The effects of synthetic cannabidiol on skeletal muscle development *in vitro*

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Abstract

Cannabidiol (CBD) is a non-intoxicating cannabinoid derived from the *Cannabis sativa* plant. CBD is becoming an increasingly popular treatment for several clinical conditions and in pain management. Consequently, this has attracted the attention of both clinical and non-clinical populations, including athletes. As of 2018, CBD is no longer prohibited by the World Anti Doping Agency (WADA) as it doesn't represent an actual or potential health risk to athletes and evades violating the spirit of sport, two of the criteria any substance must satisfy to be prohibited for use by WADA. Due to this, there has been an exponential increase in the usage of CBD products by athletes, however, little is still known about its potential benefits in this population. One major issue that arises with athletes using CBD is products which state they are free from any other cannabinoids, including THC, often still contain high concentrations of THC, with the urinary threshold for THC at 150 ng/ml , thus resulting in an Anti-Doping Rule Violation (ADRV). This is where synthetic cannabidiol (sCBD) may be a favourable alternative use to CBD for athletes. sCBD is an active pharmaceutical ingredient that is chemically identical to the naturally produced hemp-derived CBD without any other cannabinoids or chemicals present, therefore theoretically it should possess similar properties.

This thesis aimed to investigate the effects of synthetic cannabidiol on skeletal muscle development and growth by treating murine C_2C_{12} muscle cells with varying doses of sCBD at different stages of myogenesis to assess cell viability and myotube morphology. The main findings from this work suggest that high concentrations of sCBD are not tolerated by C_2C_{12} cells and cause cell death in both myoblasts and myotubes. Preliminary findings demonstrated higher doses of sCBD at 50 µM ($P = <0.0001$) and 20 µM ($P = <0.0008$) are detrimental to myoblast health as metabolic activity was reduced and cell viability was reduced at 50 µM ($P = <0.0363$) after 48-hours. Following 10 days of serum withdrawal to induce differentiation in

the presence or absence of differing concentrations of sCBD, metabolic activity was significantly reduced at 50 µM (P = <0.0001), 20 µM (P = <0.0001) and 10 µM (P = <0.0001) with reduced cell viability reported at 50 µM (P = <0.0486) and 20 µM (P = <0.0302). On the other hand, following acute treatment, where myotubes were treated with differing concentrations of sCBD at terminal differentiation, significant reductions in metabolic activity were only reported at 50 µM (P = <0.0045). Significant changes in myotube morphology were only observed after a repeated treatment of sCBD, however, a decrease in nuclear fusion index (NFI) following acute dosing was also recorded. In conclusion, this thesis provides preliminary data on the effects of sCBD on skeletal muscle myogenesis *in vitro*. Our findings indicate that higher doses of sCBD are detrimental to muscle cell health both during proliferation and differentiation, with greater negative effects reported after chronic treatment of sCBD. Athletes should avoid supplementing with CBD/sCBD due to WADA's unclear stance on synthetic and other natural cannabinoids, as well as the alleged detrimental effects.

Contents

Abbreviations

ADRV	Anti-doping Rule Violation
AEA	Anandamide
AEDs	Anti-epileptic drugs
AFF	Adverse Analytical Finding
BrdU	Bromo-deoxyuridine
BSA	Bovine Serum Albumin
CBC	Cannabichromene
CBD	Cannabidiol
CBG	Cannabigerol
CBN	Cannabinol
CBL	Cannabicyclol
CNS	Central Nervous System
DM	Differentiation Media
DMEM	Dulbecco's Modified Eagle's Medium
ECS	Endocannabinoid System
EdU	5-Ethynyl-2'-deoxyuridine
FAAH	Fatty acid amide hydrolases
FBS	Fetal Bovine Serum
FDA	Food and Drug Administration
FSC	Forward Scatter
GM	Growth Media
HS	Horse Serum
IF	Immunofluorescence Imaging
MAGL	Monoacylglycerol lipase
MTT	3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide
MyHC	Myosin Heavy Chain
NAPE-PLD	*N*-acyl-phosphatidylethanolamine-selective phospholipase D
NADPH	Nicotinamide Adenine Dinucleotide Phosphate

NBCS	Newborn Calf Serum
NFI	Nuclear Fusion Index
PBS	Phosphate Buffered Saline
PI	Propidium Iodide
PS	Penicillin Streptomycin
sCBD	Synthetic Cannabidiol
SD	Standard Deviation
SSC	Side Scatter
THC	Δ9-tetrahydrocannabinol
TNNT-1	TroponinT-1
TRI reagent	Trizol reagent
VCON	Vehicle Control
WADA	World Anti-Doping Agency
2-AG	2-arachidonoylglycerol
ABHD6	α,β-hydrolase 6

Declaration

I declare that the work in this thesis was carried out in accordance with the regulations of Liverpool John Moores University. Apart from the help and advice acknowledged, the work within was solely completed and carried out by the author.

Any views expressed in this thesis are those of the author and in no way represent those of Liverpool John Moores University and the School of Sport and Exercise Science.

This thesis has not been presented to any other University for examination either in the United Kingdom or overseas. No portion of the work referred to in this research project has been submitted in support of an application for another degree or qualification of this or any other university or institute of learning.

Signed: 

Date: 19/04/2022

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Dedication

To my wonderful family and friends, your support has inspired me throughout my research, and I owe everything to you. You remind me what is most important to me, and I hope to continue to make you proud.

Chapter 1

General Introduction & Literature Review

1.1 General Introduction

For centuries, nutraceuticals and herbal treatments have been used for numerous purposes across cultures (Close, Gillham and Kasper, 2021). A Sumerian clay slab from Nagpur, estimated to be 5000 years old, contains the oldest recorded documentation of medicinal herb use in medication production (Petrovska, 2012). It compromised 12 recipes for drug preparation, pertaining to over 250 different plants, some of which were alkaloid-rich, such as poppy, henbane, and mandrake (Petrovska, 2012). Nutmeg, pepper, garlic, onion. fig, coriander, juniper, etc, are mentioned within other ancient texts such as the Indian Holy Books and the Ebers Papyrus, for their therapeutic uses (Petrovska, 2012). Since it was first sited in the Chinese pharmacopoeia in 2,600 BC, marijuana has been credited with various psychological, physical and emotional benefits (Maroon and Bost, 2018).

In recent years, the consumption of *Cannabis sativa*-based products has been on the rise (Czégény et al., 2021). The cannabis plant is commonly used for recreational or medicinal purposes, as it supposedly has anxiolytic, antipsychotic, anti-inflammatory, and neuroprotective effects (Docter et al., 2020). At least 140 different cannabinoids are derived from the cannabis plant, with the most studied compounds being Δ9-tetrahydrocannabinol (THC) and cannabidiol (CBD) (Pagano et al., 2020). THC has traditionally been the more recognised compound as it is responsible for the psychoactive effects of cannabis and is found at high concentrations in indica/marijuana (>0.3%) and low concentrations in sativa/hemp (<0.3%). CBD has gained popularity more recently due to its apparent medicinal properties, legality surrounding its use, and lack of psychoactive effects associated with THC (McCartney et al., 2020; Close, Gillham and Kasper, 2021).

The popularity of CBD products in sport has seen significant growth, particularly as an aid to recovery. Recovery is a major performance priority for sports science and medicine teams as it is a factor in physiological and cognitive performance (Rojas-Valverde, 2021). The use of plant derived compounds to maximise recovery isn't a new strategy, however. Researchers, coaches, and athletes have reported strategies that include the intake of plant-derived products such as ginseng, green tea, and spinach (Rojas-Valverde, 2021). Polyphenols, including flavonoids derived primarily from fruits and plants, have also become of great interest to athletes, due their antioxidant and anti-inflammatory effects (Overdevest et al., 2018). Previous research conducted suggests that polyphenols derived from blueberries and cherries aids muscle soreness and improved muscle strength following eccentric exercise (Overdevest et al., 2018). Therefore, polyphenol supplementation is perceived as an effective strategy to improve exercise performance and is an alternative recovery strategy for athletes and coaches (Overdevest et al., 2018).

Due to the proposed benefits of CBD and reported anti-inflammatory and pain relief properties, there has been a drastic increase in the use of CBD within the world of elite sport over the last decade, and interest in CBD has also intensified among the general population as internet searches for 'CBD' in the USA indicate (Docter et al., 2020; Leas et al., 2019). Cannabinoids are now considered the second most commonly used substance among contact sports athletes, replacing nicotine (McDuff et al., 2019). One explanation for the rise of CBD within sports may be due to its removal from the World's Anti-Doping Agency (WADA) list of prohibited substances in 2018 (McCartney et al., 2020). However, athletes considering using CBD products must be mindful as all other cannabinoids are still prohibited by WADA. CBD products are permitted for use by athletes if they contain less than 1 mg of THC in the final product, however the issue arises for athletes when reports suggest that commercially available

CBD products contain THC levels higher than the legal threshold, thus resulting in an anti-doping rule violation (ADRV) and possibly elimination from competition (Kasper et al., 2020). Although viewed as fairly safe, CBD is still not risk-free and has shown dose-dependent adverse effects in humans such as a loss of appetite and somnolence to liver abnormalities, thus potentially questioning its removal from WADA's prohibited list, as it may represent an actual or potential risk to the athlete (Huestis et al., 2019).

The majority of CBD research is generally in conjunction with other cannabinoids, mainly THC, therefore the findings from these studies can't directly be associated to the effects of CBD, it may be due to interactions between cannabinoids often referred to as 'the entourage effect' (Kasper et al., 2020). Due to the large number of different cannabinoids present in the cannabis sativa, difficulties arise when separating CBD. As it is almost impossible to get a pure sample of natural plant derived CBD, synthetic cannabidiol (sCBD) offers a potentially safer approach for athletes, as it is a man-made compound free from THC. Therefore, it is vital to conduct research investigating the effects of sCBD on muscle, as it represents an attractive alternative to eliminate risk of an ADRV for athletes wanting to take CBD.

1.2 Literature Review

1.2.1 Historical Perspective

Cannabis Sativa is among one of the earliest plants known to be cultivated by man, with archaeological records stating its cultivation dates back to the end of the first ice age with the birth of agriculture 10,000 to 12,000 years ago (Zuardi, 2006). Historically, Central Asia and South-East Asia have been advocated as probable regions for the natural origin and primary domestication of the *Cannabis sativa* and are likely to have played a key role in its evolution (Bonini et al., 2018). Evidence suggests that cannabis was first used in China, with the fibres collected from the cannabis stems being manufactured into ropes, textiles, and paper (Zuardi,

2006). Even today, fibres from the plant are used as a constituent of fishing nets (Savo et al., 2013). Today, the *Cannabis sativa* plant is primarily used for its pharmaceutical properties. Although there is uncertainty as to when cannabis was first used for something other than textiles, in 2737 BC Chinese Emperor Sheng Nung drew up the first Chinese pharmacopeia, the pen-ts'ao in which *Cannabis sativa* is mentioned (Abel, 2013). Indications for the use of cannabis in this ancient text include: fatigue, rheumatic pain, malaria and others (Zuardi, 2006). The psychoactive properties of cannabis were also first mentioned in this text, with 'visions of the devil' and 'communicating with spirits' being observed after taking 'ma-fen' (the fruit of cannabis) (Zuardi, 2006). Due its numerous different properties, *Cannabis sativa* has been recognized as a sacred plant by several religions over the centuries (Touw, 1981).

1.2.2 Cannabis strains

The *Cannabis sativa* plant contains various chemical compounds with possible bioactive effects, including >140 cannabinoids (McCartney et al., 2020). The two most notable constituents and the most studied of the phytocannabinoids are cannabidiol (CBD) and Δ9-tetrahydrocannabinol (THC), totalling up to 40% of the extracts of the plant (Maroon and Bost, 2018). Although the exact classification has been debated (Piomelli and Russo, 2016), there are two major stains of cannabis: *Cannabis indica* and *Cannabis sativa* (see **figure 1.1**) (Kasper et al., 2020). Different levels of THC can be found in various strains, with the *Cannabis sativa L-strain*, otherwise known as hemp, containing less than 0.2-0.3% dry weight THC and other strains containing up to 30% dry weight THC, commonly referred to as marijuana (Kasper et al., 2020). Numerous countries have acknowledged the commercial value of hemp, and a legal limit of 0.2-0.3% THC is often applied, hence the emergence of *Cannabis Sativa Light*, which is a genetically modified variety of *Cannabis sativa* containing high concentrations of CBD and reduced levels of THC, lower than the 0.2% threshold (Kasper et al., 2020; Pagano et al.,

2020). The difference in levels of THC is vitally important, as marijuana is primarily grown due to the psychoactive properties of THC, while hemp is cultivated for a variety of reasons including biofuel, rope, clothes and notably to produce cannabidiol, for its perceived therapeutic properties from pain relief to treatment of epilepsy (Friedman and Sirven, 2017).

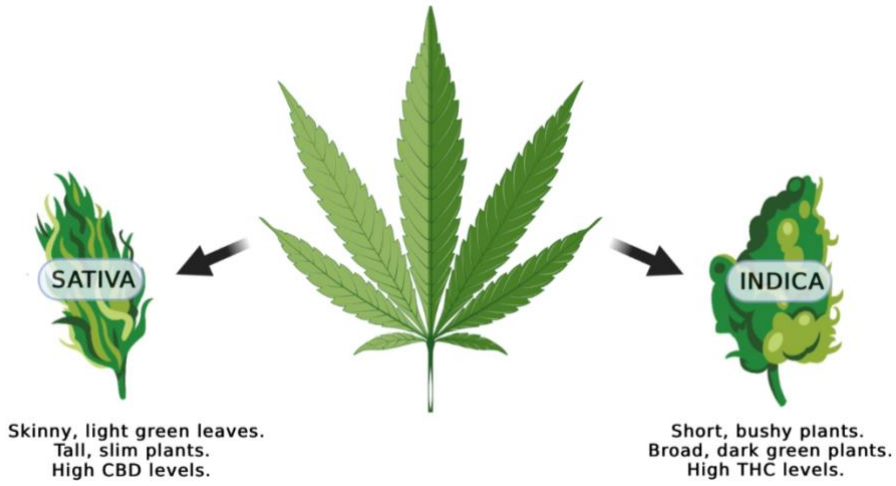


Figure 1.1 Two different strains of cannabis (*C. sativa & C. indica*) and their respective properties

1.2.3 Cannabinoids

The properties of *Cannabis sativa* were largely undefined until the last ~100 years. With advancements in biology and chemistry, the structure and properties of *Cannabis sativa* have been uncovered The identification of different cannabinoids in *Cannabis sativa* plant have provided a mechanism for how the plant exerts its effects. In recent years, cannabinoids have been significantly studied for their possible anticancer effects and symptom management in cancer patients (Dariš et al., 2019). The research conducted concluded that there is potential antitumor activity of phytocannabinoids (plant-derived) trans-Δ9-tetrahydrocannabinol (THC), cannabinol (CBN), Δ8-THC, cannabidiol (CBD) and cannabicyclol (CBL), in addition to certain synthetic cannabinoids such as WIN-55,212-2 (Dariš et al., 2019).

Amongst the cannabinoids, cannabidiol (CBD) has received the greatest amount of research attention. CBD was first isolated from *Cannabis* in 1940 by Roger Adams (Burstein, 2015). Nonetheless, since its discovery, no further research was conducted for almost 25 years until 1963 when the structure of CBD was elucidated by the group of Professor Raphael Mechoulam from the Hebrew University of Jerusalem (Crippa et al., 2018). Not only did this group clarify the exact chemical structure of CBD, but they were also responsible for the precise structure and stereochemistry of Δ9-THC and numerous other cannabinoids. Within their research, they discovered that various tetrahydrocannabinols (THC) were responsible for the psychoactive properties of the plant and that although cannabidiol has pharmacological activity, it is not psychoactive (Kalant, 2001).

Although CBD and THC are the most researched of all the cannabinoids, other cannabinoids have been shown to have therapeutic properties. Of other cannabinoids, cannabinol (CBN) has been shown to have a centrally acting effect like THC, but is much less potent (Williamson and Evans, 2000). CBN is an oxidised metabolite of THC, therefore it is a weak psychoactive compound which binds to the cannabinoid receptor, specifically having higher affinity towards CB2 (Morales, Hurst, and Reggio, 2017). Cannabigerol (CBG) is a non-psychoactive phytocannabinoid found in low concentrations of the plant, it has a low affinity for the cannabinoid CB1 and CB2 receptors, but directly impacts the endocannabinoid system because of its ability to inhibit anandamide (AEA) uptake (Morales, Hurst, and Reggio, 2017). CBG inhibits prostaglandin production at different levels and thus has anti-inflammatory and analgesic properties, it also possesses anti-proliferative properties and has been proposed for cancer therapy (Deiana, 2017). Another abundant phytocannabinoid in the plant, discovered in 1966 by Gaoni and Mechoulam, is cannabichromene (CBC) (Morales, Hurst, and Reggio,

2017). CBC does not display any significant affinities towards both CB1 and CB2 receptors, however it does directly affect the endocannabinoid system by inhibiting anandamide (AEA) uptake (Morales, Hurst, and Reggio, 2017). A study conducted by Turner and Elsohly in 1981, discovered that CBC possess anti-inflammatory properties following the treatment in rats.

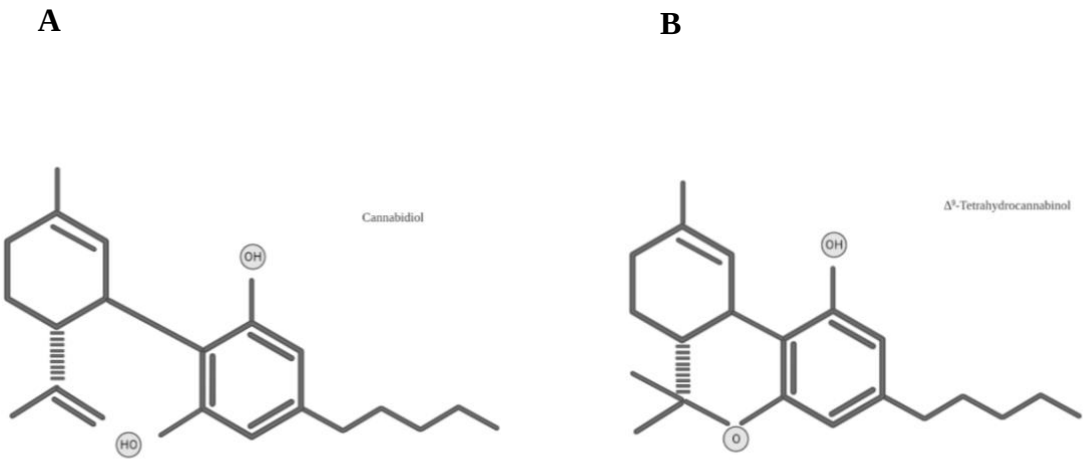


Figure 1.2 Chemical structure of CBD (**A**) and Δ9-THC (**B**). Both compounds share the exact molecular formula, C21H30O2, however structurally there is one difference. Where THC contains a cyclic ring, CBD contains a hydroxyl group and this small difference in molecular structure gives the two compounds different pharmacological properties.

1.2.4 Cannabinoid Receptors

Up to now, two cannabinoid receptors have been identified, the CB_1 and the CB_2 receptor (Grotenhermen, 2005). In the mid-1980's research conducted by Allyn Howlett's laboratory at St Louis University discovered definite evidence that cannabinoid receptors do indeed exist (Pertwee, 2006). Confirmation of these receptors came with the cloning in 1990 of the rat CB1 receptor in Tom Bonner's laboratory and the cloning in 1993 of the CB2 receptor in Seam Munro's laboratory in Cambridge (Pertwee, 2006). Activation of the CB1 receptor causes circulatory and psychotropic effects similar to marijuana intake, while activation of the CB2

receptor does not (Grotenhermen, 2005). As a result of this, selective CB2 receptor agonists are becoming a key area of interest for therapeutic uses of cannabinoids, specifically for their analgesic, anti-inflammatory and anti-neoplastic actions (Grotenhermen, 2005). Cannabinoid receptors CB1 and CB2 are class A G-protein-coupled receptors (GPCRs). CB1 receptors are highly expressed in the central nervous system (CNS) and are mainly found on neurons in the brain, spinal cord, and peripheral nerves (Grotenhermen, 2005; Li et al., 2020). CB1 regulates central and peripheral physiological processes such as pain regulation, memory, metabolism, and energy balance (Li et al., 2020). CB2 receptors on the other hand are primarily associated with elements of the immune system and are expressed in the peripheral organs of the immune system such as the spleen, tonsils, and thymus (Li et al., 2020). CB2 has been identified as a therapeutic target for inflammatory, neuroinflammation and neuropathic pain (Li et al., 2020).

Cannabinoid receptors are G protein-coupled receptors that facilitate the central and peripheral actions of extracts from the cannabis plant. Agonistic stimulation of both types of cannabinoid receptors activates the coupling to the G_i proteins and it has also been reported that the CB1 receptor couples with G_s or G_q proteins under specific conditions (Grotenhermen, 2005; Li et al., 2020). The CB1 receptor binds to the main active ingredient of *Cannabis sativa*, THC, and elicits most of its effects through the CNS (Kendall and Yudowski, 2017). CB1 receptors also bind to synthetic cannabinoids such as JWH-015, WIN55212-2 and endocannabinoids, arachidonoylethanolamide (AEA) and 2-arachidonoylglyrcerol (2-AG) (Kendall and Yudowski, 2017). Following ligand binding and receptor activation, CB1 receptors couple to $G_{i/o}$ type G proteins which leads to a fast reduction in levels of cAMP as result of inhibiting adenylate cyclase activity (Kendall and Yudowski, 2017). The CB1 receptor is also responsible for the activation of extracellular signal regulated kinase (ERK) and modulation of ion channels including Ca^{2+} and K^+ (Reddy, Maurya and Velmurugan, 2019). CB2 receptors also signal

inhibition of adenylate cyclase and regulate MAO kinase activity, through their capability to couple to $G_{i/o}$ proteins (Demuth and Molleman, 2006). The inhibitory effect it has on cAMP production, which is the product of CB2 stimulation, is alleged to underlie the regulation of immune function by cannabinoids (Demuth and Molleman, 2006).

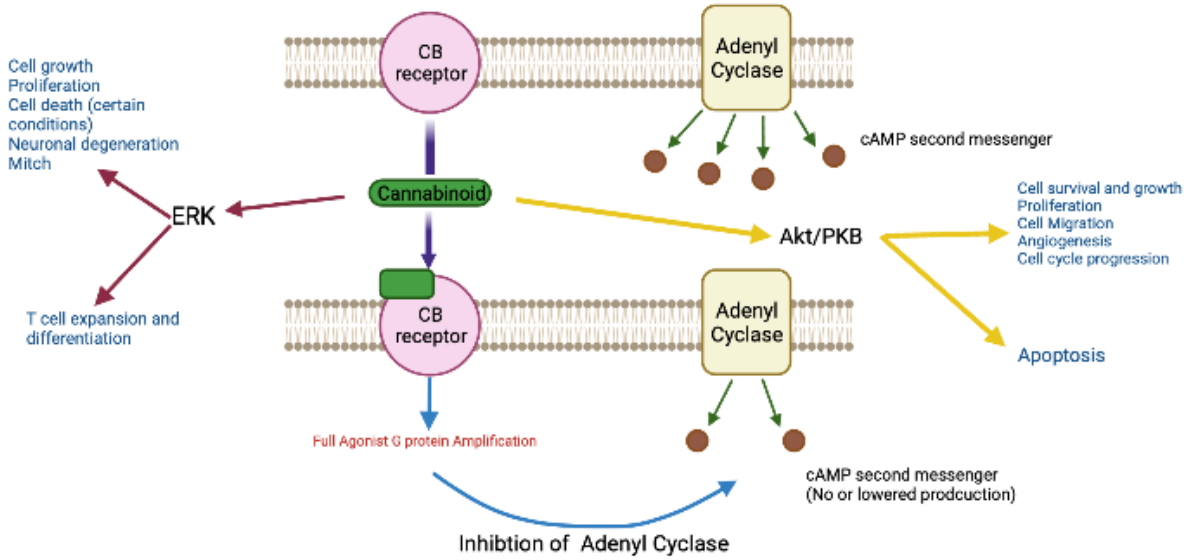


Figure 1.3 Cannabinoids mechanism of action (natural and synthetic). At cellular level, upon interacting with appropriate receptors, cannabinoids cause inhibition of adenyl cyclase, promotion of ERK and Akt/PKB signalling pathways. This results in a variety of different cellular function such as proliferation, growth, and cell death (Redrawn from Reddy, Maurya and Velmurugan, 2019).

1.2.5 The Endocannabinoid System

The endocannabinoid system (ECS) is a network of molecules that work together to regulate the metabolism of both naturally occurring and exogenously administrated cannabinoids (Reddy, Maurya and Velmurugan, 2019). The ECS comprises of cannabinoid receptors, endogenously produced cannabinoids (endocannabinoids), transport proteins (responsible for

the uptake and transport of endocannabinoids) and enzymes that are accountable for the synthesis and degradation of the endocannabinoids (Vučković et al., 2018; Mackie, 2008). Research conducted by Devan et al, identified that there are specific binding sites (cannabinoid receptors) in the brain and identified that the receptor-binding affinities of the different compounds paralleled their corresponding influences of biological activity (Devane et al., 1988). The identification of cannabinoid receptors stemmed from the desire to understand the psychoactive properties of THC (Mackie, 2008).

Due to the recent identification of cannabinoid receptors, there has been an exponential increase in studies exploring the endocannabinoid system and its regulatory functions in health and disease (Pacher, Batkai and Kunos, 2006). The discovery of cannabinoid receptors implied the presence of endogenous ligands capable of activating them, the 'endocannabinoids', the two major endocannabinoids being anandamide (AEA) and 2-arachidonoglycerol (2-AG) (Di Marzo and Matias, 2005) (Close, Gillham and Kasper, 2021). Endocannabinoids are biosynthesized from membrane phospholipids as a result of activity from numerous enzymes including *N*-acyl-phosphatidylethanolamine-selective phospholipase D (NAPE-PLD, which is involved in the biosynthesis of anandamide), diacylglycerol lipases α and β are inactivated through a reuptake process, followed by enzymatic degradation (Pagano et al., 2017). This process occurs primarily through fatty acid amide hydrolases (FAAH) and via the serine hydrolases monoacylglycerol lipase (MAGL) and α, β-hydrolase 6 (ABHD6) and 12 (Pagano et al., 2017).

