Characterisation of the anabolic effects of leucine on primary chicken muscle cells and murine C2C12 muscle cells

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Abstract

Leucine is a branched-chain amino acid, which possesses a unique ability to stimulate the rate of protein synthesis in muscle cells both *in vivo* and *in vitro*. This thesis aimed to evaluate the anabolic effects of leucine on primary chicken and continuous murine muscle cells, with the ultimate goal to develop a nutritional strategy, involving leucine intake, to improve the welfare of broiler chickens and alleviate muscle wasting in humans.

Broilers are chickens that have been genetically selected for accelerated growth, particularly the breast muscles. Such high growth rate has magnified other causes of leg deformities and increased the incidence of lameness in modern broiler chickens, which is a pressing welfare concern. Identifying the mechanisms that underlie the high growth of broiler chickens is pivotal to improve the health and welfare of these birds. Therefore, we sought to optimise the culture conditions for muscle stem cells, also known as satellite cells (SCs), isolated from the breast (pectoralis major) and leg (peroneus longus) muscles of two-week-old broilers and layers, the latter are chickens that exhibit normal growth. We also investigated the proliferation and differentiation capacities of these four populations of SCs and compared the response of SCs derived from the breast and the leg muscles to different concentrations of leucine. Results indicated that SCs isolated from the breast muscles of broiler chickens were the most proliferative. On the other hand, SCs derived from the leg muscles of layers had the highest differentiation capacity. Notably, treatment with high leucine concentrations increased the proliferation and differentiation of SCs derived from the leg, but not the breast, muscles of broiler chickens.

Muscle wasting is a debilitating condition, characterised by a progressive loss of muscle mass. In recent years, leucine has received substantial attention for its ability to treat various muscle wasting conditions. Herein, the effects of different concentrations and exposure times of leucine alone, or combined with glutamine on C2C12 cells, an *in vitro* model of skeletal muscles, were evaluated. The impact of different serum starvation protocols and incubation in media with different serum contents on C2C12 cells were also evaluated. This was achieved by measuring cell viability, protein accumulation, phosphorylation of key proteins of signalling pathways involved in protein synthesis and the expression of muscle specific microRNAs (myomiRs) following short-, medium- and long-term exposure of C2C12 cells to leucine and/or glutamine. Results indicated that short-term serum starvation, prior to leucine treatment, decreased the

viability and total protein content of cells. In line with this, long-term incubation in reduced-serum and serum-free media compromised the viability, survival and total protein of C2C12 cells. Serum was also essential for attaining the protein anabolic effects of leucine on C2C12 cells. Intriguingly, only low/physiologically relevant concentrations of leucine were capable of boosting the viability and increasing the total protein of C2C12 cells.

Our results also indicated a concentration-dependent increase in the phosphorylation of the mammalian target of rapamycin (mTOR) substrates, the eukaryotic initiation factor 4E binding protein 1 (4E-BP1) and the ribosomal protein S6 kinase (p70 S6K), following short-term exposure of differentiated C2C12 cells to leucine. However, the phosphorylation of mTOR substrates became inversely correlated with leucine concentrations following longer exposures. Another notable finding was how glutamine did not exert any additive effect on the leucine-induced phosphorylation of p70 S6K and 4E-BP1, following short-term exposure. However, upon medium-term exposure, glutamine exerted two differential effects depending on the concentration of leucine used. Specifically, glutamine increased the phosphorylation of p70 S6K and 4E-BP1 when combined with low concentrations of leucine, while it decreased the phosphorylation of p70 S6K and 4E-BP1 when combined with a high concentration of leucine. Notably, glutamine was essential for sustaining the leucine-induced phosphorylation of p70 S6K following long-term stimulation.

Stimulation of C2C12 cells with leucine also induced the phosphorylation of the extracellular signal regulated kinase 1,2 (ERK1/2), but in an inverse dose-dependent manner. Combining leucine and glutamine did not increase the phosphorylation of ERK1/2, although glutamine rescued the decline in ERK1/2 phosphorylation following long-term stimulation with leucine. Importantly, inhibition of ERK1/2 decreased, but did not prevent, the phosphorylation of mTOR substrates in C2C12 cells following leucine and/or glutamine treatment. However, inhibition of the phosphoinositide 3-kinase (PI3K)/protein kinase B (PKB or Akt) signalling prevented the phosphorylation of mTOR substrates in C2C12 cells following leucine and/or glutamine treatment. Finally, long-term stimulation with leucine increased the expression of myomiRs (miR-1, miR-133a, miR-133b, miR-206 and miR-499) in differentiated C2C12 cells.

Altogether, this thesis elucidated the differences between SCs derived from chickens selected for high growth (broilers) and chickens with normal growth rate (layers). It also elucidates the differences that exist between SCs derived from selected (breast) and unselected (leg) muscles of the same bird. The results of this thesis suggest that leg deformities of broiler chickens could

be due to poor proliferation and differentiation of leg muscle cells. They also suggest that leucine supplementation may provide added benefit in increasing the mass and strength of broilers leg muscles and consequently, may enhance the health and welfare of these birds. This thesis has also shown the negative effects associated with serum restriction and emphasised the central role of serum in promoting the protein effects of leucine on cultured muscle cells. New insights into the concentration- and time-effects of leucine on C2C12 cells have been provided. In addition, this thesis elucidated how the combined effect of leucine and glutamine is both concentration- and time-dependent. Moreover, a possible involvement of signalling pathways, other than mTOR, in mediating the protein effects of leucine on muscle cells was detected. Finally, this thesis has been the first to test the temporal expression of myomiRs in cultured muscle cells following leucine treatment. It is hoped that these findings may ultimately contribute to the development of a nutritional strategy, using leucine, to increase muscle mass and subsequently improve the health and welfare of broiler chickens and mitigate muscle wasting in humans.

Declaration

I declare that all the work in this thesis is original and has not been previously accepted in any substance for any degree in the University of Nottingham and is not concurrently submitted in candidature for any other degree elsewhere. I also declare that all the work in this thesis was obtained from the experimental work done by myself, except for the qRT-PCR runs in chapter 7 where I received help from Belinda Wang, a technician at the School of Veterinary Medicine and Science, University of Nottingham.

Signature: *Nashwa Anwar*

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List of abbreviations

- +ve Positive
- % Percentage
- µg Microgram
- µl Microlitre
- 4E-BP1 Eukaryotic translation initiation factor 4E binding protein 1
- AAs Amino acids
- AB AlamarBlue assay
- Akt Protein kinase B
- AMPK Adenosine 5′-monophosphate activated protein kinase
- ANOVA Analysis of variance
- AO Acridine orange
- AU Arbitrary unit
- BCA Bicinchoninic acid assay
- BCAAs Branched-chain amino acids
- BL Broiler’s peroneus longus muscle
- BP Broiler’s pectoralis major muscle
- BSA Bovine serum albumin
- cDNA Complementary DNA
- CEE Chicken embryo extract
- CM Collection medium
- CO_2 Carbon dioxide
- CP Crossing points
- CSCs Chicken satellite cells
- DAPI 4',6-diamidino-2-phenylindole
- dGM GM supplemented with dialysed FCS
- DMEM Dulbecco′s Modified Eagle′s Medium
- DMSO Dimethyl sulfoxide
- DNA Deoxyribonucleic acid
- DsM Dissociation medium
- EAAs Essential amino acids

- ECL Enhanced chemiluminescence
- EDTA Ethylenediaminetetraacetic acid
- EdU 5-Ethynyl-2-deoxyuridine
- elF Eukaryotic translation initiation factor
- ERK Extracellular signal regulated kinase
- FCS Foetal calf serum
- FoxO Forkhead family of transcription factors
- *g* G force
- Gln Glutamine
- GM Growth medium
- GSK Glycogen synthase kinase
- h Hour
- H&E Haematoxylin and eosin
- HCL Hydrochloric acid
- HI Heat inactivated
- HRP Horseradish peroxidase
- HS Horse serum
- HSD Honestly significant difference
- ICC Immunocytochemistry
- IGF Insulin-like growth factor
- IPC Internal positive control
- Leu Leucine
- LL Layer peroneus longus muscle.
- LP Layer pectoralis major muscle
- M Molar
- mA Milliampere
- MAFbx Muscle atrophy F-box
- MAPK Mitogen activated protein kinase
- Mcad M-cadherin
- min Minute
- MiR MicroRNA gene
- miRNA MicroRNA
- ml Millilitre

- mM Millimolar
- MPB Muscle protein breakdown
- MPS Muscle protein synthesis
- mTOR Mammalian target of rapamycin
- MuRF1 Muscle RING finger protein 1
- Myf5 Myogenic factor 5
- MyHC Myosin heavy chain
- MyoD Myoblast determination protein 1
- MyomiRs Muscle specific microRNAs
- *n* Biological replica
- No./no. Number
- nt Nucleotide
- NTC No template control
- ºC Degree centigrade
- O/N Overnight
- p70 S6K Ribosomal protein S6 kinase
- PAX3 Paired box 3
- PAX7 Paired box 7
- PBS Phosphate buffered saline
- PCR Polymerase chain reaction
- PI Propidium iodide
- PI3K Phosphoinositide 3-kinase
- PKB Protein kinase B
- PL pectoralis major muscle
- PM peroneus longus muscle.
- PVDF Polyvinylidene fluoride membrane
- RIN RNA integrity number
- RIPA Radioimmunoprecipitation assay buffer
- RNA Ribonucleic acid
- RT Room temperature
- RT- Real time
- SCCs Satellite cell cultures
- SCs Satellite cells

- SDS-PAGE Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
- Ser Serine
- SM Serum-free medium
- SPSS Statistical Package for Social Sciences
- StM Starvation medium
- T Tween
- TBS Tris-buffered saline
- Thr Threonine
- Tris-HCL Tris hydrochloride
- TSC Tuberous sclerosis complex
- V Voltage
- *v/v* Volume per volume
- VCC Viability and Cell Count assay
- -ve Negative
- *w/v* Weight per volume
- WB Western blot
- VB-48^{TM} Vitabright-48^{TM} assay
- JC-1 5, 5, 6, 6-tetrachloro-1, 1, 3, 3-tetraethylbenzimidazol -carbocyanine iodide
- 1X One time
- 3X Three times
- 10X Ten times

1. Introduction

Muscle wasting in humans, due to ageing or chronic diseases, results in progressive loss of muscle mass and function, poor quality of life and high mortality (Powers *et al*., 2016). It is widely accepted that promoting muscle growth (hypertrophy) is a primary goal to combat muscle wasting (Bamman *et al*., 2018). Muscle hypertrophy can be achieved by increasing the proliferation of satellite cells (SCs) and/or enhancing the rate of muscle protein synthesis (MPS). SCs play an indispensable role in muscle growth and regeneration (Yablonka-Reuveni, 2011; Murphy *et al*., 2011). Leucine, a branched-chain amino acid (BCAA), has received much attention in recent years for its ability to promote muscle hypertrophy through increasing the rate of protein synthesis within muscle cells (Anthony *et al*., 1999; Anthony *et al*., 2000; Shah *et al*., 2000; Vary, 2007; Wilson *et al*., 2010). Within this context, we aimed to examine the properties of SCs isolated from chickens selected for high growth rate (broilers) and chickens that exhibit normal growth rate (i.e., layers). We also aimed to characterise the anabolic effects of leucine on these primary chicken cells and on an established mouse myoblast (C2C12) cell line, an *in vitro* model of muscle cells.

1.1. Skeletal muscles

Skeletal muscles constitute 30 to 40% of the total body mass of animals and humans. For all vertebrates, skeletal muscles are essential for life and survival as they are involved in vital functions such as movement, keeping posture, breathing, metabolism and heat production (Kamei *et al*., 2020). Skeletal muscles are composed of elongated cells (muscle fibres) surrounded by plasma membrane (sarcolemma) and embedded in extracellular collagen matrix. Muscle fibres, also called myofibres, are syncytia with multiple nuclei (myonuclei) contained within the fibres' cytoplasm (sarcoplasm). Myonuclei are post mitotic and incapable of division or self-renewal. On the other hand, muscle fibres contain a small population of peripherally located cells called SCs, which are capable of proliferation upon proper stimulation (Huxley, 1964). Each muscle fibre contains hundreds of myofibrils running through and parallel to the whole fibre's length (Figure 1.1). Myofibrils are bundles of two filaments, thin filaments (mostly actin) with 7 nm diameter and thick filaments (mainly myosin) with 15 nm diameter (Huxley, 1964).

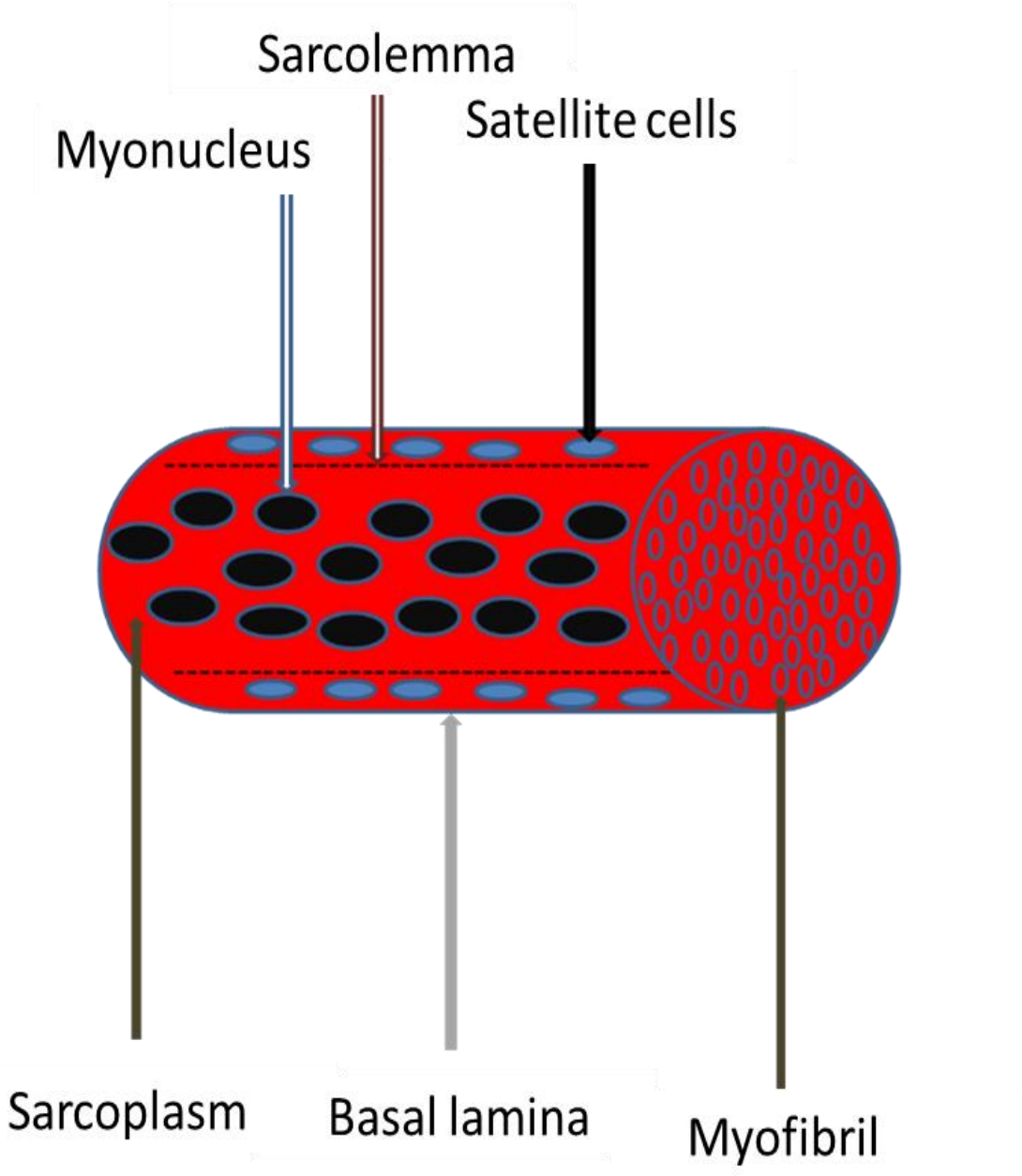


Figure 1.1. Schematic diagram illustrating the structure of a muscle fibre. A muscle fibre is an elongated muscle cell surrounded by a plasma membrane called the sarcolemma and filled with myofibrils, which run parallel to each other on the long axis of the muscle fibre and through its entire length. A muscle fibre has multiple nuclei called myonuclei contained within the fibre's cytoplasm or sarcoplasm. It also has a small population of peripheral cells wedged between the sarcolemma and the basal lamina called satellite cells. The figure is modified from Muntz, 1990.

1.2. Satellite cells

Adult myogenic progenitors or SCs were first identified in 1961 and designated on the basis of their unique peripheral position being wedged between the basement membrane (basal lamina) and plasma membrane (sarcolemma) of muscle fibres (Mauro, 1961). Earlier light microscopic observations indicated that all the nuclei visible in myofibres were true muscle fibre nuclei (myonuclei). However, the year 1961 witnessed a breakthrough in skeletal muscle biology when Mauro, by means of electron microscopy, detected a small portion of nuclei that was neither contained within the myofibre's sarcoplasm nor surrounded by its sarcolemma (Mauro, 1961). Those were the nuclei of fusiform cells intimately associated with the muscle fibre but were separated from the sarcolemma by narrow intercellular space of about 300 Ångstrom (Mauro, 1961). Those cells were named ‘satellite cells’ based on their unique anatomical position within the muscle fibres.

SCs were first identified in frogs and rats but subsequent examination of other animal species, such as bats and mice, confirmed their presence and extended the scope of these cells in more vertebrate species making them a general phenomenon in all skeletal muscles (Muir *et al*., 1965). A previous osmotic experiment was performed to elucidate how closely SCs are associated with their parent myofibres (Muir *et al*., 1965). The SCs were found to be osmotically independent from myofibres indicating a complete separation of myofibre’s sarcoplasm from the associated SCs cytoplasm (Muir *et al*., 1965). The same study revealed that SCs are fusiform, or spindle shaped with central small nuclei rich in heterochromatin. They also have high nuclear to cytoplasmic ratio because the nucleus is surrounded by a thin rim of cytoplasm containing few cytoplasmic organelles (Muir *et al*., 1965).

All the above-mentioned morphological features supported an early assumption about the quiescence of SCs. Nevertheless, upon activation, the morphology of SCs is altered. An increase in the cytoplasmic-nuclear ratio and the number of cellular organelles occurs and nuclei become low in heterochromatin (Schultz and McCormick, 1994). Following activation, SCs can be easily identified by their appearance because they enlarge, bulge from the myofibres surface and grow cytoplasmic processes from the cell pole (s) (Schultz and McCormick, 1994).

1.2.1. Distribution of satellite cells

SCs are found in all skeletal muscles regardless of the muscle fibres composition (Yin *et al*., 2013). Studies on neonatal hind limb muscles showed that SCs represent 30% of the total muscle fibre nuclei count (Snow *et al*., 1977; Bischoff, 1994). However, upon full differentiation of muscle fibres the number of SCs decline dramatically to represent only 2-7% of the total sub-laminal nuclei (Hawke and Garry, 2001; Yablonka-Reuveni, 2011). The distribution of SCs among skeletal muscles differs according to muscle type as the number of SCs is lower in fast-twitch muscle compared to slow-twitch ones. SCs are also more abundant in slow type fibres than fast ones within the same muscle (Gibson and Schultz, 1982). The distribution of SCs among skeletal muscles also differs according to the species, where SCs tend to aggregate towards the myofibre's tendenous end in chickens (Allouh *et al*., 2008). Also, the anatomical location can be a factor, where the number of SCs increases near blood capillaries (Schmalbruch and Hellhammer, 1977) and at sites of motor neuron junctions (Wokke *et al*., 1989). Additionally, age can influence SCs distribution, where a decline in SC numbers has been reported in aged animals; however, this decline varied among muscles and the most dramatic decrease was recorded in aged limb muscles (Schultz and Lipton, 1982; Shefer *et al*., 2006).

1.2.2. Satellite cells are true myogenic stem cells

Stem cells are defined as cells capable of asymmetric cell division for self-renewal and the generation of differentiated and undifferentiated progenies (Potten and Loeffler, 1990). Accumulating evidence for more than 50 years indicates that SCs are true myogenic stem cells. Initially, the evidence that SCs act as myogenic stem cells was based on experiments, which examined the distribution of labelled thymidine cells within growing and injured skeletal muscles (Moss and Leblond, 1971). However, those early findings led to an assumption that SCs only provide myonuclei to muscle fibres during development (Moss and Leblond, 1971), but maintain quiescence during postnatal life (Schultz *et al*., 1978).

The isolation and culturing of intact myofibres provided conclusive proof that SCs are capable of giving rise to myoblasts (satellite-derived myoblasts) that can differentiate into myotubes *in vitro* (Yablonka-Reuveni, 2011). Furthermore, transplantation of single myofibres into living organisms suggested that SCs are capable of proliferation into new SCs (self-renewal) and

committed myogenic progeny (myoblasts) *in vivo* (Collins *et al*., 2005; Zammit *et al*., 2006). Recently the application of genetic labelling technology has enabled tracing of SCs and their progeny through what is known as "lineage studies". These lineage studies confirmed the ability of SCs to produce myogenic cells and repair muscle injuries (Lepper *et al*., 2009). Finally, studies conducted on genetic models, mostly mice, with depleted SCs also provided evidence that SCs play a crucial role in muscle repair and supported the notion that SCs are truly myogenic stem cells (Lepper *et al*., 2011; McCarthy, 2011; Yablonka-Reuveni, 2011).

1.2.3. Activation of satellite cells

The presence of mitotic figures within the basement membrane of rat's skeletal muscle fibres was reported in the year 1964 (Macconachie *et al*., 1964). Following that a plethora of research established the crucial role of SCs in muscle growth and regeneration. Adult SCs are quiescent in normal state but upon proper stimulation such as exercising, stretching, denervation or muscle fibre crushing then, SCs become activated, begin to cycle and proliferate (Carlson and Faulkner, 1983). Depending on the extent of injury, daughter cells either differentiate into new myofibres or fuse with their parent fibres leading to hypertrophy (Yablonka-Reuveni, 1995). The activation of SCs is controlled by myriad of factors including, but not limited to, systemic, muscle niche and inflammatory response factors (Yablonka-Reuveni, 2011). The most recognised factors that control the activation of SCs are hepatocyte growth factor (HGF) and nitric oxide synthase (Tatsumi *et al*., 2001).

1.2.4. The role of satellite cells in muscle hypertrophy

It is well-established that muscle fibres are post mitotic cells incapable of any proliferative activity. Therefore, it is down to SCs to generate new muscle fibres during postnatal life. More than 100 years ago, it was noticed that skeletal muscle fibres increase in size and content without any division of the myofibre's nuclei (Lewis and Lewis, 1917). The same observations were also reported by Stockdale and Holtzer who indicated that the accretion of myofibre's content was not associated with the replication of myonuclei (Stockdale and Holtzer, 1961). A possible involvement of newly identified SCs in muscle growth and regeneration was suggested (Mauro, 1961). However, the conclusive evidence that SCs are responsible for postnatal muscle growth was provided in 1970 (Moss and Leblond, 1970). Despite a well-established notion of

SCs supplying new nuclei (DNA) to parent myofibres during muscle hypertrophy, the involvement of SCs in muscle hypertrophy has been questioned. A recent study, using a genetic mouse model with depleted SCs, showed the ability of skeletal muscles to grow in size (hypertrophy) in the absence of SCs (McCarthy, 2011). This hypertrophy was not associated with neither formation of new muscle fibres nor increase in the number of muscle fibres nuclei (McCarthy, 2011). However, this only reaffirm the indispensable role of SCs during muscle hypertrophy (Yablonka-Reuveni, 2011).

1.2.5. The regenerative capacities of satellite cells

Soon after their discovery, SCs raised immediate attention as possible controllers of muscle regeneration. It was first suggested by Mauro (Mauro, 1961) and later on by Muir and co-workers (Muir *et al*., 1965) that SCs might have regenerative capabilities in vertebrates. Although the functional evidence for this assumption was unavailable until another researcher reported the division of SCs within embryonic muscle fibres (Church, 1969). This was shortly followed by an observation of what looked like cytoplasmic bridges between growing muscle fibres and dividing SCs (Dorn, 1969), which suggests a possible fusion of daughter cells with parent muscle fibres.

