rat muscle cell line (L6-CRL 1458^{TM}) was obtained from the American Type Culture Collection (ATCC, USA). These cells were cultured in a growth medium consisting of low-glucose Dulbecco's Modified Eagle Medium (DMEM) (Pricella Biotechnology, China) supplemented with 10% fetal bovine serum (FBS) (HyClone Laboratories Inc., USA) and 1% penicillin-streptomycin (PenStrep) antibiotics (HyClone Laboratories Inc., USA).

For subculturing, once the cells reached 80%-90% confluency—typically within 3-4 days in a T25 —they were passaged using 0.25% trypsin – 0.53 mM EDTA solution and diluted in growth media. The L6 cells were then seeded at specific densities depending on the assay. For the cell viability assays, cells were diluted to 5×10^4 cells/mL and plated in a 96-well plate. For the LDH activity and glucose assay, the cells were seeded at 1×10^5 cells/mL in a 12-well plate.

Once the myoblasts reached 60%-70% confluency within 2-3 days, differentiation was induced by replacing the growth medium with differentiation medium, which contained low-glucose DMEM supplemented with 2% horse serum (Gibco, USA) and 1% PenStrep antibiotics. The differentiation medium was changed every two days until myotube formation was observed. Myotube formation (Figure 3.1B) was confirmed through morphological observation under a light microscope. The cells were maintained