These specific endocannabinoids interact with cannabinoid receptors to regulate numerous vital bodily functions and play an important role in the regulation of homeostasis within the body (Noreen et al., 2018). As well as playing a fundamental role within the regulation of

homeostasis, the ECS is existent in all body tissues to control immune system functions, digestive functions, inflammatory responses, mood and stress reactions and various other bodily functions (Pagano et al., 2017). Furthermore, when combined with frequently used anti-inflammatory drugs, endocannabinoids and synthetic cannabinoids elicit synergistic antinociceptive effects, which may be useful for pain management (Pacher, Batkai and Kunos, 2006).

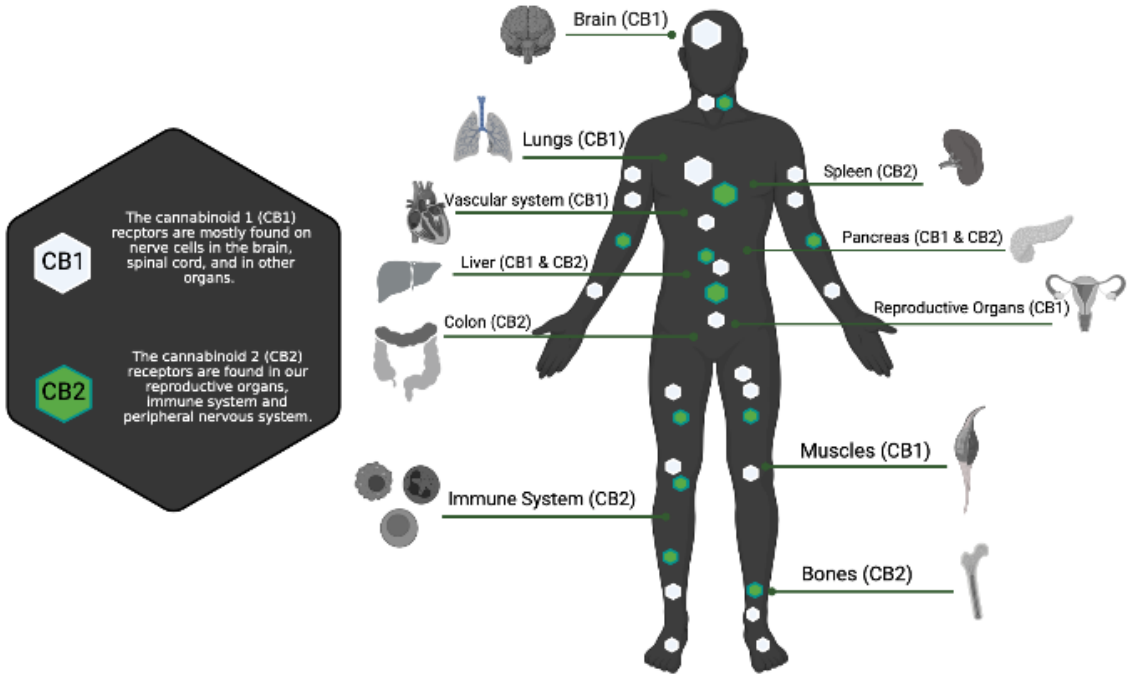


Figure 1.3 The endocannabinoid system within the body, exhibiting the location and distribution of CB1 and CB2 receptors (Reddy, Maurya and Velmurugan, 2019).

1.2.6 CBD and skeletal muscle

Exercise, specifically unfamiliar strenuous activity, can lead to muscle damage (Ebbeling and Clarkson, 1989). Although not permanent, as the tissues repair, evidence of muscle damage exists and includes morphological changes, performance decrements and delayed onset muscle soreness (Ebelling and Clarkson, 1989). Various types of exercise have been shown to induce

muscle damage (Ebelling and Clarkson, 1989) Extreme (i.e., endurance sports such as triathlon, marathon running, etc.) and/or eccentric types of exercises that are characterised by powerful lengthening contractions during which muscle fibres are overstretched, can also cause skeletal muscle fibre damage (Fatouros and Jamurtas, 2016). This is what is known as exercise-induced muscle damage (EIMD) and it decreases muscle function whilst triggering inflammatory responses in the body (McCartney et al., 2020). Although inflammation is necessary for EIMD repair and regeneration, it can also lead to continued muscle soreness and a delay in functional recovery (McCartney et al., 2020). Due to athletes heavy training and congested competition schedules, pain and recovery management is often treated with ant-inflammatory agents such as ibuprofen, a non-steroidal anti-inflammatory drug (NSAID) (McCartney et al., 2020; Burr et al., 2021). NSAID's have been reported to attenuate exercise-induced skeletal muscle adaption, although the exact mechanisms sustaining these effects have not been clarified, the prevention of inflammation may inhibit training-induced skeletal muscle hypertrophy (Machida and Takemasa, 2010). Therefore, it is no surprise that a recent study in elite rugby players discovered 26% of players had previously trailed or were currently taking CBD supplements, possibly as an alternative to NSAID's (Kasper et al, 2020).

Previous research suggests that CBD could induce changes in cortisol release which would in turn regulate inflammatory responses to injury (Rojas-Valverde, 2021). CB1 and CB2 cannabinoid receptors interact with adenosine receptors, resulting in reduced cytokine levels and reduced expression of overreactive immune cells (Rojas-Valverde, 2021). Furthermore, CBD appears to mediate mechanisms related with gastrointestinal damage prevention, due to inflammation, and improve recovery of skeletal injuries (Rojas-Valverde, 2021). CBD (300mg) has also been reported to stimulate changes in glucocorticoids such as cortisol in humans, which is one of the crucial homeostatic regulators of the inflammatory response to

injury (Zuardi, Guimarães and Moreira, 1993; Rojas-Valverde, 2021). With regards to muscle-specific inflammation, one study investigated the effect of high-dose CBD (60 mg·kg) on transcription and synthesis of pro-inflammatory markers (IL-6 receptors, TNF-α, TNF-β, and inducible nitric oxide synthase) in MDX mice (McCartney et al., 2020). The findings from this study conclude that CBD inhibited mRNA expression of each marker and a decline in plasma concentration of IL-6 and TNF-α was recorded (McCartney et al., 2020). Although CBD-related inhibitions in neuroinflammation could have therapeutic potential in sports research, current research examining the inflammatory response to CBD in humans is limited and inconclusive (Rojas-Valverde, 2021; McCartney et al., 2020).

1.2 7 Emergence of cannabinoid use in sport

Although CBD is non-psychoactive, some pre-clinical evidence suggests it may be responsible for some of the beneficial effects of cannabis for sports activities, such as reduced anxiety (Lachenmeier and Diel, 2019). More recent research has discovered that when utilized efficiently and taken as an adjunctive treatment, cannabidiol has a positive impact on diseases such as; Parkinson's disease, Alzheimer's Disease, and neuropathic pain (Maroon and Bost, 2018). CBD has also been shown to elicit neuroprotective, anti-inflammatory, and immunomodulatory benefits, as well as having a beneficial impact on pain related behaviours and post-exercise recovery, making it an interesting prospect for athletes (Maroon and Bost, 2018; Lachenmeier and Diel, 2019). Within the sporting domain, cannabis, including all cannabinoids, are prohibited by the World Anti-Doping Agency (WADA) across all sports in competition as a result of the adverse effects of Δ^9-THC as it violates the "spirit of sport" (Golombek et al., 2020). However, in 2018, WADA removed CBD from their list of prohibited substances both in and out of competition (Close, Gillham and Kasper, 2021). Since the removal of CBD from WADA's prohibited list, coupled with the proposed benefits, there has

been an exponential increase in both athletes and consumer interest in CBD (Lachenmeier and Diel, 2019).

Athletes considering using CBD must first and most importantly recognise the legal threshold of THC and other cannabinoids within cannabidiol in order to avoid an anti-doping rule violation (ADRV). THC is a 'threshold substance', with WADA's rules and regulations stating that the presence of >150 $ng \cdot ml^{-1}$ of THC in urine would result in an anti-doping rule violation. However, for other minor cannabinoids on the prohibited list, no threshold yet exists, meaning that the detection of even the smallest amount in the urine would be considered as an AFF (Mareck et al., 2021). CBD products must be used with caution as research suggests that some commercially available CBD products have been shown to contain significantly higher amounts of THC than stated on the label, with some levels being greater than the legal threshold (Gurley et al., 2020). WADA also clarified, that CBD products which state they are THC free, may still contain varying concentrations of banned cannabinoids that could lead to an adverse analytical finding (AFF) when an athlete is subjected to routine doping controls (Mareck et al., 2021). However, to obtain a pure sample of CBD is almost impossible, and because of this numerous sporting bodies are opposed to the use of CBD products.

Current research surrounding the use of CBD within sport is limited, one study however, examined the use of cannabidiol on pain relief and recovery in rugby players (Kasper et al., 2020). Due to the nature of the sport, markers of inflammation and muscle soreness are very common, therefore coaches and players constantly aim to improve recovery strategies to reduce pain, with one emerging technique being the use of cannabidiol (Kasper et al., 2020). The findings from the study conclude that of all the players who used CBD, 67% reported a perceived benefit, with 41% stating it improved sleep and 14% claiming it improved pain and aided recovery, with no adverse side effects reported (Kasper et al., 2020). One of the most

alarming findings was that only 48% of players were concerned about an ADRV, this is even more worrying when it's coupled with the fact that of all the CBD products being used, only one had been tested for other contaminants (such as cannabinoids, terpenes and flavonoids) (tested by Banned Substances Control Group, California) (Kasper et al., 2020). The key message from this study was that individuals taking CBD, especially athletes, need to be educated on the potential consequences of CBD use and highlights the need for further research to be conducted on the efficacy of CBD in pain relief and enhancing recovery.

1.2.8 Synthetic Cannabidiol (sCBD)

A potentially safer approach to CBD use is through synthetically produced cannabidiol (sCBD) since this is a synthetically manufactured compound free from natural existing THC and any other cannabinoids. Synthetic cannabinoids largely refer to synthetic compounds that display agonist, antagonist, or allosteric activity at CB1 and/or CB2 receptors, which can be structural analogues of natural cannabinoids (Duan et al., 2021). The effects of cannabinoids are exerted when they interact with the endocannabinoid system (ECS) and activate the CB1 and CB2 receptors (Duan et al., 2021). The interaction between cannabinoids and the ECS has a significant impact on numerous pathophysiological conditions such as inflammation, pain, and epilepsy and thus is often the reason why both natural and synthetic cannabinoids are widely studied as therapeutic agents (Duan et al., 2021). In the United States, synthetic cannabinoids such as nabilone and dronabinol (synthetic product of Δ9-THC) have been approved for the treatment of nausea and vomiting associated with cancer chemotherapy in patients who failed to respond to conventional therapies. (Badowski, 2017; Duan et al., 2021). Dronabinol has also been identified as a treatment for anorexia and weight loss in patients with AIDS (Badowski, 2017).

CBD and its structural analogues, like other phytocannabinoids, have traditionally been purified and isolated from a cannabis extract, but due to structural, physical, and chemical formula similarities among phytocannabinoids, this has proven difficult (Jung et al., 2019). Therefore, the chemical synthesis of CBD asserts its own advantages, considering practical difficulties in the purification and consistent quality control of natural CBD (Jung et al., 2019). The chemical compositions in the cannabis plant are different from strain-to-strain and from harvest-to-harvest, therefore, to achieve a high-quality control suitable for pharmaceutical grade is very difficult and cost's a substantial amount (Jung et al., 2019). However, the chemical structure of naturally derived CBD and synthetic CBD is largely identical; therefore, similar efficacy and tolerance is to be expected (Bisogno et al., 2001). Advantages of synthetically derived cannabinoids include easier quality control, unlimited production possibilities and reduced environmental impact, with the most important factor being that it also aids to support further investigations of its therapeutic use (Klotz et al., 2019). Studies within cell culture and animals have concluded that synthetic cannabinoids produce similar qualitative physiological, psychoactive, anti-inflammatory effects to plant-derived cannabinoids (Dariš et al., 2019). The use of synthetic cannabinoids also eliminates any extraction-associated problems, as its synthetically produced so avoids extracts such as THC (Jung et al., 2019). There is, however, a substantial gap in literature on the efficacy of sCBD, specifically on muscle cells and from an athlete's perspective and as research suggests, the supposed benefits of CBD may be due to the 'entourage effect', which is the idea that the whole cannabis plant possess greater therapeutic potential than its individual components, therefore limiting the efficacy of sCBD.

1.2.9 The Entourage Effect

The ‘entourage effect’, which proposes that the effects of the whole cannabis plant are larger than the sum of its individual components due to an interaction between its phytochemical constituents, is a rising term within the cannabis community (Anderson et al., 2021). The relationship between CBD and THC has been examined within various studies, with some showing an interaction between the two had no effect on pain relief, and other studies stating the opposite (Li et al., 2019; Cuttler, LaFrance and Craft, 2020). Research conducted by Cuttler et al, which examined the acute effects of cannabis on pain, discovered that THC, CBD, or their interaction influenced change in pain severity ratings. However, to some extent, the findings from this study conflict the findings from one previous study, which established higher levels of THC were associated with greater reduction in pain ratings, while higher levels of CBD was associated with smaller reductions (Cuttler, LaFrance and Craft, 2020).

In addition to the almost 200 various cannabinoids (including synthetic products) identified, terpenes/terpenoids and flavonoids may contribute to the overall effects of *Cannabis sativa* in way which is still unknown (Nahler, Jones and Russo, 2019). Terpenoids, as well as cannabinoids, constitute the essential oil of the plant and are responsible for the distinctive odour of cannabis (Gallily, Yekhtin and Hanuš, 2018). Physiologically, terpenoids are accountable for protecting the cannabis plant from insects who will consume the plant and attracting pollinating insects amongst other purposes (Gallily, Yekhtin and Hanuš, 2018; Russo, 2011). Pharmacologically speaking, terpenoids have been proposed to be involved in influencing the properties of different cannabinoids and contributing to the entourage effect (Gallily, Yekhtin and Hanuš, 2018; Russo, 2011). An animal study that exposed mice to terpenoid odours discovered that it had a profound effect on activity levels, signifying a direct impact on the brain, even at low concentrations (Buchbauer et al., 1993). Similar levels are comparable to THC concentrations found in humans receiving cannabis extracts for pain relief

or symptoms of multiple sclerosis in randomised controlled trails (Russo, 2011). Therefore, the unique therapeutic effects of terpenoids may contribute meaningfully to the entourage effects of cannabis-based medicinal extracts (Russo, 2011).

Another consideration is the number of different companies that manufacture CBD products and the various processes and methods used to get to the final product. The number of pre-harvest factors, post-harvest conditions and processes all influence the final profile of phytocompounds (Nahler, Jones and Russo, 2019). Other factors which have an impact include the selection of the strain, the conditions in which the plant grows, the way in which its harvest and the drying and extraction of the specific cannabinoids is likely to have the greatest influence (Nahler, Jones and Russo, 2019). In conclusion, there are numerous interactions that occur after the administration of cannabis-based products and thus impact the synergistic effect on phytocompounds. Therefore, the 'entourage effect' may not always be the same and is dependant primarily on the profile of phytocompounds (Nahler, Jones and Russo, 2019).

1.2.10 Summary

This literature review has provided an overview on cannabidiol and its potential use for athletes within elite sport. As previous research suggests, CBD possesses many properties that are deemed favourable for use by athletes. As such, since its removal from WADA's list of prohibited substances in 2018, there has been an increase in CBD use amongst athletes. However, athletes consuming CBD need to be mindful as often the levels of THC are incorrectly stated within the product and WADA state that the presence of >150 $ng \cdot ml^{-1}$ THC in urine would result in an ADRV. Although CBD is non-prohibited by WADA, their stance on synthetic cannabinoids and other natural cannabinoids is unclear, as threshold exists for THC but not for the >140 other cannabinoids present in the cannabis sativa. Therefore, an

alternative and potentially safer approach to CBD is synthetic cannabidiol, a manmade substance that is free from THC. The 'entourage effect' however, suggests that CBD is more effective when it contains other cannabinoids, something which sCBD does not possess. Although CBD use among athletes is high, most of the perceived benefits for athlete's use is anecdotal, emphasising the need for further research in practice. To address the current gap in knowledge on the direct effects of CBD and specially sCBD on skeletal muscle, we investigated the effects of sCBD cell viability and myotube morphology on C_2C_{12} muscle cells *in vitro*, with the findings potentially having great importance for use among athletes.

1.3 Aims and Objectives

1.3.1 Aims

1. To determine the effects of synthetic (sCBD) administration on C_2C_{12} myoblast viability during proliferation.
2. To determine the effects of acute and chronic sCBD administration on C_2C_{12} cell viability of myotubes during and after differentiation.
3. To determine the morphological effects of acute and chronic sCBD administration on C_2C_{12} myotube formation as expressed via immunofluorescence imaging.

1.3.2 Objectives

To achieve aim 1) sCBD across a dose range of 0.001 µM – 50 µM will be administrated to C_2C_{12} skeletal muscle myoblasts for 24 and 48-hours where cell viability will be assessed via the MTT and PI assay upon cessation of experiments. To determine the effects of sCBD on DNA synthesis in myoblasts, sCBD (0.001 µM – 50 µM) will be administrated to C_2C_{12} skeletal muscle myoblasts for 60-minutes and EdU incorporation measured.

To achieve aim 2) in an acute treatment, a single dose of sCBD (0.001 µM – 50 µM) will be administrated to C_2C_{12} myotubes at a late stage of differentiation. Secondly, in a chronic treatment protocol, a repeated dose of sCBD (0.001 µM – 50 µM) will be administrated to C_2C_{12} myotubes at various time points throughout differentiation. To determine effects on cell viability, the MTT and PI assay will be conducted upon termination of the experiment at late-stage differentiation.

To achieve aim 3) acute and chronic dosing of sCBD (0.001 µM – 5 µM) will be administrated to C_2C_{12} muscle cells at various time points throughout differentiation and immunofluorescent labelling of myosin heavy chain conducted to examine the morphological effects of sCBD treatment timing.

1.3.3 Hypotheses

Due to findings from previous research, we hypothesised that synthetic cannabidiol may be beneficial for cellular health. We hypothesised that sCBD will have a positive effect on myoblast proliferation and differentiation. Furthermore, we hypothesized that sCBD would have differing effects dependent upon the stage of differentiation it was administered. Following chronic treatment, we hypothesized that sCBD would incur greater negative effects on myotube morphology due to the early and increased exposure time to sCBD, in comparison to the acute treatment.

Chapter 2

General Methodology

2.1 Cell model of myogenesis

An *in vitro* study design was implemented for the experiments in this thesis, in which C_2C_{12} myoblasts were differentiated in a 2D culture system. The advantages of 2D cell models include single and low-cost maintenance of the cell culture and function tests can be performed. In terms of usage and analysis, 2D cultures are highly replicable and easily interpretable when it comes to experiments. The differentiation of skeletal muscle cells *in vitro* is a useful model for studying various stages of skeletal muscle myogenesis including proliferation, fusion and myotube maturation (see **figure 2.1**). Growth conditions, reagents and cell treatments are described in detail in the following sections.

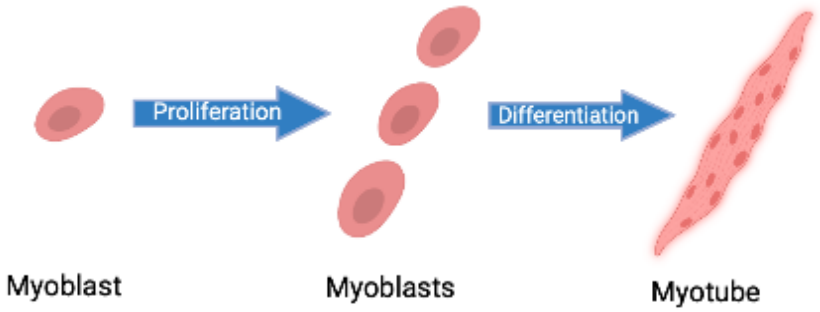


Figure 2.1 The steps of skeletal muscle myogenic differentiation, including proliferation, fusion and myotube maturation. During myogenesis, myoblasts enter the cell cycle and proliferate, where they divide and multiply. Once the growth factor is withdrawn from the myoblast culture, proliferating cells exit from the cell cycle and differentiate into myocytes. These myocytes then fuse to one another to form myotubes, which is the end step in myogenesis.

2.2 Plasticware

All cell populations were culture on T75 culture flasks (Corning, NY, USA) and experiments conducted on 6 and 12-well plates (Corning, NY, USA). Eppendorf tubes purchased

(Eppendorf, Hamburg, Germany) as well as cryogenic vials (Simport Scientific, Saint-Mathieu-de-Beloeil, Canada).

2.3 Cell Culture Reagents

0.2% Gelatin was prepared by mixing 1g porcine gelatine powder (Sigma-Aldrich Company Ltd, Dorset, UK) in 500ml distilled H_2O. Gelatin was labelled with the date, autoclaved, and stored at room temperature. Dulbecco's modified Eagle's medium (DMEM) (4.5 g/L glucose w/L-Gln) was purchased from Gibco (Life Technologies, California, US) and was used to culture C_2C_{12} skeletal muscle cells. Sera were purchased from Gibco (Life Technologies, California, US) and included: Horse serum (HS), Newborn calf serum (NBCS) and Fetal bovine serum (FBS). Penicillin and streptomycin (PS) were added to all media (1%: 50 U/mL penicillin and 50 µg/mL streptomycin). To wash the cells, phosphate buffered Saline (PBS) was used and prepared by placing 5 PBS tablets (Sigma-Aldrich, Sigma Life Sciences, St Louis MO, USA) into 1000ml of dH_2O. Growth Media (GM) for C_2C_{12} cells comprised: DMEM, 10% FBS, 10% NBCS and 1% syringe filtered penicillin-streptomycin solution (PS). Differentiation Media (DM) for C_2C_{12} cells comprised: DMEM, 2% HS and 1% syringe filtered penicillin-streptomycin solution. Synthetically produced cannabidiol (sCBD) (molecular mass 314.47 g/mol) was provided by Pureis Ltd. Galway, Ireland in powdered form and reconstituted in an industrial solvent, dimethyl sulfoxide (DMSO) (36 mg/ml).

2.4 Cell Culture

A sterile laminar flow hood (Kojair biosafety cabinet class II, Kojair, Finland) was used to conduct all cell culture. C_2C_{12} skeletal muscle myoblasts were cultured on T75 culture flasks (Corning. NY, USA), with experiments conducted on cell monolayers seeded on sterile 6 or 12-well plates (Corning. NY, USA), respectively. Once cultured, the cells were preserved in a

HEARcel 150i CO2 incubator (Thermofisher Scientific, UK) at 37ºC, 5% CO_2. Solutions containing H_2O were prepared using dH_2O from a MilliQ water purification system (Merck KGaA. Darmstadt, Germany). Porcine gelatin solution (0.2% gelatin) was added to T75 culture flasks (5ml) and multi-well plates (2ml) for cell adhesion. Myoblast cultures were grown in growth media (GM) containing: DMEM, 10% FBS, 10% NBCS and 1% PS and was replaced with fresh GM every 24-hours until desired confluency was reached.

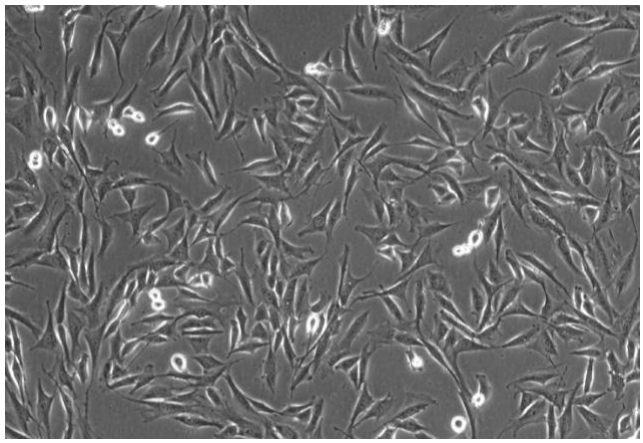


Figure 2.2 C_2C_{12} skeletal muscle cells at ~80% confluence

To induce differentiation of myoblasts into multinucleated myotubes, GM was switched to differentiation medium (DM) following aspiration of GM and three washes with warm 1X PBS solution. DM consisted of: DMEM, 2% HS and 1% PS and was topped up with fresh DM at 10% of the well volume (i.e. 200ul in 2ml) every 48-hours.

2.4.1 Propagation of myoblasts

C_2C_{12} myoblasts were raised from liquid nitrogen. $1x10^6$ myoblasts were seeded on pre-gelatinised T75 flask (Thermo Fisher Scientific, Waltham, MA, USA) containing 15ml GM. T75 flask was then placed in the incubator at 37°C, 5% CO_2 to proliferate until 80% confluency was attained (see **figure 2.2**)

2.4.2 Passaging cells

Once 80% confluent, the T75 flask was ready to split. Remaining GM was aspirated and T75 flask washed twice with 1X PBS. 1ml of trypsin EDTA was added to the confluent T75 flask, ensuring full coverage, and placed in the incubator for 5 minutes. Following this incubation period, the T75 flask was briefly checked under the microscope to ensure the cells had lifted and 4ml of GM was added to the flask to neutralise the trypsin. This 5ml cell/GM solution was then gently homogenised by pipetting in a 50ml falcon tube with 25ml fresh GM. Fifteen ml cell/GM solution was then added to two pre-gelatinised T75 flasks and placed in the incubator at 37°C, 5% CO_2 to proliferate.

2.4.3 Seeding cells onto well-plates

Once ~80% confluent, existing GM was aspirated and T75 flask washed twice with PBS. One ml of trypsin was added to the confluent T75 flask and placed in the incubator for a period of 5 minutes. Following this incubation period, the T75 flask was briefly checked under the microscope to ensure the cells had lifted and 4ml of GM was added to the flask. Twenty µL trypan blue and 20 µL cell/GM solution was added to an Eppendorf and cells counted using a haemocytometer (see section **2.4.4** of this thesis). From the cell count, the correct amount of cell solution and GM was added to a new 50ml falcon and fully homogenised.