This evidence was supported by Moss and Leblonde (1970), who demonstrated, by means of thymidine labelling, that SCs contributed new nuclei that fused with growing muscle fibres of rats. They showed that the number of SCs started to increase one hour (h) after H-thymidine injection and doubled their number at 24 h. Labelled daughter cells, generated from SCs division, started to fuse with parent muscle fibres as early as 18 h post injection and at 48 h almost half (49%) of these cells were incorporated within the myofibre's sarcoplasm (Moss and Leblonde, 1970). Interestingly, those early incorporated nuclei retained the morphological criteria of mother SCs as indicated by their dark chromatin. In contrast, cells that had been incorporated for more than 48 h had the morphological features of true myonuclei (light chromatin) (Moss and Leblond, 1970). However, more definite conclusion was only possible through a number of lineage studies, which have clarified the role of SCs during muscle regeneration. These studies traced SCs through an induced activation of transcription factor paired box 7 reporter within the muscles of adult animals (Lepper *et al*., 2009). Additionally, studies undertaking a novel approach of SCs depletion in mice (Pax7CreERT2 reporter mice) have substantiated the role of SCs in muscle regeneration (Murphy *et al*., 2011).

1.2.6. Detection and genetic markers of satellite cells and their progeny

For a long time, the electron microscope was the only reliable tool for the identification of SCs. Only recently, the identification of SCs has become feasible under light microscopy by means of immuno-staining of biomarkers expressed by SCs (Zammit *et al*., 2006). Throughout life, SCs express a range of distinctive specific biomarkers (Figure 1.2). Among those factors are paired box 3 (Pax3), paired box 7 (Pax7), c-Met, M-cadherin (Mcad), neural cell adhesion molecule 1 (NCAM1), vascular cell adhesion molecule 1 (VCAM1), cluster of differentiation 34 (CD34), syndecan 3 (SDC3) and syndecan 4 (SDC4) (Kaung *et al*., 2008).

Of special importance are the paired pox transcription factor Pax7 and its paralogue Pax3. Pax7 is a protein commonly expressed by SCs (Kuang and Rudnicki, 2008). The availability of a perfect conjugating antibody to Pax7 protein enables its detection within muscle sections under light microscope. However, the expression activity of this genetic marker changes with the activation of SCs (Yablonka-Reuveni, 2011). Pax3 is also expressed by SCs of somite origin only (Otto *et al.,* 2006). Despite that Pax3 mRNA is detectable in most muscles, its translation is inhibited during postnatal myogenesis by certain microRNAs (miRNAs) and the protein levels of Pax3 have been shown to decline with age in chickens (Kirkpatrick *et al*., 2010 and reviewed by Yablonka-Reuveni, 2011).

Both quiescent and activated SCs express myogenic factor 5 (Myf5) transcripts (Starkey *et al*., 2011). However, Myf5 protein is only detectable upon activation and proliferation of SCs and declines upon the initiation of differentiation. Satellite-derived myoblasts (daughter cells) continue to express Pax7, but they are distinct from their mother cells by their expression of myoblast determination protein 1 (MyoD). Following their withdrawal from the cell cycle; myoblasts maintain the expression levels of MyoD, however the expression level of Pax7 declines concurring with up regulation of myogenin. Shortly after differentiation, myoblasts express muscle specific proteins such as sarcomeric myosin. Subsequently, they start to fuse into myotubes concurring with up regulation of muscle regulatory factor 4 (MRF4) and low expression levels of MyoD, Myf5 and myogenin in adult myofibres.

A group of adhesion molecules such as Mcad, NCAM1 and VCAM1 are also valuable markers for the detection of SCs. Mcad is a calcium dependent cell adhesion molecule that is moderately expressed in a subpopulation of quiescent SCs (Irintchev *et al*., 1994). Because Mcad is highly

expressed during periods of muscle regeneration, it is thought to aid the migration of SCs to injury sites (Irintchev *et al*., 1994; Cornelison and Wold, 1997; Beauchamp *et al*., 2000; Hawke and Garry, 2001). Likewise, both NCAM1 and VCAM1 are expressed in quiescent and activated SCs following muscle injury (Covault and Sanes, 1986; Jesse *et al*., 1998). The role of NCAM1 and VCAM1 is thought to be mediating SCs adhesion to basal lamina during periods of quiescence (Chen *et al*., 2013). They also mediate the migration of SCs during periods of activation especially in response to injury (Hawke and Garry, 2001). C-met is a receptor for HGF and is usually expressed by inactive SCs during adult life and is considered a hallmark of SCs quiescence (Andermarcher *et al*., 1996; Cornelison and Wold, 1997).

The development of genetic reporter mice allowed the detection of SCs via the expression of beta-galactosidase (β-gal) or specific flurophore such as green fluorescent protein (GFP) (Day *et al*., 2007). Examples of these genetically altered mice are the nestin-GFP mice, which allow the isolation of SCs through fluorescent-activated cell sorting (FACS) (Ono *et al*., 2010). Also, the Myf5 nlacZ/+ mice, which allow the detection of SCs within intact muscle fibres through X-gal staining (Ono *et al*., 2010). The 3F-nlacZ-E mice are also useful in distinguishing SCs through the expression of myosin light chain 3F in myonuclei but not within SCs. Additionally, a cross of nestin-GFP mice with Myf5 nlacZ/+ mice and nestin–GFP mice with 3F-nlacZ-E mice have been useful in detecting SCs by both FACS and gal staining in which SCs are GPF positive and X-gal negative (Day *et al*., 2010).

1.3. Chicken as a model for myogenic studies

Chicken (*Gallus gallus domesticus*), or common domestic fowl, is a domesticated sub-species of the wild Red Jungle fowl, a common wild ancestor to all birds (Tadano *et al*., 2014). Chickens are the most common and widespread species of all birds and farm animals (Bennett *et al*., 2018). Billions of chickens are raised each year using intensive farming techniques all over the world aiming for high growth (broilers) and maximum egg production (layers) (Bennett *et al*., 2018). Chicken has been extensively used in scientific research for more than a century (Tadano *et al*., 2014). Chickens have been a great model for a wide range of myogenic studies.

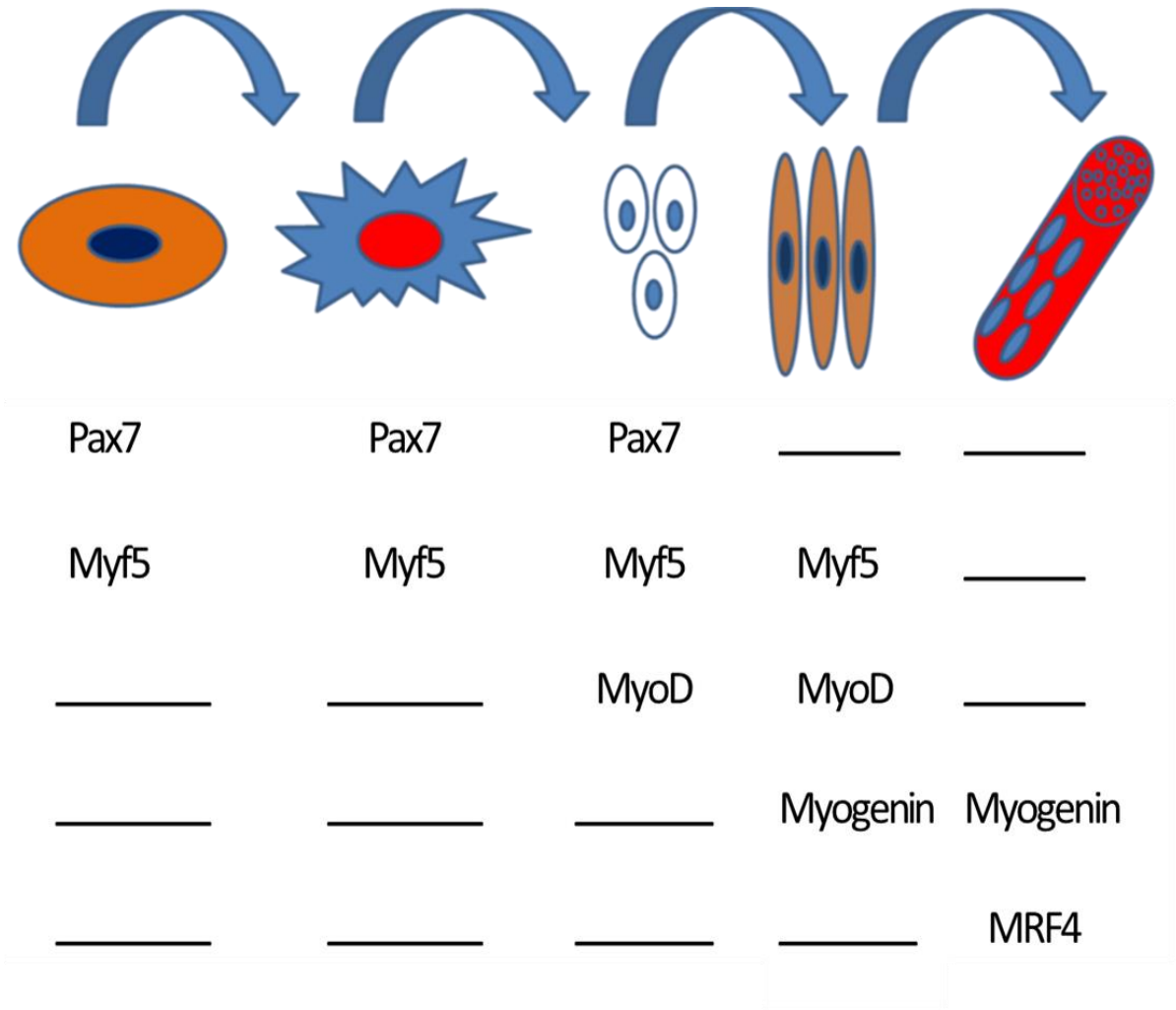


Figure 1.2. Schematic illustration showing the expression of myogenic regulatory factors during satellite cell activation. Quiescent SCs express Pax7 and Myf5. Then, the levels of Pax 7 decline in activated SCs, while the levels of Myf5 increase. Myoblasts resulted from the proliferation of activated SCs are characterised by the expression of Myf5 and MyoD. Following withdrawal from the cell cycle; cells maintain the expression of Myf5 and MyoD but start expressing myogenin. Differentiation into myofibres is characterised by decreased expression of MyoD and Myf5 and increased expression of myogenin and MRF4. Abbreviations: SCs = satellite cells; Pax7 = paired box 7; Myf5 = myogenic factor 5; MyoD = myoblast determination protein 1; MRF4 = muscle regulatory factor 4. This chart was designed based on information sourced from Kuang and Rudnicki, 2008; Yablonka-Reuveni, 2011; Starkey *et al*., 2011.

The ease of access to embryos at different embryonic stages made them ideal for studying muscle development throughout embryonic life. Also, the presence of two genetically closed chicken breeds, broilers and layers, exhibiting divergent muscle growth provided a great opportunity for understanding the different cues regulating muscle growth and hypertrophy.

1.3.1. Broiler chickens

Broilers are chickens reared for meat production. These chickens have high growth rate, high food conversion ratio and low activity levels (Buzala and Janicki, 2016). These birds were the result of active genetic selection during the 1950s when chicken breeders crossbred roosters from Cornish line with hens from Plymouth Rock and New Hampshire lines. The new hybrid had improved production traits such as favourable body type, high weight gain and high food conversion rate (Buzala and Janicki, 2016). The growth rate of broilers has improved over the years. In the 1950s, it took broilers 84 days to gain a weight of 4 pounds. However, this time was cut down to 53 days in 1970 and 49 days and 1988 (Zuidhof *et al*., 2014). Today's broilers reach their marketing weight, 4 to 5 pounds, in just 5 weeks, which is half the time the unselected chickens take to achieve the same weight (Zuidhof *et al*., 2014). It is estimated that 74% of all poultry meat across the world is produced under intensive farming conditions (Buzala and Janicki, 2016). Broilers are commercially raised in large buildings known as "grow-out houses" with limited floor space of eight-tenths square foot space per bird, minimum mobility and free access to food (Buzala and Janicki, 2016).

1.3.2. Layer chickens

Layers are chickens reared for egg production. Layers were produced by active genetic selection for high egg yield. Almost all of today's layer chickens originated from cross breeding of different leghorn chicken lines (Buzala and Janicki, 2016). Layers start to lay eggs at 16 weeks of age with massive average annual production of 300 eggs. The egg production cycle normally lasts for 17 months. However, the egg laying rate declines after 12 months of production, which is the age when most layers are slaughtered (Buzala and Janicki, 2016).

1.4. Alteration of muscle growth caused by selection

Selection for high growth within the same species can result in up to five-fold weight difference among selected and non-selected lines (Bünger *et al*. 2001). This massive weight gain was always accompanied by increased muscle mass in selected animals (Rehfeldt *et al*., 2000). Selection for high growth was shown to alter the number and the diameter of myofibres (Rehfeldt and Bünger, 1990). It is well known that the number and the diameter of myofibres are two important factors in shaping the total muscle mass (Rehfeldt *et al*., 2000).

In chickens, the number of muscle fibres is determined early in embryonic life, during the process of prenatal myogenesis. At the time of hatching, the number of muscle fibres is set in all birds. Therefore, it has been suggested that any postnatal gain can be only achieved through hypertrophy. However, many studies demonstrated that selection for rapid muscle growth leads to accelerated proliferation of SCs and myoblasts postnatally (Penney *et al*., 1983; Brown and Stickland, 1994). In mice, selection for high growth has altered the proliferative capacity of muscle cells and the rate of protein synthesis (Rehfeldt *et al*., 2002). Selection for rapid growth also increased DNA synthesis within myofibres (Knizetova *et al*., 1972; Campion *et al*., 1982; Jones *et al*., 1986; Mitchell and Burke, 1995) but without affecting the fibre's nuclear to cytoplasm ratio (Martin and White, 1979; Fowler *et al*., 1980; Campion *et al*., 1982; Penney *et al*., 1983).

1.4.1. Selection has altered the properties of broiler and layer chickens

Broiler and layer chickens show great differences in postnatal growth rate, food intake (Fujimura *et al*., 1991), feeding behaviour (Hocking *et al*., 1997) and physical activities (Stojanović *et al*., 2009). At the time of hatching, no difference in DNA content or muscle weight was apparent between broiler and layer chicks (Mizuno and Hikami, 1971). Any difference in muscle mass between broilers and layers occurs postnatally. The rate of weight gain in broiler chickens was twofold more than layers at the age of 4 days (Jones *et al*., 1986). Howver, differences in body weight between broiler and layer chickens were significantly apparent at the age of 2 weeks (Jones *et al*., 1986). At that age, broilers showed as much as double the body weight of layers (Jones *et al*., 1986). Also, at that age, the pectoralis major muscle (PM) of broiler chickens was twice the weight of layers (Jones *et al*., 1986).

It is well documented that broiler chickens have higher muscle mass than layers (Buzala and Janicki, 2016). The muscle mass is shaped by four main factors: (1) the number of myofibres, (2) the size of myofibres, (3) the rate of protein synthesis and (4) the rate of protein degradation. Many studies illustrated differences in body weight between broiler and layer lineages are mainly due to differences in both the number and size of muscle fibres. A higher number of muscle fibres was observed in the breast muscles of broilers compared to layers (Mizuno and Hikami, 1971). Also, twice the number of myofibres was recorded in the breast muscles of two broiler strains compared to layer chickens (Scheuermann *et al*., 2004). In addition, a larger diameter was observed for broiler's myofibres compared to layers (Scheuermann *et al*., 2004). Interestingly, it has been suggested that selection for high growth in chickens had altered both the number and size of myofibres but without altering the fibre types (Remignon *et al*., 1995). However, this finding was doubted by a report of a high expression of genes coding for slow type fibres in layers and a higher content of fast type fibres in broiler's muscles (Zheng *et al*., 2009).

1.4.2. Selection has altered the muscle protein turnover in broilers and layers

Muscle mass is the end result of a fine balance between protein synthesis and protein degradation. Genetic selection for chicken breeds with high meat production (broilers) and high egg yield (layers) has affected the protein turnover in both breeds. The greater muscle mass of broiler chickens is likely the result of a slow rate of protein degradation rather than an enhanced rate of MPS (Jones *et al*., 1986; Maeda *et al*., 1990). The rate of protein degradation, indicated by factional breakdown rate in the breast muscles of layers was 1.3-fold higher than broiler's (Jones *et al*., 1986). However, Duclos *et al*. (1996) reported no differences between the rates of protein degradation between the two chicken breeds. *In vitro*, 45% of the protein synthesised in the breast muscles of broilers was retained compared to only 16% protein retention in the breast muscles of layers (Jones *et al*., 1986). This finding was attributed to lower concentration of proteases in broiler's cultures (Orcutt and Young, 1982). On the other hand, the rate protein synthesis measured by fractional synthesis rate, within the breast muscles of broilers was higher than that of layers (Jones *et al*., 1986). Also, the fractional accretion rate of the breast muscles of broiler's was higher than the leg muscles of broilers and the breast and leg muscles of layers (Jones *et al*., 1986).

1.4.3. Selection for high growth has altered the properties of satellite cells

Selection for high growth has altered the proliferation capacities of SCs. The SCs of animals selected for high growth were more proliferative indicated by increased numbers of myonuclei and DNA content of muscle fibres (Knizetova *et al*., 1972; Penney *et al*., 1983; Brown and Stickland, 1994; Rehfeldt *et al*., 2002). This higher proliferation capacity of SCs was reflected by the formation of *de novo* myofibres with embryonic myosin heavy chain (MyHC) phenotype in selected animals (McFarland*et al*., 1995). Notably, the response of SCs to different mitogens varied among fast- and slow-growing chicken lines. Cultured SCs isolated from fast-growing chickens synthesised more DNA in response to foetal calf serum (FCS), insulin and insulin-like growth factor-1 (IGF-1) supplementation compared to SCs isolated from slow-growing chickens (Duclos *et al*., 1996). Similar results were also reported in SC cultures of heavy weight and light weight turkeys (MacFarland *et al*., 1995; Merly *et al*., 1998) and sheep (Mathison *et al*., 1989).

The higher responsiveness of SCs from fast-growing animals to the mitogenic powers of IGF-1 was proposed to be the result of higher numbers of IGF-1 receptors in these animals (Mathison *et al*., 1989). On the other hand, SCs from mice selected for higher growth were insensitive to the mitogenic effects of insulin and IGF-1 (Rehfeldt *et al*., 2000). Another plausible hypothesis of how selection might have altered SCs is that selection for higher growth might have favoured one compartment of SCs. Normally skeletal muscle has two populations of SCs; the first is highly dividing and represent about 80% of the SC pool while the second accounts for only 20% and represents slowly dividing (reserve) cells (Schultz, 1996). Muscles of fast-growing animals contain more dividing SCs than reserve cells (Feldman and Stockdale, 1991; Dusterhoft and Pette, 1993; Merly *et al*., 1998).

In chickens, SCs are first evident as a distinct population during mid-embryonic to late foetal phases, from embryonic day 12 to 18 when myofibres are surrounded by basal lamina (Ishikawa, 1966; Ontell and Kozeka, 1984; Feldman and Stockdale, 1991; Hartley *et al*., 1992). An earlier study, examined the differences between broiler and layer SCs *in vitro*, showed more nuclei per myotubes in layer cultures at any tested time point (Orcutt and Young, 1982). Also, the fusion rate in layers cultures was higher than broilers. However, a contradictory result was obtained by others who recorded a higher nuclei number in broilers myogenic cultures (Ridpath *et al*., 1984). This disagreement might have resulted from applying different culturing

conditions in both studies (Rehfeldt *et al*., 2000).

1.5. The murine C2C12 myoblast cell line as an *in vitro* model for myogenic studies

The C2C12 is a mouse myoblast cell line that has been extensively used as a model for myogenic studies (Burattini *et al*., 2009). C2C12 cells under appropriate *in vitro* conditions, transforms from proliferating mononucleated cells (myoblasts) into fusing multinucleated syncytia (myocytes). Myocytes under serum restriction differentiate into multinucleated syncytia (myotubes). These multinucleated myotubes retain all the morphological and functional properties of skeletal muscles (Murphy *et al*., 2016).

1.6. Myogenesis

Myogenesis, also known as 'muscle formation', is a vital biological process in all animals and humans. Myogenesis is an orchestrated process that involves two phases: (i) embryonic myogenesis, which takes place during prenatal life and results in muscle formation and (ii) postnatal myogenesis, which accompanies muscle hypertrophy and regeneration following birth and throughout life (Muntz, 1990). Embryonic (or developmental) myogenesis is a dynamic multistep process which starts with the commitment of early embryonic precursor cells to the myogenic lineage followed by their proliferation and differentiation into myocytes and ends up with the fusion of myocytes into syncytial myotubes and the expression of muscle specific genes (Muntz, 1990). On the other hand, postnatal myogenesis only occurs upon stimulation, exercise or injury. It involves the activation and proliferation of SCs followed by the fusion of their progeny with parent myofibres, leading to muscle hypertrophy or formation of new fibres, resulting in muscle regeneration (Muntz, 1990).

1.6.1. Prenatal myogenesis

Skeletal muscles develop when embryonic mesoderm (somite, paraxial and prechordal mesoderm) gives rise to pluripotent stem cells. The latter generate primitive myogenic precursors that are committed to the myogenic lineage. The proliferation of primitive myogenic precursors establishes a pool of myoblasts that continue to proliferate actively until special

signals induce them to halt proliferation, exit the cell cycle and differentiate (Rehfeldt *et al*., 2000). Cells that fail to withdraw from the cell cycle are usually eliminated by apoptosis except for a small proportion of undifferentiated myoblasts, which are retained as SCs. Following their differentiation, mononucleated myoblasts express muscle specific proteins and fuse to form multinucleated myotubes (Rehfeldt *et al*., 2000). Newly formed myotubes acquire myofibrils and form muscle fibres (myofibres) (Christ and Ordahl, 1995).

Studies on chicken embryos revealed that myogenesis occurs in two waves of myoblast differentiation: an early embryonic and a late foetal. During the early embryonic wave, embryonic myoblasts fuse and give rise to primary myotubes between the 4^{th} and 7^{th} embryonic day. These primary myotubes have central nuclei and serve as a framework for a larger population of myotubes "secondary myotubes" (Miller *et al*., 1993). Secondary myotubes are much smaller than primary ones. They are formed during the second differentiation wave, between the 8^{th} and 16^{th} ED, from the differentiation of foetal myoblasts (Beermann *et al*., 1978; Miller and Stockdale, 1986; Crow and Stockdale, 1986; Miller *et al*., 1993; Lee *et al*., 2004). The significance of these two different myogenic lineages remains not fully understood and they are not related to fibres' composition (Hughes and Blau, 1992). Notably, SCs are a small population of undifferentiated myoblasts that are associated with the muscle fibres and became dominant during late embryonic life (Moss, 1968). SCs are mitotically active during foetal and early postnatal life, as they provide nuclei to growing myofibres. However, SCs enter quiescence shortly after birth and remain inactive throughout the postnatal life (Moss and Leblond, 1971).

1.6.2. Postnatal myogenesis

Postnatal myogenesis is an orchestrated process that involves several steps in a highly sequential manner. Postnatal myogenesis starts with the activation of quiescent muscle stem cells or SCs and the subsequent production of myoblasts, which continue to proliferate and differentiate to form new myofibres (Le Grand and Rudnicki, 2007). In birds, the total number of muscle fibres is fixed at hatching (Smith, 1963). This means that any postnatal muscle growth occurs only through hypertrophy, achieved mainly by the activation of protein synthesis signalling pathways. Postnatal muscle growth could also be achieved through the activation and proliferation of resident SCs. This results in the production and addition of new nuclei and

newly synthesised DNA to muscle fibres (Moss and Leblond, 1971; Carlson and Faulkner, 1983).

1.6.3. Prenatal versus postnatal myogenesis

Prenatal and postnatal myogenesis are different. Embryonic myogenesis generates multinucleated myotubes that develop into myofibres (Muntz, 1990) while postnatal myogenesis is mainly attributed to the addition and fusion of cells to existing muscle fibres resulting in their growth or repair. Prenatal myogenesis is best described as hyperplasia and formation of new myofibres, while postnatal muscle growth is mainly a hypertrophy of existing muscle fibres (Smith, 1963). Another important difference between the two myogenic processes is the cellular components involved. Somite, specifically dermomyotome, derived myogenic progenitors are responsible for the initiation of the myogenic process during embryonic life. On the other hand, SCs are the main cells concerned with postnatal muscle growth and regeneration (Le Grand and Rudnicki, 2007). There is some evidence that cells other than SCs are involved in muscle regeneration. Some of those possible myogenic stem cells are skeletal muscle side population cells, bone marrow stem cells, bone marrow side population stem cells, pericytes and mesangioblasts (Aziz *et al*., 2012).