Seeding density was calculates using the following equation:

Required ml trypsinized cells = Required seed (no.cells/ml) / cell count (no.cells/ml) x Required ml of media

Depending on the experiment, 6 or 12 well-plates were pregelatinized and 1 or 2ml of cell/GM solution was added to each well and placed in the incubator at 37°C, 5% CO_2 to proliferate.

2.4.4 Cell Counting by Trypan Blue Exclusion

A Neubauer haemocytometer was used for cell counting. This consists of a glass cover slip resting on a counting chamber. The cells were prepared as a 1:1 suspension in 0.4% trypan blue stain and loaded into the haemocytometer. Cells in the four corner grids were counted under a microscope (Olympus CKX31 Microscope) at × 10 magnification. Viable cells were recognized as small, round, and clearly visible, while non-viable cells were misshapen, slightly larger and had lost their membrane integrity, hence were trypan blue positive. The resultant mean of 4 grids was calculated, which represented average cell numbers occupied 0.1 mm^3. This value was then multiplied by 2 to take account of the dilution factor of 1:1. A further multiplication by 10^4 was undertaken to extrapolate the number of cells in 0.1mm^3 to 1 cm^3 (equivalent to 1 ml of cell suspension). The total number of cells contained in the cell suspension could be calculated by multiplying by the total volume of cell suspension (ml).

2.5 Microscopy and Live Imaging

Live images of the cells were captured using a Leica DMII6000B live Imaging Microscope (Leica Biosystems, Wetzlar, Germany). Images of cell monolayers were taken using the 10x objective and 0.5 magnification c-mount fitted to the camera. Image inspection and processing was conducted using Leica Application Suite for Windows, Biosystems, Wetzlar, Germany and Image J (1.53a, National Institutes of Health, USA).

2.6 Statistical Analysis

All data was prepared, analysed, and represented in Prism (GraphPad Prism version 9.1.1 for Windows, GraphPad Software, San Diego, California USA).Data are presented as mean ± standard deviation (SD). Prior to analysis, all data was analysed for normal distribution using a Shapiro-Wilk test. If normality was confirmed a parametric one-way ANOVA was used. If

normality was not confirmed, then a non-parametric Kruskal-Wallis test was used to assess difference in all independent variables, with the threshold of significance set at $P < 0.05$.

2.7 MTT Cell Viability Assay

2.7.1 General Principal

The MTT assay is a colorimetric assay used to measure cellular metabolic activity as an indicator of cell viability, proliferation, and cytotoxicity. It is based on the ability of nicotinamide adenine dinucleotide phosphate (NADPH)-dependent cellular oxidoreductase enzymes to reduce the tetrazolium dye MTT to its insoluble formazan, which has a purple colour. The absorbance of this coloured solution can be quantified by measuring at a certain wavelength (570 nm). The assay measures cellular activity as the enzymatic conversion of the MTT solution to formazan crystals occurring in the mitochondria.

2.7.2 General Protocol

Tetrazolium dye was added to PBS at a concentration of $5mg.ml^{-1}$ to generate an MTT solution. MTT solution (5mg/ml) was added to each well (amount of MTT solution was equal to 10% of total media). Plates were then incubated for 180-minutes, during this time the MTT should interact with NADPH-dependant cellular oxidoreductase enzymes to form visible purple formazan crystals at the bottom of the well. Following this incubation period, existing media was aspirated before a second incubation period lasting 6-minutes, with the lid of the plate removed. Thereafter, DMSO (500 µL) was added to cell monolayers resulting in a purple-coloured solution. Plates were then placed on a plate rocker for 2-minutes at 120 rpm until all cells were lifted from the base of the well. Plates were then positioned into a Spark multimode microplate reader (Tecan, Mannedorf, Switzerland) and measured at a wavelength of 570 nm. Cell viability data was generated and analysed in Microsoft Edexcel (Version 16.61.1).

2.8 EdU Assay

2.8.1 General Principal

A primary way for assessing cell health and evaluating different drug treatments is to measure a cell's ability to proliferate. The most accurate method of doing this is by directly measuring DNA synthesis. Initially this was performed by incorporation of radioactive nucleosides (for example, H-thymidine). This method was then later replaced by antibody-based detection of the nucleoside analogue bromo-deoxyuridine (BrdU). The Click-iT EdU Assay (the assay used within this study) provides a superior alternative to BrdU assays for measuring cell proliferation. EdU (5-Ethynyl-2'-deoxyuridine) is a thymidine analogue which is incorporated into the DNA of dividing cells and fluorescently labelled with a bright, photostable Alex Fluor dye in a quick, highly-specific click reaction.

2.8.2 General Protocol

C_2C_{12} skeletal muscle cells were plated in the early evening (~5pm) on pre-gelatinised 12-well plates. Remaining media was aspirated ~15-hours later to reduce cell proliferation and over confluence. Following this, a solution containing GM/sCBD/EdU was added to cell monolayers and placed in the incubator for 60-minutes at 37°C, 5% CO_2. Thereafter, existing media was aspirated, and cell monolayers fixed with 4% Paraformaldehyde (PFA) and incubated for a 15-minute period at room temperature. Cell monolayers were then washed twice with 1 mL of 3% bovine serum albumin (BSA) in PBS before 1 mL of the permeabilization buffer (0.5% Triton X-100 in PBS) was added to monolayers and incubated at room temperature for 20 minutes.

Permeabilization buffer was removed, and cell monolayers were washed with 3% BSA in PBS. The Click-iT reaction cocktail was then added to monolayers, ensuring the reaction cocktail

was evenly distributed over the well, this was then incubated for a 30-minute period at room temperature whilst protected from the light. The reaction cocktail was then aspirated, and cell monolayers washed twice with PBS. Nuclear counterstaining was then conducted by adding 1X Hoechst 33342 (5 µg/mL in PBS) to monolayers and incubated at room temperature for 30-minutes. Existing media was removed, cell monolayers washed once with PBS and stored for imaging.

A **B**

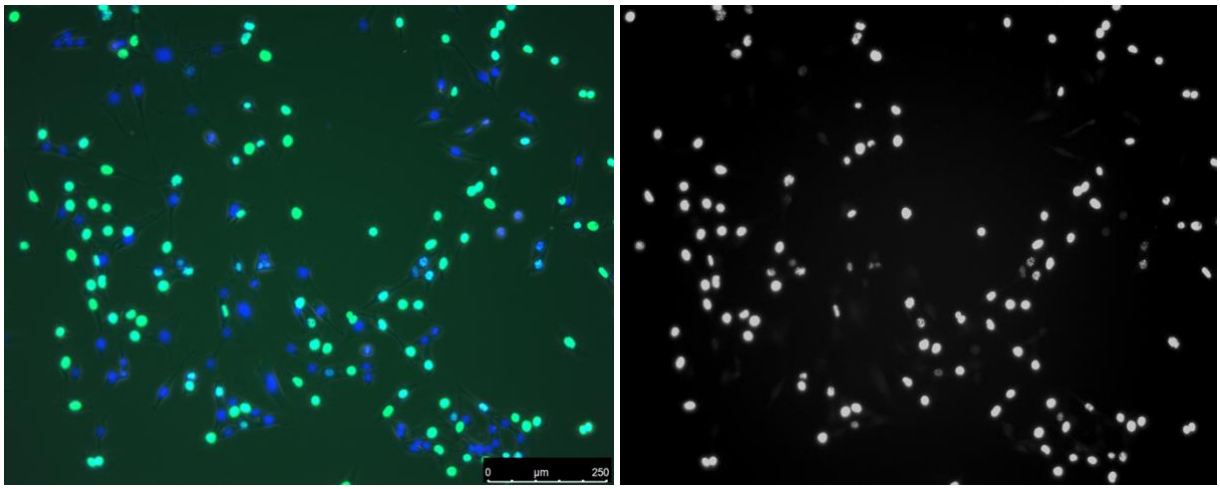


Figure 2.3 EdU staining of proliferating cells. DNA (blue) was staining with Hoechest 33342. Green cells show EdU/Hoechest positive cells. The two overlapped channels are displayed in image **A**, with only the green channel present in image **B**. Nuclei is counted in both images and the difference calculated indicating the percentage of EdU incorporation.

2.9 Flow Cytometry

Flow cytometry is a fast and reliable method used to quantify viable cells. Determining cell viability is a vital factor when evaluating a cells response to specific supplements or other environmental factors. The basic idea of flow cytometry is that as a molecule travels through a known beam of light, it scatters and/or emits light, which can then be measured using an array of detectors (see **figure 2.4**).

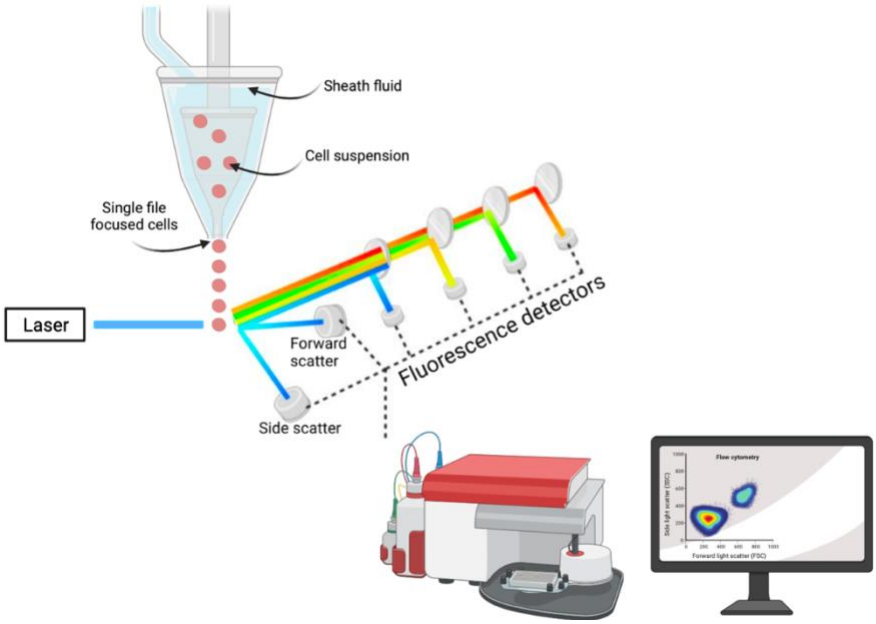


Figure 2.4 The basic layout of a flow cytometer apparatus, illustrating the fluidic, optical, and electronic systems. Cells in suspension flow in single file through an illuminated volume where they scatter light and emit fluorescence. This is then detected by the detectors which pick up a combination of scattered and fluorescent light. This data is then analysed by a computer that is attached to the flow cytometer using special software, where data such as the physical and chemical structure of the cells is generated.

To precisely determine the properties of individual cells, first the suspension must be dispensed through a stream of fluid (sheath fluid), which hydrodynamically aligns the cells into single file as they pass the laser. Before any fluorescent probe analysis, the first thing the flow will measure will be two dynamics of the light, forward scatter (FSC) and side scatter (SSC) (see **figure 2.5**). The FSC detector lies in line with the beam of light from the laser and as the cells pass through it, it deflects the light, casting a 'shadow' on the detector behind it. This information is then used to calculate the cell size; the larger the cell, the larger the shadow generated. The SSC detector, on the other hand, is perpendicular to the laser beam, allowing light to be dispersed by internal cellular components as it enters the cell. Essentially, the more

light that is scattered the more complex the cell. The two components are then combined in a dot plot, where populations of comparable sized cells, as well as cells of similar complexity, will group together. From the plot created, the nature of the cellular population can be determined as cells that are healthy will cluster together in a specific region on the graph and cells experiencing apoptosis, tend to shrink in size, making them more complex and shifting them into another region on the graph.

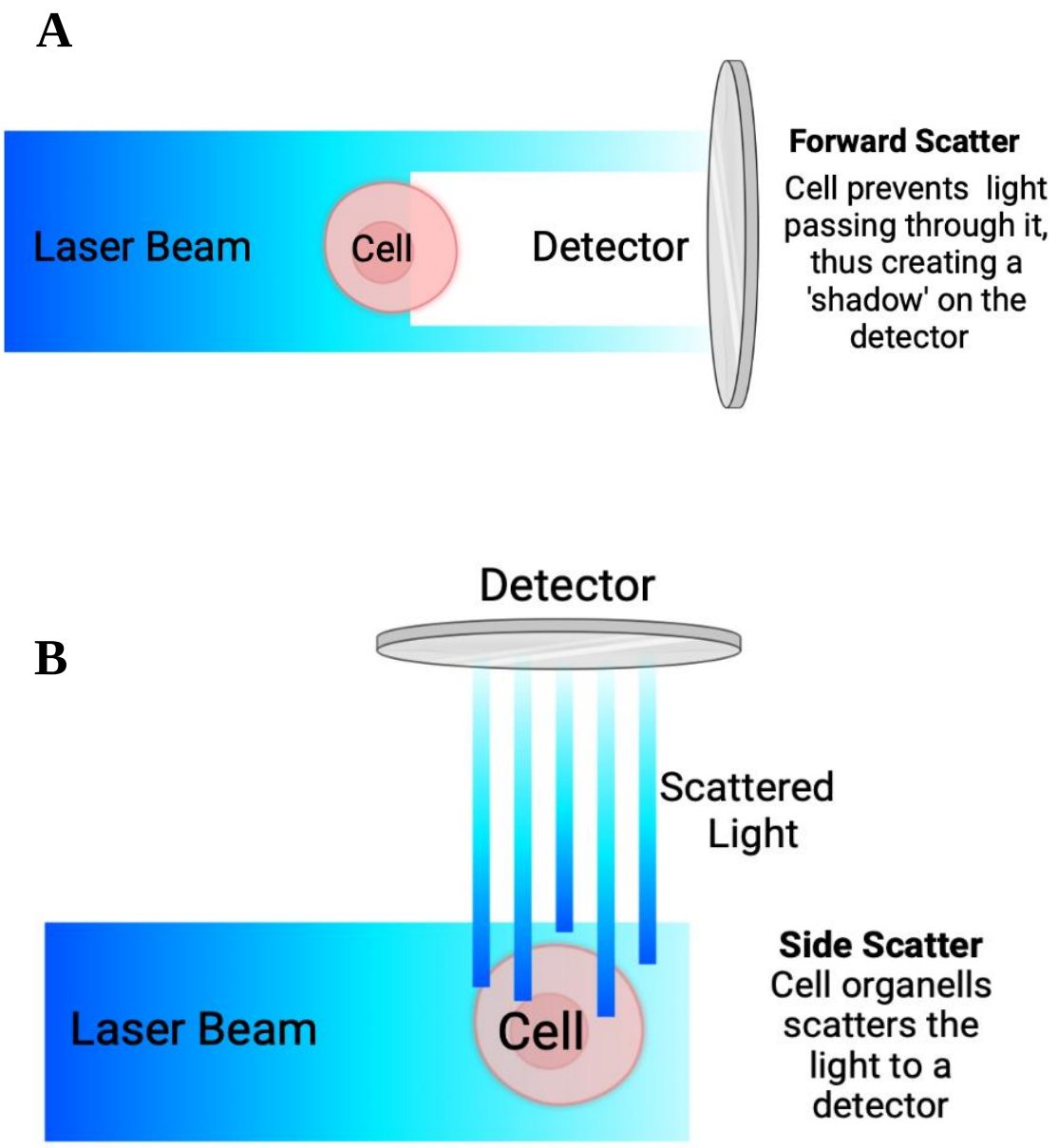


Figure 2.5 Forward Scatter (**A**) VS Side Scatter (**B)** on the flow cytometer.

Each flow cytometer can be equipped with a range of different detectors, with some having up to sixteen different channels. The flow cytometer used within the current study was a BD accuri C6 with four channels for fluorescence intensity.

2.10 Propidium Iodide (PI) Assay

2.10.1 General Principle

Propidium Iodide (PI) is a red-fluorescent dye which penetrates only damaged cellular membranes. Upon entry, PI binds to double-stranded DNA. PI is not membrane-permeable meaning it cannot cross intact plasma membrane and therefore will only be present in DNA of cells where the plasma membrane has been compromised/permeabilized. Therefore, making it useful to differentiate between necrotic, apoptotic, and healthy cells based on membrane integrity. PI is used more often than other nuclear stains as it is reasonable, stable and a suitable indicator of cell viability, based on its capacity to exclude dye in living cells (Rieger et al., 2011).

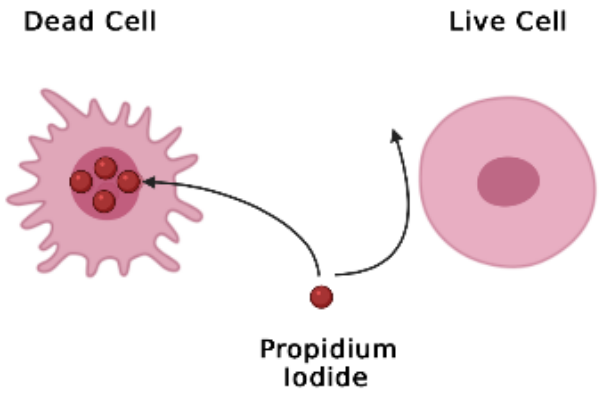


Figure 2.6 Propidium Iodide only being able to enter the cell membrane of a dead cell.

2.10.2 General Protocol

Remaining media on cell monolayers was pipetted into corresponding labelled Eppendorf's, i.e., media from cell monolayers dosed with 5 µL sCBD, was pipetted into an Eppendorf labelled 5 µL. Monolayers were then washed twice with PBS, before 200 µL of trypsin was added and placed in the incubator for 5 minutes. The media collected in the first step was then used to neutralize the trypsin, ensuring that the corresponding Eppendorf was used for each well. The solution of trypsin and media was homogenized before being pipetted back into the Eppendorf. Eppendorf's were then centrifuged for 5 minutes at 3000 rcf. existing media was aspirated and a small white pellet present at the bottom of the Eppendorf. 200µl of fresh DM was then added to each Eppendorf and vortexed fully until the pellet dissolved.

Samples were then prepared to be run through the low. 20 µL of PI solution (ThermoFisher Scientific) was added to each sample and vortexed. Analysis of samples needed to be done quickly, as longer incubation periods can lead to increased cellular death results. For samples containing myoblasts, the flow was set to fast and for samples containing myotubes, the flow was set at speed 22 ul/min and the core size set to 13.

2.11 Immunofluorescence (IF) Imaging

2.11.1 General Principal

Immunocytochemistry (ICC/IF) is a technique used to assess the presences of a specific protein/antigen in cells by use of a specific antibody that binds to it. The antibody allows visualization of the protein under a microscope. Immunocytochemistry is a valuable tool to study the presence of sub-cellular localization of proteins. The general ICC/IF protocol involves fixation (maintaining structures within the cell whilst rendering them dead), followed by a blocking step, primary antibody incubation and secondary antibody incubation.

2.11.2 General Protocol

Remaining media was aspirated, and cell monolayers washed 3 times with PBS. To fix the cells, paraformaldehyde (PFA 4%) solution was added to monolayers and incubated at room temperature for 10 minutes. Thereafter, the fixative solution was removed, and wells washed 3 times with PBS. The fixed sample can be stored for several days at 5˚C. Existing PBS was aspirated and permeabilization buffer (PBS + 0.1% Triton X-100) added to each well and incubated at room temperature for 15 minutes. Permeabilization buffer was then removed, and monolayers washed twice with cool PBS before the blocking buffer (10% Goat Serum in PBS, concentration ratio 1:500) was added and incubated at room temperature for a further 30 minutes. Monolayers were the washed once with PBS and the primary antibody (MF-20 in BSA, concentration ratio 1:300) added to each well, ensuring the lights dimmed, as MF-20 is light sensitive. Plates were then wrapped in parafilm and placed in the fridge (5°C) overnight.

Following this overnight period, the primary antibody was removed, and wells washed 3 times with cool PBS, leaving PBS on for 5 minutes with each wash. Then, the second antibody (Alexa-fluor goat anti mouse 488 in BSA, concentration ratio 1:400) was applied and plates covered in foil and left at room temperature for 60 minutes. Second antibody was then removed, and cell monolayers washed twice with PBS. The last step was nuclear counterstaining, DAPI, which is a blue-fluorescent DNA stain, in H_20 (concentration ratio 1:100) was added to monolayers and incubated at room temperature, avoiding light exposure, for 15 minutes. DAPI solution was then aspirated, a small volume of PBS was added to wells and plates were wrapped in tin foil before being stored in the fridge until required for imaging.

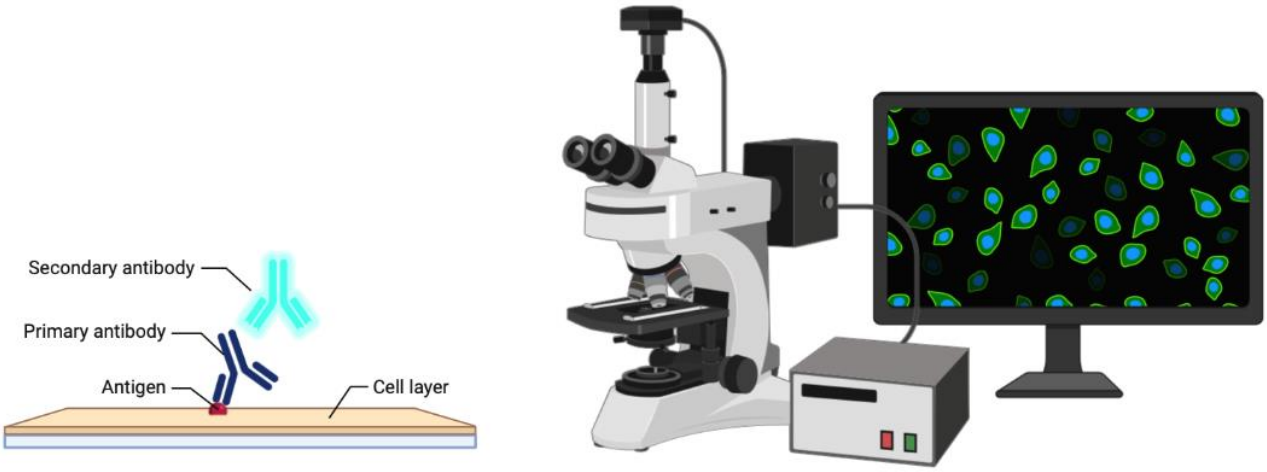


Figure 2.7 A schematic representation of antigen immunolabeling and fluorescent microscopy, demonstrating the binding of a primary and secondary antibody to an antigen and the visualisation of fluorescence emitting cells through a fluorescent microscope.

All stained monolayers were captured under a Leica DM116000B Microscope (Leica Biosystems, Wetzlar, Germany) at 10x objective. Blue colour channels (wavelength ~ 490-450nm) were used as an indicator of DAPI and green colour channels (wavelength ~ 560-520nm) used as an indicator for MyHC (Myosin Heavy Chain). Twelve images were taken per condition.

2.11.3 Measuring Myotube Number and Area

Myotube counting is a fundamental part of analysis as it helps to quantify myotubes formed during culture and is of interest to many investigations. In the present study, myotube number and area was measured in ImageJ (Rasband, W.S., ImageJ, U.S. National Institutes of Health, Bethesda, Maryland, USA). All images were scaled appropriately according to the level of magnification used during imaging, set to 8-bit and displayed a binary state. The free hand tool was used to differentiate between myotubes and blasts. Myotubes were classified as multi

nucleic structures with an observable outer membrane. Images then analysed, and results displayed in Prism.

2.11.4 Measuring Number of Nuclei

Number of nuclei was counted per image, and this was later used to measure EdU incorporation and calculate nuclear fusion index. In the present study, nuclei number was measured in ImageJ. Images were inverted, the appropriate threshold set (majority of nuclei highlighted) and the watershed setting applied. The smallest nuclei within the image was identified and the mean value calculated. The image was then analysed, and all nuclei equal to or greater than the mean size of the smallest nuclei outputted. The data was then analysed in Prism.

2.11.5 Measuring Number of Nuclei Per Myotube

To accurately calculate the average number of nuclei per myotube, the multi-point tool in ImageJ was used. Individual blue DAPI channel images were overlayed onto the corresponding green MyHC channel images and the number of nuclei within each myotube counted. To calculate the average number of nuclei per myotube, the number of nuclei within myotubes was divided by the number of myotubes.

Chapter 3

The Dose-Response Effects of Synthetic Cannabidiol on C_2C_{12} Myoblast Proliferation and Cell Viability

3.1 Abstract

Purpose – Research investigating the effects of synthetic cannabidiol (sCBD) on skeletal muscle cell proliferation and cell health and survival is limited. As such, little is known about the dose-response effects of sCBD. Therefore, the aim of this study was to assess the dose response (50 µM-0.001 µM) effect of sCBD on C_2C_{12} cell proliferation and survival for 24 and 48-hours.

Methods - C_2C_{12} skeletal muscle cells were seeded on gelatinised plates in growth media for 24-hours until 80% confluent. Following this period, cells were dosed at varying concentrations of sCBD (50 µM-0.001 µM) and left to proliferate for 24 and 48-hours. MTT and PI assays were then conducted after 24 and 48-hours to assess metabolic activity and cell health. To assess DNA synthesis, an accurate measure of cell proliferation, cells were cultured overnight and then treated with sCBD and labelled with EdU for 60-minutes.

Results – Cellular metabolic activity (MTT assay) after 24-hours was significantly reduced at 50 µM sCBD (P = <0.0001) compared to CON. After 48-hours, a significant reduction in metabolic activity was reported at 50 µM sCBD (P = <0.0001) and 20 µM (P = <0.0008) compared to CON. No significant difference in cell viability was recorded after 24-hours in sCBD. After 48-hours cell viability was significantly reduced at 50 µM (P = <0.0363) compared to CON. No significance was recorded in EdU incorporation at any sCBD dose compared to CON indicating no apparent effect on cell proliferation.

Conclusions – Higher doses of sCBD at 50 µM and 20 µM are detrimental to metabolic activity, with 50 µM also having a negative effect on cell viability at 48-hours. Significance was reported at higher doses of sCBD after 48-hours, implying sCBD is detrimental to myoblast health in a concentration and time-dependent manner

3.2 Introduction

When working with natural and synthetic products with unknown effects on the cell type of interest, it is vital to conduct dose response procedures to examine the effects on cell health. Cell-based assays are frequently used to measure cellular proliferation, cell viability and cytotoxicity as they monitor the response and health of cells in culture after treatment with various stimuli. This specific study investigated the effects of sCBD on cell proliferation, thus the assays conducted monitor the number of cells over time, the number of cellular divisions, cellular metabolic activity, and DNA synthesis, as these all occur during proliferation. To accurately assess the effects of sCBD on cell health, 3 cell viability assays were conducted: MTT assay, PI assay and EdU assay.