1.7. Muscle wasting

Muscle wasting (or muscle atrophy) is a debilitating condition characterised by an altered balance between the rates of MPS and muscle protein breakdown (MPB) (Bonaldo and Sandri, 2003). Also, it is well accepted that muscle loss is a direct outcome of protein degradation (Ferreira *et al*., 2008). During muscle atrophy, degraded muscles proteins are utilised for the synthesis of stress-related proteins and/or energy production via gluconeogenesis (Attaix *et al*., 2005; Hasselgren *et al*., 2005). A variety of physiological and pathological conditions trigger muscle atrophy such as ageing, sepsis, nutrition restriction, denervation, immobilisation, hereditary muscular disorders, diabetes and cancer. (Vellas *et al*., 2016; Dumitru *et al*., 2018). Over the past 30 years researchers have identified different approaches to attenuate and reverse muscle atrophy. Among many approaches, nutritional and drug interventions were the most important tools in fighting different forms of muscle atrophy.

1.7.1. Pharmacological interventions for treatment of muscle wasting

Although being limitative, various drugs have been used to counteract muscle atrophy in humans.

1.7.1.1. Myostatin inhibitors

Myostatin negatively affects muscle size. Elevated levels of myostatin have been reported in humans and animals with muscle atrophy (Sakuma and Yamaguchi, 2012, 2015), which makes myostatin an attractive candidate for treatment of muscle wasting. Indeed, blockade of endogenous myostatin attenuated muscle atrophy in mdx mice, a model of Duchenne muscular dystrophy (DMD) (Bogdanovich *et al*., 2002).

Also, transgenic mice expressing follistatin or a dominant-negative form of the activin IIB receptor, both are myostatin inhibitors, exhibited an increase in muscle size (Lee and McPherron, 2001). In addition, animal models of various muscle disorders, such as DMD, amyotrophic lateral sclerosis, cancer cachexia and limb girdle muscular dystrophy 2F showed marked improvement after receiving antibodies for myostatin (Bogdanovich *et al*., 2002; Holzbaur *et al*., 2006; Murphy *et al*., 2011). Moreover, genetically manipulated mice lacking *myostatin* showed an increased number of SCs, muscle hypertrophy and improved muscle regeneration (Siriett *et al*., 2006).

Blockade of myostatin was also helpful in controlling sarcopenia. Aged mice treated with PF-354, a myostatin inhibitor, showed improvement in both the size and the performance of their skeletal muscles (Lebrasseur *et al*., 2009; Murphy *et al*., 2010). Similarly, injection of elder humans with LY2495655, a myostatin antibody, increased their lean body mass (LBM) and improved their physical performance (Becker *et al*., 2015). However, despite the potential of myostatin inhibitors in treating muscle atrophy, their use has been showing adverse effects such as urticaria, confusion and septic meningitis (Morley, 2016).

1.7.1.2. Testosterone

Testosterone, the main male sex hormone, is an anabolic steroid with pleiotropic roles inside the human body (Tyagi *et al*., 2017). Testosterone supplementation increases muscle mass by inducing hypertrophy of type I and II muscle fibres and by enhancing the proliferation of SCs

(Sinha-Hikim *et al*., 2002; Sinha-Hikim *et al*., 2003; Sinha-Hikim *et al*., 2006). Elderly men receiving testosterone showed a dose-dependent increase in both the cross-sectional area (CSA) of skeletal muscle fibres and the number of SCs possess proliferating cell nuclear antigen, both are indicative of muscle hypertrophy (Sinha-Hikim *et al*., 2006). Similarly, mice treated with testosterone showed muscle hypertrophy (Brown *et al*., 2009).

Testosterone has been shown to modulate various signalling pathways that affect muscle mass, such as the IGF-1, myostatin and Wnt signalling pathways (Ferrando *et al*., 2002; Mendler *et al*., 2007; Singh *et al*., 2009). However, testosterone induced-muscle hypertrophy is mainly achieved by the activation of p38 signalling (Brown *et al*., 2009). Testosterone induced-muscle hypertrophy was also associated with the suppression of the c-Jun N-terminal kinase, a central player in stress signalling pathways (Brown *et al*., 2009). Overall, there is a conclusive agreement on testosterone's ability in encouraging muscle hypertrophy and/or reversing muscle atrophy. However, its use has been hindered by its potential risks for increasing prostate cancer and cardiovascular diseases in elder population (Morley, 2016).

1.7.1.3. Soy isoflavones

The role of estrogen, the primary female sex hormone, in maintaining muscle mass is well documented (Dionne *et al*., 2000; Sorensen *et al*., 2001) and the activation of the protein kinase B (PKB or Akt)/ the mammalian target of rapamycin (mTOR) signalling pathways in a variety of tissues by estrogen is a strong proof of estrogen’s ability to induce protein synthesis (Pedram *et al*., 2002; Patten *et al*., 2004). Soy isoflavone is a flavonoid that exerts the same functions of estrogen because of the apparent structural similarity (Tham *et al*.,1998).

Over the years, soy isoflavone has been successfully used to attenuate muscle atrophy. Supplementation of soy isoflavone prevented fat accumulation (Kurrat *et al*., 2015) AND increased muscle mass in ovariectomised mice (Beekmann *et al*., 2015). Also, high doses of soy isoflavone preserved muscle mass and muscle fibres CSA following denervation induced muscle loss in mice (Abe *et al*., 2013). Similarly, lower doses of isoflavone decreased the effect of denervation on muscle fibres CSA (Tabata *et al*., 2019). The anti-atrophy action of soy isoflavones has been attributed to a total inhibition of E3 ubiquitin ligase Cbl-b and a partial inhibition of muscle atrophy F-box (MAFbx) and muscle RING finger protein 1 (MuRF1) (Abe *et al*., 2013). Soy isoflavones were also shown to suppress the apoptotic-dependent signalling (Tabata *et al*., 2019).

1.7.1.4. Ghrelin receptor agonists

Ghrelin is a hormone produced mainly by the stomach and to a lesser extent by the small intestine, the pancreas and the hypothalamus (Kojima and Kangawa, 2004). Ghrelin has been shown to increase food intake and to stimulate the secretion of growth hormone (Kojima and Kangawa, 2004). Agonists of the ghrelin receptor are useful in counteracting muscle atrophy. Treatment with anamorelin, a selective ghrelin receptor agonist (Bach *et al*., 2004) increased food intake, body weight and GH levels in rats (Pietra *et al*. 2014). It also increased the levels of IGF-1 and growth hormone in pigs (Pietra *et al*. 2014).

In humans, capromorelin, a ghrelin receptor agonist, increased LBM in elderly people with sarcopenia (White *et al*., 2009). However, the most recognised role of ghrelin receptor agonists is their ability to counteract muscle loss associated with cachexia (Akamizu and Kangawa, 2010; Temel *et al*., 2016; Bai *et al*., 2017). Despite the ability of ghrelin receptor agonists to increase food intake and muscle mass, their use in treatment of muscle atrophy was not associated with improved muscle function (Morley, 2016). Additionally, the use of ghrelin receptor agonists was associated with serious side effects such as atrial fibrillation and dyspnea (Morley, 2016).

1.7.1.5. Deacetylase inhibitors

The acetylation of histone is an important mechanism by which muscle mass is maintained. Disruption of this mechanism, during catabolic conditions, leads to degradation of muscle proteins (Sakuma and Yamaguchi, 2018). Trichostatin A (TSA) is a well-recognised deacetylase inhibitor. The potential anabolic effect of TSA on muscles was first investigated *in vitro*, where TSA encouraged the differentiation of C2C12 myoblasts into myotubes (Iezzi *et al*., 2004). This effect was concomitant with the upregulation of MyHC, follistatin, retinoblastoma tumor suppressor protein (pRb) and glycogen synthase (Iezzi *et al*., 2004). In another study, treatment of nutrient-deprived C2C12 myotubes with TSA resulted in the suppression of the FoxO signalling and the consequent downregulation of its target genes; microtubule-associated protein light chain 3, MuRF1 and MAFbx (Beharry *et al*., 2014). *In vivo,* TSA promoted muscle recovery following injury indicated by *de novo* fibre formation and the expression of embryonic and neonatal myosin (Iezzi *et al*., 2004). TSA also increased the number and size of myofibres

in mice with spinal muscular atrophy (Avila *et al*., 2007; Bricceno *et al*., 2012). Likewise, this effect was mediated by the downregulation of MuRF1 and MAFbx (Avila *et al*., 2007; Bricceno *et al*., 2012).

1.7.1.6. Angiotensin-converting enzyme inhibitors

Angiotensin II is a peptide hormone with vital biological activities such as maintaining sodium-water balance (Hall, 1986) and regulation of blood pressure (Fyhrquist *et al*., 1995). The negative role of angiotensin II on muscle mass was first demonstrated in 1996 when its infusion in rats resulted in the loss of skeletal muscle mass (Brink *et al*., 1996). The catabolic actions of angiotensin II on muscles were also evident by its increased levels in the plasma of patient with muscle atrophy (Masson *et al*., 1998; Simoes e Silvia *et al*., 2006). Studies suggested that angiotensin II does not affect the rate of MPS, however it accelerates the rate of MPB (Brink *et al*., 2001).

Angiotensin II enhances protein degradation by suppressing the protein anabolic IGF-1/Akt signalling (Brink *et al*., 1996; Brink *et al*., 2001; Song *et al*., 2005; Maggio *et al*., 2006). It also upregulates the expression of MuRF1 and MAFbx and activates caspase-3 within skeletal muscles (Song *et al*., 2005). The use of angiotensin converting enzyme (ACE) inhibitors to prevent muscle loss, especially during the course of chronic illnesses, is promising (Maggio *et al*., 2006). ACE inhibitors decreased muscle loss in elderly women suffering high blood pressure (Onder *et al*., 2002). They also improved muscle function in the functionally impaired elder population (Sumukadas *et al*., 2007) and prevented weight loss associated with heart failure (Anker *et al*., 2003; Dossegger *et al*., 1993). However, the use of ACE inhibitors is not totally safe and side effects such as muscle cramps, hypotension and hyperkalemia were associated with their use (Morley, 2016).

1.7.1.7. Cyclooxygenase inhibitors

Cyclooxygenase is an enzyme expressed in the skeletal muscles and catalyses the production of prostanoids including prostaglandins (Sudbo *et al*., 2003). Prostaglandins play a central role in the development of inflammatory responses and were shown to control the rates of MPS and MPB (Rodemann and Goldberg, 1982; Palmer, 1990). Three forms of COX have been identified, COX1, COX2 and COX3, with two forms, COX1 and COX2, present in skeletal

muscles (Vane *et al*., 1998). It has been reported that COX2 was associated with inflammation (Davis *et al*., 2004). In support of this finding, high levels of COX2 have been recorded in the lungs of patients with muscle atrophy induced by chronic pulmonary diseases (Shi *et al*., 2017). There is a strong evidence that the use of COX2 inhibitors such as celecoxib, rofecoxib, nimesulide and meloxicam can attenuate muscle wasting associated with cachexia. Indeed, treatment with celecoxib reversed muscle loss in mice and humans with late-stage cancer (Davis *et al*., 2004; Lai *et al*., 2008; Romero *et al*., 2010).

Also, rofecoxib and nimesulide, reduced cancer-mediated muscle wasting in mice (Baumgarten *et al*., 2007). Aside from cachexia, COX2 inhibitors were also helpful in attenuating muscle wasting during the course of different inflammatory conditions. For example, celecoxib was successful in decreasing muscle wasting associated with inflammation in rats with induced emphysema (Roh *et al*., 2010). Meloxicam also attenuated arthritis-mediated muscle wasting in rats (Granado *et al*., 2007). *In vitro*, meloxicam decreased the rate of protein degradation within C2C12 myoblasts following exposure to a proteolysis-inducing factors (Hussey and Tisdale, 2000). It is worth to note that the use of COX-2 inhibitors was associated with elevated risk of strokes and cardiovascular diseases especially myocardial infarction (Das, 2005).

1.7.1.8. Growth hormone

Growth hormone (GH) is a peptide produced by the anterior pituitary gland (Florini *et al*., 1996). GH is an anabolic hormone that controls the growth of various tissues including skeletal muscles (Florini *et al*., 1996) and low levels of GH is usually associated with muscle weakness (Rabijewski *et al*., 2016; Swiecicka *et al*., 2017). During early life GH is secreted in large amounts, however its secretion declines after the age of 30 (Hermann and Berger, 2001; Moran *et al*., 2002). A large body of literature documented the anti-atrophic effects of GH. For example, administration of GH increased the LBM in elder humans (Rudman *et al*., 1990). In rats, GH prevented muscle loss during hind limb-suspension (Allen *et al*., 1997). GH also reversed muscle loss and restored muscle force in a rat model with chronic heart failure (Dalla Libera *et al*., 2003). Additionally, knocking out GH receptors in mice led to reduction in muscle mass and myofibres' CSA (Sotiropoulos *et al*., 2006).

GH exerts its anabolic effects by affecting the IGF-1, Phosphoinositide 3-kinase (PI3K) and nuclear factor-κB signalling pathways (Allen *et al*., 1997; Jeay *et al*., 2000; Baixeras *et al*., 2001; Dalla Libera *et al*., 2003). GH also controls apoptosis by increasing the levels of anti-

apoptotic compounds such as B-cell lymphoma 2 (Bcl2) and Bcl2-associated athanogene 1 (Allen *et al*., 1997; Jeay *et al*., 2000; Baixeras *et al*., 2001; Dalla Libera *et al*., 2003). However, treatment with GH has a high cost and serious adverse effects were reported following the administration of GH, such as carpal tunnel syndrome, edema and hyperglycemia (Liu *et al*., 2007; White *et al*., 2009).

1.7.2. Nutritional interventions for treatment of muscle wasting

Nutrition plays a fundamental role in maintaining muscle mass under physiological state. It is also important in decreasing and/or reversing muscle loss during pathological conditions. Nutrition is more effective in enhancing the rate of MPS than decreasing the rate of MPB. This can be explained by the fact that the mTOR signalling, responsible for protein synthesis, is very sensitive to nutrient provision (Owens, 2018).

1.7.2.1. Vitamin D

Vitamin D, especially its active form calcitriol (1,25-dihydroxyvitamin D), is a steroid hormone that has been long known for its pivotal role in mineralisation of bones (Hutchison and Bell, 1992). The link between vitamin D and muscle health has been established many years ago (Stroder and Arensmeyer, 1965; Smith and Stern, 1967). However, more attention has been given to its role in muscles following the generation of VDR null (−/−) mice (Li *et al*., 1997). Vitamin D regulates different biological aspects of skeletal muscles, such as development (Endo *et al*., 2003), contractility (Vasquez *et al*., 1997) and regeneration (Camperi *et al*., 2017). Studies have also shown a direct association between vitamin D levels and muscle mass (Visser *et al*., 2003; Sato *et al*., 2005; Scott *et al*., 2010; Marantes *et al*., 2011). Deficiency of vitamin D was associated with lower back pain and paraspinal muscle atrophy in women (Zadro *et al*., 2017; Bang *et al*., 2018).

The mechanism by which vitamin D controls the mass and function of skeletal muscles is not completely understood (Dzik and Kaczor *et al*., 2019). Vitamin D also alters the activity of protein synthesis signalling pathways. Vitamin D induced the phosphorylation of Akt and mitogen activated protein kinase (MAPK) and suppressed the activity of FoxO1 signalling pathways in C2C12 cells (Buitrago *et al*., 2012; Chen *et al*., 2016). The anti-atrophic actions of vitamin D are attributed to its ability in controlling and suppressing E3 ligases MuRF1 and

MAFbx. The availability of vitamin D was associated with decreased expression levels of MuRF1 and MAFbx in human myotubes (Hayakawa *et al*., 2015). It is well documented that vitamin D regulates many aspects of mitochondrial function such as oxidative phosphorylation, calcium uptake and oxygen consumption, which could contribute to muscle hypertrophy (Sinha *et al*., 2013; Ryan *et al*., 2016). Vitamin D also controls oxidative stress by affecting the generation of reactive oxygen species (ROS) (Bhat and Ismail, 2015; Dzik *et al*., 2018; Dzik and Kaczor *et al*., 2019). Supplementation of vitamin D is increasingly recognised for treating muscle wasting. In general, the use of vitamin D is safe, however cases of vitamin D intoxication with serious manifestations like encephalopathy and renal dysfunction have been reported (Koul *et al*., 2011).

1.7.2.2. Omega-3 fatty acids

The fish oil-derived n-3 polyunsaturated fatty acids (PUFAs), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), are a class of long chain fatty acids that have pivotal biological functions inside the body (Lunn and Theobald, 2006). The first evidence that n-3 PUFAs alter the rate of protein turnover in skeletal muscles was obtained in 2007, where infusion of n-3 PUFAs increased the rate of MPS and improved protein anabolism in steer cattle (Gingras *et al*., 2007). Following this, a large body of studies has reported positive effects of n-3 PUFAs on protein turnover within muscles. For example, n-3 PUFAs attenuated muscle loss due to hindlimb immobilisation in rats (You *et al*., 2010). Also, short-term supplementation of n-3 PUFAs decreased leg-immobilisation mediated muscle wasting in women (McGlory *et al*., 2019). Similarly, long-term supplementation of n-3 PUFAs attenuated muscle wasting associated with sarcopenia in elder adults (Smith *et al*., 2015). *In vitro*, incubation of C2C12 myotubes with n-3 PUFAs increased the rate of MPS and lowered the rate of MPB (Kamolrat and Gray, 2013).

n-3 PUFAs have been extensively used to combat muscle wasting associated with cancer (cachexia) because of their anti-inflammatory properties (Gorgio *et al*., 2019). Ingestion of n-3 PUFAs decreased muscle loss in tumour-bearing mice and rats (Whitehouse *et al*., 2001; Iagher *et al*., 2013). Also, the provision of n-3 PUFAs preserved LBM in cachexic humans (Ryan *et al*., 2009; Murphy *et al*., 2011). The mechanism by which n-3 PUFAs exert their protein anabolic effects has been suggested to occur via enhancing the rate of MPS mediated by the activation mTOR signalling, in particular the phosphorylation of the ribosomal protein S6

kinase (p70 S6K) (Gingras *et al*., 2007; You *et al*., 2010; Smith *et al*., 2011; Kamolrat and Gray, 2013; Kamolrat *et al*., 2013). The anabolic effects of n-3 PUFAs are also mediated, in part, by the activation of Akt signalling (Gingras *et al*., 2007; You *et al*., 2010). The suppression of E3 ubiquitin ligases MuRF1 and MAFbx was also suggested as a possible mechanism for n-3 PUFAs' protein anabolic actions (You *et al*., 2010; Whitehouse *et al*., 2001).

1.7.2.3. Ursolic acid

Ursolic acid is a pentacyclic tri-terpenoid acid found in the peels of certain fruits and possesses antioxidant and anti-inflammatory powers (Tsai and Yin, 2008). Long-term supplementation with ursolic acid induced muscle hypertrophy and improved muscle function in obese mice (Kunkel *et al*., 2012). Ursolic acid was proven useful in attenuating muscle atrophy. It attenuated muscle loss, triggered by starvation and denervation, in humans and mice (Kunkel *et al*., 2011; Kunkel *et al*., 2012). It also prevented muscle loss due to chronic kidney disease in mice (Yu *et al*., 2017). Additionally, ursolic acid reversed muscle wasting and induced muscle hypertrophy in rats subjected to hind limb immobilisation (Kim *et al*., 2018). *In vitro*, ursolic acid increased the rate of MPS and decreased the rate of MPB in dexamethasone treated C2C12 myotubes (Yu *et al*., 2017). These effects of ursolic acid were mediated by the activation of the insulin/IGF-1 signalling and the subsequent downregulation of MuRF1 and MAFbx (Kunkel *et al*., 2011; Kim *et al*., 2018). Ursolic acid also maintained the phosphorylation state of Akt and p70 S6K for 6 h after an acute bout of exercise (Ogasawara *et al*., 2013). Reduction in the phosphorylation of p38 MAPK and nuclear factor-kappa B along with the suppression of inflammatory cytokines and myostatin were proposed as possible mechanisms for the actions of ursolic acid (Yu *et al*., 2017).

1.7.2.4. Epigalocatechin-3-gallate

Epigalocatechin-3-gallate (EGCG) is a catechin that possess powerful anti-inflammatory and antioxidant properties and has been useful in attenuating muscle wasting (Alway *et al*., 2014, Nakae *et al*., 2012). Administration of EGCG reversed muscle atrophy and promoted the recovery of rats following hind-limb suspension (Alway *et al*., 2014). EGCG also decreased signs of muscle dystrophy in mdx mice (Nakae *et al*., 2012) and increased protein levels in aged rats during muscle unloading (Takahashi *et al*., 2017). The anti-atrophy powers of EGCG were related to its ability to block the ubiquitin-proteasome signalling (Wimmer *et al*., 2015). EGCG

has been also shown to control the autophagy signalling by suppressing autophagy-related genes Beclin1 and LC3-II/LC-I, which could be another mechanism by which EGCG reduces muscle atrophy (Takahashi *et al*., 2017).

1.7.2.5. Amino acids

The role of protein supplementation in controlling muscle atrophy is well documented (Little and Phillips, 2009; Mourtzakis and Bedbrook, 2009; Glover and Phillips, 2010). Amino acids (AAs) provide the building blocks needed for protein synthesis by mTOR (Owens, 2018). Over the past 3 decades, researchers documented the ability of AAs to enhance the rate of MPS (Bennet *et al*., 1989; Volpi *et al*., 1998; Tipton *et al*., 1999). For example, supplementation of AAs has attenuated muscle wasting during prolonged periods of immobilisation and disuse-induced atrophy (Stein and Blanc, 2011; Dardevet *et al*., 2012). Also, intravenous infusion of AAs maintained the net protein balance in critically ill patients (Sundström-Rehal *et al*., 2017). Additionally, oral administration of a mixture of BCAAs to rats attenuated muscle atrophy induced by hind limb suspension (Maki *et al*., 2012). Notably, the ability of AAs to enhance the rate of MPS was attributed to leucine, which is a powerful stimulator of mTOR signalling (Rieu *et al*., 2006; Katsanos *et al*., 2006). Leucine on its own was capable of attenuating muscle atrophy associated with ageing (Rieu *et al*., 2006). It was also capable of reversing denervation-associated atrophy in rats (Ribeiro *et al*., 2015).

1.8. Branched-chain amino acids

BCAAs are a family of three proteinogenic AAs leucine, valine and isoleucine. All BCAAs have a side aliphatic chain, which resembles a branch hence they were called “branched-chain” AAs. They constitute ~ 40% of the skeletal muscles AA pool. They are indispensable AAs meaning that they cannot be synthesised by the body and need to be supplemented in diet. BCAAs are unique as they are mainly oxidised in the skeletal muscles, an extra-hepatic tissue, unlike other AAs that are catabolised in the liver (Skeie *et al*., 1990). BCAAs have diverse roles insides the body. They are essential for metabolism, normal brain function and immunity. They are also required for fatty acid oxidation, glucose uptake and glycogen synthesis. In addition, BCAAs are good sources of energy since the metabolism of leucine provides more energy, in the form of adenosine triphosphate (ATP), than glucose (reviewed by Monirujjamanand and

Ferdouse, 2014). However, the most recognised role of BCAAs is their ability to enhance the rate of MPS and/or slowing the rate of MPB. They exert this effect through the activation of the mTOR signalling pathway (Buse and Reid, 1975; Lynch *et al*., 2003; Tokunaga *et al*., 2004; Stipanuk, 2007).

1.8.1. Leucine alters the rate of protein synthesis

It is commonly accepted that BCAAs have protein anabolic properties. Interestingly, all the protein anabolic powers of BCAAs are attributed to leucine alone (Buse and Reid, 1975; Li and Jefferson, 1978; Escobar *et al*., 2006; Rieu *et al*., 2006; Katsanos *et al*., 2006). The first recognition of leucine's ability to stimulate MPS was in the 1970s when two research groups reported how leucine altered the protein turnover rate within the skeletal muscles of rats by increasing the rate of MPS (Buse and Reid, 1975; Li and Jefferson, 1978). Since then, a large body of research documented the anabolic protein properties of leucine. Leucine enhanced the rate of protein synthesis in the skeletal muscle of rats (Hong and Layman, 1984; Lynch *et al*., 2002; Crozier *et al*., 2005; Norton *et al*., 2009), neonatal pigs (Escobar *et al*., 2005; Wilson *et al*., 2010; Escobar *et al*., 2006) and humans (Greiwe *et al*., 2001; Drummond *et al*., 2008).

Leucine also demonstrated an ability to reverse protein loss in post-absorptive rats (Anthony *et al*., 2000), rats injected with glucocorticoids (Shah *et al*., 2000) and rats with sepsis (Vary, 2007). Additionally, leucine treatment helped muscle recovery in rats following exercise (Anthony *et al*., 1999). Interestingly, the protein anabolic properties of leucine were evident in tissues other than muscles. Leucine treatment enhanced the rate of protein synthesis in the liver and adipose tissue of rats (Lynch *et al*., 2002). Leucine also increased protein synthesis in the liver, pancreas and jejunum of neonatal pigs (Torrazza *et al*., 2010). *In vitro*, leucine enhanced the rate protein synthesis in cultured pancreatic beta-cells (Xu *et al*., 1998; Xu *et al*., 2001). Notably, the protein anabolic properties of leucine were only evident with high doses. Low leucine concentration of 1 mM/kg failed to elicit protein synthesis in the skeletal muscles, the liver, the jejunum or the heart of rats (McNurlan *et al*., 1982). However, a higher dose of leucine (10 mM/kg) was capable of reversing protein breakdown in the muscles of rats (Anthony *et al*., 1999).

1.8.2. Leucine alters the rate of protein breakdown

Unlike the well-established role of leucine in enhancing protein synthesis, the effect of leucine on protein degradation is not well defined and remains controversial. Leucine, combined with insulin, decreased the rate of protein breakdown in the skeletal muscles of humans (Essén *et al*., 1994; Ferrando *et al*., 1995), chickens (Klasing *et al*., 1985) and rats (Busquets *et al*., 2000; Combaret *et al*., 2005). This effect was achieved by decreasing the proteasome-dependent proteolysis (Combaret *et al*., 2005) and the ATP-ubiquitin-dependent proteolysis signalling pathways (Busquets *et al*., 2000). However, leucine on its own did not alter the rate of protein degradation in chickens (Klasing *et al*., 1985) or weanling rats (Homg and Layman, 1984).

1.8.3. The mTOR signalling meditates the leucine-induced protein synthesis

Leucine has a well-documented role in activating the mTOR signalling pathway. However, the mechanism by which leucine modulates the activity of mTOR is not fully understood. Leucine was proposed to affect mTOR signalling by modulating the binding of mTOR with Raptor and GβL. Disrupting the expression of GβL using small interfering RNAs (siRNA) decreased the phosphorylation of mTOR after provision of AAs and insulin (Kim *et al*., 2003). Also, attenuating the expression of Raptor by siRNA reduced the phosphorylation of p70 S6K, a downstream target of mTOR, post-stimulation with leucine (Kim *et al*., 2002). Additionally, leucine activates mTOR signalling by altering the activities of Rheb, tuberous sclerosis complex 1 (TSC1) and tuberous sclerosis complex 2 (TSC2) (Kimball and Jefferson, 2004). The overexpression of both TSC1 and TSC2 decreased the phosphorylation of p70 S6K in response to AAs availability (Tee *et al*., 2002). In contrast, the lack of either TSC1 or TSC2 resulted in the activation of p70 S6K (Zhang *et al*., 2003).

1.8.4. The relationship between leucine and insulin

Initially leucine was thought to induce protein synthesis by enhancing the sensitivity of cells and tissues to insulin (Malaisse, 1984). This notion was further supported by the failure of a mixture of AAs, including leucine, to increase the rate of protein synthesis in rats treated with anti-insulin serum (Preedy and Garlick,1986) and somatostatin, an inhibitor of insulin (Anthony *et al*., 2002). Leucine induced glucose uptake by the skeletal muscles of healthy rats under insulin free conditions (Nishitani *et al*., 2002). Also, dietary leucine improved glucose tolerance

in rats (Macotela *et al*., 2011) and in type 2 diabetic patients (Gannon *et al*., 2003). In addition, co-stimulation with leucine and insulin increased the rate of protein synthesis in different cells (Anthony *et al*., 2000b; Greiwe *et al*., 2001; Camillo *et al*., 2014). Finally, leucine withdrawal improved the sensitivity of human hepatoma cells and mouse primary hepatocytes to insulin (Xiao *et al*., 2011). Intriguingly, leucine was able to enhance protein synthesis independently from insulin (Anthony *et al*., 1999). This finding was reinforced by subsequent studies, which documented the ability of leucine to induce protein synthesis in the absence of insulin (Anthony *et al*., 2000; Anthony *et al*., 2002; Bolster *et al*., 2004).

Overall, leucine is capable of inducing protein synthesis independently from insulin. However, insulin is required for potentiating the protein powers of leucine (Garlick and Grant, 1988; Anthony *et al*., 1999; Anthony *et al*., 2000). Leucine enhanced glucose uptake by affecting the activity of PI3K. In support of this, inhibition of PI3K abrogated glucose uptake by rat's skeletal muscle cells (Nishitani *et al*. 2002). Likewise, stimulation of human skeletal muscle cells and rats L6 cells with leucine induced the phosphorylation of PI3K and glycogen synthase kinase 3 β (GSK-3β) (Camillo *et al*., 2014; Peyrollier *et al*., 2000). Leucine also increased the phosphorylation of PI3K and Akt in the skeletal muscles of rats and mice (Saha *et al.*, 2010; O'Neill *et al*., 2010). Interestingly, the activation of PI3K following leucine treatment was not associated with PI3K/Akt activation in humans (Glynn *et al*., 2010), neonatal pigs (Suryawan *et al*., 2008; Suryawan *et al.,* 2012), rats (Vary, 2007) and L6 cells (Peyrollier *et al*., 2000). The abovementioned facts indicate that leucine enhances glucose uptake and glycogen synthesis within muscle cells via the activation of PI3K signalling pathway, although this is not usually associated with the phosphorylation of PKB/Akt.

1.8.5. miRNAs that mediate the effects of leucine on muscles

miRNAs represent a class of non-coding RNAs that are highly conserved in almost all organisms including plants, animals and viruses. miRNAs are small regulatory RNA molecules, approximately 21-23 nucleotides in length, which silence gene expression by interfering with post-transcriptional events (Hamilton and Baulcombe, 1999; Reinhart *et al*., 2000). Several miRNAs have been reported to control the expression of a wide range of genes in metazoans (Vasudevan *et al*., 2007). A number of miRNAs have been identified as specific to the muscular tissue such as miR-1, miR-133a, miR-133b, miR-206, miR-208a, miR-208b, miR-486 and miR-499 (McCarthy and Esser, 2007; Callis *et al*., 2008; van Rooij *et al*., 2008; van Rooij *et al*.,

2009). These miRNAs control the expression of genes that regulate different aspects of muscle biology (Chen *et al*., 2006; Kim *et al*., 2006). They also control intracellular signalling transduction in muscle cells. The disruption of miR-1 and miR-486 altered the IGF-1 and PI3/Akt signalling pathways, respectively (Elia *et al*., 2009; Small *et al*., 2010).

Studies reporting alterations in the expression of miRNAs post leucine treatment are very limited. Moreover, most of available studies evaluated the effect of essential amino acids (EAAs) rather than evaluating the sole effect of leucine. One study evaluated the effect of two anabolic stimuli, exercise and ingestion of EAAs on the expression of primary and mature miRNAs in aged and young men (Drummond *et al*., 2008). However, in that study, the exact contribution of EAA could not be identified. Another study assessed the effect of ingesting leucine rich EAA mixture on the expression of primary and mature miRNAs within the skeletal muscles of young and aged human subjects (Drummond *et al*., 2009). Results of that study showed that ingesting 10 grams of EAA mixture increased the expression of miR-499, miR-208b, miR-23a, miR-1 and pri-miR-206 (Drummond *et al*., 2009).

miR-499 and miR-208b have been previously reported to alter muscle fibre phenotype by affecting repressors of the *β MyHC*, thus favouring a slow muscle fibre phenotype (Rooij *et al*., 2008). miR-499 and miR-208b were also suggested to target the myostatin gene, a negative controller of muscle growth, hence aid muscle hypertrophy (Callis *et al*., 2009; Drummond *et al*., 2009). miR-23a targets myocyte enhancing factor 2 (MEF2), a promoter for slow muscle phenotype (Michel *et al*., 2004) and some genes involved in muscle metabolism (McGee *et al*., 2004). miR-23a was also reported to increase muscle hypertrophy and decrease muscle atrophy in cardiac and skeletal muscles (Wada *et al*., 2008; Lin *et al*., 2009). miR-1 was shown to control the expression of muscle specific genes such as MyoD and myogenin and MEF2 (Drummond *et al*., 2009). *In vitro,* incubation of C2C12 myoblasts with leucine induced the expression of miR-27a. miR-27a promoted the proliferation of C2C12 cells and inhibited the expression of *Mstn* (Chen *et al*., 2013). Taken together, despite the paucity of studies, it is clear that leucine alone or combined with other EAA alters the expression of miRNAs within muscle cells both *in vivo* and *in vitro.* It is likely that leucine achieves its protein anabolic effect by upregulating miRNAs that target *Mstn*.

1.9. Glutamine

Glutamine is the most abundant extracellular AA. Glutamine was typically classified as a non EAA, however the high need for it during clinical conditions made many researchers consider glutamine as a "conditional EAA" (Lacey and Wilmore, 1990). Interestingly, certain cells and tissues such as the intestines, kidneys and neurons of the central nervous system, immune cells and pancreatic β cells possess a high need for glutamine (Lacey and Wilmore, 1990). Glutamine plays a vital role in maintaining the viability, growth, survival and integrity of cells both *in vivo* and *in vitro* (Newsholme *et al*., 2003). Glutamine mediates a lot of biochemical reactions *in vivo* and its conversion to alanine yields flavin adenine dinucleotide (FADH2) and nicotinamide adenine dinucleotide (NADH) that aid in energy (ATP) production (Gstraunthaler *et al*., 2000).

Glutamine is a nitrogen carrier to the liver where it is converted to urea or glucose through gluconeogenesis (De-Souza *et al*., 2001). Also, the metabolism of glutamine in the kidneys generates hydrogen ions (H^+) which, upon their release into blood stream, help in maintaining the acid-base balance (Taylor and Curthoys, 2004). Additionally, glutamine alters the immune response by enhancing the proliferation of T-cells, the responsiveness of B lymphocytes and the phagocytic activity of macrophages (Newsholme, 2001). Furthermore, glutamine is essential for the growth, proliferation and survival of various cells *in vitro*. The first deduction of glutamine's pivotal role in supporting the growth of cultured cells was proposed in 1949 (Ehrensvard *et al*., 1949) and was further supported by the work of Eagle and co-workers (1956). Normally, culture media contain 19 AA supplemented at their physiological levels except for glutamine, which is supplemented at 10-100 times more than the levels of other AAs (Newsholme *et al*., 2003).

1.9.1. The role of glutamine in supporting cultured cells

Glutamine has been shown to support the proliferation of various cultured cells. Exposure to glutamine enhanced the proliferation of two human carcinoma (Caco-2 and SW620) and three ovarian cancer cell lines (HEY, SKOV3 and IGROV-1) in a dose-dependent manner (Turowski *et al*., 1994; Yuan *et al*., 2015). Also, glutamine, combined with epidermal growth factor, enhanced the proliferation of rat's intestinal (IEC-6) cells (Ko *et al*., 1993). On the other hand, glutamine deprivation has been reported to halt cell proliferation, induce cell cycle arrest,

stimulate apoptosis and increase oxidative stress in cultured cells (Zhu *et al*., 2014; Yuan *et al*., 2015).

Glutamine starvation reduced the cell number, induced the accumulation of autophagosomes and increased the expression of autophagy markers in porcine intestinal epithelial cells (Zhu *et al*., 2014). Glutamine restriction also decreased the growth and survival of experimental tumours (Tannock *et al*., 1986). In addition, glutamine withdrawal resulted in cell cycle arrest and apoptosis along with decreased ATP production in three ovarian cancer cell lines (Yuan *et al*., 2015). It also increased intracellular oxidative stress in prostate cancer cells, indicated by increased production of ROS and glutathione (Fu *et al*., 2006; Yuan *et al*., 2015). Finally, an enhanced expression of Poly (ADP-ribose) polymerase and Calnexin, both are suggestive of endoplasmic reticulum stress, was reported in neoplastic cells following glutamine starvation (Crespo *et al*., 2012; Yuan *et al*., 2015).

1.9.2. The role of glutamine in protein synthesis

An increased rate of protein synthesis was detected in the skeletal muscles of rats perfused with glutamine (MacLennan *et al*., 1987). It also reversed muscle loss in diabetic rats by activating the protein-synthetic Akt signalling and inhibiting the expression of protein degradative MuRF-1 and MAF-bx (Lambertucci *et al*., 2012). Glutamine, mixed with other AA, increased fractional synthesis rate of dermal tropocollagen in malnourished rats (Murakami *et al*., 2013). Additionally, glutamine increased the rate of DNA and protein synthesis within hepatocytes of rats (Yoshida *et al*., 1995). On the other hand, glutamine starvation decreased the rate of protein synthesis in porcine intestinal epithelial cells (Zhu *et al*., 2014).

1.9.3. The effect of glutamine on the mTOR/S6K and the MAPK/ERK signalling pathways

A growing evidence suggests that glutamine exerts its favourable biological effects through the mTOR and MAPK signalling pathways. Glutamine supplementation increased the phosphorylation of both mTOR and the extracellular signal regulated kinase 1,2 (ERK1/2), members of MAPK signalling, in porcine small intestinal epithelial (IPEC-1) cells (Zhu *et al*., 2015). On the other hand, glutamine deprivation decreased the phosphorylation of both signalling pathways in the same cells (Zhu *et al*., 2015). Glutamine induced a dose-dependent

phosphorylation of S6K, a target of mTOR, and ERK1/2 in ovarian cancer cell lines (Yuan *et al*., 2015). It also activated the MAPK signalling in human dental pulp cells (HDPCs) (Kim *et al*., 2014). However, the role of glutamine in mediating protein synthesis is controversial. Glutamine did not induce the phosphorylation of mTOR within muscle (C2C12) cells (Deldicque *et al*., 2008). Moreover, it resulted in the suppression of mTOR and MAPK signalling in rats intestinal (IEC-18) and human colonic (Caco-2) cells (Sakiyama *et al*., 2009).

1.9.4. The combined effect of leucine and glutamine on mTOR signalling

BCAAs play a central role in nitrogen metabolism, and they are the main nitrogen donors for the biosynthesis of nitrogen carriers such as glutamine. Consequently, the catabolism/oxidation of BCAA is halted by the availability of glutamine (Holeck, 2002). Glutamine and leucine are usually supplemented together. The combined effect of leucine and glutamine on mTOR signalling is inconclusive. Glutamine antagonised the leucine-induced phosphorylation of mTOR and its downstream elements in enterocytes (Nakajo *et al.*, 2005). However, it did not alter the leucine-mediated phosphorylation of mTOR in C2C12 myotubes (Deldicque *et al.*, 2008).

Also, glutamine promoted the leucine-induced phosphorylation of mTOR and its downstream substrates in hepatocytes (Krause *et al.*, 2002) and pancreatic cells (Xu *et al.*, 2001). Interestingly, a few studies suggested that glutamine is needed for a full activation of mTOR following leucine treatments. This full activation of mTOR by leucine and glutamine combination was attributed to the inhibition of the adenosine monophosphate kinase (AMPK) signalling, a well-known down regulator of mTOR, by glutamine (Gleason *et al*., 2007; Xu *et al*., 2001; Nicklin *et al*., 2009).

1.10. Research questions

In light of the abovementioned evidence, it is clear that SCs play an important role in muscle growth and regeneration. A plethora of research has been undertaken in order to unravel the mechanisms that control the functions of chicken satellite cells (CSCs). However, most of the previous research has been conducted on SCs derived from the breast muscles of chickens selected for high growth. Very little is known about SCs derived from the leg muscles of chickens. Also, a few studies have compared the characteristics of SCs derived from chickens

selected for high growth (broilers) to those derived from chickens selected for high egg production (layers). Additionally, despite the presence of a protocol for the isolation and cultivation of SCs from the breast muscles of adult broiler chickens, information regarding the specific cultivation conditions for SCs isolated from the breast muscles of layer chickens, or the leg muscles of both chicken breeds, is lacking.

Leucine, a BCAA, possesses exceptional protein anabolic properties on muscle cells. However, our knowledge about the anabolic effects of leucine has been derived from studies conducted on differentiated, rather than proliferating, muscle cells. Most of the previous studies have tested the effects of low doses of leucine (>2 mM) under serum-free or reduced-serum conditions and following a prolonged serum starvation of cells. However, the impact of serum withdrawal on cell viability was overlooked. Also, no attention has been paid to evaluate the effects of serum-free and reduced-serum conditions on the anabolic effects of leucine on cells. Additionally, very little is known about the dose- and time-effects of leucine. Furthermore, data on the combined effects of leucine and glutamine, a conditional EAA, on muscle cells are both limited and inconclusive. Finally, the role of miRNAs in mediating the response of muscle cells to leucine supplementation remains poorly defined. Therefore, the present thesis sought to address these gaps by achieving the following aims

1.11. General aims

The present thesis encompasses two broad objectives, each involves several aims. The first objective was aimed to compare the growth and differentiation abilities of SCs of breast and leg muscles of young broiler and layer chickens and to investigate the differential response of these primary cells to leucine supplementation. To achieve this objective, we have explored the following aims:

1. Establishing and optimising an *in vitro* culture system for SCs isolated from the breast (pectoralis major) and leg (peroneus longus) muscles of two-week-old broiler and layer chickens.
2. Comparing the proliferative and differentiation capacities of SCs isolated from the breast and leg muscles of two weeks old broiler and layer chickens.
3. Comparing the response of SCs derived from selected (breast) and unselected (leg) muscles of two-week-old broiler chickens to different concentrations of leucine.

In the second objective, we studied the effects of increasing concentrations of leucine and/or glutamine over different treatment durations and under different culture conditions, on various parameters in murine C2C12 cells. To achieve this objective, we have explored the following aims:

4. Evaluating the effects of different nutrient starvation protocols on the viability, proliferation and total protein content of C2C12 cells.
5. Evaluating the effects of media with different serum contents on the proliferation, viability and total protein content of C2C12 cells.
6. Evaluating the short- and long-term effects of different concentrations of leucine on the proliferation, viability and total protein content of C2C12 cells.
7. Evaluating the short- and long-term effects of different concentrations of leucine combined with glutamine on the proliferation, viability and total protein content of C2C12 cells.
8. Profiling the phosphorylation and cleavage of 18 intracellular signalling proteins, using a PathScan® Intracellular Signaling Array, following stimulation of C2C12 cells with leucine alone or in combination with glutamine.
9. Assessing the phosphorylation of key proteins in the mTOR and the MAPK signalling pathways following short-, medium- and long-term stimulation of C2C12 cells with different concentrations of leucine alone or in combination with glutamine.
10. Assessing the expression of muscle-specific miRNAs in C2C12 cells following short-, medium- and long-term exposure of C2C12 cells to leucine alone or in combination with glutamine.

2. Materials and methods

2.1. Primary chicken satellite cells

2.1.1. Strains of chickens and housing conditions

Thirty-two one-day-old, male broiler chicks (ROSS 308 strain) were purchased from PD Hook Hatcheries Ltd, Oxfordshire, England. Twenty-eight one-day-old, male layer chicks (Light Sussex X Rhode Island Red) were purchased from G Potter & Son Company, North Yorkshire, England. Both broiler and layer chicks were raised in the Bio Support Unit, Sutton Bonington Campus, University of Nottingham until they were 15±1 day-old. All birds were maintained under the same standard housing conditions with 12:12 h of light-dark cycles and were provided access to food (standard broiler starter ration, Target Feeds, Scotland) and drinking water *ad libitum*.

2.1.2. Ethical note

All procedures and handling of animals were carried out in accordance with the University of Nottingham policy on the use of animals in research. Culling of birds was carried out by a trained technician at the Bio Support Unit.

2.1.3. Weight of chickens

The body weight of broiler and layer chicks, in grams, was recorded on the 1st, 7th and 14th day of age using a top loading bench scales with a digital display (Salter, A&B Industrial Services, UK). The weights of all birds were rounded to three decimal places.

2.1.4. Dissection of birds and muscle sampling

First, broiler and layer chickens were killed by cervical dislocation. Next, taking into consideration all aseptic precautions, a longitudinal incision was made through the midline of each bird and the skin was pulled back to reveal underlying muscles (Figure 2.1). Then, the whole peroneus longus (PL) muscle and approximately one fifth of the PM muscle were excised, rinsed thoroughly with sterile phosphate buffered saline (PBS) (Gibco, Thermo Fisher

Scientific, UK) and transferred into collection medium (CM). The CM was composed of high glucose Dulbecco's Modified Eagle's Medium (DMEM) (Gibco, Thermo Fisher Scientific) supplemented with 1% *v/v* antibiotic mixture (10.000 U/ml penicillin G, 10.000 mg/ml streptomycin, Thermo Fisher Scientific) and 1% *v/v* amphotericin B (Thermo Fisher Scientific).

2.1.5. Isolation of chicken satellite cells

SCs were isolated from the PM and PL muscles according to a protocol previously described by Baquero-Perez and colleagues (2012) with some modifications (Figure 2.2). First, muscle samples were rinsed with sterile PBS. Then, visible connective tissue and large blood vessels were carefully removed, muscle samples were chopped into small pieces, transferred into 50 ml conical centrifuge tubes (Greiner, Thermo Fisher Scientific) and washed for 15 minutes (min) with CM. Next, chopped muscle samples were incubated in 30 ml dissociation medium (DsM) at 37 ºC in a water bath (Nickel-Electro Ltd., UK) with frequent agitation. The DsM was composed of DMEM/Ham's F12 1:1 mixture + 15 mM HEPES (Sigma-Aldrich, UK) supplemented with 1.4 mg/ml Pronase E (Sigma-Aldrich), 1% *v/v* antibiotic mixture (Thermo Fisher Scientific) and 1% *v/v* amphotericin B (Thermo Fisher Scientific).

After enzymatic dissociation, muscle fragments were collected by centrifugation at 300 xg for 5 min on a benchtop centrifuge (Beckman-Coulter, USA), washed three times with PBS and transferred into fresh CM. Then, muscle fragments were subjected to several cycles of trituration using 25-, 10- and 5-ml plastic serological pipettes (Thermo Fisher Scientific) to release cells from the muscle fibres. Liberated cells were collected by centrifugation at 300 xg for 5 min, resuspended in fresh CM and filtered through 40 μm nylon cell strainers (Millipore, Sigma-Aldrich). The flow through was diluted in PBS and spun at 1200 xg for 10 min to pellet cells. Finally, cell pellets were washed three times with PBS before being suspended in growth medium (GM). GM contained high glucose DMEM Glutamax^{TM} (Gibco, Thermo Fisher Scientific) supplemented with 10% *v/v* horse serum (HS) (Sigma-Aldrich), 5% *v/v* chicken embryo extract (CEE) (EGG Tech, UK), 1% *v/v* antibiotic mixture (Thermo Fisher Scientific) and 1% *v/v* amphotericin B (Thermo Fisher Scientific).