Assays that measure metabolic activity are appropriate when analysing proliferation, viability, and cytotoxicity. Here we used a tetrazolium dye reduction assay as one measure of cell health, by measuring metabolic activity. The decrease in tetrazolium salts such as MTT to coloured formazan compounds only occurs in metabolically active cells. Actively proliferating cells increase their metabolic activity, whilst cells exposed to toxins have decreased activity. Viable cells with active metabolism convert MTT into a purple formazan solution with an absorbance maximum near 570 nm (Riss et al., 2004). On the other hand, dead cells lose the ability to convert MTT into formazan, thus colour formation aids as a useful and appropriate marker of only viable cells (Riss et al., 2004). Therefore, the MTT assay can be used as a quick and simple measurement of cell metabolic activity.

Since the introduction of flow cytometry-based methods, research focusing on cellular apoptosis has been significantly impacted. Propidium Iodide (PI) is a nuclear stain which is used to determine cell viability through differences in plasma membrane integrity and

permeability (Rieger et al., 2011). PI cannot enter live cells due to the presence of an intact plasma membrane but can enter dead cells because of damaged cell membrane (Rieger et al., 2011). Therefore, the PI assay is used as it's a suitable marker of cell viability due to its ability to exclude dye in living cells.

To assess cell proliferation, several methods can be adopted (see sections 2.7, 2.8 and 2.10). One approach is to quantify the percentage of cells synthesizing DNA during a specific time (Mead and Lefebvre, 2014). By employing 5-ethynyl-2´-deoxyuridine (EdU) staining, EdU incorporated into newly synthesized DNA can be detected with a fluorescent azide and quantified by fluorescent microscopy. Therefore, the EdU assay is used as it is a fast and reliable method which quantifies the percentage of DNA-synthesizing cells and essentially measures cell proliferation.

Research investigating the effects of synthetic cannabidiol is limited, as such, little is known at what dosage or if sCBD becomes cytotoxic to C_2C_{12} skeletal muscle cells. The purpose of this study was to examine the effects of sCBD on proliferation to identify which, if any doses of sCBD are cytotoxic to myoblast cell health. As previous research suggests, CBD elicits many benefits to cell health, such as having an antioxidant and anti-inflammatory effect (Overdevest et al., 2018). Therefore, due to these reported benefits, we hypothesized that sCBD would have a positive effect on myoblast health during proliferation.

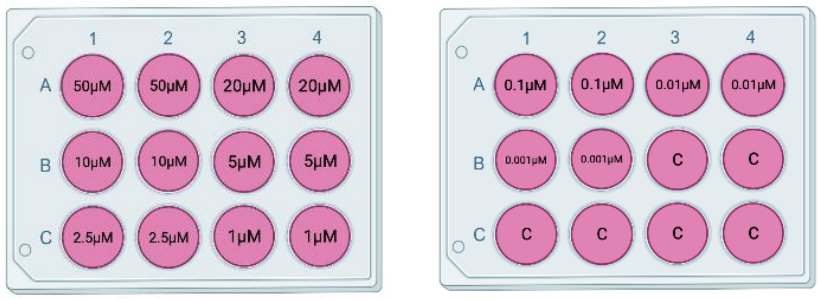
3.3 Procedures

To examine the effects of sCBD on myoblasts, 24hr and 48hr viability assays were conducted. For the MTT and PI assays, low passage C_2C_{12} cells were seeded at $3x10^4$ cells·ml^{-1} in pre-gelatinised 12-well plates in growth media (GM). Plates were then incubated at 37˚C 5% CO_2

for 24-hours. Once cell confluency reached 80%, GM was removed, and cell monolayers were washed twice with PBS. Growth media dosed with sCBD, concentrations ranging from 0.001 µM to 50 µM was then added to cell monolayers (see **figure 3.1.**). For control (CON), cell monolayers were treated with just GM. Monolayers were then incubated for either 24 or 48-hour periods. Upon cessation of these time points, cell viability assays were conducted.

For the EdU assay, C_2C_{12} skeletal muscle cells, passage number 8, were seeded at $3x10^4$ cells·ml^{-1} on pre-gelatinised 12-well plates in growth media (GM) and left to adhere overnight. The following morning media was changed to GM containing sCBD at doses ranging from 0.001 µM to 50 µM or vehicle control, and 2 wells received no EdU solution as this was the negative control (see **figure 3.1**). All media contained of EdU solution, apart from the media used for the negative control. Cultures were then incubated at 37˚C for 60-minutes. Following this incubation period, cells were then fixed and stained for imaging (see section 2.10.4).

A



B

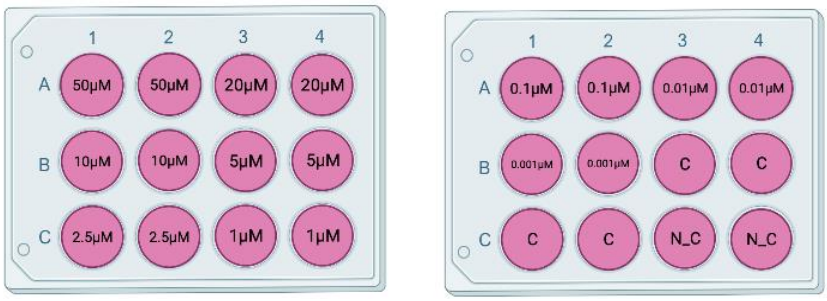


Figure 3.1 Schematic representation of the four 12-well plate study design with corresponding sCBD doses (0.001 µM to 50 µM) or vehicle control (CON) for MTT and PI Assay (**A**) and the appropriate sCBD doses (0.001 µM to 50 µM) or vehicle control (CON) and negative control(N_C) for EdU Assay (**B**).

3.3.1 MTT Assay

Following the 24- and 48-hour treatment of sCBD/GM, cell media was aspirated, and monolayers treated with a tetrazolium dye solution as described in section 2.6.2 of this thesis. Cellular metabolic activity was then measured using a plate reader (570nm wavelength) as described in section 2.6.2. Sample size of n=4 per condition.

3.3.2 PI Assay

Following the 24 and 48-hour treatment of sCBD/GM, cell media and monolayers were aliquoted into labelled Eppendorf's and prepared for analysis via the flow cytometer as described in section 2.9.2 of this thesis. Propidium Iodide solution was then added to the cell suspension 5 minutes prior to analysis conducted by flow cytometry, see section 2.9.2. Sample size of n=4 per condition.

3.3.3 EdU Assay

Prior to conducting this study, the EdU assay was optimized. Following the first incubation period of 6-hours with EdU and sCBD at varying concentrations (0.001 µM – 50 µM) cell death was reported at 0.001 µM. Therefore, a pilot study was conducted where cells were treated with EdU solution at different time points (1, 2, 3 and 4-hours), images captured and the effects on proliferation analyzed. All time points revealed no negative effect on cell health with proliferation across all doses recorded best at an incubation period of 60-minutes as cells looked subjectively healthy and confluent. Therefore, in all studies within this thesis, treatment

of EdU was for 60-minutes. Following the 60-minute incubation period with EdU solution, cells were fixed and stained for imaging. ImageJ software was used to calculate EdU cell incorporation. Four images per well were captured and 2 channels, EdU and DAPI analyzed. The number of EdU positive nuclei and the number of DAPI positive nuclei were counted to calculate the percentage of cells that had undergone cell division during the treatment period. Sample size of n=4 per condition.

3.4 Results

3.4.1 MTT Assay

A Shapiro-Wilk test was conducted on both data sets to assess normality and lognormality. Both data sets were normally distributed, and a parametric one-way ANOVA used (see **figure 3.2**). The findings from the 24-hour treatment of sCBD conclude that metabolic activity at 50 µM sCBD was significantly lower (46.61 % ± 4.5 %, *P* = <0.0001) compared to control (100% ± 6.24 %).. Metabolic activity at 50 µM was significantly lower compared to 20 µM (109.3 % ± 9.15 %, *P* = <0.0001), 10 µM (115.6 % ± 12.41 %, *P* = <0.0001), 5 µM (125.9 % ± 8 %, *P* = <0.0001), 2.5 µM (124.1 % ± 12.67 %, *P* = <0.0001), 1 µM (128.3 % ± 14.11 %, *P* = <0.0001), 0.1 µM (131.7 % ± 15.28 %, *P* = <0.0001), 0.01 µM (114.2 % ± 21.28 %, *P* = <0.0004) and 0.001 µM (128.2 %± 17.53 %, *P* = <0.0001).

The findings from the 48-hour treatment of sCBD conclude that metabolic activity at 50 µM sCBD (35.91 % ± 4.79 %, *P* = <0.0001) and 20 µM (79.42 % ± 3.15 %, *P* = <0.0008) was significantly lower compared to control (100 % ± 14.01 %). Significance was also reported in between sCBD doses; Metabolic activity at 50 µM was significantly lower compared to 20 µM (*P* = <0.0001), 10 µM (93.69 % ± 6.03 %, *P* = <0.0001), 5 µM (93.3 % ± 8.23 %, *P* = <0.0001), 2.5 µM (94.84 % ± 4.4 %, *P* = <0.0001), 1 µM (98.22 % ± 3.64 %, *P* = <0.0001), 0.1 µM (100.8 % ± 5.25 %, *P* = <0.0001), 0.01 µM (103.4 % ± 4.76 %, *P* = <0.0001) and 0.001 µM (

105.4 % ± 3.17 %, *P* = <0.0001). Metabolic activity at 20 µM was also significantly lower compared to 1 µM (*P* = <0.0100), 0.1 µM (*P* = <0.0023), 0.01 µM (*P* = <0.0005) and 0.001 µM (*P*= <0.0001).

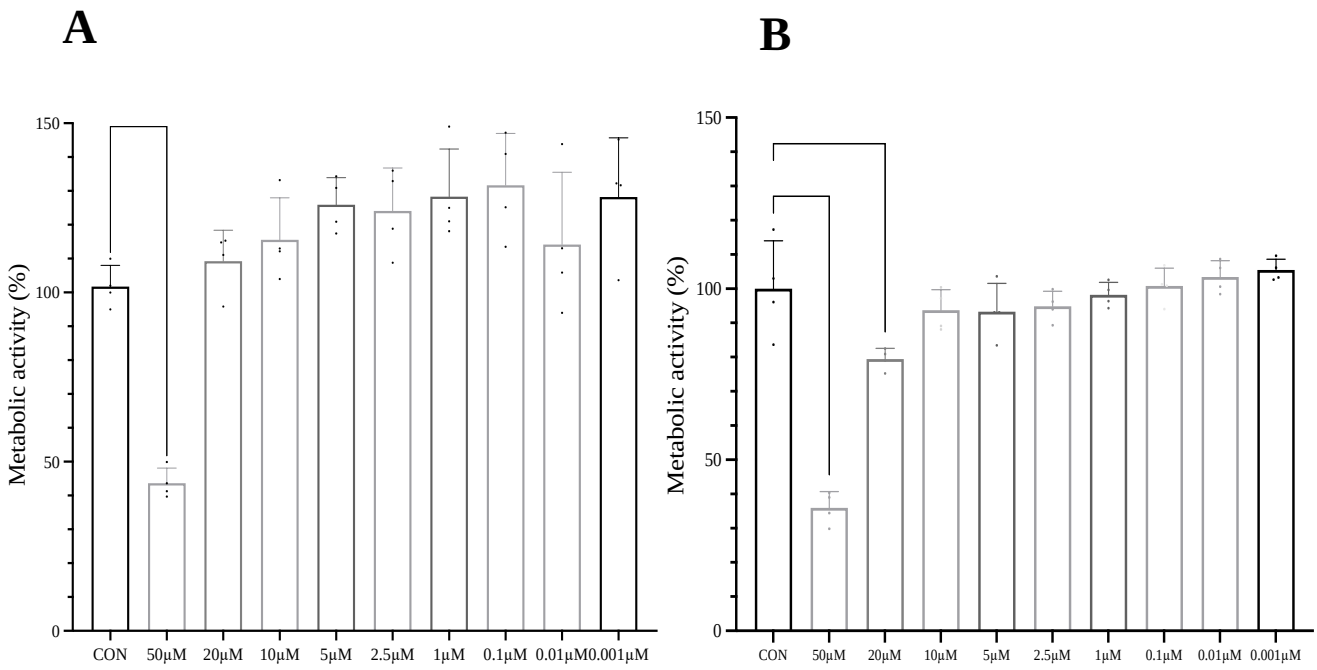


Figure 3.2 Data collected following the MTT assay. Cellular metabolic activity (%) after 24-hours in GM/sCBD (**A**) and 48-hours in GM/sCBD (**B**). Pairwise comparisons represented.

3.4.2 PI Assay

A Shapiro-Wilk test was conducted on both data sets to assess normality and lognormality. Normality was not confirmed on both sets of data, so a Kruskal-Wallis test was run as this is a non-parametric equivalent of ANOVA. The findings from the 24-hour treatment of sCBD conclude that there was so significant difference in cell viability across all doses compared to control (CON). No significant differences were reported in between doses. The findings from the 48-hour treatment of sCBD conclude that 50 µM (86.67 % ± 6.3, *P* = <0.0363) was significantly lower compared to CON (97.99 ± 0.08). No other significant differences were reported.

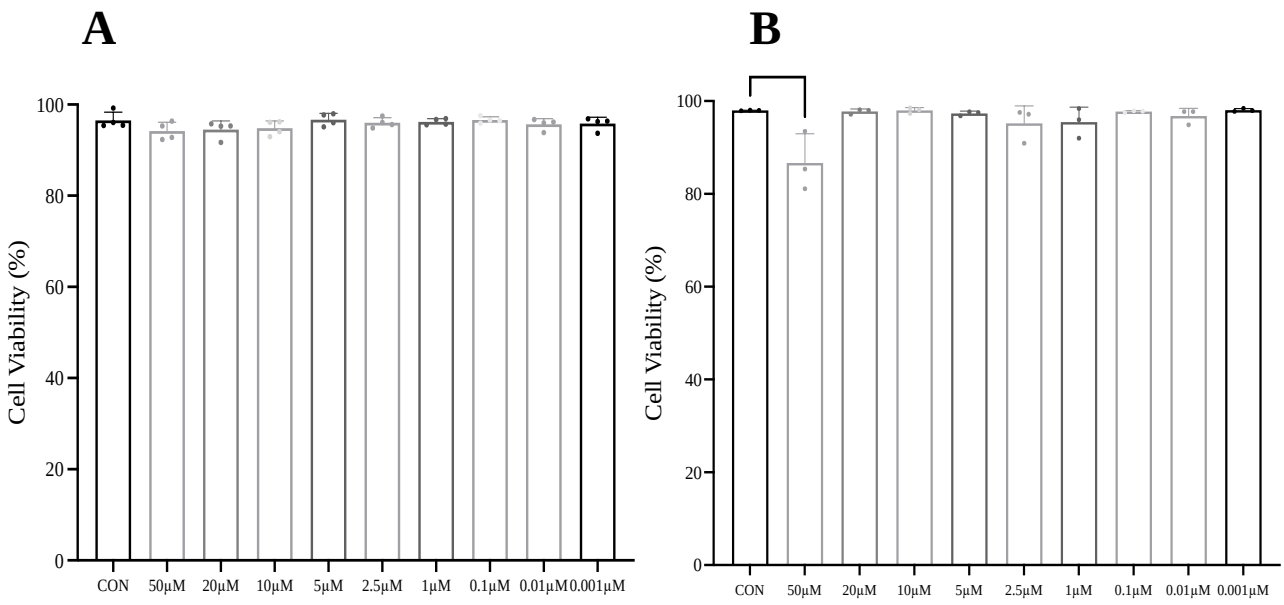


Figure 3.3 Data collected following the PI assay**.** Cell viability (%) after 24-hours in GM/sCBD (**A**) and 48-hours in GM/sCBD (**B**). Pairwise comparisons reported.

3.4.3 EdU Assay

A Shapiro-Wilk test was conducted on both data sets to assess normality and lognormality. Normality was not confirmed, so a Kruskal-Wallis test was run. No significant difference in EdU incorporation (%) was recorded across all doses compared to control (CON) (58.63 ±4.92, P = >0.9999).

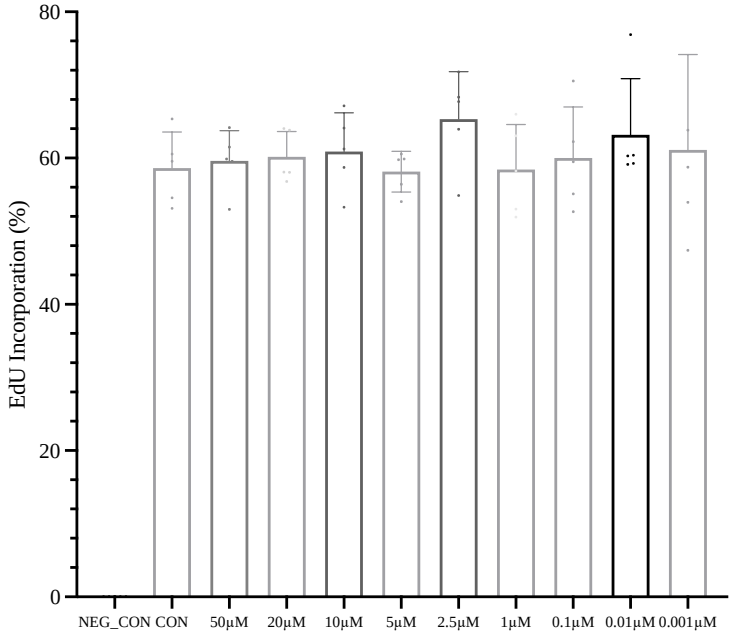


Figure 3.4 Data collected following the EdU assay. EdU incorporation (%) across all doses and CON.

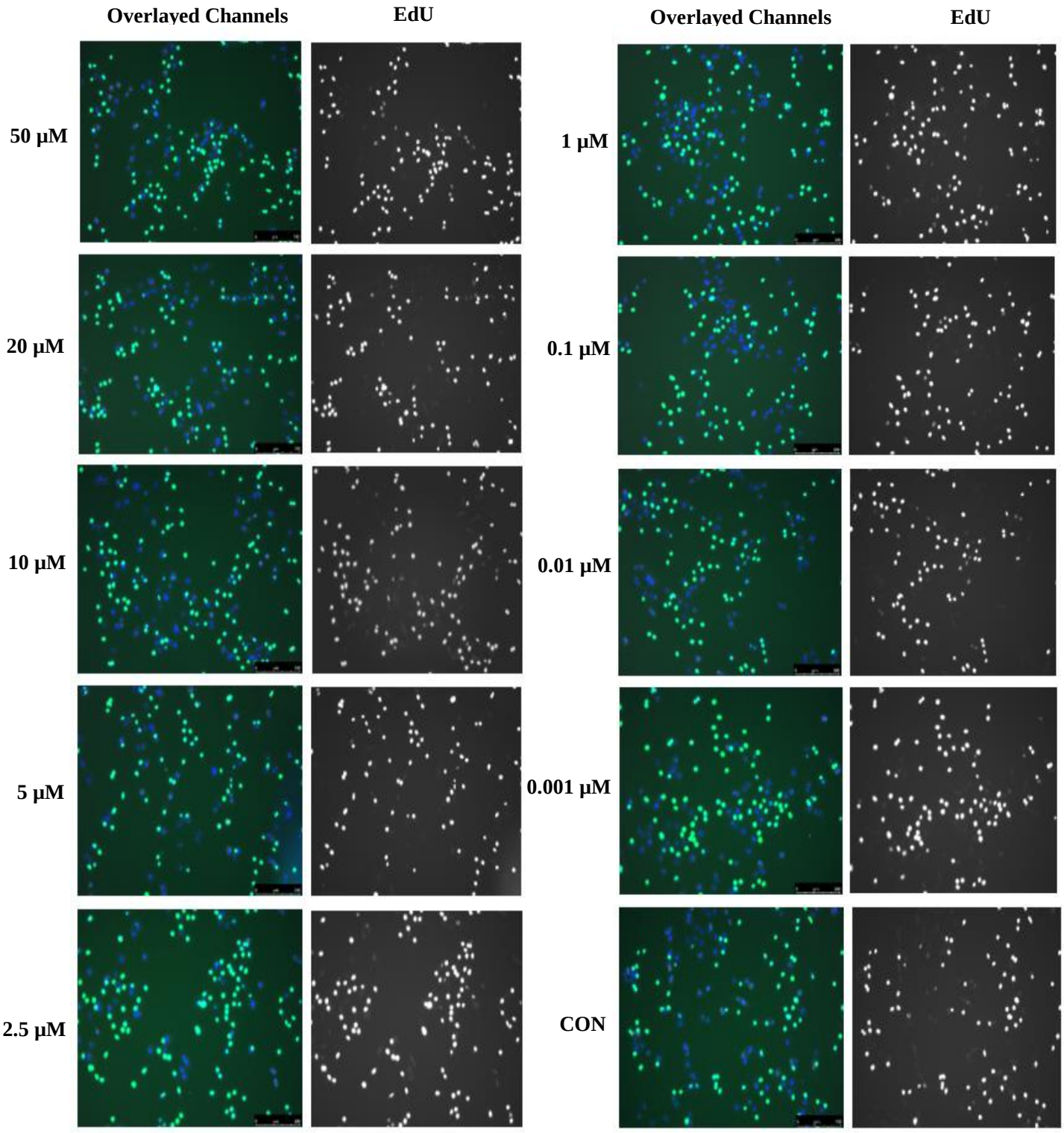


Figure 3.5 Images captured following EdU assay. Overlayed channel displaying both DNA (blue) stained cells with Hoechst 33342 and EdU/Hoechst (green) positive cells. EdU/Hoechest positive cells present in EdU channel. See section 2.8.2 to calculate percentage of EdU incorporation in proliferating cells.

3.5 Discussion

The main aim of this investigation was to identify the effects of sCBD on cell proliferation and to identify at what concentration of sCBD is cytotoxic. In the current study, C_2C_{12} cells were left to proliferate for 24 and 48-hours in the presence of different sCBD concentrations and we hypothesized that sCBD would have a positive effect on cellular health during proliferation. The main findings of the investigation allow us to reject the hypotheses. It was observed that following the 24hr treatment of sCBD, metabolic activity was significantly reduced at 50 µM, implying high doses of sCBD *in vitro* are detrimental to metabolic activity. Following the 48-hour treatment of sCBD, metabolic activity was also significantly reduced at 50 µM and 20 µM compared to control, with reductions being greater than those seen at 24-hours, implying a longer exposure to sCBD had a greater impact on metabolic activity during proliferation. These findings are similar to a study conducted by Oláh et al who investigated the effects of CBD on human immortalized SZ95 sebocytes (Oláh et al., 2014). They discovered that administration of 50 µM CBD evoked apoptosis-driven cytotoxicity, hence reducing cell viability (Oláh et al., 2014).

The results from our study also conclude that lower concentrations of sCBD at 10 µM to 0.001 µM had no impact on metabolic activity. A study conducted by Aviello et al (2012) investigated the potential chemopreventive effect of cannabidiol in an experiment model of colon cancer. The results from the MTT following the 24hr treatment of cannabidiol during proliferation

(concentrations 0.01-10μM) on CACO-2 and HCT116 cell lines concluded that CBD was not cytotoxic and had no effect on cell proliferation, in agreement with the findings from the present study (Aviello et al., 2012). Oláh et al. (2014) observed inhibited proliferation in SZ95 cells at 1 μM, 5 μM and 10 μM after 48-hours of CBD treatment, however at these concentrations, no decrease in cellular viability or an induce in apoptosis was recorded (Oláh et al., 2014).

Whilst these results suggest that higher doses of sCBD administration likely decreases myoblast cell viability, the MTT assay alone is an inadequate to confirm these findings as it only measures metabolic activity, not specifically cellular viability. Research has identified a direct association between glucose concentrations in cell media and the MTT reductive rate, one of numerous limitations of this assay as section 6.2 of this thesis describes. Therefore, the propidium iodide assay was also used in the current study. No effect on cell viability was recorded across all doses of sCBD after 24-hours, with only 50 μM significantly decreasing cell viability after 48-hours. Previous research has indicated that within different cell lines, a relationship exists between autophagy and CBD-mediated cell death. Research examining the effects of CBD on MDA-MB-31 breast cancer cells, observed nuclear condensation and margination which are typical characteristics of apoptosing cells (Shrivastava et al., 2011). Morphological changes such as membrane whorls, increased vacuolization and less intracellular organelles were also observed, findings consistent with autophagy (Shrivastava et al., 2011). Thus, suggesting the coexistence of apoptosis and autophagy in CBD-treated cells (Shrivastava et al., 2011). A study conducted by Vrechi and colleagues (2021), which employed the PI assay to monitor nuclear DNA content in SH-SY5Y cell populations in the presence or absence of CBD, aimed to explore the possibility between autophagy and CBD-mediated cell death (Vrechi et al., 2021). In this study, cells were treated with CBD (1, 2, 5,

10, 25, 50 and 100 µM) or control, for 24 and 48-hours and the sub-G_0/G_1 fraction was used as an indicator of cell death (Vrechi et al., 2021). They discovered that CBD significantly increased the sub-G_0/G_1 fraction in both a concentration and time-dependent manner (Vrechi et al., 2021). Their findings revealed that following a 24hr exposure to 50 µM and 100 µM CBD, there was a significant increase in the number of cells in the sub-G_0/G_1 fraction, as well as following a 48hr exposure to 25 µM (Vrechi et al., 2021). To measure autophagy, varying concentrations of CBD (5, 10 and 50 µM) was administrated to SH-SY5Y cells, and a lysosome inhibiter added. LC3-II protein expression was measured as this is a standard marker for autophagosome accumulation, and the results revealed CBD increased LC3-II levels in a concentration-dependent manner, confirming CBD treatment modulated autophagic flux of SH-SY5Y cells. These findings suggest that the decrease in cell viability reported after 48-hours at 50 µM may be due to apoptosis and autophagy, although further research needs to be conducted to confirm this. Our findings also support that with increases in CBD concentrations and exposure time, greater impact on cell health is reported, as no significance was reported after 24-hours but after 48-hours, cell viability was significantly reduced after 50 µM.