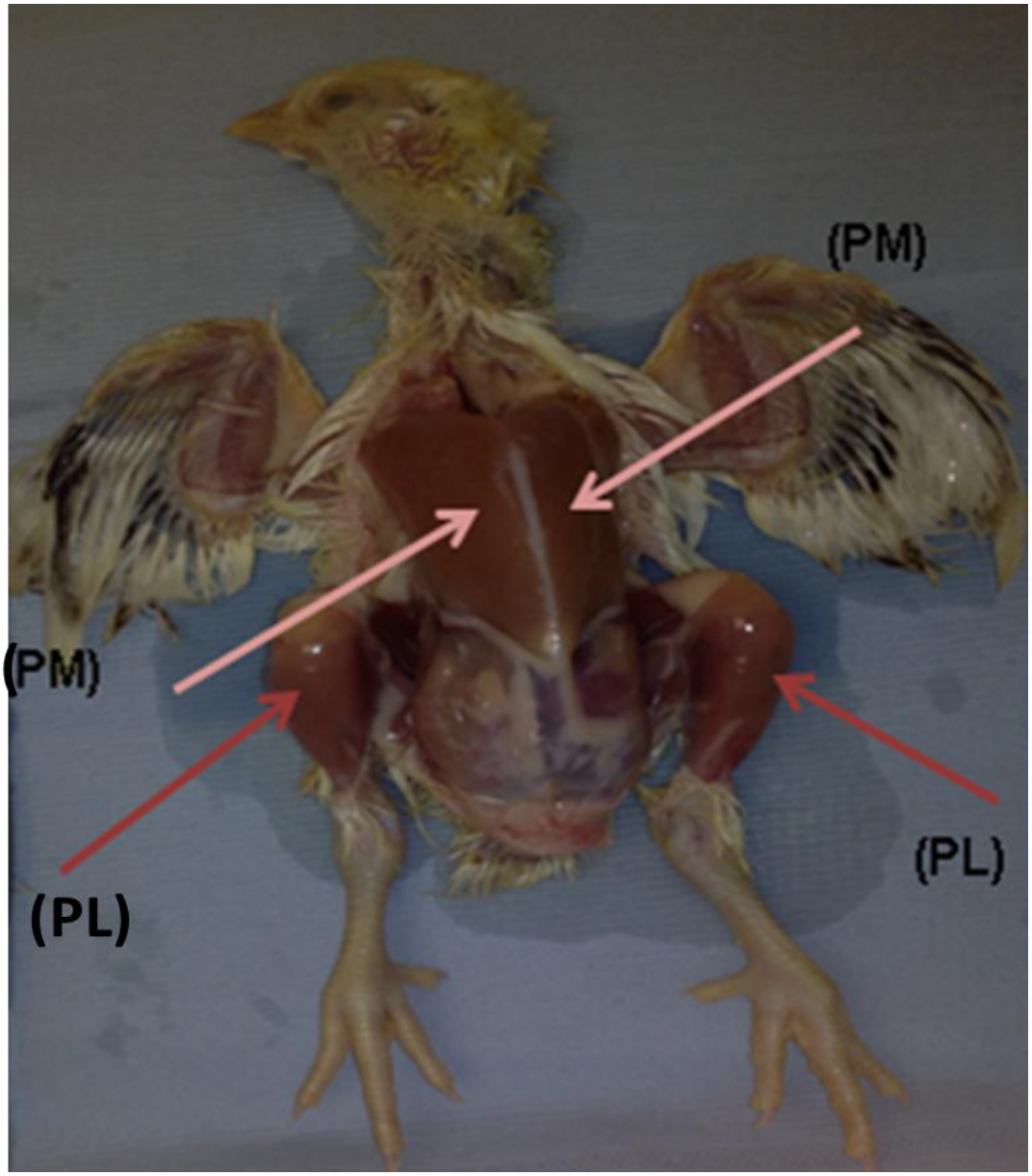


Figure 2.1. Illustration of chicken dissection and muscle sampling. A two-week-old broiler chick was killed by cervical dislocation, and an incision was made along its midline. Next, sideway incisions were made, and the skin was pulled back to reveal underlying skeletal muscles. Pink arrows point to the breast (PM) muscles. Red arrows point to the leg (PL) muscles. Abbreviations: PM = pectoralis major muscle; PL = peroneus longus muscle.

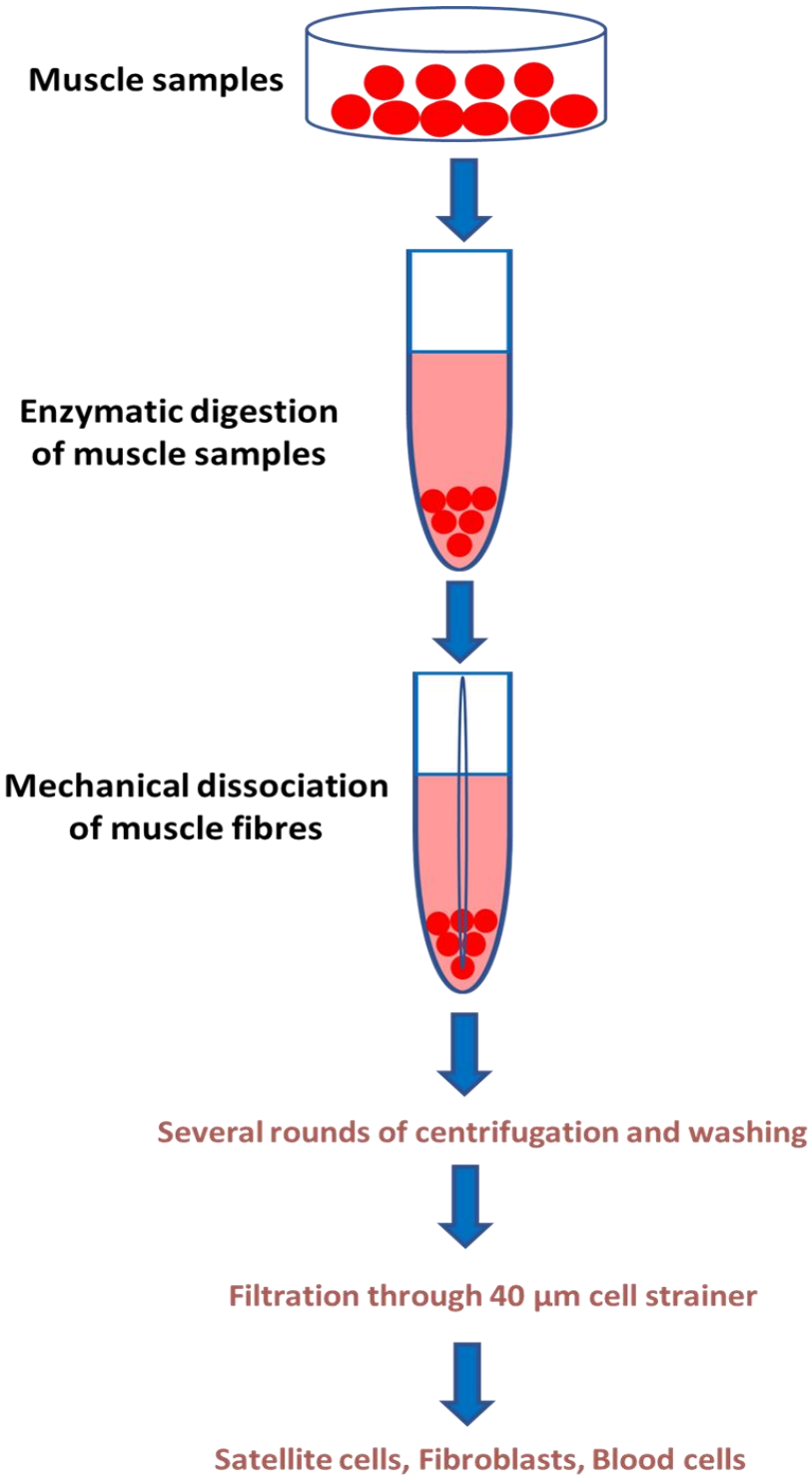


Figure 2.2. Schematic illustration showing the steps involved in the isolation of chicken satellite cells. The breast and leg muscles of two-week-old broiler and layer chickens were subjected to enzymatic digestion with Pronase E. This was followed by mechanical dissociation of muscle fibres using pipettes of different sizes to liberate cells. Enrichment of liberated SCs was achieved by differential centrifugation and filtration through cell strainers. Abbreviations: µm = micrometre. The chart was designed based on information sourced from Baquero-Perez *et al*., 2012.

2.1.6. Exposure to Pronase

We sought to optimise the enzymatic digestion of broilers and layers muscles by incubating chopped muscles samples with Pronase E in DsM for 30-, 45- or 60- min inside a 37 ºC water bath. The isolation of SCs was carried out as described earlier. Isolated cells were maintained in GM with daily microscopic observation to check for cell growth.

2.1.7. Mechanical dissociation of muscle fibres using gentleMACS™ Dissociator

Enzymatically digested muscle fragments from the PM and PL muscles of broiler chickens were transferred into gentleMACS^{TM} C tubes (Miltenyi BioTec, Germany) and subjected to either 1 or 2 rounds of mechanical dissociation using the M (muscle) setting of a gentleMACS™ Dissociator (Miltenyi BioTec).

2.1.8. Collagen coating of culture vessels

Small volumes of 2% collagen type I solution (Sigma-Aldrich) were used to cover the bottom of cell culture flasks, dishes and well plates (Nunc, Thermo Fisher Scientific). Next, cell culture vessels were left to dry overnight (O/N) inside a laminar flow hood (Herasafe^{TM} Class II, Thermo Electron Corporation, Germany) Al collagen-coated vessels were gently rinsed with PBS solution prior to use. All steps were carried out using sterile equipment and reagents at room temperature (RT).

2.1.9. Differential plating

We sought to enrich SCs by plating freshly isolated muscle cell suspension on un-coated tissue culture vessels inside a standard 37 °C humidified tissue culture incubator with 5% CO_2 in air (Heracell^{TM}, Thermo Electron Corporation) for 3-4 h. Following this, the media, containing non-adherent cells, were decanted into 50 ml centrifuge tubes (Greiner, Thermo Fisher Scientific) and centrifuged at 300 xg for 5 min to pellet cells. Cell pellets were re-suspended in GM and plated on collagen coated tissue culture vessels with daily microscopic observation to check for cell growth.

2.1.10. Seeding densities of satellite cells

SCs were counted using a haemocytometer (Corning™ Counting Chamber, Thermo Fisher Scientific) and seeded at three different densities

- Low density (~ 1,500 cells/cm^2)
- Medium density (~ $5x10^3$ cells/cm^2)
- High density (~ $3x10^4$ cells/cm^2)

2.1.11. Maintenance of satellite cell cultures

SCs were maintained in GM inside a standard 37 °C humidified tissue culture incubator. All satellite cell cultures (SCCs) were washed with PBS after 24 and 48 h of plating to remove debris and dead cells. GM was replenished daily.

2.1.12. Attachment assay

To determine the efficiency of the isolation protocol, all primary cultures were stained for SCs markers Pax7 and Mcad after 24 h of seeding, using indirect immunohistochemical staining.

2.1.13. Subculture of chicken satellite cells

Sub-culturing SCs was undertaken to avoid overgrowth and spontaneous differentiation. Briefly, media were collected from ~ 60% confluent SCCs and cells were rinsed three times with PBS. Next, cells were incubated in different concentrations (0.05 X, 0.25 X, 0.5 X or 1X) of Trypsin-EDTA (Sigma-Aldrich) for 1 min. Detached cells were collected into GM and spun at 300x *g* for 1 min. Then, cell pellets were washed with PBS, reconstituted in GM and plated onto collagen coated culture dishes with daily microscopic observation using Leica inverted microscope (Leica Microsystems Ltd., UK) to check for cell growth.

2.1.14. Cryopreservation of chicken satellite cells

Cryopreservation of SCs was essential to maintain availability of these primary cells. First, SCs isolated from the PM and PL muscles of broiler and layer chickens were enzymatically detached

from the surface of the culture vessels using Trypsin-EDTA. Then, cells were resuspended in two different freezing media (FM). The first was a traditional high serum FM (90% *v/v* HS (Sigma-Aldrich) + 10% *v/v* Dimethyl sulfoxide (DMSO) (Thermo Fisher Scientific). The second was a commercial stem cell cryopreservation medium (CELLBANKER®1, amsbio., UK). Following this, cells were aliquoted into cryovials (Nunc, Thermo Fisher Scientific), placed inside a freezing container with isopropanol (Mr. Frosty™, Thermo Fisher Scientific) and transferred to a -80 ºC freezer for short-term storage. Subsequently, all cryovials were transferred into liquid nitrogen for long-term storage.

2.1.15. Inducing the differentiation of chicken satellite cells

To assess the effect of serum-restriction on CSCs, ~70% confluent cultures derived from the PM and PL muscles of broiler chickens, were shifted into low serum differentiation medium (DM). DM was composed of high glucose DMEM Glutamax™ (Gibco, Thermo Fisher Scientific) supplemented with 2% *v/v* HS (Sigma-Aldrich) + 5% *v/v* CEE (EGG Tech) + 1% *v/v* penicillin/streptomycin mixture (Thermo Fisher Scientific) + 1% *v/v* amphotericin B (Thermo Fisher Scientific) for 48 h. Myogenesis was judged morphologically and by assessing the expression of MyHC-*fast* using indirect immunohistochemical staining.

2.1.16. Exposure of chicken satellite cells to leucine

To determine the long-term effect of leucine on CSCs, ~60% confluence SCCs derived from the PL and PM muscles of broiler chickens were incubated with 0 mM (control), 1-, 2-, 3-, 4- or 5 mM leucine in GM. The effect of leucine on cells was assessed morphologically, following 24 h of exposure.

2.1.17. Immunocytochemistry

At specified time points (24, 72, 96, 120 and 192 h) SCs plated in 24 well plates (Nunc, Thermo Fisher Scientific) were stained for the detection of myogenic markers Pax7, Mcad, desmin, MyoD and MyHC-*fast* using indirect immunocytochemistry. In brief, media were decanted from wells and cells were fixed and permeabilised at one step in 1:1 *v/v* methanol/acetone mixture (Thermo Fisher Scientific) for 10 min at – 20 ºC. Next, cells were blocked in 5% *v/v*

normal goat serum (Thermo Fisher Scientific) in PBS for 20 min. Then, cells were washed three times (3X) with PBS and incubated with specific primary antibodies diluted in PBS for 4 h, see Appendix 1 for list of antibodies and their dilutions. This was followed by three washes with PBS and incubation with biotinylated secondary antibodies specific against the host species of the primary antibody used; Goat anti-Rabbit and Goat anti-Mouse (Thermo Fisher Scientific) for 1 h. Cells were washed 3X with PBS and were incubated with Vectastatin reagent A and B (Vectastatin Elite ABC Kit, Vector Laboratories, UK) for 30 min. Finally, cells were washed 3X with PBS, incubated with peroxidase substrate solution (Vector VIP Substrate Kit for Peroxidase, Vector Laboratories) for 5 min, washed 3X with PBS and 1X with deionised water. Unless otherwise stated, all staining steps took place RT.

2.1.18. Staining primary chicken myotubes

Fully differentiated myotubes originated from the PM and PL muscles of broiler chickens were stained with haematoxylin and eosin (H&E) as follows: first cells were fixed in 70% ethanol (Thermo Fisher Scientific) for 10 min. Next, cells were incubated with Alum haematoxylin solution (Raymond A Lamb, Thermo Fisher Scientific) for 10 min, followed by 5 min of rinsing with tap water. After this, cells were incubated in eosin solution (VWR Chemicals, UK) for 2 min, followed by brief rinsing with deionised water. All steps took place at RT.

2.2. The murine C2C12 cells

The C2C12 cell line (mouse C3H muscle myoblasts ECAXX 91031101) was purchased from Sigma-Aldrich. Only cells from passages 4-10 were used in this work.

2.2.1. Media compositions

The compositions of full growth medium (full GM), growth medium (GM), full differentiation medium (full DM), differentiation medium (DM), serum-free medium (SM), starvation medium (StM), glutamine-free growth medium (GM-Gln) and growth medium supplemented with dialysed FCS (dGM) are detailed in Appendix 2.

2.2.2. Cell culture conditions for C2C12 cells

C2C12 myoblasts were seeded at a density of $5x10^3$ cells/cm². Cells were routinely maintained in full GM at 37 °C inside a humidified tissue culture incubator with 5% CO_2 in air. Differentiation into myotubes was induced by shifting ~80% confluent cultures into full DM for 8 days.

2.2.3. Incubation of proliferating and differentiated C2C12 cells in different media

2.2.3.1. Incubating C2C12 myoblasts in glutamine-free medium

To test the effect of glutamine deprivation on proliferating C2C12 cells, C2C12 myoblasts were expanded in full GM to ~ 50% confluence. Then, cells were either kept in full GM (control) or shifted into GM-Gln for the required times.

2.2.3.2. Incubating C2C12 myoblasts in serum-free medium

To test the effects of short-term serum starvation and long-term incubation in serum-free medium on proliferating C2C12 cells, C2C12 myoblasts were expanded in full GM until reaching ~ 50%. Then, cells were either kept in full GM (control) or shifted into SM for the required times.

2.2.3.3. Incubating C2C12 myoblasts in dialysed FCS

To test the effect of dialysed FCS on proliferating C2C12 cells, C2C12 myoblasts were expanded in full GM until reaching ~ 50% confluence. Then, cells were either kept in full GM (control) or shifted into dGM for the required times.

2.2.3.4. Incubating C2C12 myoblasts in media with different serum compositions

To compare the effects of media with different serum contents on proliferating C2C12 cells, C2C12 myoblasts were expanded in full GM until reaching ~ 50% confluence. Then, cells were either maintained in full GM (control) or shifted into dGM or SM for the required times.

2.2.3.5. Incubating C2C12 myotubes in serum-free medium

To test the effects of short-term serum starvation and long-term incubation in serum-free medium on differentiated C2C12 cells, C2C12 myotubes were either kept in full DM (control) or shifted into SM for the required times.

2.2.4. Starving C2C12 cells prior to leucine and/or glutamine treatment

Prior to leucine and/or glutamine treatment, C2C12 cultures were rinsed three times with PBS. Then, C2C12 myoblasts were starved of serum, leucine and glutamine by 1 h incubation in StM. C2C12 myotubes were starved of serum, leucine and glutamine by 4 h incubation in StM.

2.2.5. Preparation of stock solutions of L-leucine and L-glutamine

L-leucine powder (Sigma-Aldrich) and L-glutamine powder (Gibco, Thermo Fisher Scientific) were dissolved in double distilled water to make a 100 mM and 200 mM stock solutions, respectively. Next, both solutions were filtered through membrane syringe filters with 0.22 μm pore size (Millipore, Sigma-Aldrich) and dispensed into aliquots and stored at -20 °C. Leucine and glutamine were diluted in media of choice prior to treatment.

2.2.6. Exposure of C2C12 cells to leucine and/or glutamine

Proliferating and fully differentiated C2C12 cells were exposed to leucine and/or glutamine as described below.

2.2.6.1. Exposure of C2C12 myoblasts to leucine in media with different serum contents

For the purposes of chapter 4, C2C12 myoblasts were expanded in full GM until reaching ~ 50% confluence then, cells were starved for 1 h. Following this, C2C12 myoblasts were incubated with 0 mM (negative control), 2 mM, 5 mM or 10 mM L-leucine in GM, dGM or SM for the required times.

2.2.6.2. Exposure of C2C12 myoblasts to leucine and/or glutamine in growth medium

For the purposes of chapter 5, C2C12 myoblasts were expanded in full GM until reaching ~

50% confluence and starved for 1 h. The cells were then shifted into GM devoid of leucine and glutamine (0 mM), negative control; GM supplemented with 2 mM L-glutamine (glutamine group); GM supplemented with 2-, 5- or 10 mM L-leucine (leucine groups); or GM supplemented with the previous concentrations of leucine combined with 2 mM L-glutamine (leucine + glutamine groups) for the required times.

2.2.6.3. Exposure of C2C12 myoblasts to leucine and/or glutamine in starvation medium

For the purposes of chapter 6, C2C12 myoblasts were expanded in full GM until reaching ~50% confluence. Then, cells were either maintained in full GM (positive control) or starved for 1 h. Following starvation, cells were incubated in StM devoid of leucine and glutamine (0 mM), negative control; StM supplemented with 2 mM L-glutamine (glutamine group), StM supplemented with 2-, 5- or 10 mM L-leucine (leucine groups); or StM supplemented with the previous concentrations of leucine combined with 2 mM L-glutamine (leucine + glutamine groups) for short- (10 and 30 min), medium-term (60 min) exposures.

2.2.6.4. Exposure of C2C12 myotubes to leucine in media with different serum contents

For the purposes of chapter 4, C2C12 myotubes were starved for 4 h followed by incubation in either DM or SM devoid of leucine (0 mM); negative control, or DM and SM supplemented with 2-, 5- or 10 mM L-leucine (leucine groups) for the required times.

2.2.6.5. Exposure of C2C12 myotubes to leucine and/or glutamine in differentiation medium

For the purposes of chapter 5, differentiated C2C12 myotubes were starved for 4 h. Starvation was followed by incubating cells in DM devoid of leucine and glutamine (0 mM); negative control, DM supplemented with 2 mM L-glutamine (glutamine group), DM supplemented with 2-, 5- or 10 mM L-leucine (leucine groups) or DM supplemented with the previous concentrations of leucine combined with 2 mM L-glutamine (leucine + glutamine groups) for the required times.

For the purposes of chapter 7, C2C12 myotubes were starved for 4 h. Subsequently, C2C12 myotubes were shifted into full DM (control/baseline), DM devoid of leucine and glutamine (0

mM), DM supplemented with 5 mM L-leucine (leucine group), or DM supplemented with 5 mM L-leucine combined with 2 mM L-glutamine (leucine + glutamine group) for short- (3 h), medium- (16 h) or long-term (24 h) exposures.

2.2.6.6. Exposure of C2C12 myotubes to leucine and/or glutamine in starvation medium

For the purposes of chapter 6, C2C12 myotubes were either maintained in full DM (positive control) or starved for 4 h. Following starvation, cells were incubated in StM devoid of leucine and glutamine (0 mM), negative control; StM supplemented with 2 mM L-glutamine (glutamine group); StM supplemented with 2-, 5- or 10 mM L-leucine (leucine groups); or StM supplemented with the previous concentrations of leucine combined with 2 mM L-glutamine (leucine + glutamine groups), for short- (10 and 30 min), medium- (60 min) or long-term (24 h) exposures.

2.2.7. Assessment of cell viability

The viability of C2C12 cells were evaluated following different treatments using a number of viability assays as described below.

2.2.7.1. The AlamarBlue viability assay

The viability of C2C12 myoblasts, plated in 96 well plates (Nunc, Thermo Fisher Scientific), was assessed using the AlamarBlue (AB) assay (Invitrogen, USA). First, 10 µl of AB reagent was added to each of blank, control and treated wells. Then, the plates were wrapped in aluminium foil (Bryson Packaging™, Thermo Fisher Scientific) and incubated for 1 h inside a tissue culture. At the end of incubation, the absorption of blank, control and treated wells was measured at 540 and 600 nm wavelength using an ELISA plate reader spectrophotometer (Flurostar optima, BMG Labtech, Germany). Cell viability was calculated according to the following formula

$$\text{Percentage difference between control and treated cells} = \frac{(O2 \times A1) - (O1 \times A2)}{(O2 \times P1) - (O1 \times P2)} \times 100$$

Where:

O_1 = molar extinction coefficient of oxidized AB at 540 nm

O_2= molar extinction coefficient of oxidized AB at 600 nm

A_1 = absorbance of test wells at 540 nm

A_2 = absorbance of test wells at 600 nm

P1 = absorbance of positive control well at 540 nm

P2 = absorbance of positive control well at 600nm

2.2.7.2. The VitaBright-48™ Assay

The vitality of C2C12 myoblasts was assessed using the VitaBright-48™ (VB-48™) vitality assay (ChemoMetec Inc., Denmark) according to the manufacture's protocol. First, cells were harvested by trypsinisation and centrifuged at 300 xg for 5 min. Next, cell pellets were re-suspended in PBS and stained by mixing 19 parts of cell suspension with 1 part of Solution 5 (ChemoMetec Inc.). Then, 10 µl of this mixture were loaded into each chamber of NC-Slide A8™ (ChemoMetec Inc.). Finally, the fluorescence intensity of stained cells was assessed using a NucleoCounter® NC-3000™ cell analyser (ChemoMetec Inc.).

2.2.7.3. The Viability and Cell Count Assay

The viability of C2C12 cells was assessed using the Viability and Cell Count (VCC) assay (ChemoMetec Inc.). First, cells were harvested by trypsinisation, centrifuged at 300 xg for 5 min and cell pellets were re-suspended in PBS. Then, a small volume (~10 µl) of cell suspension was drawn into Via1-Cassette™ (ChemoMetec Inc.), which is lined with acridine orange and DAPI stains. Next, the fluorescence intensity of stained cells was assessed using a NucleoCounter® NC-3000™ cell analyser (ChemoMetec Inc.). Total, live and dead count were assessed by the NucleoCounter® NC-3000™ software (ChemoMetec Inc.).

The viability of cells was calculated as follows:

$$\% \text{ Viability} = \frac{C_t - C_{nv}}{C_t} \text{ X } 100$$

Where:

% viability = the percentage of viable cells

C_t = the total concentration of cell

C_{nv}= the concentration of non-viable cells

2.2.7.4. Measuring the diameter of C2C12 cells

The diameter of C2C12 myoblasts and C2C12 myotubes was measured using a VCC assay (ChemoMetec Inc.). In brief, cells were washed with PBS, harvested by trypsinisation, centrifuged in benchtop microcentrifuge (Eppendorf® 5417, Thermo Fisher Scientific) at 300 xg for 5 min and re-suspended in PBS. After this, a small volume of cell suspension (~10 µl) was loaded into Via1-Cassette™ (ChemoMetec Inc.) for image cytometry using a NucleoCounter® NC-3000™ advanced image cytometer (ChemoMetec Inc.). Cell diameter was assessed by the NucleoCounter® NC-3000™ software (ChemoMetec Inc.).

2.2.7.5. Assessment of mitochondrial trans-membrane potential

The mitochondrial membrane potential of C2C12 myoblasts was evaluated using a Mitochondrial Potential assay (ChemoMetec Inc.). First cells were harvested by trypsinisation and counted using a haemocytometer (Corning™ Counting Chamber, Thermo Fisher Scientific). Next, ~ 1 x 10^6 cell were suspended in 1 ml PBS. Then, cells were stained in 12.5 µl of Solution 7 (ChemoMetec Inc.) for 30 min at 37 ºC. After that, cells were centrifuged at 400 xg for 5 min and cell pellets were carefully washed with PBS, the last step was repeated three times. At the end, cell pellets were resuspended in 250 µl of Solution 8 (ChemoMetec Inc.), and 10 µl of cell suspension were loaded into each chamber of a NC Slide-A8™ (ChemoMetec Inc.). The fluorescence intensity of stained cells was assessed using a NucleoCounter® NC-3000™ cell analyser (ChemoMetec Inc.).

2.2.8. The Click-iT EdU proliferation assay

The proliferation of C2C12 myoblasts was measured using a Click-iT® EdU Alexa Fluor® 555 Imaging Kit (Invitrogen) according to the manufacturer's instructions. C2C12 myoblasts were incubated with 10 µM of EdU working reagent (WR) in GM for 24 h. Next, media were

discarded, and cells were fixed and permeabilised in one step by 10 min incubation with 1:1 *v/v* acetone /methanol (Thermo Fisher Scientific) mixture at -20 ºC, followed by a quick rinse with 3% *w/v* bovine serum albumin (BSA) (Thermo Fisher Scientific) in PBS. Then, cells were incubated with 500 µl of Click-iT® Reaction Cocktail Mix (Invitrogen) (see Appendix 3) for 30 min in the dark. This was followed by three washes with PBS. After this, cells were incubated with 1 ml of 5 µg/ml Hoechst 33342 solution (Invitrogen) for 30 min, followed by three washes with PBS. Fluorescence was detected at 555 nm excitation and 565 nm emission, using an ELISA plate reader spectrophotometer (Flurostar optima). Unless otherwise stated, all staining steps took place at RT.

2.2.9. Indirect immunofluorescence

SCs and C2C12 cells, plated in 24 well plates, were stained for the detection of Pax7, desmin and MyHC-*fast* using indirect immunofluorescence. In brief, cells were fixed and permeabilized by 10 min incubation in 1:1 *v/v* acetone /methanol (Thermo Fisher Scientific) mixture at -20 ºC. Cells were then blocked in 3% *w/v* BSA in PBS for 30 min. Subsequently, cells were incubated for 4 h with primary antibodies diluted in PBS (see Appendix 1 for list of antibodies and their dilutions). After three washes with PBS, cells were incubated with a secondary antibody (Alexa Fluor 488 conjugated Goat anti-Mouse IgG, Thermo Fisher Scientific) for 1 h. Finally, Fluoroshield™ with DAPI (Gene Tex, USA) was used to stain nuclei and mount cells. Cells were examined, and images were obtained, at 495 nm emission and 519 nm excitation using Leica epifluorescence microscope equipped with a charge-coupled-device camera (Leica Microsystems Ltd.). Unless otherwise stated, all staining steps took place at RT.

2.2.10. Preparation of cell lysates

At the end of specific time points, C2C12 cells were lysed in Radioimmunoprecipitation assay (RIPA) lysis buffer (50 mM Tris-HCl, 150 mM sodium chloride, 1% Igepal CA-630, (NP-40), 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulphate) (Sigma-Aldrich) supplemented with 1X Halt Phosphatase Inhibitor Cocktail (Thermo Fisher Scientific) and 1X Halt Protease Inhibitor Cocktail (Thermo Fisher Scientific). First, media were decanted, and cells were rinsed with ice-cold PBS. Next, cells were incubated in cold lysis buffer for 10 min on ice. Then, cells were scraped from plates using cell scrapers with pivoting blades (Greiner, Sigma-Aldrich) and

collected in microcentrifuge tubes (Thermo Fisher Scientific) for further 20 min incubation on ice with constant agitation. At the end, all tubes were centrifuged at 12,000 xg for 15 min at 4 ºC using a refrigerated centrifuge (Avanti J-E Centrifuge, Beckman Coulter Inc., USA) and the supernatants were collected.

2.2.11. Protein quantification

The total protein content of C2C12 cells was determined using the Bicinchoninic acid (BCA) method for microplates (Pierce BCA protein assay kit, Thermo Fisher Scientific) according to the manufacturer's instructions. First, BSA standards were prepared as serial dilutions from 2mg/ml BSA stock solution (Thermo Fisher Scientific) in RIPA lysis buffer (Sigma-Aldrich)- see Appendix 4 for more details. Next, WR was prepared by mixing 50 parts of reagent A with one part of reagent B according to the following equation:

Total volume of WR in µl = (number of standards + number of samples) x (number of replicates) x 200.

Then, either 10 or 25 µl of each BSA standard and protein sample were pipetted per well of a flat bottom microplate (Nunc, Thermo Fisher Scientific). Subsequently, 200 µL of WR was added to each standard and sample well and the contents of each well were thoroughly mixed. Then, plates were wrapped in aluminium foil (Bryson Packaging™, Thermo Fisher Scientific), and incubated at 37 ºC inside a tissue culture incubator for 30 min. The absorption of protein standards and unknown protein samples was measured in an ELISA plate reader spectrophotometer (Flurostar optima) at 595 nm wavelength. The values of BSA standards were used to plot a standard curve, then the concentration of sample's protein was calculated by interpolation on this standard curve.

2.2.12. Gel electrophoresis

Equal amounts of protein (10 µg for C2C12 myoblasts and 20 µg for C2C12 myotubes) were subjected to electrophoresis using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Briefly, proteins were denatured and reduced by incubation with 2X Tris-Glycine SDS sample buffer (Invitrogen) and 10X NuPAGE reducing agent (Invitrogen) in a block heater (Grant Bio.) for 2 min at 85 ºC according to Appendix 5. Next, proteins were separated on 4-20% Novex Tris-Glycine gels (Novex™, Life Technologies, UK) at constant voltage (125 V)

for 2 h in 1X Tris-Glycine SDS running buffer (Novex™, Life Technologies).

2.2.13. Protein transfer

Following electrophoresis, protein bands were blotted onto microporous (0.2 µm) polyvinylidene fluoride (PVDF) membranes (GE Healthcare Life Sciences, Sweden). First, PVDF membranes and filter paper sheets (Whatman 3 mm Paper, Sigma-Aldrich) were cut to the gel size. Next, PVDF membranes were activated in 100% methanol (Thermo Fisher Scientific) for 1 min. Then, the PVDF membranes, filter papers and gel were equilibrated in 1X Tris-Glycine Transfer Buffer (Cell Signaling Technology, USA) for 5 min. After this, a blot sandwich was assembled with careful elimination of air bubbles using a glass rod. Proteins were transferred at 80 mA for 1 h using a Trans-Blot® SD Semi-Dry Transfer Cell (Bio-Rad Laboratories, USA).

2.2.14. Immunoblotting

Following protein transfer, blots were blocked with 5% skimmed milk (Marvel, Tesco, UK) in Tris-buffered saline (TBS) with Tween-20 (TBS-T) (Thermo Fisher Scientific), for 1 h at RT. Then blots were probed O/N at 4 ºC with monoclonal primary antibodies diluted in 5% BSA/TBS-T – antibodies and their dilutions are provided in Appendix 1. Next, blots were washed 3X, 5 min each, with TBS-T and incubated for 1 h at RT with horseradish peroxidase (HRP) conjugated secondary antibody (Cell Signaling Technology) diluted at 1:1000 in blocking buffer. At the end blots were washed 3X, 5 min each, with TBS-T.

2.2.15. Visualization of protein bands

Protein bands were visualised by incubating blots with Enhanced Chemiluminescent substrate (ECL) (Cell Signaling Technology) for 5 min. Excess ECL was removed, blots were wrapped in Saran Wrap plastic films (Dow Chemicals, UK) and placed inside an X-ray cassette. Blots were then exposed to X-ray films (Kodak® BioMax®, Merck, Sigma-Aldrich) in a darkroom with a safe light for various exposure times. At the end, X-ray films were developed using an automatic film processor (Konica Minolta Medical & Graphic Inc., Japan).

2.2.16. Quantification of protein bands

Digital copies of developed X-ray films were used to quantify the intensities of protein bands using ImageJ (Java-based image-processing and analysis) software downloaded from http://rsb.info.nih.gov/ij/index.html. First, digital images were converted to 8-bit format and background subtraction was carried out using the rolling ball radius method as described by Gassmann and co-workers (2009). Next, the "Rectangular Tool" of ImageJ was used to draw a frame around each protein band. Then, measurements were made by selecting the "Gels" function followed by the "Plot lanes" function from the "Analyze" menu. Then, histograms, indicating the intensities of protein bands, appeared and quantification of peak areas was made as arbitrary area values. The levels of all proteins were compared to an internal loading control (β-actin) to correct for loading variation and to maximise accuracy of quantification. The levels of phosphorylated target proteins were normalised with the total levels of target proteins except for GSK-3 β and Akt-1 that were normalised to β-actin. Data are expressed as percentage change from controls.

2.2.17. Stripping antibodies and reprobing

A mild stripping buffer was used to remove antibodies from immunoblots in order to reprobe them with new antibodies. PVDF membranes were incubated for 1 h in 0.1 M glycine stripping buffer at RT- see Appendix 6 for the compositions of the stripping buffer. This was followed by multiple washings in PBS and TBS. Blocking of blots and incubation with primary and secondary antibodies were carried out as detailed earlier.

2.2.18. The PathScan® Intracellular Signaling Arrays

The PathScan® Intracellular Signaling Array kit (Cell Signaling Technology) was used to screen the phosphorylation and cleavage of 18 signalling pathways, according to manufacture recommendation. First, C2C12 myotubes were starved for 4 h. Following starvation, cells were left in StM (0 mM leucine); control or exposed to leucine alone (5 mM L-leucine) or leucine combined with 2 mM L-glutamine (5 mM + Gln) in StM for 30 min. Cell lysates were prepared by first decanting media from culture plates and rinsing cells twice with ice-cold PBS. Next, 1 ml of ice-cold cell lysis buffer (Cell Signaling Technology) supplemented with 1 mM phenylmethylsulfonyl fluoride (PMSF) was added to each plate and cells were incubated with

lysis buffer for 10 min on ice. Then, cells were scrapped off plates using cell scrapers (Greiner, Sigma-Aldrich), collected into microcentrifuge tubes (Thermo Fisher Scientific), centrifuged at 12,000 xg for 15 min at 4 ºC and the supernatants were carefully collected into new microcentrifuge tubes.

The protein contents of supernatants were determined using a BCA assay, as detailed earlier and approximately 0.4 mg of protein was used per each membrane array. In brief, one membrane array was placed in a well of a multi-chamber plastic dish (Cell Signaling Technology) and blocked in 2 ml of Membrane Array Diluent Buffer (Cell Signaling Technology) for 1 h at RT. Then, 1,500 µl of Cell Lysate Buffer (Cell Signaling Technology) diluted in Membrane Array Diluent Buffer, were added per membrane for an O/N incubation at 4 °C. On the following day, array membranes were washed 3 times, 5 min each, with 1X Array Wash Buffer (Cell Signaling Technology). Membranes were then incubated with 1,500 µl of 1X Detection Antibody Cocktail (Cell Signaling Technology) for 1 h at RT followed by three washes, 5 min each, with 1X Array Wash Buffer (Cell Signaling Technology). Next, membranes were incubated with 1500 µl of 1X HRP-linked Streptavidin for 30 min at RT (Cell Signaling Technology). This step was followed by three washes, 5 min each, with 1X Array Wash Buffer. Finally, membranes were incubated with 10 ml 1X chemiluminescent reagent (Cell Signaling Technology) for 2 min, placed inside a chemiluminescent development folder (Cell Signaling Technology) and exposed to X-ray films (Kodak® BioMax®, Mereck, Sigma-Aldrich).

2.2.19. Blocking the ERK1/2 signalling

The U0126 (Cell Signaling Technology), a specific inhibitor of MEK 1 and MEK 2, was used to block the ERK1/2 signalling in C2C12 myotubes, according to the manufacture's recommendation. A 10 mM stock solution of U0126 was prepared in methanol (Thermo Fisher Scientific). Then, C2C12 myotubes were either incubated with methanol (control vehicle) or 10 µM U0126, in methanol, for 2 h prior to leucine and/or glutamine exposure.

2.2.20. Blocking the PI3K/Akt signalling

LY294002 (Cell Signaling Technology), a potent PI3K inhibitor, was used to block the PI3K/Akt signalling pathway in C2C12 myotubes according to the manufacturer's instructions. First, a 10 mM stock solution of LY294002 was prepared in DMSO (Thermo Fisher Scientific).

Then, C2C12 cells were incubated with either DMSO (control vehicle) or 25 µM LY294002, in DMSO, for 2 h prior to leucine and/or glutamine exposure.

2.2.21. Isolation of microRNAs

MiRNAs were isolated from C2C12 myotubes using the miRNeasy mini kit (Qiagen, Germany), according to instructions provided by the manufacture. First, media were decanted and 700 µl of Qiazol lysis reagent were added to cells. Next, cells were scrapped off wells and the cell/Qiazol suspension was transferred to RNase-free microcentrifuge tubes (Qiagen), vortexed vigorously for 15 seconds (Stuart vortex mixer, BioCote Ltd., UK) and left at RT for 5 min. Then, 140 µl of chloroform (WEB SCIENTIFIC, UK) was added to each tube, followed by vigorous shaking for 15 seconds, and 2-3 min of incubation at RT. All tubes were centrifuged at 12,000 xg for 15 min at 4 °C and the aqueous phase was carefully transferred into RNase free collection tubes (Qiagen). Absolute ethanol (Thermo Fisher Scientific) was added to the aqueous phase at 3:2 ratio and mixed well by pipetting. After that, 700 µl of the aqueous phase/ethanol homogenate was transferred into mini spin columns (Qiagen), spun at 8,000 xg for 15 seconds at RT and the flow through was discarded. Next, 700 µl of RWT Buffer (Qiagen) was added to spin columns, followed by 15 seconds of centrifugation at 8,000 xg and the flow through was discarded. Then, spin columns were washed by 500 µl of RPE Buffer (Qiagen), span for 15 seconds at 8,000 xg and the flow through was discarded. After this, 500 µl of RPE Buffer was added to each spin column and spin columns were dried by centrifugation at 8,000 xg for 2 min at RT. Finally, spin columns were transferred into new 1.5 ml collection tubes (Qiagen) followed by eluting the columns membranes with 40 µl RNase free water (Qiagen), centrifugation at 8,000 xg for 1 min and ended by collecting the flow through (i.e., total RNA). Unless otherwise stated, all staining steps took place at RT.

2.2.22. Quantification of RNA

The purity and concentrations of isolated RNAs, including miRNAs, were assessed using the NanoDrop 8000 spectrophotometer (Thermo Fisher Scientific). After cleaning the optical surfaces of the spectrophotometer with molecular grade water and lint-free wipes (Kimwipes™, Kimberly-Clarke Professional™ Kimtech Science™, Thermo Fisher Scientific), a blank measurement was made using 1 µl of RNase free water (Qiagen). Next, 1 µl of RNA sample

was dispensed into each well of the spectrophotometer's lower pedestal. Sample concentration and purity were measured using the nucleic acid program of NanoDrop ND-8000 software. Only RNA samples with A260/280 optical density (O.D.) ratio close to 2.00 were used for cDNA synthesis.

2.2.23. Assessing the integrity of miRNAs

The integrity of isolated RNAs, including miRNAs, was assessed using the Agilent RNA 6000 Nano Kit (Agilent Technologies, USA) according to the manufacture's recommendations. The electrodes of Agilent 2100 Bioanalyzer were decontaminated by filling out one of the wells of an electrode cleaner with 350µl RNAseZap (Ambion, Inc., USA) and placing it inside the Bioanalyzer for 1 min. Next, the electrodes of Agilent 2100 Bioanalyzer were cleaned by filling one of the wells of an electrode cleaner with 350 µl RNase-free water (Qiagen) and placing it inside the Bioanalyzer for 10 seconds. After this, the lid of the Bioanalyzer was left open for 10 seconds to dry the electrodes. Then, the reagents were prepared as follows: first the RNA 6000 Nano Ladder (Agilent Technologies) was incubated in a block heater (Grant Bio, UK) at 70 °C for 2 min, this was followed by snap cooling on ice. Second, RNA 6000 Nano dye concentrate (Agilent Technologies) was vortexed (Stuart vortex mixer, BioCote Ltd.) for 10 seconds. Next, 550 µl of RNA 6000 Nano gel matrix was allocated into a spin filter (Agilent Technologies) and the spin filter was centrifuged at 1,500 xg for 10 min. Then, 65 µl of filtered gel matrix were combined with 1 µl of RNA dye in an RNase free microcentrifuge tube (Thermo Fisher Scientific). This tube was vortexed thoroughly and centrifuged at 13,000 xg for 10 min. Finally, miRNA samples were denatured at 70 °C for 2 min and cooled on ice.

Chip loading started by placing a new RNA Nano chip (Agilent Technologies) in the chip priming station and dispensing 9 µL of gel-dye mix into the bottom of wells marked with circled G symbol. Next, the chip priming station was closed, and the plunger was pressed until it was held by the syringe clip. After 30 seconds, the clip was released, and the plunger was slowly pulled back into the 1 ml position. Then, 9 µL of gel-dye mix was dispensed into the bottom of wells marked with un-circled G symbol. After this, 1 µL of RNA 6000 Nano Ladder was dispensed into wells marked with a ladder symbol, while 5 µl of RNA 6000 Nano Marker were dispensed into all wells (sample wells and wells marked with ladder symbol). Finally, 1 µL of RNA/miRNA sample was dispensed into each of the 12 sample wells, and the chip was vortexed for 1 min. The RNA chip was carefully placed into the receptacle of an Agilent 2100

BioAnalyzer instrument (Agilent Technologies). Then, “Chip Run” was initiated using the Agilent 2100 Expert Software (Agilent Technologies). Only samples with RNA Integrity Number (RIN) ≥ 9.0 were used for cDNA synthesis.

2.2.24. Complementary DNA synthesis

Reverse transcription of miRNA was achieved by using a miScript II RT kit (Qiagen). In brief, RNA samples and the miScript Reverse Transcriptase Mix (Qiagen) were thawed on ice, while the 10X miScript Nucleics Mix (Qiagen) and the 5X miScript HiSpec Buffer (Qiagen) were thawed at RT. Next, reverse transcription master mix was prepared according to Appendix 7. Then, ~ 1µg of total RNA was added to each tube containing reverse transcription master mix, followed by brief centrifugation to mix the tubes content. After this, all tubes were incubated in a block heater (Grant Bio.) at 37 ºC for 60 min. The reaction was ended by incubating all tubes in a block heater (Grant Bio.) at 95 ºC for 5 min, to inactivate the miScript Reverse Transcriptase Mix. The complementary DNA (cDNA) was stored at –20 ºC until further analysis.

2.2.25. Quantitative real-time polymerase chain reaction

The expression levels of five muscle-specific miRNAs (miR-1, miR-133a, miR-133b, miR-206 and miR-499) were quantified using a quantitative real-time polymerase chain reaction (qRT-PCR).

2.2.25.1. Setting up the reaction

This part of the experimental work was carried out by Belinda Wang, a technician at the School of Veterinary Medicine and Science. The qRT-PCR reaction is set up as follows: first, QuantiTect SYBR Green Mix (HotStarTaq® DNA Polymerase, QuantiTect SYBR Green PCR Buffer, dNTP mix including dUTP, SYBR Green, ROX™ passive reference dye and 5 mM MgCl2, Qiagen), a reverse primer (miScript Universal Primer, Qiagen), specific forward primers (miScript primer assays, Qiagen), listed in Appendix 8, template cDNA and RNase free water (Qiagen) were mixed according to Appendix 9. Then, 25 µl of this master mix were dispensed into each well of a 96-well plate (Roche Applied Science, Germany). Plates were tightly sealed with plastic films (Roche Applied Science), centrifuged for 1 min at 2000 xg and amplified according to the manufacture’s recommendation (see Appendix 10 for qRT-PCR

cycling conditions) inside a LightCycler® 480 Real-Time PCR System 96-well Plate Platforms (Roche Applied Science). Hs_RNU6B (Qiagen) was used as internal control/reference gene.

2.2.25.2. Data analysis

The mean crossing point values (CP) were calculated from the duplicate reactions. Next, the relative expression of each miRNA was calculated using the $2^{(-\Delta\Delta Ct)}$ method (Livak and Schmittgen, 2001). The expression levels of target miRNAs were normalised against the expression levels of Hs_RNU6B (reference gene) using the LightCycler® 480 software, release 1.5.1 (Roche Applied Science). Results are expressed as fold changes with respect to the values of the control/baseline samples.

2.2.26. Statistical analysis

The data in this work derived from at least three biologically distinct (independent) replicates (n = 3-4) representing measurements of separate samples from the same source that were subjected to identical treatments, to account for any biological variation. Each biological replicate involved three technical replicates, which are repeated measurements of the same biological sample, to account for any variation due to the equipment used. Data are presented as mean of values ± standard deviation (SD). Differences between two groups were tested by a paired two-tailed Student's *t*-test using Excel software (Microsoft office for Windows 2016 and 2019). Statistical significance of more than two groups was analysed by one-way analysis of variance (ANOVA) using Excel software (Microsoft office for Windows 2016 and 2019). Differences among more than two groups were compared by Tukey honestly significant difference (HSD) *post hoc* test using Statistical Package for Social Sciences (SPSS) Statistics for Windows, version 20 (IBM Corp., Armonk, N.Y., USA). Image analyses, scatter plots and histograms for vitality and viability experiments were obtained using a NucleoView™ software (Chemometec Inc.). Quantile-quantile (Q-Q) plots were generated using GraphPad Prism 7.0 software downloaded from www.graphpad.com. An alpha level of significance was set at 0.05 and P values less than 0.05 indicate statistically significant differences. P values are represented as follows: $^{*}P < 0.05$, $\underline{^{*}}P < 0.05$, $^{+}P < 0.05$, $\underline{^{+}}P < 0.05$, $^{**}P < 0.01$, $\underline{^{**}}P < 0.01$, $^{++}P < 0.01$, $\underline{^{++}}P < 0.01$, $^{***}P < 0.05$, $\underline{^{***}}P < 0.001$, $^{+++}P < 0.001$ and $\underline{^{+++}}P < 0.001$.

3. Comparing the proliferation and differentiation of muscle stem cells isolated from two chicken hybrids with different growth rates

3.1. Introduction

The genetic similarities, yet divergent growth rates, between two chicken breeds, namely "broilers" and "layers", have led to several studies aimed at delineating the molecular mechanisms underlying this dichotomy. Broilers are chickens that have been selected for fast growth and high meat deposition, particularly in the breast muscles (Buzala and Janicki, 2016). In contrast, layers are chickens selected for high egg production, though their growth rate is not affected by this selection (Buzala and Janicki, 2016).

It has been previously reported that selection for high growth in animals favoured the presence of fast-proliferative type of SCs (Feldman and Stockdale, 1991; Dusterhoft and Pette, 1993; Molnar and Dodson, 1993; Merly *et al*., 1998). This was further supported by an enhanced proliferation of SCs derived from fast-growing (selected) animals, when compared with SCs derived from slow-growing lines (Penney *et al*., 1983; Brown and Stickland, 1994; Rehfeldt *et al*., 2002). It has been also suggested that most of the muscle gain in broiler chickens occurs after hatching (Jones *et al*., 1986). It is known that SCs are the main factor in postnatal muscle growth (Yablonka-Reuveni, 2011). Therefore, it is likely, that selection for high growth has changed the properties of broilers' SCs.