One study, which examined the pharmacological effects of plant-derived versus synthetic cannabidiol, explored the effects of CBD in the ovarian cancer cell line SKOV-3 (Maguire et al., 2021). Cells were treated with either vehicle or CBD (10-50 µM) and allocated into 2 time points: 24 and 48-hours (Maguire et al., 2021). The findings conclude that 10 µM CBD reduced cell viability in proliferating cells and 50 µM CBD was cytotoxic in both proliferating and confluent cells (Maguire et al., 2021). Greater effects were also reported at 48-hours (Maguire et al., 2021). Although no significant reductions in metabolic activity and cell viability were reported at 10 µM sCBD, both metabolic activity and cell viability were significantly decreased at 50 µM sCBD in our findings. Therefore, this research supports our findings as 50 µM sCBD

was detrimental to cell health and greater effects were reported after 48-hours. This study also discovered that none of the purified CBD samples differed in their effectiveness across concentrations or time points which implies differences in CBD origin has no impact on its antiproliferative/cytotoxic effects on SK0V-3 cells (Maguire et al., 2021).

The reduction in cell viability after 48-hours indicates the possibility that treatment with sCBD induces apoptosis. Findings by Fisher et al, concluded that following the 24 and 48-hour treatment with CBD (24 µM and 32 µM) there was an increase in the early apoptotic cell population in a time-dependent manner (Fisher et al., 2016). Microscope analysis supported these findings as treatment with 32 µM CBD affected cell morphology as the number of cells that appeared to have denatured increase (Fisher et al., 2016). Although microscopic analysis was not conducted in this study to observe the morphological effects of sCBD on myoblasts, the reduction in cell viability could potentially be associated with sCBD treatment being attributable to apoptotic cell death in a dose and time dependant manner. Cannabidiol was also tested for its ability to induce apoptosis in three separate cells lines: SiHA, HeLa and ME-180 cells (Lukhele and Motadi, 2016). Cells were treated with CBD at various concentrations (0, 50, 100 and 150 µg/ml) for 24 hours and analysis conducted via the flow cytometer. The results confirmed that following the PI assay, the type of cell death induced was apoptosis (Lukhele and Motadi, 2016). Within the different cells, cannabidiol induced 51.35 % apoptosis in SiHA cells, 43.3 % in HeLa and 28.6 % in ME-180 cell lines (Lukhele and Motadi, 2016). Further findings conclude that cannabidiol induced early apoptosis in all three cell lines (Lukhele and Motadi, 2016).

As mentioned previously, the differences in results across all studies could potentially be due to the different cell lines in question. Research conducted by Chen et al examined the effect of

a 48hr exposure to 12 µM CBD on T24, UM-UC-3 and 5637 cells (Chen et al., 2021). The findings from the PI flow cytometry assay concluded that exposure to 12 µM CBD for 48-hours elevated apoptotic cell population compared to control (Chen et al., 2021). On the other hand, no pro-apoptotic effect was recorded on UM-UC-3 and 5637 cells, implying that various doses of CBD effect the health of different cell lines in dissimilar ways (Chen et al., 2021).

Specific markers of cellular apoptosis would strengthen our findings and confirm whether sCBD induces apoptosis at higher doses *in vitro*. For example, conjugates of annexin V, the human vascular anticoagulant annexin V, Ca^{2+}-dependent phospholipid-binding protein that has a high affinity for the anionic phospholipid phosphatidylserine (PS) can be used to detect apoptotic cells. In normal healthy cells, PS is located on the cytoplasmic surface of the plasma membrane. However, during apoptosis, the plasma membrane undergoes structural changes that include translocation of PS from the inner to the extracellular side of the plasma membrane. In addition, different gene families such as caspases, inhibitor of apoptosis proteins, B cell lymphoma (Bcl)-2 family of genes, tumor necrosis factor (TNF) receptor gene superfamily, or p53 gene are also involved in the process of apoptosis and can be detected by RT-qPCR (Kira et al, 2016).

The final findings from this study conclude that no significance was reported in EdU incorporation across all doses of sCBD compared to control, proposing sCBD has no inhibitory or stimulatory effect on proliferation. The effects of CBD on proliferation were also investigated on HT-29 cells by measuring the incorporation of BrdU into DNA and they discovered similar findings (Sainz-Cort, Müller-Sánchez and Espel, 2020). They observed no changes in DNA synthesis after 3 days of incubation with any concentration of CBD (0.5, 1, 2, 4 and 10 µM) (Sainz-Cort, Müller-Sánchez and Espel, 2020). On the other hand, there was

a significant decrease compared to control in DNA synthesis following the treatment of 10 µM CBD on SW480 cells, thus suggesting that CBD effects DNA synthesis in certain cell lines (Sainz-Cort, Müller-Sánchez and Espel, 2020).

Chapter summary

In summary, the data presented from this investigation suggests that synthetic cannabidiol is detrimental to myoblast cell health in a concentration and time dependent manner. Higher doses at 50 µM and 20 µM, seem to have a damaging impact on metabolic activity, whereas cell viability is only reduced at 50 µM after 48-hours, suggesting the effects of sCBD on metabolic activity precedes cell death. Synthetic cannabidiol could potentially induce apoptosis, which would explain why cell viability is reduced at 50 µM, however further analysis would be needed to confirm this. Interestingly, no significance was recorded in EdU incorporation, proposing sCBD does not inhibit DNA synthesis. However, monolayers were only incubated for 60-minutes with sCBD and EdU solution, therefore it may be due to this short exposure period why no change in EdU incorporation was recorded. Therefore, our results conclude that sCBD is cytotoxic to C_2C_{12} myoblasts at higher doses (50 µM and 20 µM) and with an increased exposure time (48-hours).

This study primarily focused on the effects of sCBD on proliferation, hence future work should utilise these different cellular viability assays and perform them at different time points throughout the time course of differentiation. Therefore, a greater understanding of the effects of sCBD on myogenesis, not just proliferation, can be achieved.

Chapter 4

The Effects of Acute and Chronic Exposure to Synthetic Cannabidiol on C2C12 Myotube Viability and Metabolic Activity

4.1 Abstract

Purpose – The findings from chapter 3 demonstrate the cytotoxic effect sCBD has on myoblast viability at certain doses. Therefore, this chapter aims to study the effects of sCBD on metabolic activity and cell viability during differentiation of myoblasts to myotubes.

Methods – C_2C_{12} skeletal muscle cells were cultured in growth media for 24-hours until 80% confluent then switched to low serum media to induce differentiation. For the chronic treatment of sCBD, cells were treated with sCBD at 0 hours of differentiation and maintained in sCBD treated media during 10 days of differentiation. For acute treatment with sCBD, cells were left to differentiate in DM for 8 days and then treated with sCBD for 48-hours once mature myotubes were already formed. MTT and PI assays were conducted upon conclusion of termination of experiments to determine cellular metabolic activity and viability.

Results – Metabolic activity compared to CON was significantly lower at 50 µM (P = <0.0001), 20 µM (P = <0.0001) and 10µM sCBD (P = <0.0001) after chronic treatment. After acute treatment, only 50 µM was significantly lower (P = <0.0045) compared to CON. Cell viability compared to CON was significantly lower at 50 µM (P = <0.0486) and 20µM sCBD (P = <0.0302) after chronic treatment. No significance in cell viability was recorded following acute treatment of myotubes.

Conclusions – Detrimental effects on metabolic activity were observed following the chronic treatment of sCBD at higher doses (50 µM, 20 µM and 10 µM) and significant decrease in cell viability was found at 50 and 20 µM. Following acute treatment however, only metabolic activity was affected, with reductions in metabolic activity reported at 50 µM sCBD. No significance in cell viability was recorded after acute treatment, implying increased exposure to sCBD leads to amplified damaging effects on cell health. Similar effects were also recorded in myoblasts, with 50 µM reducing metabolic activity and cell viability after 48-hours

suggesting higher concentrations of sCBD are detrimental to both myotube and myoblast health.

4.2 Introduction

Differentiation of skeletal muscle from myoblasts to myotubes *in vitro* is a useful tool to facilitate investigation of the mechanisms which regulate muscle mass maintenance and growth (Murphy et al., 2016). Myogenesis is a regulated process which ends in the formation of new muscle fibres (Sanvee, Bouitbir and Krähenbühl, 2021). Within this process, satellite cells are activated to mononucleated precursor cells (myoblasts), which then, in the presence of myogenic factors involved in gene regulation, proliferate and fuse with one another to form elongated, multinucleated myotubes (Sanvee, Bouitbir and Krähenbühl, 2021). In study 1, cell viability of myoblasts was assessed during proliferation which identified higher concentrations of sCBD at 50 µM were cytotoxic to cell health, with greater effects reported after 48-hours. In this study, following sCBD treatment at different time points throughout differentiation, cell viability of myotubes will be measured. As this is the next step in myogenesis, it is just as important to investigate the effects on myotubes as well as myoblasts, as similar findings could potentially have implications on cell growth moving forward.

The overall aim of this study was to assess myotube health and viability, following both an acute and chronic treatment of sCBD. Cell-based assays were conducted on terminally differentiated myotubes. Although detrimental effects were reported on cell health at high concentrations of sCBD during proliferation, we hypothesised that sCBD at lower doses would have a positive impact on myotubes during differentiation as previous research suggests lower concentrations of CBD (range 1-5 µM) had no negative effect on anabolic signalling pathways such as the mTORC1-axis (Langer, Avey and Baar, 2021). However, at higher concentrations of 50 µM sCBD and with increased exposure time, we hypothesised that negative effects on

cellular health would be reported as previous research suggests following both a high dose (50 µM) and a long exposure to CBD (10 µM for 6 days), a decrease in cell viability of SZ95 cells is reported (Oláh et al., 2014).

4.3 Procedures

To examine the effects of chronic treatment of sCBD, the following procedure was followed. Low passage C_2C_{12} cells were seeded at $3x10^4$ cells·ml^{-1} in pre-gelatinised 12-well plates in growth media (GM). Plates were then incubated at 37˚C 5% CO_2 for 24-hours. Once cell confluency reached 80%, GM was aspirated, and cell monolayers washed twice with PBS. Monolayers were then treated with sCBD in differentiation medium, with doses ranging from 0.001 µM to 50 µM or CON (see **figure 4.1**). Media was then topped up every 48-hours with the respective treatment at 10% of the volume of media in the well. After 10 days in sCBD or vehicle control, the experiment was concluded.

To examine the effects of acute treatment of sCBD, the same procedures were followed to expand the myoblasts population as described earlier, except when cell confluency reached 80%, all plates were switched to DM with no treatment. Cells were then left to differentiate in DM for 8 days with media topped up every 48 hours. On day 8 of differentiation, myotubes were treated with sCBD with doses ranging from 0.001 µM to 50 µM or vehicle CON (see **figure 4.1**), for a 48-hour period. The experiment terminated on day 10 at which stage MTT and PI assays were performed

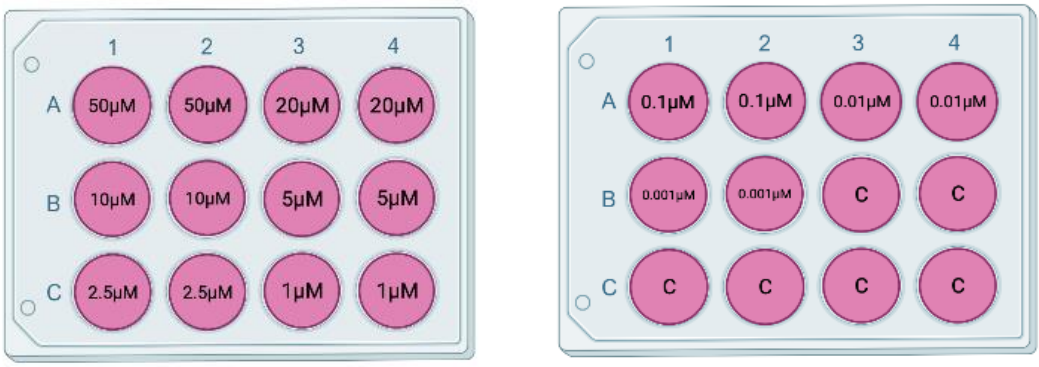


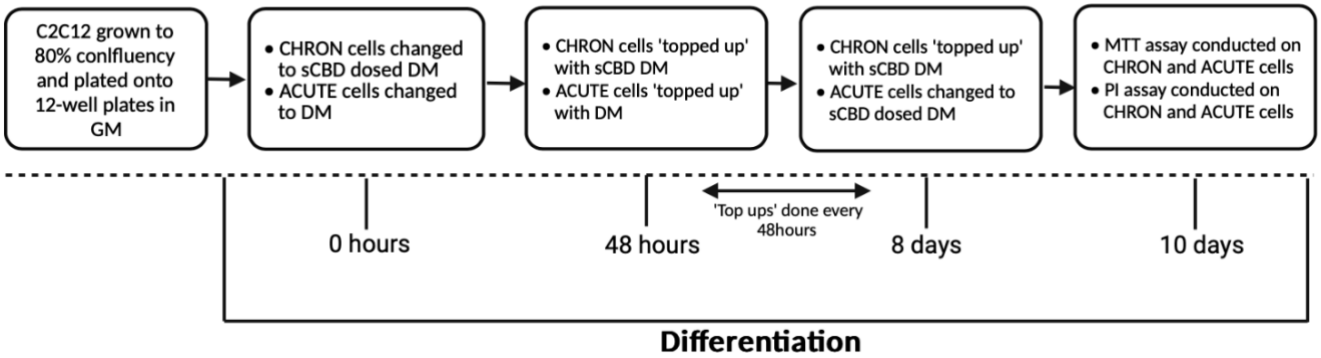
Figure 4.1 Schematic representation of the experimental design; two 12-well plates with the corresponding sCBD doses (0.001 µM to 50 µM) or CON for both the acute and chronic treatment of sCBD.

4.3.1 MTT Assay

Upon cessation of both acute and chronic experiments, cell media was aspirated, and monolayers treated with a tetrazolium dye solution as described in section 2.6.2 of this thesis. Cellular metabolic activity was then measured using a plate reader (570 nm wavelength) as described in section 2.6.2. Sample size of n=4 per condition.

4.3.2 PI Assay

Upon cessation of both acute and chronic experiments, cell media and monolayers were pipetted into labelled Eppendorf's and prepared to be run through the flow as described in section 2.9.2 of this thesis. Propidium Iodide solution was then added to the cell suspension 5



minutes prior to analysis conducted by flow cytometry, see section 2.9.2. Sample size of n=4 per condition.

Figure 4.2 Schematic representation of the experimental design used to examine the effects of chronic and acute treatment of sCBD on the differentiation of muscle cells.

4.4 Results

4.4.1 MTT Assay

A Shapiro-Wilk test was conducted on both data sets to assess normality and lognormality. Normality was confirmed on the data produced from the chronic study and a parametric one-way ANOVA used. On the other hand, normality was not confirmed on the data produced from the acute study, so the non-parametric equivalent, Kruskal-Wallis test was run.

Following chronic treatment with sCBD, cellular metabolic activity of myotubes was significantly lower at doses 50 µM (9.72 % ± 0.35 %, *P* = <0.0001), 20 µM (8.89 % ± 0.53 %, *P* = <0.0001) and 10 µM (76.94 % ± 5.15 %, *P* = <0.0001) compared to CON (112.8 % ± 8.95 %). Metabolic activity at 50µM was significantly lower compared to 10 µM (*P*= <0.0001), 5 µM (107.9 % ± 6.18 %, *P* = <0.0001), 2.5 µM (95.34 % ± 8.66 %, *P* = <0.0001), 1 µM (106 % ± 6.47 %, *P* = <0.0001), 0.1 µM (99.37 % ± 8.25 %, *P* = <0.0001), 0.01 µM (95.22 % ± 1.99 %, *P* = <0.0001) and 0.001 µM sCBD (116.4 % ± 15 %, *P* = <0.0001). Metabolic activity at 20 µM was significantly lower compared to 10 µM (P = <0.0001), 5 µM (*P* = <0.0001), 2.5 µM (*P* = <0.0001), 1 µM (*P* = <0.0001), 0.1 µM (*P* = <0.0001), 0.01 µM (*P* = <0.0001) and 0.001 µM sCBD (*P* = <0.0001). Significance was also reported at 10 µM, as metabolic activity at this dose was significantly lower compared to 1 µM (*P* = <0.0037), 0.1 µM (*P* = <0.0380) and 0.001 µM (*P* = <0.0001).

Following acute treatment of sCBD, cellular metabolic activity compared to CON (111 % ± 19.09 %) was only significantly lower at a dose of 50 µM (5.56 % ± 0.5 %, *P* = <0.0045). All other doses were not significantly different compared to CON.

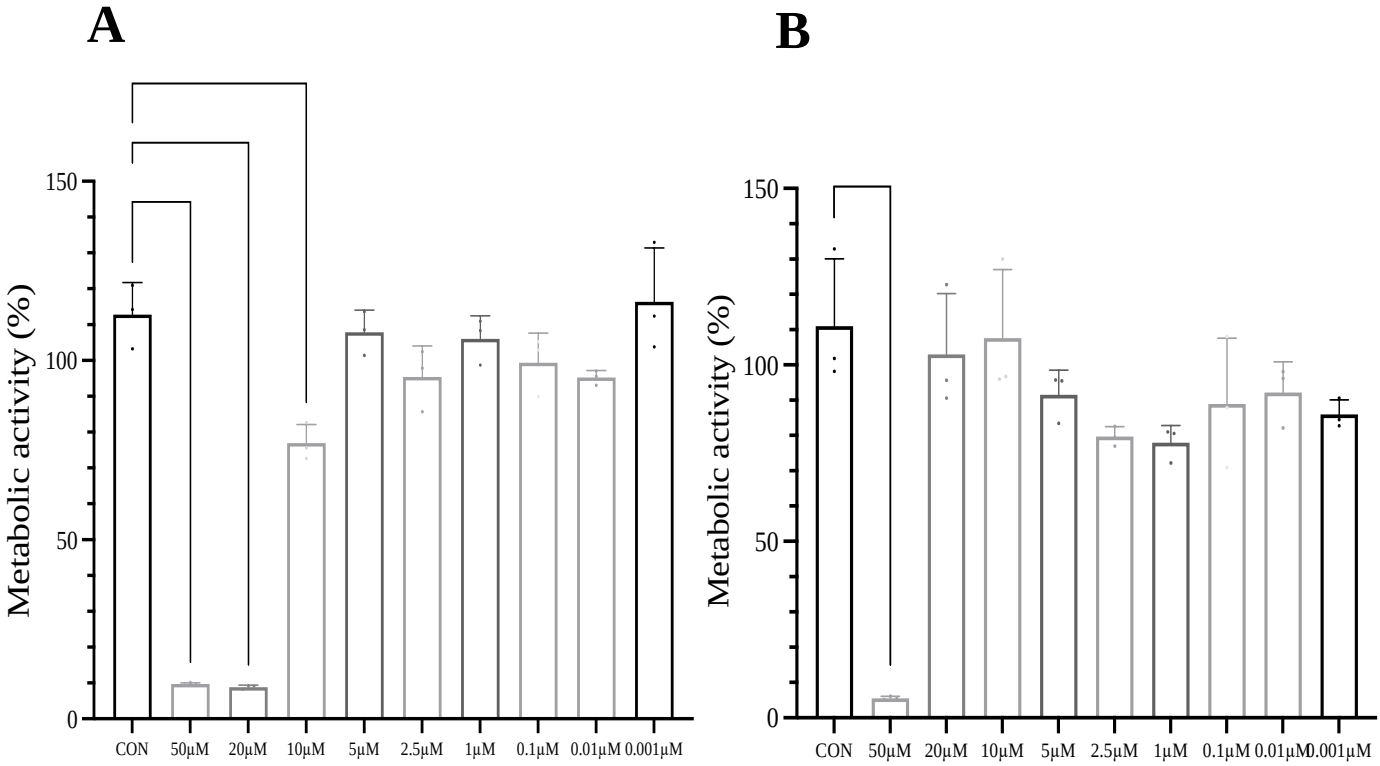


Figure 4.3 Data collected following the MTT assay. Cellular metabolic activity (%) following chronic (**A**) and acute (**B**) treatment of sCBD after 10 days. Pairwise comparisons represented.

4.4.2 PI Assay

A Shapiro-Wilk test was conducted on both data sets to assess normality and lognormality. Both data sets were not normally distributed, so a Kruskal-Wallis test was performed.

Following chronic treatment of sCBD, cell viability was significantly lower at 50 µM (45.27 % ± 11.19 %, *P* = <0.0486) and 20 µM (42.86 % ± 8.24 %, *P* = <0.0302) compared to control (CON) (92.70 % ± 0.93 %). Cell viability at 50 µM was significantly lower compared to 0.01 µM (94.05 % ± 0.83 %, *P* = <0.0161) and 0.001 µM (94.19 % ± 1.07 %, *P* = <0.0101). Cell

viability at 20 µM was also significantly lower compared to 0.01 µM (P = <0.0090) and 0.001 µM (P = <0.0055).

Following acute treatment of sCBD, no significant differences in cell viability were reported across all doses compared to control (CON).

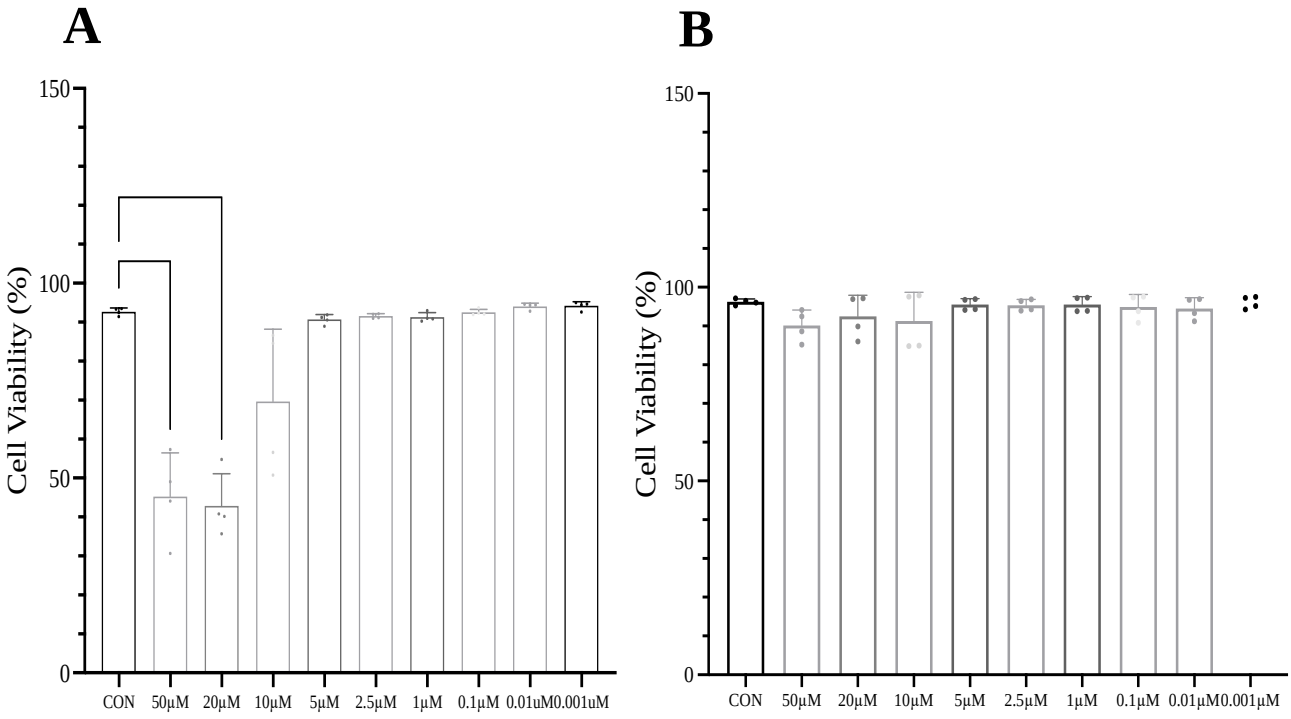


Figure 4.4 Cell viability (%) following chronic (**A**) and acute (**B**) treatment of sCBD after 10 days. Pairwise comparisons represented.

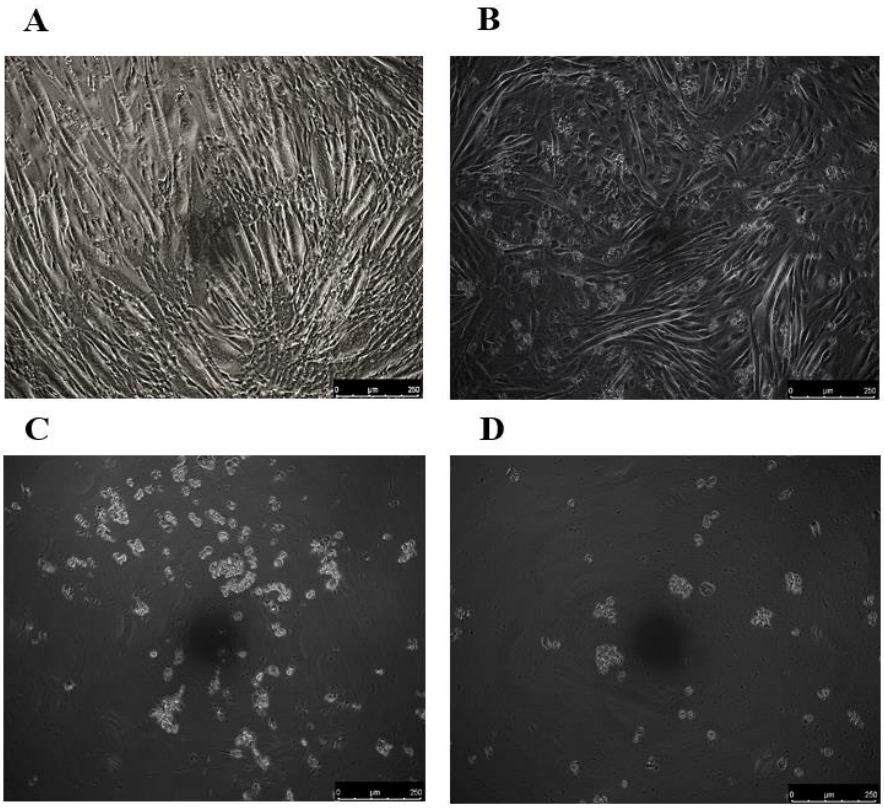


Figure 4.5 Images captured at 10 days following the chronic treatment of sCBD in **A**) control **B**) of 10 µM sCBD, **C**) 20 µM sCBD and **D**) 50 µM sCBD.