Nutrition is the second most important factor in postnatal muscle growth, after genetics. Selection for high growth has led to changes in the sensitivity of SCs to different nutritional stimuli, as SCs from fast-growing animals synthesized more DNA in response to FCS, insulin and IGF-1 (Mathison *et al*., 1989; MacFarland *et al*., 1995; Duclos *et al*., 1996; Merly *et al*., 1998). It is likely that such selection for high growth has altered the response of CSCs to the provision of leucine, a BCAA with an established role in enhancing protein synthesis within muscle cells (Crozier *et al*., 2005; Norton *et al*., 2009; Escobar *et al*., 2005; Wilson *et al*., 2010; Escobar *et al*., 2006; Drummond *et al*., 2008).

The application of muscle tissue culture techniques – ranging from primary, clonal and single

myofibres – to study the physiology, adaptation and regulation of muscle stem cells has been a valuable tool in satellite cell research for more than four decades. The first use of myogenic cell culture took place in 1960, before the identification of SCs and their role in muscle growth and regeneration (Konigsber, 1960). The greatest advance in this field took place in 1974, when Bischoff successfully liberated and cultivated SCs from the skeletal muscles of rats (Bischoff, 1974). Culturing single muscle fibres, for the purpose of monitoring the behaviour of SCs within their anatomical niche, was a further milestone in this field (Bischoff, 1986). Most of the information on the development, biology and regenerative capacities of SCs has been gained from primary cell cultures. *In vitro* cultivation of SCs enables their examination by excluding the myriad of niche, intrinsic and systemic factors that affect their behaviour and response *in vivo*.

The growth and molecular biology of broiler-derived SCs has been investigated (Orcutt and Young, 1982; Ridpath *et al*., 1984), however, most studies were conducted on SCs isolated from the breast muscles of broiler chickens at their marketing age. As a result, little is known about the culture conditions required for the cultivation of SCs derived from younger broiler chickens, SCs derived from the leg muscles of broiler chickens and SCs derived from the breast and leg muscle of other chicken breeds such as layers. Therefore, we established a sustained *in vitro* culture system to cultivate four SC populations derived from the breast (PM) and leg (PL) muscles of young broiler and layer chickens and investigated the proliferation, differentiation and response of these cells to different stimuli.

3.2. Research hypotheses

The hypotheses tested in the present study were: selection for high growth has altered the proliferative and differentiation capacities of broiler and layer SCs, selection for high growth has altered the proliferative and differentiation capacities of SCs derived from the breast and leg muscles of the same bird and SCs derived from the breast and leg muscles respond differently to nutritional stimuli.

3.3. The aims of this chapter

1. To establish and optimise a sustained *in vitro* culture system for SCs isolated from the breast (PM) and leg (PL) muscles of two-week-old broiler and layer chickens.

2. To monitor the proliferative and differentiation capacities of these different populations of SCs.
3. To assess the response of SCs isolated from selected (breast) and unselected (leg) muscles of two-week-old broiler chickens to different concentrations of leucine.

3.4. Results

3.4.1. Weight gain in broiler and layer chickens

Results indicated that the hatching weight of broiler chicks (47.8 ± 3.5 g) was significantly higher than layers (34.7 ± 3.8 g) ($P < 0.001$). In the same line, the weight of broilers at the end of the first (119 ± 9.3 g) and second (271 ± 31 g) week of age was significantly higher than layers at the end of the first (73 ± 8.7 g) and second (154 ± 10.8 g) week of age ($P < 0.001$) (Figure 3.1). A significant increase in the weight of broilers, compared with broilers on the hatching day, was evident at the end of the first and second week of age ($P < 0.001$) (Figure 3.1). Also, a significant increase in the weight of layer chickens, compared with layers on the hatching day, was evident at the end of the first and second week of age ($P < 0.001$) (Figure 3.1). The weights of broiler and layer chickens are listed in Appendix 11.

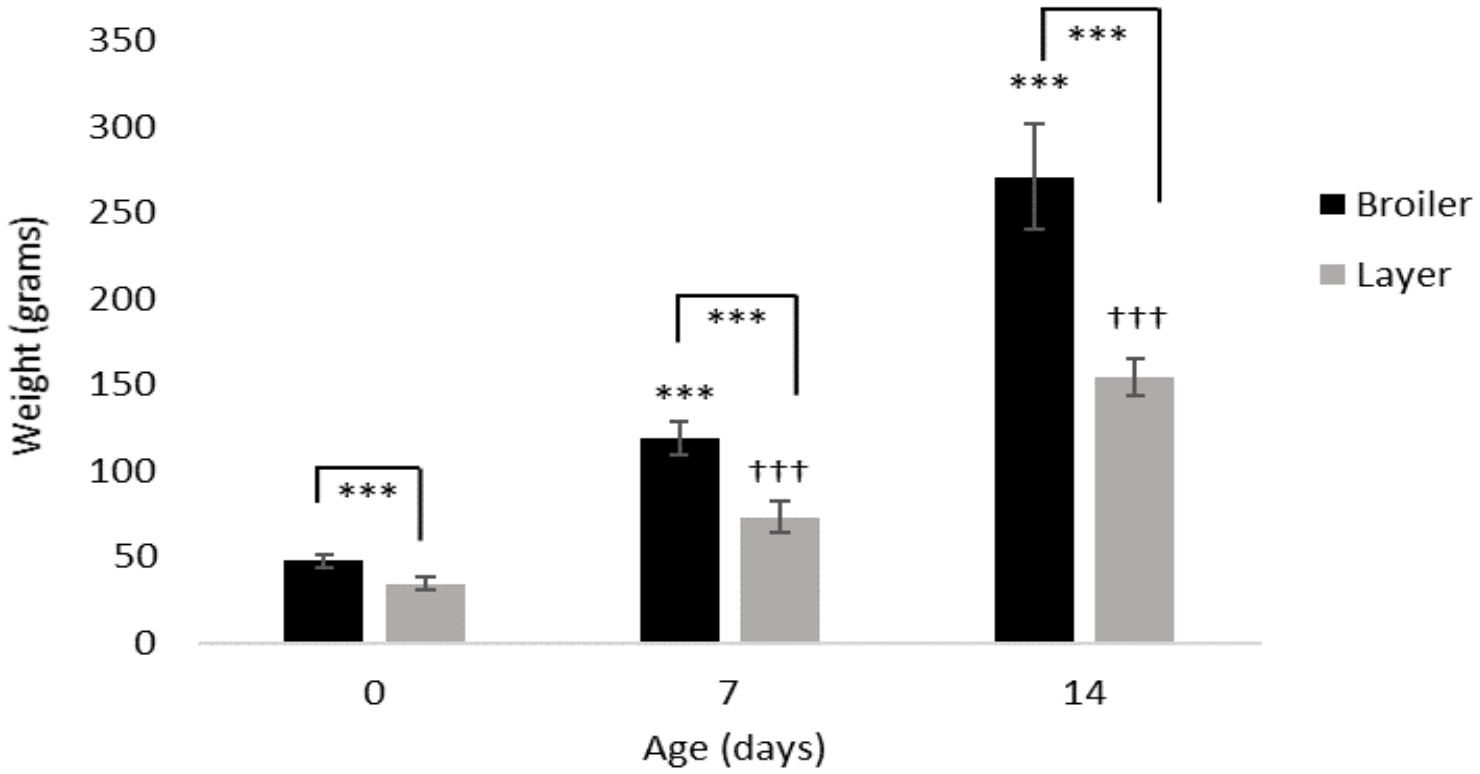


Figure 3.1. Weight of broiler and layer chickens at hatching (0 days), 7th and 14th days of life. Data are presented as means ± SD. (n = 50; 25 broilers and 25 layers). Analysed by one-way ANOVA using Microsoft Excel followed by Tukey's HSD *post hoc* test using SPSS Statistics 20. Asterisks indicate significant increase compared to broilers at hatching (0 days); positive control and significant differences between broiler and layer groups $^{***}P < 0.001$. Crosses indicate significant increase compared to layers at hatching (0 days) $^{+++}P < 0.001$. The weight of broilers was higher than layers on 0, 7th and 14th days of life. A significant increase in the weight of broiler chickens, compared with broilers on the hatching day, was evident on the 7th and 14th days of life. A significant increase in the weight of layer chickens, compared with layers on the hatching day, was evident on the 7th and 14th days of life.

3.4.2. Attachment assay

To assess the myogenicity of cell preparations, primary chicken cultures were stained for the expression of the myogenic markers Pax7 and Mcad. Results revealed that ~ 80 ± 5% of cells were myogenic (i.e., Pax7 +ve/Mcad +ve) (Figures 3.2-4).

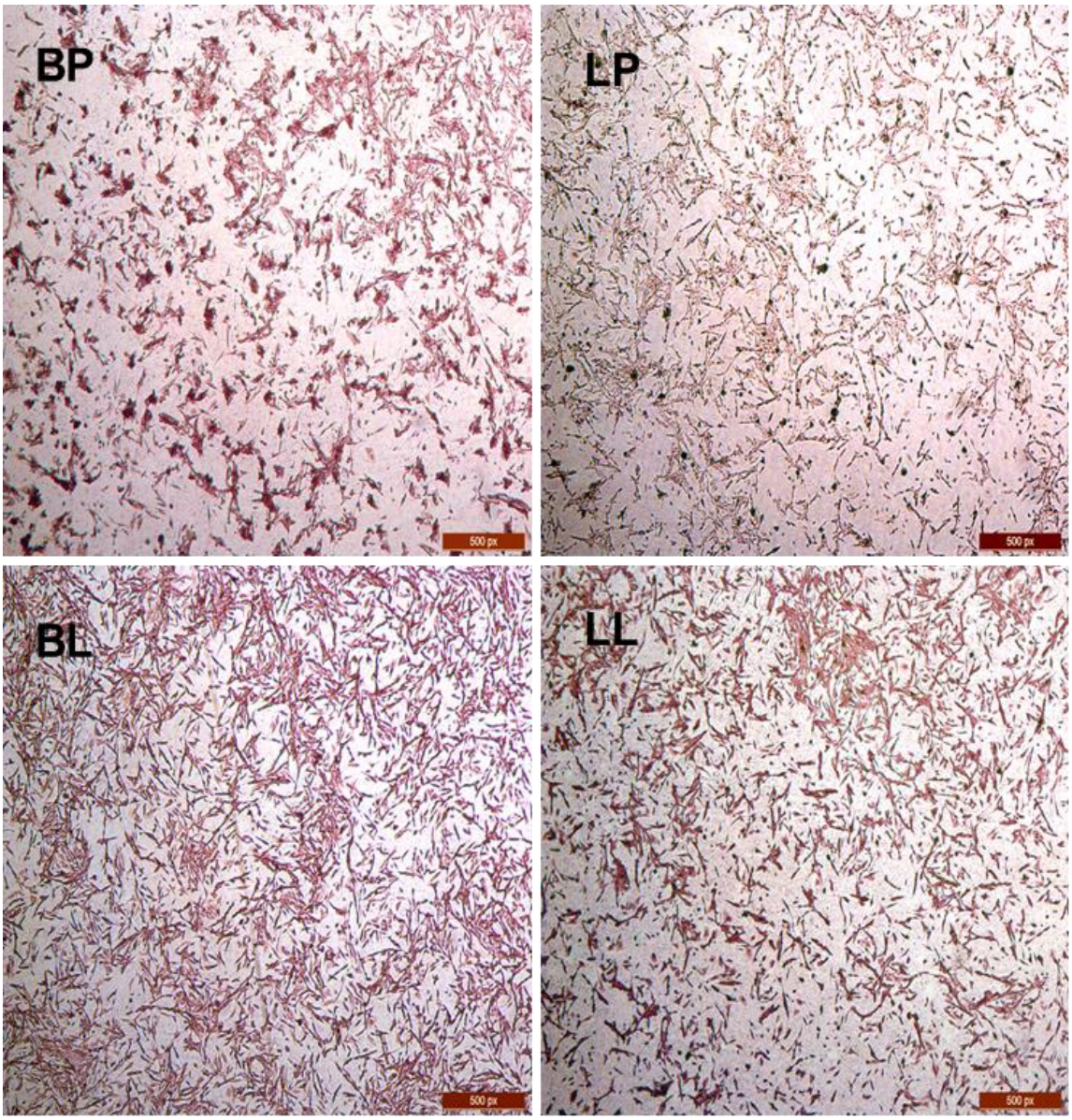


Figure 3.2. Expression of Pax7 in 1-day-old satellite cultures derived from broiler and layer chickens, assessed using indirect immunocytochemistry. SCs cells isolated from the breast (pectoralis major) and leg (peroneus longus) muscles of broiler and layer chickens were plated on collagen coated culture dishes and maintained in GM. Micrographs were taken 24 h after plating. Abbreviations: BP = broiler pectoralis major muscle; LP = layer pectoralis major muscle; BL = broiler peroneus longus muscle; LL = layer peroneus longus muscle.

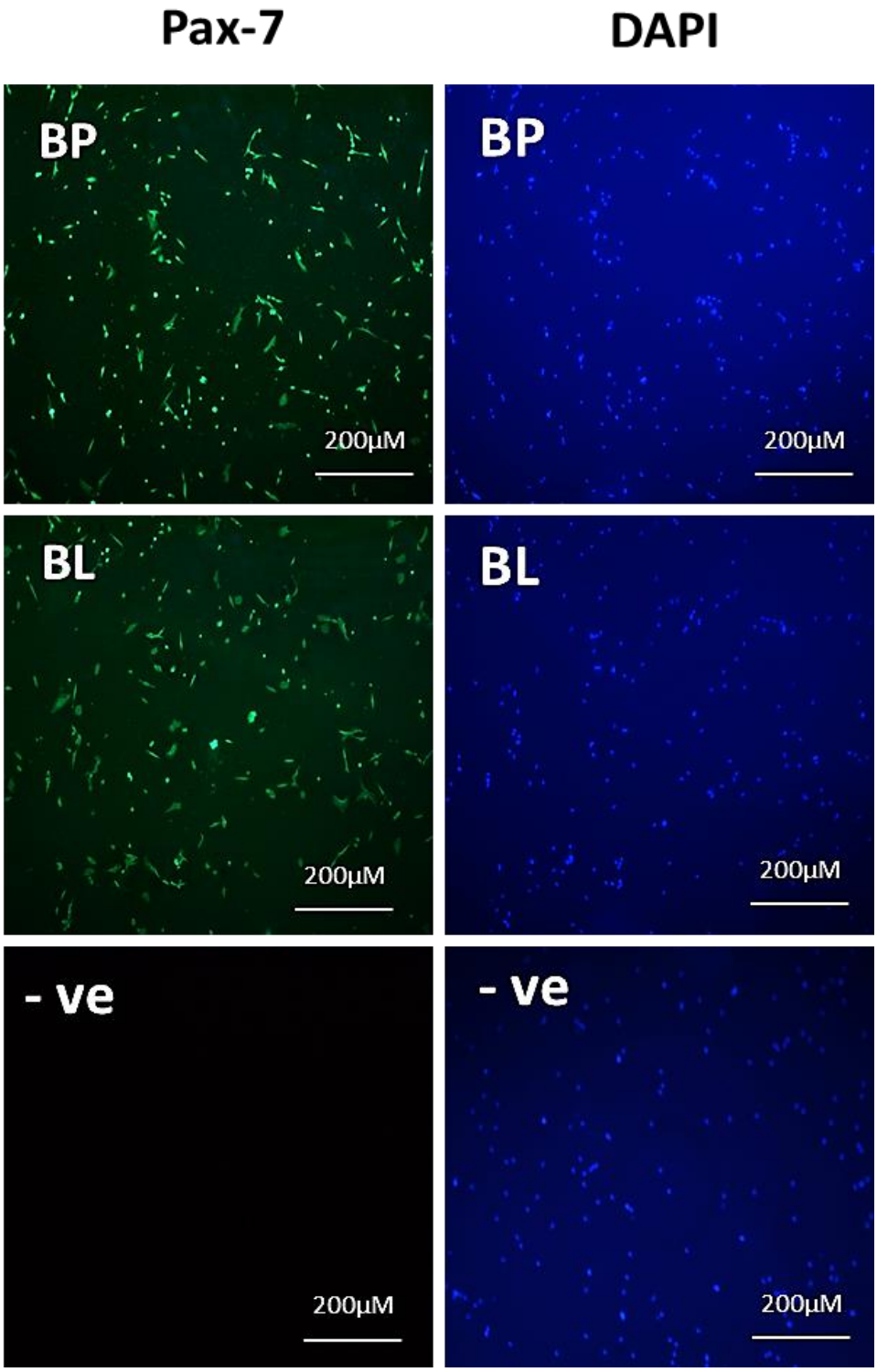


Figure 3.3. Expression of Pax7 in 1-day-old satellite cultures derived from broiler chickens; assessed using indirect immunofluorescence. SCs isolated from the breast (pectoralis major) and leg (peroneus longus) muscles of broiler chickens were plated on collagen coated culture dishes and maintained in GM. Micrographs were taken 24 h after plating. Abbreviations: BP = broiler pectoralis major muscle; BL = broiler peroneus longus muscle; Pax7 = paired box 7; -ve = negative (no primary antibody) control; µM = micrometre.

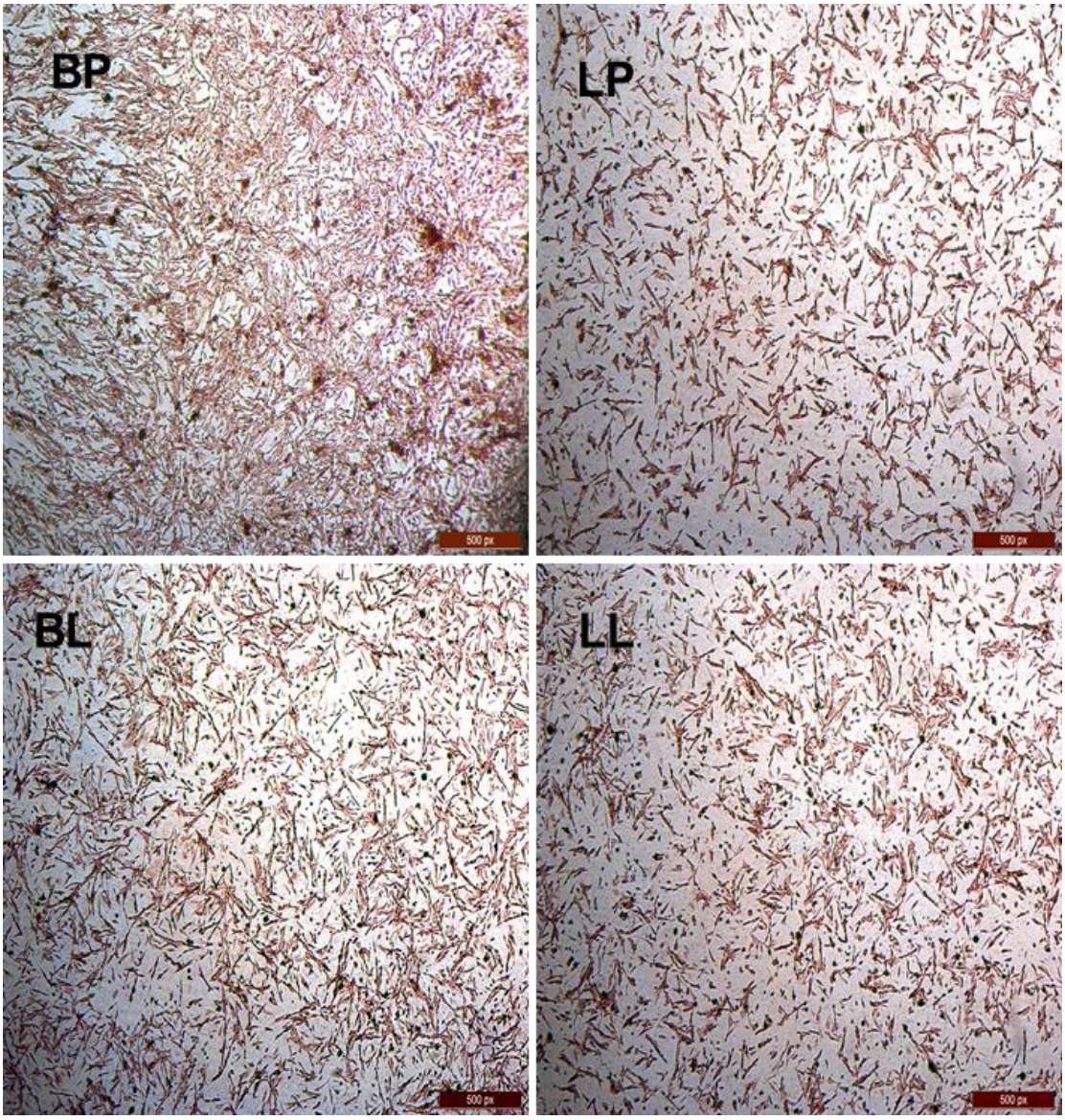


Figure 3.4. Expression of Mcad in 1-day-old satellite cultures derived from broiler and layer chickens; assessed using indirect immunocytochemistry. SCs isolated from the breast (pectoralis major) and leg (peroneus longus) muscles of broiler and layer chickens were plated on collagen coated culture dishes and maintained in GM. Micrographs were taken 24 h after plating. Abbreviations: BP = broiler pectoralis major muscle; LP = layer pectoralis major muscle; BL = broiler peroneus longus muscle; LL= layer peroneus longus muscle; µM = micrometre.

3.4.3. Exposure to Pronase

To determine the optimum duration of enzymatic digestion; muscle samples dissected from the PM muscles of broiler chickens were subjected to 30-, 45- or 60- min of incubation in Pronase and the growth of cells was evaluated morphologically. Results showed that 30 min of enzymatic digestion produced low numbers of SCs that failed to form a monolayer following 4 days of incubation. However, 45 and 60 min of exposure to Pronase resulted in viable SCs that propagated in culture and formed a monolayer by the 4^{th} day of incubation.

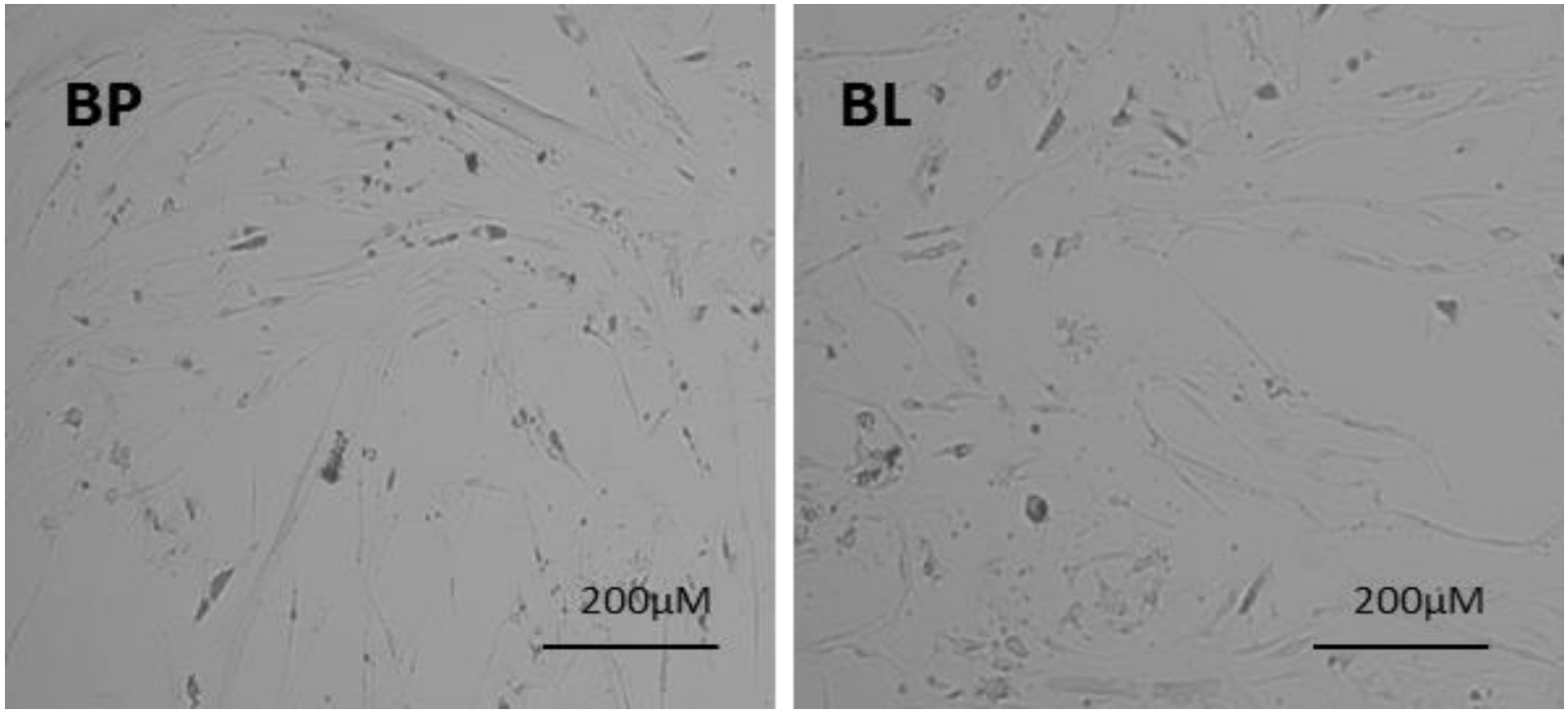


Figure 3.5. Phase-contrast micrographs comparing the growth of chicken satellite cells recovered from differential plating. SCs cells isolated from the breast (pectoralis major) and leg (peroneus longus) muscles of broiler chickens were plated on uncoated culture dishes for 4 h. Then, cells were plated on collagen-coated culture dishes and maintained in GM. Micrographs were taken on the 5th day of incubation. Abbreviations: BP = broiler pectoralis major muscle; BL = broiler peroneus longus muscle; µM = micrometre.