4.5 Discussion

The main aim of the investigation was to assess cell health and viability of myotubes, following chronic and acute treatment of synthetic cannabidiol. We hypothesized that sCBD at lower doses would benefit cellular health during differentiation, however our findings do not align with this therefore the hypothesis can be rejected. We also hypothesised that higher doses of sCBD and an increase in exposure time would have detrimental effects on cellular health as study 1 identified cytotoxic effects on myoblasts at higher doses of sCBD.

The main findings from this study partly support the proposed hypotheses. Following both the chronic and acute treatment of sCBD, metabolic activity was significantly reduced at 50 µM compared to control, thus suggesting that this concentration of sCBD is detrimental to myotube health. However, metabolic activity in the acute treatment was only significantly reduced at 50

µM in comparison to the chronic treatment where doses of sCBD at 20 µM and 10 µM sCBD significantly reduced metabolic activity. No positive effect on cellular health was reported following both acute and chronic sCBD treatment, therefore we can reject the hypothesis that sCBD at lower doses would benefit cellular health. As identified in chapter 3, sCBD seems to have a detrimental effect on myoblasts during proliferation at higher doses and this also increases with exposure time. In the chronic treatment, where cells are treated with sCBD after 24-hours and exposed throughout differentiation, the effects on metabolic activity and cell viability are significantly greater compared to an acute treatment. Therefore, it appears that sCBD is detrimental to cell health during the early stages of differentiation which is, in turn, reducing metabolic activity and cell viability of myotubes.

In comparison to metabolic activity and cell viability of myoblasts at 24 and 48-hours, there is a significant decrease in metabolic activity and cell viability after a chronic treatment of sCBD on myotubes. One explanation for this difference could potentially be due to the serum within the growth media having a protective effect against sCBD. A previous study assessed the viability and proliferation of cancer cells treated with CBD at different concentrations in the presence of serum (Sainz-Cort, Müller-Sánchez and Espel, 2020). HT-29 cells were incubated in media with 0.5% FCS and the 24-hour treatment of 10 µM CBD significantly reduced cell viability (Sainz-Cort, Müller-Sánchez and Espel, 2020). Analysis on the morphology of cells identified that treatment with 10 µM CBD led to changes in cell form, such as cell rounding, cellular detachment and wrinkled cells, all characteristics of dead cells (Sainz-Cort, Müller-Sánchez and Espel, 2020). On the other hand, CBD did not inhibit the viability of HT-29 cells even after 3 days in media containing 10% FCS.. FCS comprises a variety of growth factors, proteins and nutrients, and differences in FCS source could have a significant impact on cultured cell viability, proliferation, and differentiation (Sainz-Cort, Müller-Sánchez and

Espel, 2020). Therefore, 10% FCS serum may contain a greater amount of growth factors and nutrients which possibly may be having a protective effect on cells. In the current study, 10% FCS was present in the growth media used to proliferate myoblasts but was not present in the differentiation media (Sainz-Cort, Müller-Sánchez and Espel, 2020). Therefore, this could potentially explain why in the chronic study, where cells were treated at 24-hours with sCBD in DM, the significant reduction in metabolic activity and cell viability, as FCS serum was not present in the media. However, the reduction in cell viability could be the result of other factors such as the repeated dosing monolayers received and the increase in exposure time.

When examining the literature, cell viability primarily is assessed over acute periods during proliferation. Few studies have examined cell viability following a longer exposure to CBD and have found similar findings to our data. Research examining the effects of CBD on SZ95 cells, reported a decrease in cell viability following both a high dose (50 µM) and with a long-time exposure (10 µM for 6 days) (Oláh et al., 2014). The administration of 50 µM CBD induced apoptosis-driven cytotoxicity (Oláh et al., 2014). Following the repeated dosing of 10 µM CBD for 6 days, a decrease in cell number (cell viability) and lipogenesis was recorded (Oláh et al., 2014). Another study, which investigated the anti-inflammatory effectivity of CBD on mesenchymal stromal cells (MSCs), discovered a reduction in cell viability following a 7-day treatment of 3 µM CBD (Ruhl, Kim and Beier, 2018). Therefore, longer incubation periods with CBD seem to have greater detrimental effects on cell health, as other findings discovered following a longer incubation time (i.e 5 days), a great amount of cell death was reported at 4 µM CBD (Sainz-Cort, Müller-Sánchez and Espel, 2020). Although these findings may not be identical to ours, it still supports a role for exogenous CBD to negatively impact cellular health as exposure time to CBD increases.

Chapter summary

In summary, this chapter has demonstrated the detrimental effect sCBD has on myotube health at high concentrations and increased exposure time. It can be concluded that 50 µM sCBD is damaging to cell health following both an acute and chronic treatment. Greater reductions in metabolic activity and cell viability were recorded following chronic treatment of sCBD, especially at 50, 20 and 10 µM implying that an early and increased exposure to sCBD impacts cell health and effects myotube formation, possibility due to having an inhibitory effect on myogenin expression levels at high doses. Although metabolic activity was significantly reduced at 50 µM following acute treatment, no significant reductions in cell viability were reported across all doses, reinstating the idea that the effects of sCBD on metabolic activity precede cell death.

Forthcoming studies conducted within this thesis will use concentrations of sCBD at 5 µM to 0.001 µM, as higher doses have proved to be detrimental to cell health, therefore the morphological effects on lower doses, that have been proven to not be cytotoxic, will be analyzed to determine whether sCBD has an impact on myotube formation.

Chapter 5

The Effects of Acute and Chronic Treatment with Synthetic Cannabidiol on C_2C_{12} Myotube Formation

5.1 Abstract

Purpose – The purpose of the study was to assess the effects of both an acute and chronic treatment of sCBD on myotube area, number and nuclear fusion index (NFI).

Methods - C_2C_{12} skeletal muscle cells were plated in growth media for 24-hours until 80% confluent. For the sCBD chronic treatment, cells were dosed with sCBD and topped up with treated media every 48-hours for 10 days. For the acute treatment of sCBD, cells were left to differentiate in DM for 8 days and then dosed with sCBD for 48-hours. Monolayers were then fixed and stained for imaging.

Results –No significant difference in myotube area and number were recorded after the acute treatment. NFI was significantly lower 5 µM (P = <0.0011), 2.5 µM (P = <0.0273), 1 µM (P = <0.0003), 0.1 µM (P = <0.0281) and 0.001 µM (P = <0.03280) sCBD. After the chronic treatment, there was a significant decrease in myotube area at 5 µM (P = <0.0001), 2.5 µM (P = <0.0002), 0.1 µM (P = <0.0186) and 0.01 µM (P = <0.0029) sCBD. There was also a significant decrease in myotube number at 5 µM (P = <0.0006) and 2.5 µM (P = <0.0011) sCBD. There was a significant increase in NFI at doses 5 µM (P = <0.0001), 2.5 µM (P = <0.0001), 1 µM (P = <0.0021) and 0.1 µM (P = <0.0428).

Conclusions – Once myotubes are fully formed, an acute treatment of sCBD appears to have no effect on myotube area and number but does reduce NFI across most doses (5, 2.5, 1, 0.1 and 0.001 µM). On the other hand, myotube area, number and NFI are significantly reduced at higher doses of sCBD (5 µM to 1 µM) following chronic treatment, implying an increased exposure to high concentrations of sCBD effects muscle morphology in dose-response manner.

5.2 Introduction

Current research examining the effects of sCBD and CBD on myotube morphology is limited, as such little is known of its effects on myotube formation. Previous research has investigated the effects of CBD on cell morphology during proliferation (Fisher et al., 2016). The findings concluded that following a treatment with 10 µM CBD, the number of cells in the medium which had lost their normal shape and become round and swollen, had increased (Fisher et al., 2016). These results confirmed that CBD treatment might induce the appearance of typical characteristics of apoptosis (Fisher et al., 2016). As seen in chapter 4, sCBD at high concentrations seems to be detrimental to myotube health, implying morphological changes are occurring during differentiation. Therefore, analysing myotube morphology following the treatment of sCBD, might be useful, as it will help to give an insight into cellular behaviour during differentiation.

Precise measurements of cell number and size are essential to the investigation of cellular growth and development *in vitro* (Agley et al., 2012). A frequently used indicator of muscle cell size and stage of differentiation is the fusion index. The nuclear fusion index (NFI) describes the number of nuclei within myotubes as a percentage of the total number of nuclei in an image (Agley et al., 2012). In the current study, NFI, as well as myotube number and area, was measured to assess any morphological changes present following the treatment of sCBD at varying doses. Monolayers were also fixed and immunostained for MF-20, which is a maker of myosin heavy chain, seen only in differentiating myoblasts and myotubes.

This study aimed to investigate the anabolic effects of sCBD on C_2C_{12} skeletal muscle myotubes following an acute and chronic sCBD treatment. To measure the effects of sCBD on the morphology of muscle cells, myotube number, area and nuclear fusion index (NFI) was analysed. Previous research has investigated the effects of CBD on anabolic and inflammatory

signalling in C_2C_{12} myotubes through analysis on mTORC1 signalling under fasted conditions and following addition of insulin-like growth factor 1 (IGF-1) (Langer, Avey and Baar, 2021). The findings determined CBD (range of 1-5 µM) had no effect on anabolic signalling through the mTORC1-axis (Langer, Avey and Baar, 2021). Research investigating the effects of cannabidiol and cannabinol on C_2C_{12} myoblast proliferation and differentiation, discovered no significant increases in myotube diameter, surface area and myonuclear index, following exposure to 1 and 5 µM CBD (Lau, 2019). The previous chapters within this thesis, indicate that sCBD seems to have only a detrimental effect on myoblast and myotube health, especially at higher concentrations. Therefore, we hypothesized that following both acute and chronic exposure to sCBD, no significant increases in myotube formation would be reported. As findings in the previous chapter indicate, we also hypothesized that greater effects of CBD on myotube morphology will be reported following chronic treatment, as monolayers were repeatedly dosed during differentiation.

5.3 Procedures

Low passage C_2C_{12} myoblasts were seeded at $8x10^4$ cells·ml^{-1} in pre-gelatinised 6-well plates in growth media (GM). Cell monolayers were then incubated at 37˚C, 5% CO_2 for a 24-hour period until cells were ~80% confluent. Once cell confluency reached 80%, GM was aspirated, and cell monolayers washed twice with PBS.

To examine the effects of acute sCBD treatment, cell monolayers were induced to exit the cell cycle and differentiate with differentiation media (DM) and topped up every 48-hours with 10% of well concentration for 8 days. On day 8, existing media was removed, and cell monolayers were treated for 48-hours with sCBD in differentiation medium, with doses ranging from 0.001 µM to 5 µM (see **figure 5.2**). To examine the effects of chronic sCBD

treatment, cell monolayers were treated with sCBD in differentiation medium at 0-hours, with doses ranging from 0.001 µM to 5 µM, see **figure 5.2**. Monolayers were topped up every 48-hours with 10% of well concentration of their respective doses for 10 days. Upon termination of the experiment on day 10, monolayers were fixed using paraformaldehyde (PFA 4%) and prepared for immunofluorescence (IF) imaging, see section 2.11.

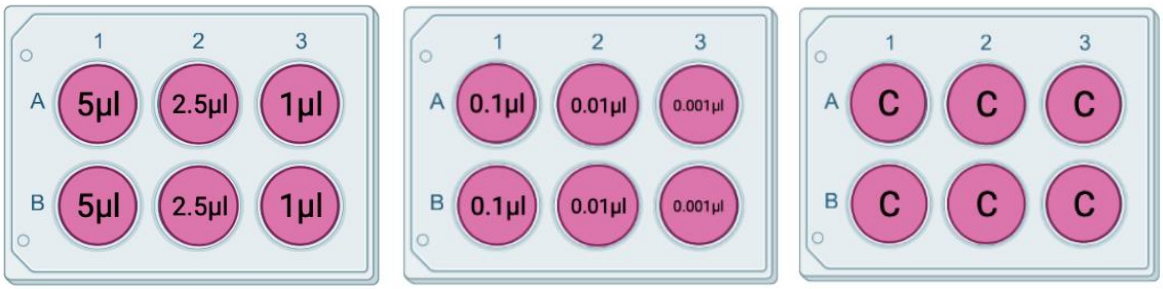


Figure 5.1 3 x 6 well-plates with the appropriate sCBD doses (5 µM – 0.001 µM) or control, for the acute treatment and chronic treatment.

5.3.1 Immunocytochemistry

After 10 days of differentiation, monolayers were fixed and immunostained for MF20, a marker of myosin heavy chain. Nuclei were counterstained with DAPI, see section 2.11. Stained samples were then imaged and myotube number, area and nuclear fusion index measured and calculated as described in sections 2.11.3-5. Sample size of n=12 per condition.

5.3.2 Live Imaging

Following 10 days of differentiation, monolayers were fixed and stained and myotubes live imaged under a microscope at 10x objective as described in section 2.11.2. Myotube number, are and NFI were measured as outlined in sections 2.11.3-5.

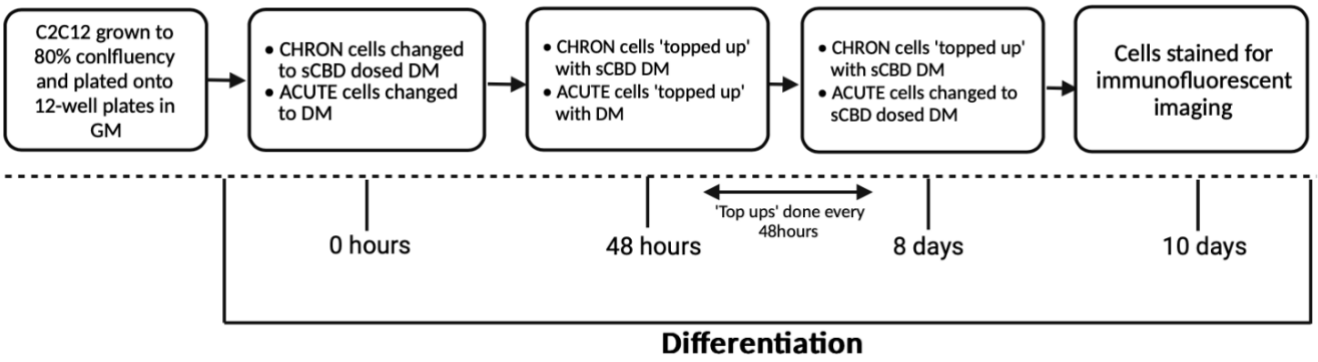


Figure 5.2 Schematic representation of the experimental design used to examine the effects of chronic and acute treatment of sCBD on the morphology of muscle cells.

5.4 Results

5.4.1 Acute treatment of sCBD

A Shapiro-Wilk test was conducted on all data sets to assess normality and lognormality. Normality was not confirmed on all sets of data, therefore a Kruskal-Wallis test was performed as a non-parametric equivalent of the ANOVA.

Average myotube area in control (CON) was 155.6 ± 226.6 μm^2. There was no significant difference in average myotube area across all doses in comparison to control, see **figure 5.3**. No significant differences were recorded when sCBD doses were compared to one another. Average myotube number in CON was 40.17 ± 5.95. Across all doses, there was no significant difference in myotube number when compared to control, see **figure 5.3**. Comparable with average myotube area, no differences were reported when sCBD doses were compared to one another. Nuclear Fusion Index (NFI) in CON was 32.98 ± 3.62. Acute treatment of sCBD resulted in a decreased NFI when monolayers were treated with; 5 µM (26.73 ± 5.76, P = <0.0011), 2.5 µM (28 ± 4.19, P = <0.0273), 1 µM (25.86 ± 3.54, P = <0.0003), 0.1 µM (28.20

± 2.17, *P* = <0.0281) and 0.001 µM (28.21 ± 2.57, *P* = <0.0328), all data represented in **table 1**. There was a significant decrease in NFI when monolayers were dosed with 1 µM in comparison to 0.01 µM (31.45 ± 4.42, *P* = <0.0460).

Table 5.1. Standard deviations and mean values for average myotube area, number and NFI for all doses and CON.

sCBD-DM Dose	Average Myotube Area (µm^2)	Average Myotube Number	Nuclear Fusion Index (NFI)
CON	155.6 ± 226.6	40.17 ± 5.95	32.98 ± 3.62
5 µM	190.6 ±3 41.7	41.08 ± 7.53	26.73 ± 5.76
2.5 µM	183.8 ± 279.9	42.92 ± 9.02	28 ± 4.19
1 µM	215.3 ± 446.7	37.17 ± 5.72	25.86 ± 3.54
0.1 µM	182.3 ± 409.8	41.92 ± 7.42	28.20 ± 2.17
0.01 µM	192 ± 370.8	41.83 ± 7.29	31.45 ± 4.42
0.001 µM	165.9 ± 298.3	41.67 ± 6.85	28.21 ± 2.57

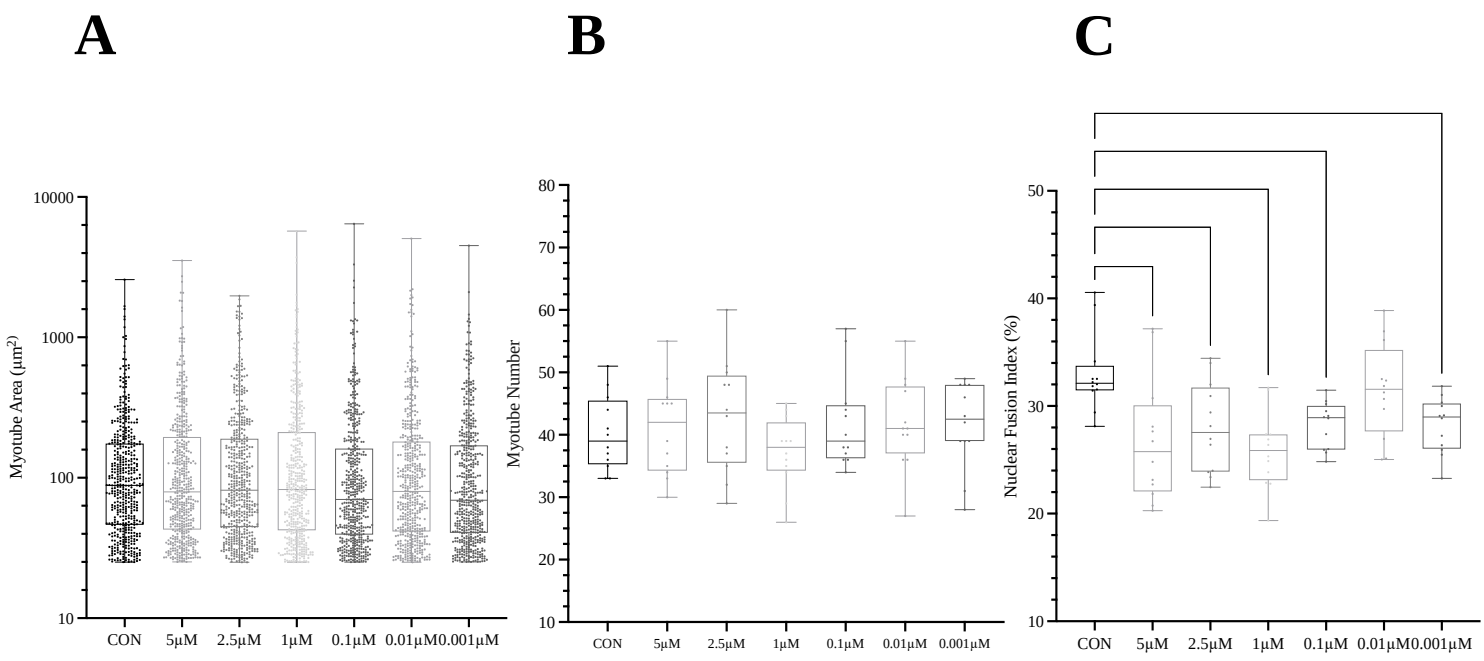


Figure 5.3 The effects of acute treatment of sCBD at varying doses on **A**) myotube area, **B**) myotube number and **C**) nuclear fusion index (NFI). Pairwise comparisons represented.

5.4.2 Chronic treatment of sCBD

A Shapiro-Wilk test was conducted on all data sets to assess normality and lognormality. Normality was confirmed on the data produced on myotube number and an ordinary one-way ANOVA was run. Normality was not confirmed on both sets nuclear fusion index and myotube area, therefore a Kruskal-Wallis test was run as this is a non-parametric ANOVA.

Average myotube area in control (CON) was 155.6 ± 226.6 μm^2. Chronic treatment of sCBD resulted in a decreased average myotube area when monolayers were treated with; 5 µM (120.2 ± 183.2, P = <0.0001), 2.5 µM (116.9 ± 164.2, P = <0.0002), 0.1 µM (123.8 ± 159.8, P = <0.0186) and 0.01 µM (120.6 ± 150.6, P = <0.0029), all data represented in **table 5.1**. There was also a significant difference in between doses, with average myotube area at 5 µM (120.2 ± 183.2) significantly lower in comparison to 0.001 µM (149 ± 254.9, P = <0.0166). No other significance was reported in between doses. Average myotube number in CON was 40.17 ± 5.95). Following chronic treatment of sCBD, doses of 5 µM (28.75 ± 4.21, P = <0.0006) and 2.5 µM (29.25 ± 7.75, P = <0.0011) had an inhibitory effect on average myotube number when compared to control, see **figure 5.4**. Differences in myotube number within doses was also recorded. There was a significant decrease in average myotube number when monolayers were treated with 5 µM in comparison to 0.1 µM (38.83 ± 6.35, P = <0.0090) and 0.01 µM (40.92 ± 6.43, P = < 0.0007). Doses of sCBD at 2.5 µM also had an inhibitory effect on myotube number when compared to doses 0.1 µM (P = <0.0156) and 0.01 µM (P = <0.0014). Nuclear Fusion Index (NFI) in CON was 19.31 ± 1.97. Compared to control, there was a significant decrease in NFI at doses 5 µM (26.12± 2.6, P = <0.0001), 2.5 µM (26 ± 3.03, P = <0.0001)

and 1 µM (27.73 ± 1.46, P = <0.001) all data represented in **table 5.2**. No significant differences in NFI were reported within doses.

Table 5.2. Standard deviations and mean values for average myotube area, number and NFI for all doses and CON.

sCBD-DM Dose	Average Myotube Area	Average Number of Myotubes	Nuclear Fusion Index (NFI)
CON	155.6 ± 226.6	40.17 ± 5.95	33.31 ± 2.95
5 µM	120.2 ± 183.2	28.75 ± 4.21	26.12 ± 2.6
2.5 µM	116.9 ± 164.2	29.25 ± 7.75	26 ± 3.03
1 µM	149 ± 206.1	35.83 ± 8.08	27.73 ± 1.46
0.1 µM	123.8 ± 159.8	38.83 ± 6.35	28.89 ± 1.97
0.01 µM	120.6 ± 150.6	40.92 ± 6.43	29.20 ± 1.67
0.001 µM	149 ± 254.9	36.67 ± 8.08	29.07 ± 2.3

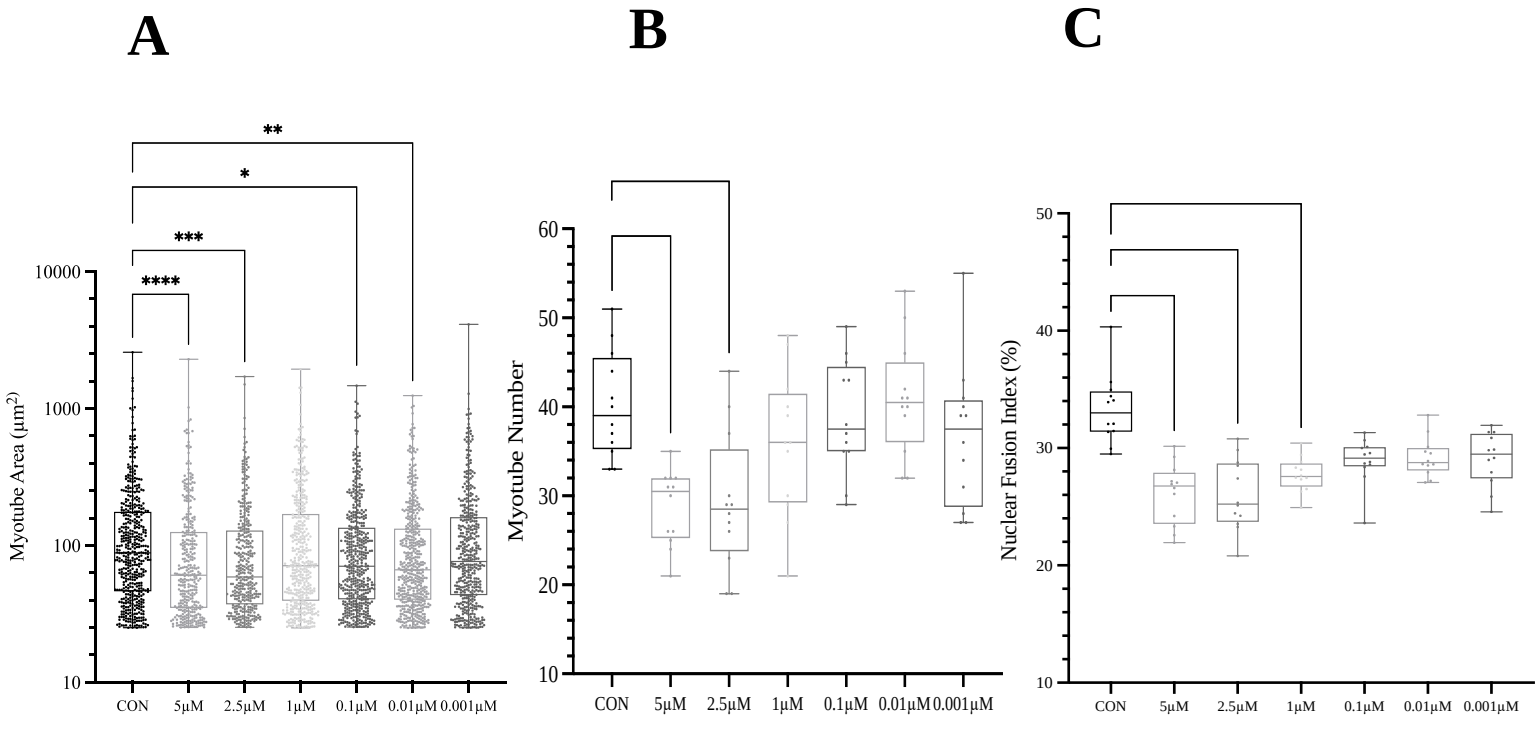


Figure 5.4 The effects of chronic treatment of sCBD at varying doses on **A**) myotube area, **B**) myotube number and **C**) nuclear fusion index (NFI). Pairwise comparisons represented.

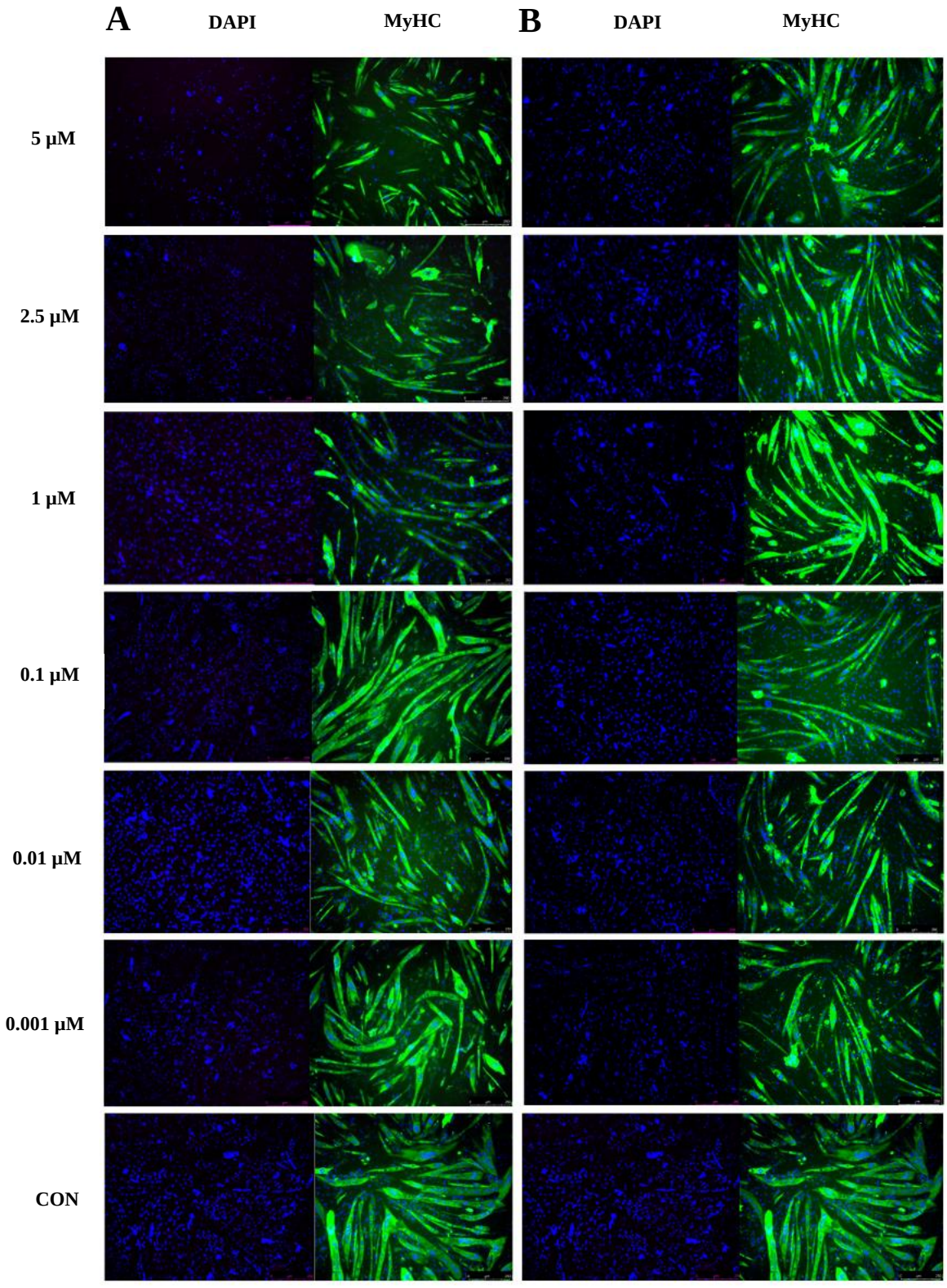


Figure 5.3 Immunofluorescent images captured at 10days following **A**) chronic **B**) acute treatment of sCBD at doses 5 µM to 0.001 µM and CON. DAPI and MyHC channels displayed.

5.5 Discussion

This study aimed to investigate the anabolic effects of acute and chronic dosing of synthetic cannabidiol on myotube formation through morphological analysis. Due to previous literature, we hypothesised that following both acute and chronic treatment of sCBD, no significant increases in myotube formation would be reported. Findings from chapters 3 and 4 in this thesis, illustrate the detrimental effect CBD appears to have on both myoblasts and myotubes, therefore, no beneficial impact on myotube formation is to be expected. In chapter 4, a greater detrimental impact on cell viability was reported after chronic exposure to CBD. Therefore, within this study, we also hypothesized that greater changes in myotube morphology would be reported following chronic treatment, as exposure time is increased.

Following acute treatment of sCBD, no significant differences were reported in myotube area and number across all doses compared to CON. There was, however, a significant decrease in NFI across all doses (except 0.01 µM) compared to CON. On the other hand, significant decreases in myotube area, number and NFI were all recorded after the chronic treatment. Myotube area was significantly reduced compared to CON at doses 5, 2.5, 0.1 and 0.01 µM, with significant decreases in myotube number at 5 and 2.5 µM observed. NFI was significantly reduced at 5, 2, 5 and 1 µM respectively. Therefore, we can accept our hypothesis as no significant increases in myotube area, number and NFI were reported following both acute and chronic treatment with sCBD. Greater detrimental effects on myotube morphology were also reported after the chronic treatment, implying increases in exposure time to sCBD, has damaging consequences on myotube formation.

A study examining the effects of cannabidiol and cannabinol on C_2C_{12} myoblast proliferation and differentiation, investigated changes in myotube morphology following CBD administration (Lau, 2019). C_2C_{12} cells were treated with CBD or CBN for 7 days, with top ups every 48-hours and on the final day, immunocytochemical analysis was conducted (Lau, 2019). Myotube diameter, surface area and myonuclear index were all used as indicators of myotube differentiation. No significant increases were seen after exposure to 5 µM CBD or CBN compared to CON (Lau, 2019). Likewise, after supplementation with 1 µM CBD, no significant increases were recorded compared to CON (Lau, 2019). The results from this study suggest CBD and CBN have no benefit on muscle differentiation, which is supported by our findings as no significant increases in myotube area, number and NFI were identified after CBD supplementation in both an acute and chronic manner.

Cannabidiol was also investigated for its effect on anabolic and inflammatory signaling in C_2C_{12} skeletal muscle cells *in vitro* (Langer, Avey and Baar, 2021). The effect of CBD was examined on mTORC1 signaling under a fasted condition, with the addition of amino acids and IGF-1 (Langer, Avey and Baar, 2021). Myotubes were plated in 6-well plates and cultured in DM for 5 days until fully formed, when myotubes were then fasted for 15 minutes by replacing DM with starvation media (SM) and increasing dosages of CBD (0, 1, 2.5 and 5 µM) (Langer, Avey and Baar, 2021). Following this 15-minute period, SM in half the wells was removed and fresh DM containing IGF-1 and increasing doses of CBD was added to monolayers for 30 minutes before harvesting cells (Langer, Avey and Baar, 2021). The results from this experiment concluded that CBD, across concentrations of 1 to 5 µM, had no effect on anabolic signaling through the mTORC1-axis (Langer, Avey and Baar, 2021). mTORC1 is one of two distinct complexes that exist from the mechanistic target of rapamycin (mTOR),

which is the master regulator of cellular metabolism (Deleyto-Seldas and Efeyan, 2021). mTORC1 is a master switch responsible for often all energy-intensive functions in the cell, promoting cell growth and building cellular biomass when nutrients are abundant and permitting autophagic recycling of cellular components, when nutrients are few (Deleyto-Seldas and Efeyan, 2021). Although the effects of sCBD on anabolic signaling was not investigated within this thesis, these findings potentially elucidate the mechanisms involved in myotube formation and how an effect on mTORC signaling, could potentially lead to a detrimental impact on myotube morphology.

In an investigation into the effects of non-euphoric plant cannabinoids on muscle quality, C_2C_{12} cells were treated with either an acute or prolonged exposure to CBD to measure the effects on differentiation (Iannotti et al., 2019). Differentiation was identified by quantifying the expression of two typical maker genes of muscle differentiation: myogenin and troponin T-1 (TNNT-1) (Iannotti et al., 2019). The results concluded that myoblasts exposed to CBD (1 µM) showed an increase in mRNA expression of the two differentiation markers and this effect was more prominent following an acute exposure rather than prolonged (Iannotti et al., 2019). The effects of prolonged exposure were established by mRNA expression levels of myosin heavy chain which is a late indicator of myotube formation. The results indicated that concentrations lower than 1 µM were ineffective where 3 µM CBD, inhibited myogenin expression levels (Iannotti et al., 2019). In the current study, gene expression was not measured, however, the findings from Iannotti and colleagues help to give an insight into the effects of CBD on the markers that augment differentiation. The reduction in myogenin expression at 3 µM CBD does potentially explain the reduction in myotube area, number and NFI at 2.5 µM sCBD within our study, as inhibiting myogenin expression levels could

hypothetically have a detrimental impact on myotubes, which is what our findings possibly indicate.

It should be noted however the incubation periods between Iannotti et al's study and ours are significantly different. Iannotti and colleagues added CBD to DM for 5/15 mins/3-hours and replaced it with DM for 72-hours for acute exposure and for prolonged exposure, CBD was added to DM for 72-hours, whereas in our study, in the acute treatment, cells were left to differentiate for 8 days before being administrated with sCBD for 48-hours and in the chronic treatment, treated with sCBD after 24-hours and topped up every 48-hours for 10 days. Therefore, the exposure time of CBD was significantly different, which resulted in examining changes in myotubes at different periods of growth. The difference in compounds, CBD and sCBD, also need to be considered when comparing our findings to previous literature.

Within the same study, they discovered that CBD, CBDV and THCV enhance primary human satellite cell differentiation (Iannotti et al., 2019). Cells from healthy donors were induced to differentiate for 5 days in the presence of 1 μM CBD, 3 μM CBDV or 3 μM THCV (Iannotti et al., 2019). Analysis conducted revealed significantly higher transcript levels of myogenin TNNT-1 and MyHC compared to CON (Iannotti et al., 2019). The findings were supported by immunofluorescence analysis which confirmed the induction of MyHC expression and staining with DAPI also identified myotube formation as numerous nuclei was observed within each myotube (Iannotti et al., 2019). The findings do not align with ours, although transcriptional analysis was not conducted within this study, morphological analysis confirmed that across all doses compared to CON, no significant increases in myotube number, area and NFI were reported, implying no increases in transcript levels of MyHC.

Chapter Summary

In summary, this chapter has demonstrated the effects of both acute and chronic sCBD treatment on the morphology of myotubes. From our findings, we can conclude that following acute treatment of sCBD, across all doses (5 µM – 0.001 µM) compared to CON, no significant differences in myotube area and number were recorded. Significant reductions in NFI were reported across all doses except 0.01 µM compared to CON, suggesting acute treatment of sCBD leads to a reduction in the proportion of the total cell populace that has fused, as the number of nuclei within myotubes decreased. Following chronic treatment, significant reductions across myotube area, number and NFI were all recorded. Myotube area was significantly reduced across all doses compared to CON except at 1 and 0.001 µM sCBD, with significant reductions in myotube number reported at 5 and 2.5 µM sCBD and NFI was also significantly reduced at 5, 2.5 and 1 µM sCBD. Greater morphological effects were reported following chronic treatment of sCBD, which suggests repeated dosing and an increase in exposure time to sCBD leads to detrimental effects on myotube formation, reducing myotube area, number and NFI.

This study provides preliminary data on the morphology of myotubes following an acute and chronic exposure to sCBD. A limitation of this study, however, was the lack of inclusion of transcriptional analysis. Although morphological analysis can provide data on myotube features, as well as identifying characteristics of apoptosis, transcriptional analysis would further support our findings and give an insight into the expression of genes that augment myotube formation, as previous research already suggests CBD has an impact on these genes.

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Chapter 6

Thesis Synthesis

6.1 Realisation of Aims

Aim 1 – To determine the effects of sCBD treatment on C_2C_{12} myoblast viability during proliferation.

C_2C_{12} myoblasts were treated with sCBD (50 µM to 0.001 µM) or CON and induced to proliferate for 24 or 48-hours. We hypothesized that sCBD would have a beneficial impact on cellular health during proliferation due to pervious research, with greater effects reported after increased exposure time (48-hours).

The findings from our study do not support the hypothesis. Three separate cell-based assays were conducted to assess cell health: MTT, PI and EdU assay. The findings from the MTT assay indicate that metabolic activity was significantly reduced after 24-hours at 50 µM and after 48-hours at 50 µM and 20 µM. Cell viability was significantly reduced after 48-hours at 50 µM as indicated by increased PI entry into myoblasts. No changes were recorded in EdU incorporation suggesting that sCBD does not impact proliferation. Therefore, we can reject our hypothesis and conclude sCBD is cytotoxic to cell health during proliferation at high concentrations (50 µM and 20 µM) and these effects are greater with increases in exposure time.

Aim 2 – To determine the effects of acute and chronic sCBD treatment on C_2C_{12} cell viability of myotubes during and after differentiation.

C_2C_{12} cells were left to proliferate for 24-hours before receiving either an acute dose of sCBD(50 µM – 0.001 µM) for 48 hours following 8 days of differentiation or chronic treatment with sCBD for 10 days, with media topped up every 48-hours. We hypothesised that lower

doses of sCBD would have a positive effect on cellular health during differentiation, with higher concentrations being cytotoxic to cell health, as findings from study 1 indicated.

The findings from our study partly support our hypotheses. Following both acute and chronic treatment of sCBD, significant reductions in metabolic activity were detected at 50 µM. No significant reductions in cell viability were recorded after acute treatment, however reductions at 50, 20 and 10 µM were observed. Greater reductions in metabolic activity and cell viability were recorded after chronic treatment. Metabolic activity was significantly reduced at 50, 20 and 10 µM compared to CON and cell viability was significantly reduced at 50 µM and 20 µM. On the other hand, at lower concentrations of sCBD, no positive effect of sCBD on cellular health was reported, suggesting we reject the hypothesis stated from the outset. Therefore, the findings from this study conclude that 50 µM is detrimental to both myoblast and myotube health after an acute and chronic treatment of sCBD. It can be declared that sCBD treatment early on in life and with an increased exposure, is detrimental to myotube health at higher doses (50 µM, 20 µM and 10 µM.

Aim 3 – To determine the morphological effects of acute and chronic sCBD treatment on C_2C_{12} myotube formation as expressed via immunofluorescence imaging.

Morphological and immunofluorescence analysis was conducted to determine the effects of sCBD on myotube area, number and NFI, as well as myosin heavy chain expression, a marker of myoblast differentiation. We hypothesised that following both acute and chronic exposure to sCBD no increases in myotube morphology would be observed, as previous research suggests. As earlier chapters in this thesis demonstrate the cytotoxic effects of sCBD,

especially following chronic treatment, a greater detrimental impact on myotube morphology was also hypothesized after chronic exposure to sCBD.

The findings from our study do support the hypotheses stated at the outset of the experiment. No significant increase in myotube area, number and NFI were recorded across all doses following acute and chronic treatment of sCBD compared to CON. In fact, after chronic treatment of sCBD, significant reductions in myotube area, number and NFI were recorded across doses from 5 µM to 0.01 µM. After acute treatment, no significant differences were measured in myotube area and number compared to CON but there was a significant decrease in NFI across all doses, except 0.01 µM.

6.2 Experimental Limitations

2D Cell Culture Models

Evidence suggests there are limitations to using a two-dimensional (2D) culture system when assessing cell health *in vitro*. In a standard 2D culture system, cells adhere and grow as a monolayer on a flat surface, such as a glass or polystyrene Petri dish, which is attached to a plastic surface (Kapałczyńska et al., 2016). Cell development in 2D monolayers allows for equal access to growth agents and nutrients present in the medium, resulting in homogenous growth and proliferation under tightly controlled conditions (Dessauge et al., 2021). Due to this, numerous primary cultures of myoblasts have been developed in humans, rats, frog and many more species (Dessauge et al., 2021). However, if these cultures are well-known and under control, they also possess several limitations, including long-term viability, a lack of native muscle architecture, and the inability to create impulse contractions (Dessauge et al., 2021). Therefore, they do not entirely replicate the anatomical structure and function of adult muscle which is necessary for muscle contraction and activity (Dessauge et al., 2021).

Furthermore, cell culture in standard *in vitro* systems presents major constraints due to its low surface to volume ratio which leads to a lack in pH, gas, and metabolite concentration and as such is not scalable (Dessauge et al., 2021). As a result, 3D culture systems are now being considered, as they display qualities that are closer to the complex *in vivo* conditions (Ravi et al., 2015). Though 2D cell lines provide good homogenous study material, culturing them as 3D models encourages them to behave in a more realistic manner in natural conditions (Ravi et al., 2015). Therefore, the use of 3D bioengineered muscle models would be useful to measure the effects of sCBD treatment after differentiation.

MTT assay for cell viability

The MTT assay alone is not an inadequate measure of cell viability. The MTT assay's reliance on metabolic function can influence the results, as there is a direct association between the glucose concentration in cell culture media and the MTT reductive rate (van Tonder, Joubert and Cromarty, 2015). The rate of conversion to the formazan has been shown to be linked to the metabolic activity of the cell and thus decreased intracellular glucose concentrations may result in a decrease in the amount of formazan crystals produced (van Tonder, Joubert and Cromarty, 2015). Another factor which is reported to influence the conversion to formazan is the number of mitochondria present in the cell, as larger cells with more mitochondria have a higher tetrazolium conversion rate (van Tonder, Joubert and Cromarty, 2015). Taking these factors into consideration, this could possibly lead to inaccurate estimations of metabolic activity and cell number being recorded.

Protein and gene data

The expression of signalling proteins implicated in the activity of sCBD were not studied in these experiments. Studying the effects of a novel substance on skeletal muscle is useful as it

can determine the amount or concentration of a specific protein or an array of different proteins within a sample. Previous research conducted identified that upon acting on the CB1 and CB2 receptor, CBD inhibits the activity of adenylyl cyclase, activates potassium channels, and activates mitogen activated protein kinase (MAPK), AKT and mTOR signalling pathways (Atalay, Jarocka-Karpowicz and Skrzydlewska, 2019). The AKT and mTOR pathway is responsible for physiological protein synthesis and the induction of other pathways such as the MAPK pathway which regulates proliferation, cell survival and apoptosis (Atalay, Jarocka-Karpowicz and Skrzydlewska, 2019). The expression of the primary CBD receptor CB1 could have been determined prior to all experiments, as previous research has demonstrated C_2C_{12} myotubes do not exhibit CB1 protein, which potentially may explain the reduced effects of CBD on myotubes in cell culture (Langer, Avey and Baar, 2021). Therefore, if protein data was collected within this study, it may have been useful as it could have potentially explained the reasons why sCBD induced apoptosis in cells at certain doses. Future work should incorporate the analysis of protein data as it provides additional information on the effects of sCBD.

Transcriptional analysis was also not performed within this thesis. Previous research has examined the effects of CBD on mRNA expression of two skeletal muscle differentiation markers, myogenin and TNNT-1(Iannotti et al., 2019). In chapter 5, myotube morphology was examined following both an acute and chronic exposure to sCBD, the inclusion of transcriptional analysis, specifically analysing the mRNA expression of both myogenin and TNNT-1, would have provided additional detail of the effects of sCBD on the genes responsible for differentiation. To further support the findings in chapter 5, genes specific to cannabidiol and synthetic cannabidiol should have been analysed. Prior findings suggest CBD binds to the vanilloid 1 receptor (TPRV1), which is responsible for the regulation of body

temperature and senses heat and pain, in a dose-dependent manner (Iannotti et al., 2014). The inclusion of analysing more genes, and collecting additional RNA, would have further supported our findings, and improved the quality of data produced. Therefore, future work should incorporate the analysis of more genes.

6.3 Conclusions and Implications

The main objective of this thesis was to investigate the effect of sCBD on cell health and viability of murine myoblasts and myotubes and to determine the morphological changes following different dosing techniques. The primary finding from this thesis is that synthetic cannabidiol confers no beneficial effects to myoblast proliferation and differentiation and is cytotoxic to myoblast and myotube health at a high *in vitro* dose of 50 µM. This effect was exaggerated following chronic treatment, where monolayers received repeated sCBD doses, as significant reductions in metabolic activity were reported at 50, 20 and 10 µM and cell viability reduced at 50 µM and 20 µM respectively. However, following acute treatment of sCBD, only metabolic activity was reduced at 50 µM. Previous research has discovered that in proliferating myoblasts and differentiating myotubes, a concentration of 50 µM CBD induces apoptosis-driven cytotoxicity in different cell lines (Shrivastava et al., 2011; Oláh et al., 2014). Although both myoblasts and myotubes were not examined within this thesis for features of apoptosis, we can converse with findings from other research and propose that sCBD at higher doses, causes apoptosis in cells *in vitro* in a time and concentration manner.

Morphological changes were reported after chronic treatment, with reductions in myotube area, number and NFI all recorded following administration of sCBD at certain doses, only NFI was reduced in the acute treatment at certain doses. Transcriptional analysis would need to be conducted to support our findings, however, due to findings from prior research, the detrimental impact sCBD appears to be having on myotube formation after chronic exposure

may be due to its inhibitory effect on genes myogenin and TNNT-1, which are both markers of skeletal muscle differentiation. Inhibition of these genes would impact myotube formation, with reductions in myotube area, number and NFI likely to be observed, which is what our results show. In chapter 5, monolayers were immunostained for DAPI and MyHC, with the findings determining significant reductions in NFI across most concentrations of sCBD. This reduction potentially indicates sCBD impedes the growth of myotubes later during differentiation following both acute and chronic exposure to sCBD. As mentioned previously, chronic treatment with sCBD confers greater detrimental effects on both cell viability and myotube morphology as opposed to acute treatment, implying that sCBD, like CBD, effects C_2C_{12} skeletal muscle cells in a concentration and time dependent manner.

A consideration when interpreting findings from *in vitro* studies is that the environment the cell line in question is cultured in does not truly replicate the natural environment within the body. Previous literature investigating the effects of CBD have often used concentrations of 50 and 20 µM when studying its effect on cell viability and lower doses of 1 to 5 µM when closer examining its impact on myotube formation. Therefore, the different concentrations of sCBD used within this thesis replicate former studies. However, we cannot validate that the doses of sCBD used within our studies replicate tissue concentrations of CBD. Moreover, it is not known how or if CBD accumulates in blood following supplementation so comparisons to circulating concentrations also cannot be made. CBD has also been examined for its possible different effects between humans and animal, as even the same blood concentrations may lead to differing effects (Iffland and Grotenhermen, 2017; Deiana et., 2011). Mice were administered 120mg/kg CBD and plasma levels were measured at 2.2 µg/ml, and humans administered 10mg/kg and blood levels of 0.01 µg/ml measured (Iffland and Grotenhermen, 2017; Deiana et., 2011). This corresponds to human blood levels of 0.12 µg/ml when 120mg/kg

CBD was given to humans (Iffland and Grotenhermen, 2017; Deiana et., 2011). Hence, the findings demonstrated that even when mice and humans are given the same CBD dose, additional amounts of the compound become available in the mouse organism and this higher bioavailability, in turn, causes greater CBD effects (Iffland and Grotenhermen, 2017; Deiana et., 2011).

A surprising and interesting finding from this thesis was that sCBD appeared to have no positive effect on C_2C_{12} skeletal muscle cells *in vitro*. Contrary to previous research by Iannaotti and colleagues (2018) who discovered that 1 µM CBD induced greater myotube formation and mRNA expression of myogenin in C_2C_{12} myoblasts, our results conclude sCBD at concentrations ranging 5 µM - 0.001 µM had no benefit on myotube formation as no increases in myotube area, number and NFI were reported after both acute and chronic treatment. Other unpublished research conducted in our laboratory investigated the effects of broad-spectrum CBD on C_2C_{12} muscle cells and discovered similar findings as CBD appeared to have no positive impact on muscle cells.

One study, which aimed to investigate whether naturally derived CBD or synthetically produced CBD would behave similarly in 3 human cell models of disease, discovered interesting and similar findings to ours (Maguire et al., 2021). Their results determined that all CBD samples (natural and synthetic) produced similar effects at their respective doses and time points throughout all studies (Maguire et al., 2021). Although some minor variability existed in the amount of effect and percentage purity of CBD samples, none of these variances resulted in one CBD sample being superior to any others (Maguire et al., 2021). Synthetic cannabidiol is often less favoured over natural CBD, as a recent study in Germany asked 153 epileptic patients if they worry over the origin of CBD, with 73% favouring natural CBD due to its

botanical origin, with sCBD perceived as inferior (von Wrede et al., 2020; Maguire et al., 2021). The perception that full spectrum CBD is therapeutically superior is due to the 'entourage hypothesis', which states the effects of CBD are enhanced because of the interaction between all compounds within the cannabis plant, though our research and other findings suggest that the effects of CBD are similar, no matter the origin of the extract.

Although the interest in CBD among athletes is increasing, there is no evidence for any beneficial effects on muscle, both pre-clinical and clinical. However, there are proven benefits of CBD in epilepsy, which has recently culminated in US Food and Drug Administration (FDA) approval of Epidiolex, for the treatment of Lennox-Gastaut and Dravet syndromes (Mascal et al, 2019). A series of studies investigated THC and CBD for its ability to control seizures in different animal models of epilepsy (Wallace et al., 2001). Their findings concluded both THC and CBD produced anticonvulsant effects in rodents (Wallace et al., 2001). CBD has demonstrated various benefits, including the capacity to control seizures as effectively as currently available anti-epileptic drugs (AEDs) and a lower risk of side effects when compared to currently used AEDs in therapy (Dos Santos et al., 2015). In conclusion, numerous *in vitro* and *in vivo* studies support the role of CBD in the management of epilepsy.

Whilst our research suggests sCBD might not have much of an impact on skeletal muscle cells under normal conditions, previous research has demonstrated CBD to have no detrimental effects at lower doses, especially when compared to substances used for similar purposes, such as NSAID's. CBD at ranging doses of 1-5 μM was discovered to have no detrimental effects on C_2C1_2 skeletal muscle cells, as the effects of CBD on anabolic or inflammatory signalling *in vitro* were small (Langer, Avey and Baar, 2021). On the other hand, previous research into the effects of NSAID's on EIMD have discovered that they possibly impair the adaptive

response to exercise (Schoenfeld, 2012). COX enzymes are very important and often necessary to achieve maximal skeletal muscle hypertrophy as they have been demonstrated to stimulate satellite cell proliferation, differentiation, fusion and increase muscle protein synthesis (Schoenfeld, 2012). As NSAID's allegedly exert their actions by inhibiting COX, there is the potential whereby these drugs may have detrimental effects on muscle growth and development (Schoenfeld, 2012). Other research has indicated NSAID's such as ibuprofen have a blunting effect on the protein synthesis response observed after high-intensity eccentric exercise, thus leading to possible adverse effects on skeletal muscle development following both acute and chronic exercise (Trappe et al., 2002)). Therefore, CBD and sCBD may be used an alternative to NSAID's, as it appears to have no adverse effects on anabolic and inflammatory signalling.

Synthetic cannabinoids differ from naturally occurring cannabinoids in terms of chemistry and pharmacology. Due to their structural dissimilarity to THC, they can avoid regulatory constraints and are less likely to be detected by routine drug screenings. In the area of study of synthetic cannabinoids, there is vast amount of room for research as well as a great need for it, as a thorough understanding on these compounds is still lacking. Our research provides information on the cytotoxicity of sCBD on myoblasts and myotubes at higher doses and discloses at lower doses, sCBD has no positive impact on myotube formation, therefore the lack of any positive effect should discourage athletes who are considering supplementing with it. As reported, our research identified no positive effects of sCBD on skeletal muscle, therefore a more sceptical approach to future studies is advised. Future *in vitro* research needs to include a more in-depth investigation into the effects of sCBD on skeletal muscle cells, as including protein and transcriptional analysis would provide a greater understanding on the reported findings. Then to further develop the findings, research should include animal trails, to establish if findings reported *in vitro* are translated *in vivo*. Subject to the findings, clinical studies should also be conducted. The study should include participants supplementing with

sCBD for a chronic period (10-12 weeks) at a safe dosage and muscle biopsies taken at different time points throughout the course of the study. Samples should then be analysed for protein content and transcriptional analysis conducted to determine if sCBD has any effect on the genes responsible for muscle growth and development. In regard to EIMD, research should explore whether supplementation with both sCBD and NSAID's following muscle damage has differing effects and whether anabolic and inflammatory signalling is affected by the mechanisms of which these supplements elicit their effects. This research would provide a greater understanding of the effects of sCBD on skeletal muscle, and possible discoveries may have great significance for athletes wishing to supplement with an alternative substance to NSAID's.

Currently WADA's stance on sCBD is unclear, as only CBD is non-prohibited, with other natural cannabinoids prohibited. Our data, together with previous studies, imply that the effects of sCBD/CBD supplementations on skeletal muscle are detrimental. Even though sCBD/CBD appears to have less of an adverse effect on inflammatory responses to exercise than NSAID's, no beneficial effects are reported, and research is limited. With our findings suggesting sCBD is cytotoxic to cell health at higher concentrations and has a significant negative impact on myotube formation at lower concentrations, evidence recommending athletes to supplement with it is considerably lacking. As a result, we highly advise athletes to refrain from supplementing with sCBD/CBD until WADA provides a clearer stance on cannabinoids in competition.

References

1. Abel, E.L. (2013) *Marihuana: the first twelve thousand years*. Springer Science & Business Media.

2. Agley, C.C., Velloso, C.P., Lazarus, N.R. and Harridge, S.D., 2012. An image analysis method for the precise selection and quantitation of fluorescently labeled cellular constituents: application to the measurement of human muscle cells in culture. *Journal of Histochemistry & Cytochemistry*, *60*(6), pp.428-438.

3. Anderson, L.L., Etchart, M.G., Bahceci, D., Golembiewski, T.A. and Arnold, J.C. (2021) Cannabis constituents interact at the drug efflux pump BCRP to markedly increase plasma cannabidiolic acid concentrations. *Scientific Reports*, 11 (1), 1-14.

4. Atalay, S., Jarocka-Karpowicz, I. and Skrzydlewska, E., 2019. Antioxidative and anti-inflammatory properties of cannabidiol. *Antioxidants*, *9*(1), p.21.

5. Aviello, G., Romano, B., Borrelli, F., Capasso, R., Gallo, L., Piscitelli, F., Di Marzo, V. and Izzo, A.A., 2012. Chemopreventive effect of the non-psychotropic phytocannabinoid cannabidiol on experimental colon cancer. *Journal of molecular medicine*, *90*(8), pp.925-934.

6. Badowski, M.E., 2017. A review of oral cannabinoids and medical marijuana for the treatment of chemotherapy-induced nausea and vomiting: a focus on pharmacokinetic variability and pharmacodynamics. *Cancer chemotherapy and pharmacology*, *80*(3), pp.441-449.

7. Bisogno, T., Hanus, L., De Petrocellis, L., Tchilibon, S., Ponde, D.E., Brandi, I., Moriello, A.S., Davis, J.B., Mechoulam, R. and Di Marzo, V. (2001) Molecular targets for cannabidiol and its synthetic analogues: effect on vanilloid VR1 receptors and on the

cellular uptake and enzymatic hydrolysis of anandamide. *Br J Pharmacol*, 134 (4), 845-852.

8. Bonini, S.A., Premoli, M., Tambaro, S., Kumar, A., Maccarinelli, G., Memo, M. and Mastinu, A. (2018) Cannabis sativa: A comprehensive ethnopharmacological review of a medicinal plant with a long history. *J Ethnopharmacol*, 227, 300-315.

9. Buchbauer, G., Jirovetz, L., Jager, W., Plank, C. and Dietrich, H., 1993. Fragrance compounds and essential oils with sedative effects upon inhalation. *Journal of pharmaceutical sciences*, *82*(6), pp.660-664.

10. Burr, J.F., Cheung, C.P., Kasper, A.M., Gilham, S.H. and Close, G.L., 2021. Cannabis and athletic performance. *Sports Medicine*, *51*(1), pp.75-87.

11. Burstein, S. (2015) Cannabidiol (CBD) and its analogs: a review of their effects on inflammation. *Bioorganic & medicinal chemistry*, 23 (7), 1377-1385.

12. Chen, S., Deng, C., Zheng, W., Li, S., Liu, Y., Zhang, T., Zhang, C., Fu, Y., Miao, H., Ren, F. and Ma, X., 2021. Cannabidiol Effectively Promoted Cell Death in Bladder Cancer and the Improved Intravesical Adhesion Drugs Delivery Strategy Could Be Better Used for Treatment. *Pharmaceutics*, *13*(9), p.1415.

13. Chen, S., Zhao, M., Wu, G., Yao, C. and Zhang, J., 2012. Recent advances in morphological cell image analysis. *Computational and mathematical methods in medicine*, *2012*

14. Close, G.L., Gillham, S.H. and Kasper, A.M. (2021) CANNABIDIOL (CBD) AND THE ATHLETE: CLAIMS, EVIDENCE, PREVALENCE AND SAFETY CONCERNS.

15. Crippa, J.A., Guimarães, F.S., Campos, A.C. and Zuardi, A.W. (2018) Translational investigation of the therapeutic potential of cannabidiol (CBD): toward a new age. *Frontiers in immunology*, 9, 2009.

16. Cuttler, C., LaFrance, E.M. and Craft, R.M. (2020) A Large-Scale Naturalistic Examination of the Acute Effects of Cannabis on Pain. *Cannabis and Cannabinoid Research*.

17. Czégény, Z., Nagy, G., Babinszki, B., Bajtel, Á., Sebestyén, Z., Kiss, T., Csupor-Löffler, B., Tóth, B. and Csupor, D., 2021. CBD, a precursor of THC in e-cigarettes. *Scientific Reports*, 11(1).

18. Dariš, B., Tancer Verboten, M., Knez, Ž. and Ferk, P., 2019. Cannabinoids in cancer treatment: Therapeutic potential and legislation. *Bosnian Journal of Basic Medical Sciences*, 19(1), pp.14-23.

19. Deiana, S., 2017. Potential medical uses of cannabigerol: a brief overview. *Handbook of Cannabis and Related Pathologies*, pp.958-967.

20. Deleyto-Seldas, N. and Efeyan, A., 2021. The mTOR-autophagy axis and the control of metabolism. *Frontiers in Cell and Developmental Biology, 9, p.1519.*

21. Demuth, D.G. and Molleman, A., 2006. Cannabinoid signalling. *Life sciences*, *78*(6), pp.549-563.

22. Dessauge, F., Schleder, C., Perruchot, M.H. and Rouger, K., 2021. 3D in vitro models of skeletal muscle: myosphere, myobundle and bioprinted muscle construct. *Vetinary Research, 52*(1), pp.1-12.

23. Devane, W.A., Dysarz, F.A., 3rd, Johnson, M.R., Melvin, L.S. and Howlett, A.C. (1988) Determination and characterization of a cannabinoid receptor in rat brain. *Mol Pharmacol*, 34 (5), 605-613.

24. Di Marzo, V. and Matias, I. (2005) Endocannabinoid control of food intake and energy balance. *Nat Neurosci,* 8 (5), 585-589.

25. Docter, S., Khan, M., Gohal, C., Ravi, B., Bhandari, M., Gandhi, R. and Leroux, T., 2020. Cannabis use and sport: a systematic review. *Sports health*, *12*(2), pp.189-199.

26. Dos Santos, R.G., Hallak, J.E.C., Leite, J.P., Zuardi, A.W. and Crippa, J.A.S., 2015. Phytocannabinoids and epilepsy. *Journal of clinical pharmacy and therapeutics, 40*(2), pp.135-143.

27. Duan, W., Sun, Y., Wu, M., Zhang, Z., Zhang, T., Wang, H., Li, F., Yang, L., Xu, Y., Liu, Z.J. and Hua, T., 2021. Carbon-silicon switch led to the discovery of novel synthetic cannabinoids with therapeutic effects in a mouse model of multiple sclerosis. *European Journal of Medicinal Chemistry, 226*, p.113878.

28. Ebbeling, C.B. and Clarkson, P.M., 1989. Exercise-induced muscle damage and adaptation, *Sports medicine, 7*(4), pp.207-234.

29. Fatouros, I.G. and Jamurtas, A.Z., 2016. Insights into the molecular etiology of exercise-induced inflammation: opportunities for optimizing performance. *Journal of inflammation research, 9*, p.175.

30. Fisher, T., Golan, H., Schiby, G., PriChen, S., Smoum, R., Moshe, I., Peshes-Yaloz, N., Castiel, A., Waldman, D., Gallily, R. and Mechoulam, R., 2016. In vitro and in vivo efficacy of non-psychoactive cannabidiol in neuroblastoma. *Current oncology*, *23*(s1), pp.15-22.

31. Friedman, D. and Sirven, J.I., 2017. Historical perspective on the medical use of cannabis for epilepsy: ancient times to the 1980s. *Epilepsy & Behaviour, 70*, pp.298-301.

32. Gallily, R., Yekhtin, Z. and Hanuš, L.O., 2018. The anti-inflammatory properties of terpenoids from cannabis. *Cannabis and cannabinoid research*, *3*(1), pp.282-290

33. Golombek, P., Muller, M., Barthlott, I., Sproll, C. and Lachenmeier, D.W. (2020) Conversion of Cannabidiol (CBD) into Psychotropic Cannabinoids Including Tetrahydrocannabinol (THC): A Controversy in the Scientific Literature. *Toxics*, 8 (2).

34. Grotenhermen, F., 2005. Cannabinoids. *Current Drug Targets-CNS & Neurological Disorders, 4*(5), pp.507-530.

35. Gurley, B.J., Murphy, T.P., Gul, W., Walker, L.A. and ElSohly, M. (2020) Content versus Label Claims in Cannabidiol (CBD)-Containing Products Obtained from Commercial Outlets in the State of Mississippi. *J Diet Suppl*, 17 (5), 599-607.

36. Huestis, M.A., Solimini, R., Pichini, S., Pacifici, R., Carlier, J. and Busardò, F.P., 2019. Cannabidiol adverse effects and toxicity. *Current neuropharmacology*, *17*(10), pp.974-989.

37. Iannotti, F.A., Pagano, E., Moriello, A.S., Alvino, F.G., Sorrentino, N.C., D'Orsi, L., Gazzerro, E., Capasso, R., De Leonibus, E., De Petrocellis, L. and Di Marzo, V., 2019. Effects of non-euphoric plant cannabinoids on muscle quality and performance of dystrophic mdx mice. British journal of pharmacology, 176(10), pp.1568-1584.

38. Jung, B., Lee, J.K., Kim, J., Kang, E.K., Han, S.Y., Lee, H.Y. and Choi, I.S., 2019. Synthetic Strategies for (−)-Cannabidiol and Its Structural Analogs. *Chemistry–An Asian Journal*, *14*(21), pp.3749-3762.

39. Kalant, H. (2001) Medicinal use of cannabis: history and current status. *Pain Res Manag*, 6 (2), 80-91.

40. Kasper, A.M., Sparks, S.A., Hooks, M., Skeer, M., Webb, B., Nia, H., Morton, J.P. and Close, G.L. (2020) High Prevalence of Cannabidiol Use Within Male Professional Rugby Union and League Players: A Quest for Pain Relief and Enhanced Recovery. *Int J Sport Nutr Exerc Metab*, 1-8.

41. Kendall, D.A. and Yudowski, G.A., 2017. Cannabinoid receptors in the central nervous system: their signaling and roles in disease. *Frontiers in cellular neuroscience*, *10*, p.294.

42. Klotz, K.A., Hirsch, M., Heers, M., Schulze-Bonhage, A. and Jacobs, J. (2019) Effects of cannabidiol on brivaracetam plasma levels. *Epilepsia*, 60 (7), e74-e77.

43. Lachenmeier, D.W. and Diel, P. (2019) A Warning against the Negligent Use of Cannabidiol in Professional and Amateur Athletes. *Sports (Basel)*, 7 (12).

44. Langer, H.T., Avey, A. and Baar, K., 2021. Cannabidiol does not impact acute anabolic or inflammatory signalling in skeletal muscle in vitro. *Cannabis and Cannabinoid Research.*

45. Lau, S., 2020, 'The effects of cannabidiol and cannabinol on C2C12 myoblast proliferation and differentiation'. Masters thesis, McMaster University, Hamiltion, Ontario.

46. Li, X., Shen, L., Hua, T. and Liu, Z.J., 2020. Structural and functional insights into cannabinoid receptors. *Trends in Pharmacological Sciences*.

47. Li, X., Vigil, J.M., Stith, S.S., Brockelman, F., Keeling, K. and Hall, B. (2019) The effectiveness of self-directed medical cannabis treatment for pain. *Complementary Therapies in Medicine*, 46, 123-130.

48. Lukhele, S.T. and Motadi, L.R., 2016. Cannabidiol rather than Cannabis sativa extracts inhibit cell growth and induce apoptosis in cervical cancer cells. *BMC complementary and alternative medicine*, *16*(1), pp.1-16.

49. Machida, M. and Takemasa, T., 2010. Ibuprofen administration during endurance training cancels running-distance-dependent adaptations of skeletal muscle in mice. *Journal of Physiology and Pharmacology*, *61*(5), p.559

50. Mackie, K., 2008. Cannabinoid receptors: where they are and what they do. *Journal of neuroendocrinology*, *20*, pp.10-14.

51. Maguire, R.F., Wilkinson, D.J., England, T.J. and O'Sullivan, S.E., 2021. The Pharmacological Effects of Plant-Derived versus Synthetic Cannabidiol in Human Cell Lines. *Medical Cannabis and Cannabinoids*, *4*(2), pp.86-96.

52. Mareck, U., Fussholler, G., Geyer, H., Huestis, M.A., Scheiff, A.B. and Thevis, M. (2021) Preliminary data on the potential for unintentional antidoping rule violations by permitted cannabidiol (CBD) use. *Drug Test Anal*, 13 (3), 539-549.

53. Maroon, J. and Bost, J. (2018) Review of the neurological benefits of phytocannabinoids. *Surg Neurol Int*, 9, 91.

54. Mascal, M., Hafezi, N., Wand, D., Hu, Y., Serra, G., Dallas, M.L and Spencer, J.P., 2019. Synthetic, non-intoxicating 8, 9-dihydrocannabidiol for the migration of seizure. *Scientific reports*, *9*(1), PP.1-6.

55. McCartney, D., Benson, M.J., Desbrow, B., Irwin, C., Suraev, A. and McGregor, I.S., 2020. Cannabidiol and sports performance: a narrative review of relevant evidence and recommendations for future research. *Sports Medicine-Open*, *6*(1), pp.1-18.

56. McDuff, D., Stull, T., Castaldelli-Maia, J., Hitchcock, M., Hainline, B. and Reardon, C., 2019. Recreational and ergogenic substance use and substance use disorders in elite athletes: a narrative review. *British Journal of Sports Medicine*, 53(12), pp.754-760.

57. Mead, T.J. and Lefebvre, V. (2014) Proliferation assays (BrdU and EdU) on skeletal tissue sections. *Methods Mol Biol*, 1130, 233-243.

58. Morales, P., Hurst, D.P. and Reggio, P.H., 2017. Molecular targets of the phytocannabinoids: a complex picture. *Phytocannabinoids*, pp.103-131.

59. Murphy, S.M., Kiely, M., Jakeman, P.M., Kiely, P.A. and Carson, B.P. (2016) Optimization of an in vitro bioassay to monitor growth and formation of myotubes in real time. *Biosci Rep*, 36 (3).

60. Nahler, G., Jones, T.M. and Russo, E.B., 2019. Cannabidiol and contributions of major hemp phytocompounds to the "entourage effect"; possible mechanisms. *J. Altern. Complementary Integr. Med*, *5*.

61. Noreen, N., Muhammad, F., Akhtar, B., Azam, F. and Anwar, M.I. (2018) Is Cannabidiol a Promising Substance for New Drug Development? A Review of its Potential Therapeutic Applications. *Crit Rev Eukaryot Gene Expr*, 28 (1), 73-86.

62. Oláh, A., Tóth, B.I., Borbíró, I., Sugawara, K., Szöllõsi, A.G., Czifra, G., Pál, B., Ambrus, L., Kloepper, J., Camera, E. and Ludovici, M., 2014. Cannabidiol exerts sebostatic and

antiinflammatory effects on human sebocytes. *The Journal of clinical investigation*, *124*(9), pp.3713-3724.

63. Overdevest, E., Wouters, J.A., Wolfs, K.H., van Leeuwen, J.J. and Possemiers, S. (2018) Citrus flavonoid supplementation improves exercise performance in trained athletes. *Journal of sports science & medicine*, 17 (1), 24.

64. Pacher, P., Batkai, S. and Kunos, G. (2006) The endocannabinoid system as an emerging target of pharmacotherapy. *Pharmacol Rev*, 58 (3), 389-462.

65. Pagano, E., Orlando, P., Finizio, S., Rossi, A., Buono, L., Iannotti, F.A., Piscitelli, F., Izzo, A.A., Di Marzo, V. and Borrelli, F. (2017) Role of the endocannabinoid system in the control of mouse myometrium contractility during the menstrual cycle. *Biochem Pharmacol*, 124, 83-93.

66. Pagano, S., Coniglio, M., Valenti, C., Federici, M.I., Lombardo, G., Cianetti, S. and Marinucci, L., 2020. Biological effects of Cannabidiol on normal human healthy cell populations: Systematic review of the literature. *Biomedicine & Pharmacotherapy*, *132*, p.110728.

67. Pertwee, R.G., 2006. Cannabinoid pharmacology: the first 66 years. *British journal of pharmacology*, *147*(S1), pp.S163-S171.

68. Petrovska, B.B., 2012. Historical review of medicinal plants' usage. *Pharmacognosy reviews*, *6*(11), p.1.

69. Piomelli, D. and Russo, E.B. (2016) The Cannabis sativa Versus Cannabis indica Debate: An Interview with Ethan Russo, MD. *Cannabis Cannabinoid Res*, 1 (1), 44-46.

70. Ravi, M., Paramesh, C., Kaviya, S.R., Anuradha, E. and Solomon, F.P., 2015. 3D cell culture systems: advantages and applications. *Journal of cellular physiology*, *230*(1), pp.16-26.

71. Rieger, A., Nelson, K., Konowalchuk, J. and Barreda, D., 2011. Modified Annexin V/Propidium Iodide Apoptosis Assay For Accurate Assessment of Cell Death. *Journal of Visualized Experiments*, (50).

72. Riss, T.L., Moravec, R.A., Niles, A.L., Duellman, S., Benink, H.A., Worzella, T.J. and Minor, L. (2004) Cell Viability Assays. In: Markossian, S., Grossman, A., Brimacombe, K., Arkin, M., Auld, D., Austin, C. P., Baell, J., Chung, T. D. Y., Coussens, N. P., Dahlin, J. L., Devanarayan, V., Foley, T. L., Glicksman, M., Hall, M. D., Haas, J. V., Hoare, S. R. J., Inglese, J., Iversen, P. W., Kales, S. C., Lal-Nag, M., Li, Z., McGee, J., McManus, O., Riss, T., Saradjian, P., Sittampalam, G. S., Tarselli, M., Trask, O. J., Jr., Wang, Y., Weidner, J. R.,Wildey, M. J., Wilson, K., Xia, M. and Xu, X. (ed.) *Assay Guidance Manual.* Bethesda (MD).

73. Rojas-Valverde, D., 2021. Potential Role of Cannabidiol on Sports Recovery: A Narrative Review. *Frontiers in Physiology*, 12.

74. Ruhl, T., Kim, B.S. and Beier, J.P., 2018. Cannabidiol restores differentiation capacity of LPS exposed adipose tissue mesenchymal stromal cells. *Experimental cell research*, *370*(2), pp.653-662.

75. Russo, E.B., 2011. Taming THC: potential cannabis synergy and phytocannabinoid-terpenoid entourage effects. *British journal of pharmacology*, *163*(7), pp.1344-1364.

76. Sainz-Cort, A., Müller-Sánchez, C. and Espel, E., 2020. Anti-proliferative and cytotoxic effect of cannabidiol on human cancer cell lines in presence of serum. *BMC Research Notes*, *13*(1), pp.1-6.

77. Sanvee, G.M., Bouitbir, J. and Krähenbühl, S., 2021. C2C12 myoblasts are more sensitive to the toxic effects of simvastatin than myotubes and show impaired proliferation and myotube formation. *Biochemical Pharmacology*, *190*, p.114649.

78. Savo, V., La Rocca, A., Caneva, G., Rapallo, F. and Cornara, L. (2013) Plants used in artisanal fisheries on the Western Mediterranean coasts of Italy. *J Ethnobiol Ethnomed*, 9, 9.

79. Schoenfeld, B.J., 2012. The Use of Nonsteriodal anti-inflammatory drugs for exercise-induced muscle damage. *Sports medicine*, *42*(12), pp.1017-1028.

80. Shrivasta, A., Kuzontkoski, P.M., Groopman, J.E and Prasad, A., 2011. Cannabidiol induces programmed cell death in breast cancer cells by coordinating the cross-talk between apoptosis and autophagy. *Molecular cancer therapeutics*, *10*(7), pp.1161-1172.

81. Solinas, M., Massi, P., Cantelmo, A.R., Cattaneo, M.G., Cammarota, R., Bartolini, D., Cinquina, V., Valenti, M., Vicentini, L.M., Noonan, D.M. and Albini, A., 2012. Cannabidiol inhibits angiogenesis by multiple mechanisms. *British journal of pharmacology*, *167*(6), pp.1218-1231.

82. Touw, M. (1981) The religious and medicinal uses of Cannabis in China, India and Tibet. *J Psychoactive Drugs*, 13 (1), 23-34.

83. Trappe, T.A., White, F., Lambert, C.P., Cesar, D., Hellerstein, M. and Evans, W.J., 2002. Effect of ibuprofen and acetaminophen on postexercise muscle protein synthesis. A*merican Journal of Physiology-Endocrinology and Metabolism*, *282*(3), pp.E551-E556.

84. Turner, C.E. and ELSOHLY, M.A., 1981. Biological activity of cannabichromene, its homologs and isomers. *The Journal of Clinical Pharmacology*, *21*(S1), pp.283S-291S.

85. Van Tonder, A., Joubert, A.M. and Cromarty, A.D., 2015. Limitations of the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl-2H-tetrazolium bromide (MTT) assay when compared to three commonly used cell enumeration assays. *BMC research notes*, *8*(1), pp.1-10.

86. Volkow, N., 2018. Cannabidiol: barriers to research and potential medical benefits.

87. Vrechi, T.A., Leão, A.H., Morais, I.B., Abílio, V.C., Zuardi, A.W., Hallak, J.E.C., Crippa, J.A., Bincoletto, C., Ureshino, R.P., Smaili, S.S. and Pereira, G.J., 2021. Cannabidiol induces autophagy via ERK1/2 activation in neural cells. *Scientific reports*, *11*(1), pp.1-13.

88. Vučković, S., Srebro, D., Vujović, K.S., Vučetić, Č. and Prostran, M., 2018. Cannabinoids and pain: new insights from old molecules. *Frontiers in pharmacology*, *9*, p.125.

89. Wallace, M.L., Wiley, J.L., Martin, B.R. and DeLorenzo, R.J., 2001. Assessmnet of the role of CB1 receptors in cannabinoid anticonvulsant effects. *European journal of pharmacology*, *428*(1), pp.51-57.

90. Williamson, E.M. and Evans, F.J., 2000. Cannabinoids in clinical practice. *Drugs*, *60*(6), pp.1303-1314.

91. Zuardi, A.W. (2006) History of cannabis as a medicine: a review. *Braz J Psychiatry*, 28 (2), 153-157.