3.4.4. Differential plating

The aim of this experiment was to enrich SCs and reduce the presence of non-myogenic cells in whole muscle cell preparations. Results indicated that cells recovered from differential plating proliferated poorly and failed to form a monolayer after 5 days of incubation in GM (Figure 3.5).

3.4.5. Dissociation of muscle fibres

The aim of this experiment was to test the efficiency of mechanically dissociating chicken muscle fibres using a gentleMACs Dissociator to liberate their cellular contents. Results showed that short dissociation for 1 round yielded a low number of cells. However, the number of liberated cells increased after subjecting muscle fragments to two rounds of mechanical dissociation (Figure 3.6).

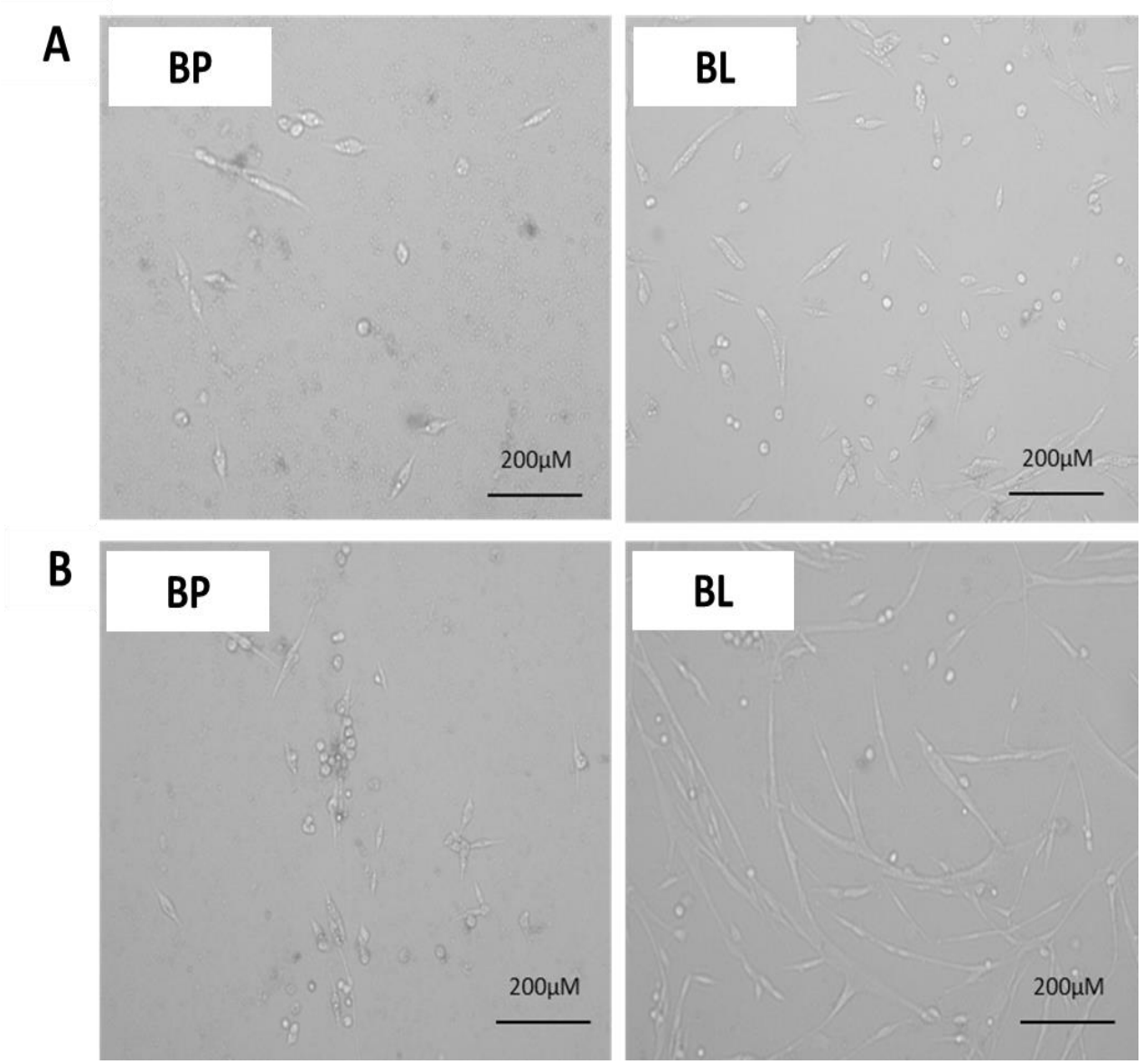


Figure 3.6. Phase-contrast micrographs depicting the growth of chicken satellite cells liberated by mechanical dissociation using a gentleMACS™ Dissociator. Enzymatically digested muscle fragments, obtained from the breast (pectoralis major) and leg (peroneus longus) muscles of broiler chickens, were subjected to 1 (A) or 2 (B) rounds of mechanical dissociation using the muscle (M) program of a gentleMacs™ Dissociator. Liberated cells were then plated on collagen-coated culture dishes and maintained in GM. Micrographs were taken on the 3rd day of incubation. Abbreviations: BP = broiler pectoralis major muscle; BL = broiler peroneus longus muscle; µM = micrometre.

3.4.6. Optimising the seeding density of chicken satellite cells

In this experiment we determined the optimal seeding density of primary CSCs. Results revealed that cultures seeded at a low density proliferated poorly and failed to form a monolayer after 8 days of incubation in GM (Figure 3.7 A). In contrast, cells seeded at a medium density reached confluence by the 5th incubation day with myotubes evident by the 8th day of incubation (Figure 3.7 B). Finally, cells seeded at a high density fused at a high rate and differentiated into myotubes on the 4th incubation day (Figure 3.7 C).

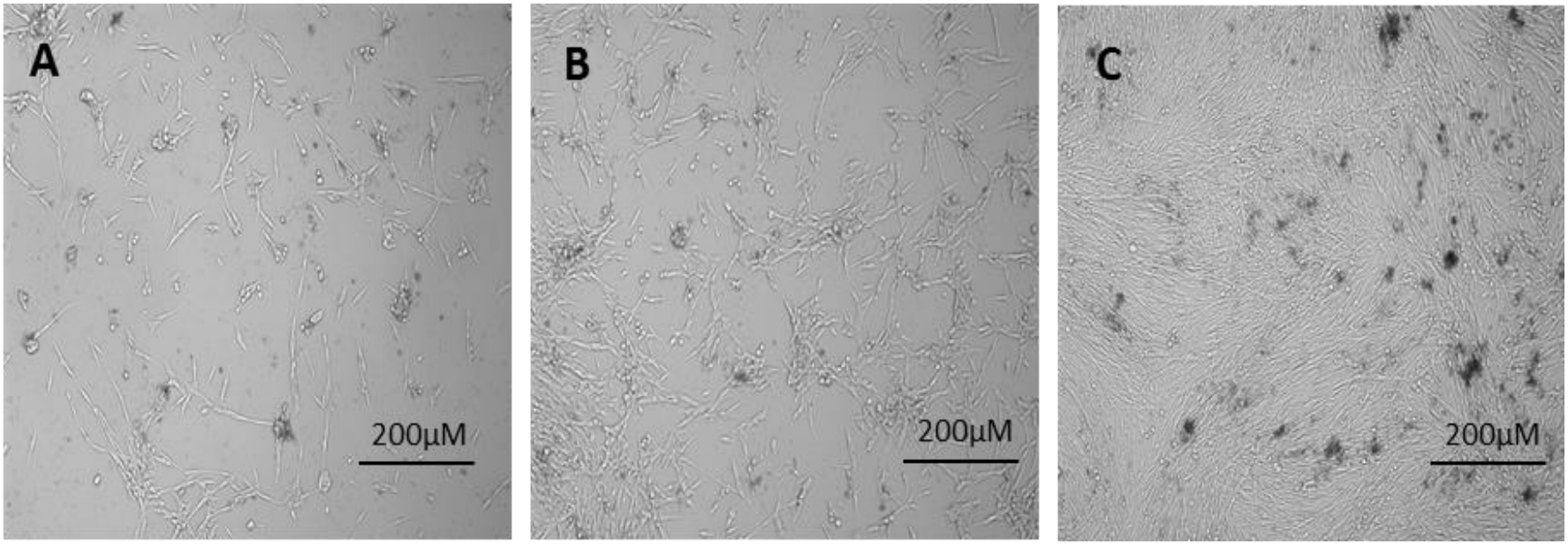


Figure 3.7. Phase-contrast micrographs depicting the morphology of chicken satellite cells plated at different densities. SCs isolated from the breast (pectoralis major) muscle of broiler chickens were plated at a low (A), medium (B) and high (C) densities on collagen-coated culture dishes and maintained in GM. Micrographs were taken on the 4th day of incubation. Abbreviation: µM = micrometre.

3.4.7. Sub-culturing chicken satellite cells

The aim of this experiment was to avoid overgrowth and spontaneous differentiation of SCCs. Results indicated that SCs harvested by 1X and 0.5 X Trypsin-EDTA failed to grow (Figure 3.8). On the other hand, cultures subjected to 0.05X Trypsin yielded viable SCs that continued to proliferate and fused in culture (Figure 3.8). Nonetheless, repeated passaging of SCCs (passage ≥ 3) did not yield viable cells.

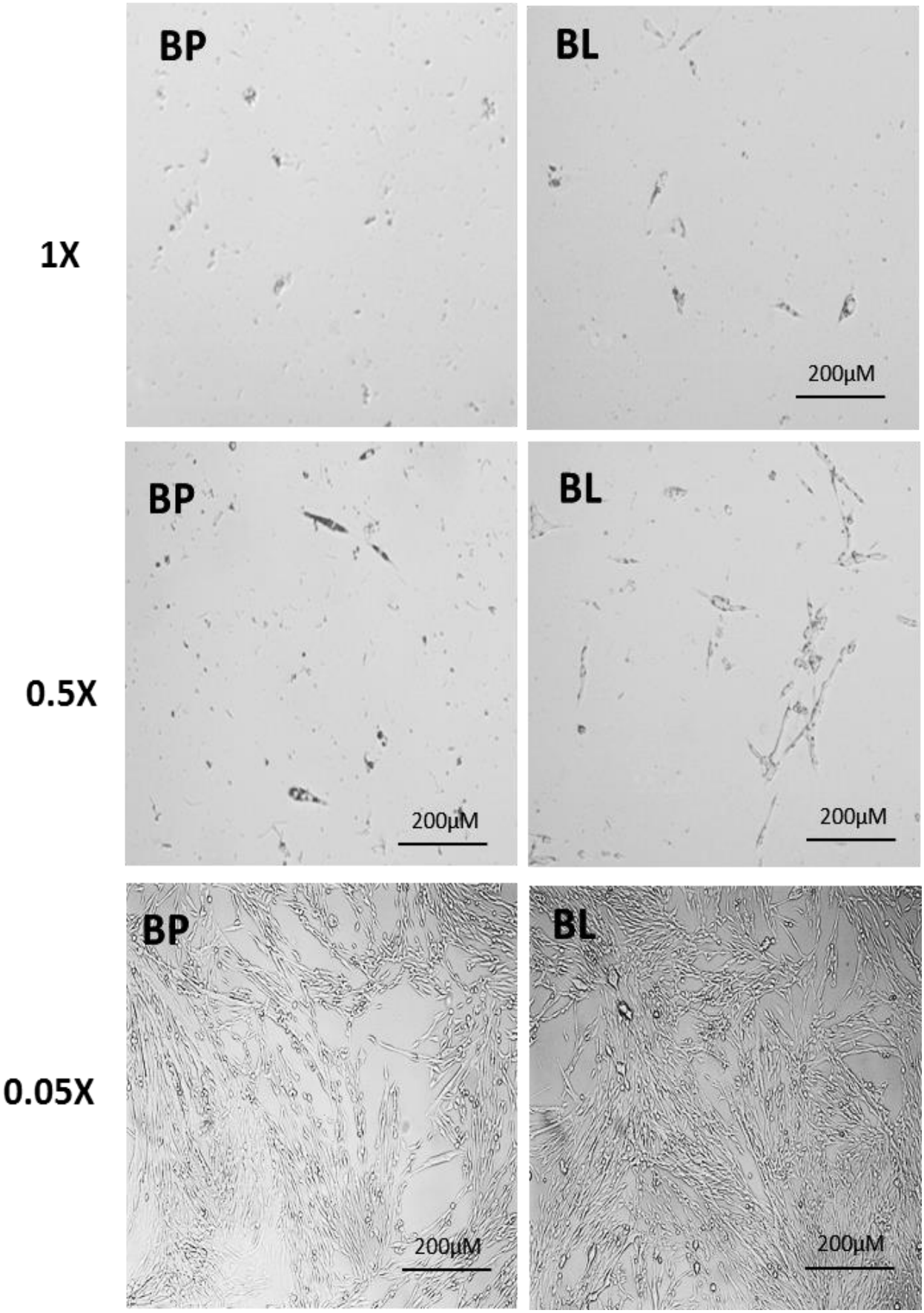

1X
BP
BL
200µM
0.5X
BP
BL
200µM
200µM
0.05X
BP
BL
200µM
200µM

Figure 3.8. Phase-contrast micrographs depicting the growth of chicken satellite cells harvested using different concentrations of Trypsin-EDTA. SCs isolated from the breast (pectoralis major) and leg (peroneus longus) muscles of broiler chickens were expanded in GM to ~50-60% confluence, then sub-cultured using 1X, 0.5X or 0.05X Trypsin-EDTA. Harvested cells were maintained in GM. Micrographs were taken on the 5th day of incubation. Abbreviations: BP = broiler pectoralis major muscle; BL = broiler peroneus longus muscle; µM = micrometre.

3.4.8. Cryopreservation of chicken satellite cells

Long-term storage was essential to maintain the availability of isolated CSCs. Cryopreservation of SCs, isolated from the breast and leg muscles of broiler and layer chickens, was attempted using either a conventional FM or a commercially available FM for stem cells. Resuscitation of cells stored in traditional FM revealed that SCs derived from the leg muscle failed to grow, while breast-derived SCs grew poorly in culture, after a lag phase of 4 days after retrieving from liquid nitrogen. On the other hand, SCs stored in stem cell specific FM grew normally following resuscitation.

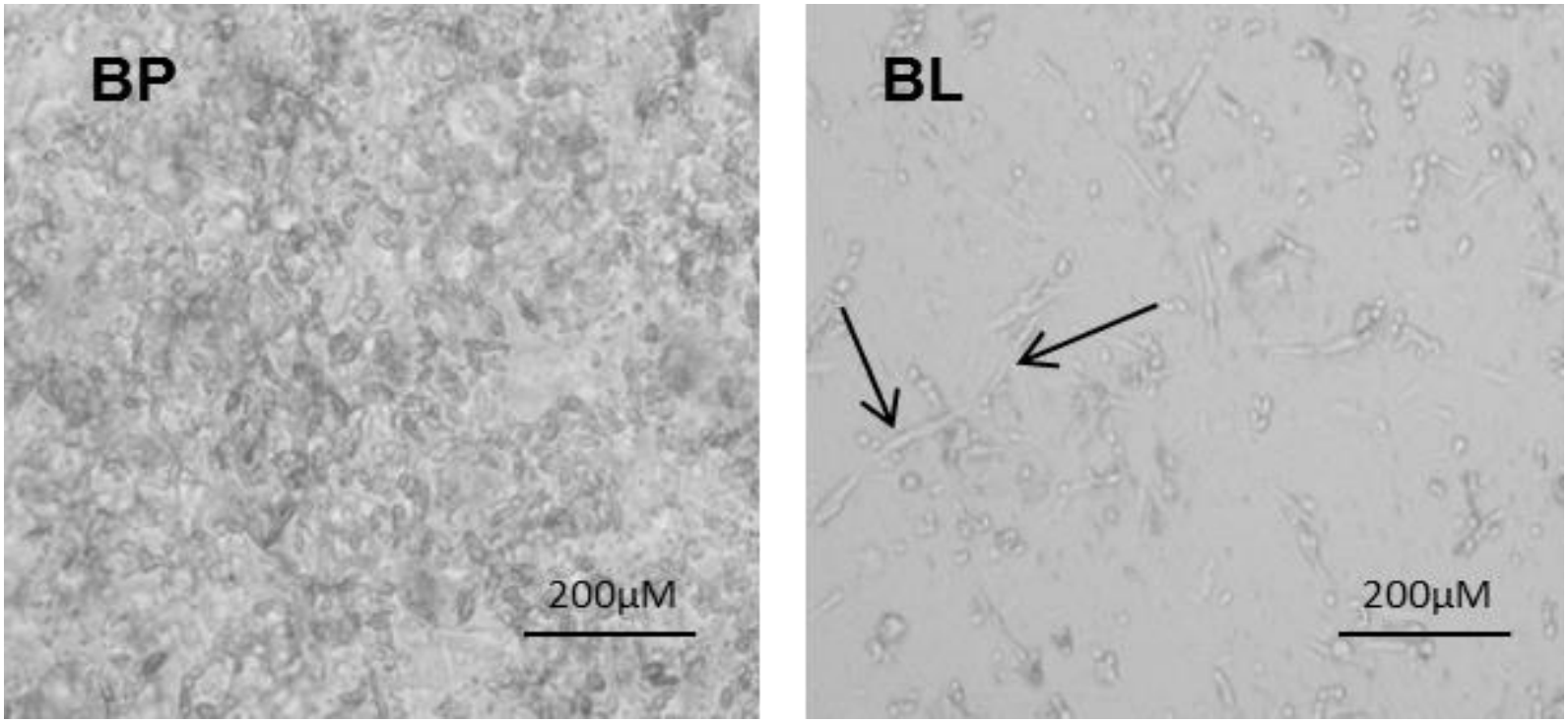


Figure 3.9. Phase-contrast micrographs depicting the morphology of chicken satellite cells after 1 day in culture. SCs were isolated from the breast (pectoralis major) and leg (peroneus longus) muscles of broiler chickens and maintained in GM. Arrows indicate muscle cells. Abbreviations: BP = broiler pectoralis major muscle; BL = broiler peroneus longus muscle. Abbreviation: µM = micrometre.

3.4.9. Evaluating the proliferative and differentiating capacities of chicken satellite cells

The aim was to evaluate and compare the proliferation and differentiation capacities of SCs derived from the breast and leg muscles of broiler and layer chickens. Results indicated that the proliferation of SCs isolated from breast (PM) muscles of both chicken breeds proliferated at higher rates, but fused into myotubes less efficiently, when compared with cells isolated from the leg (PL) muscles. The general order for proliferation rates was as follows: BP > LP> BL > LL (Figures 3.9-13). Results also showed that the differentiation of SCs, indicated by the formation of multinucleated myotubes, derived from the leg muscles of both chicken breeds formed myotubes at higher rates, when compared with cells isolated from the breast muscles. The general order of differentiation rates was as follows: LL> BL > BP > LP (Figures 3.9-13). To gain further insight into the differentiation patterns of breast- and leg-derived SCs, 8-day-old cultures derived from the breast and leg muscles of broiler chickens were stained with H&E. Results showed that cells derived from the leg muscle differentiated and aligned into myotubes. However, breast-derived SCs remained unaligned after 8 days in culture (Figure 3.14).

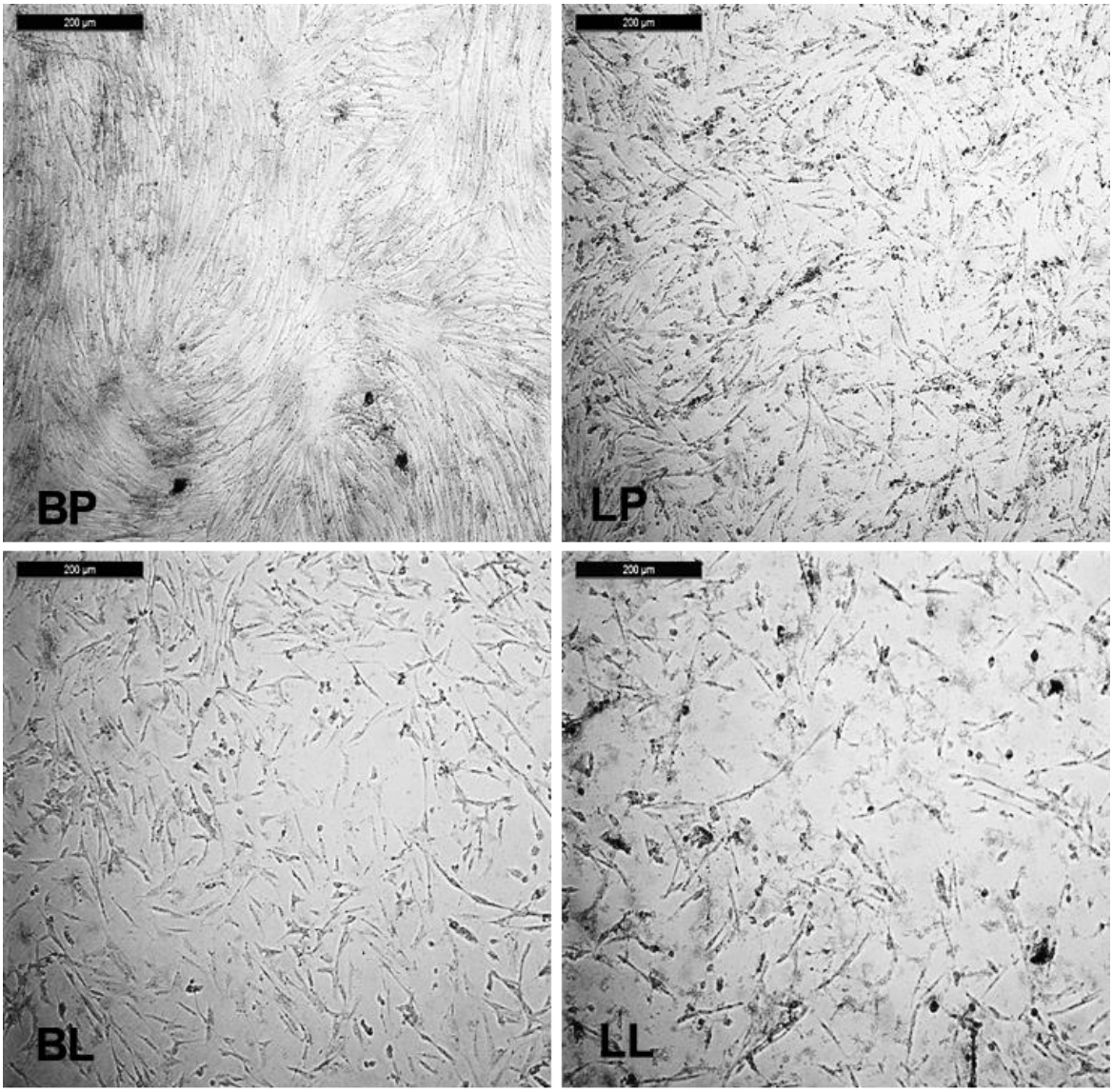


Figure 3.10. Phase-contrast micrographs depicting the morphology of chicken satellite cells following 3 days in culture. SCs were isolated from the breast (pectoralis major) and leg (peroneus longus) muscles of broiler and layer chickens and maintained in GM. Micrographs were taken on the 3rd day of incubation. Abbreviations: BP = broiler pectoralis major muscle; LP = layer pectoralis major muscle; BL = broiler peroneus longus muscle; LL = layer peroneus longus muscle; µm = micrometre.

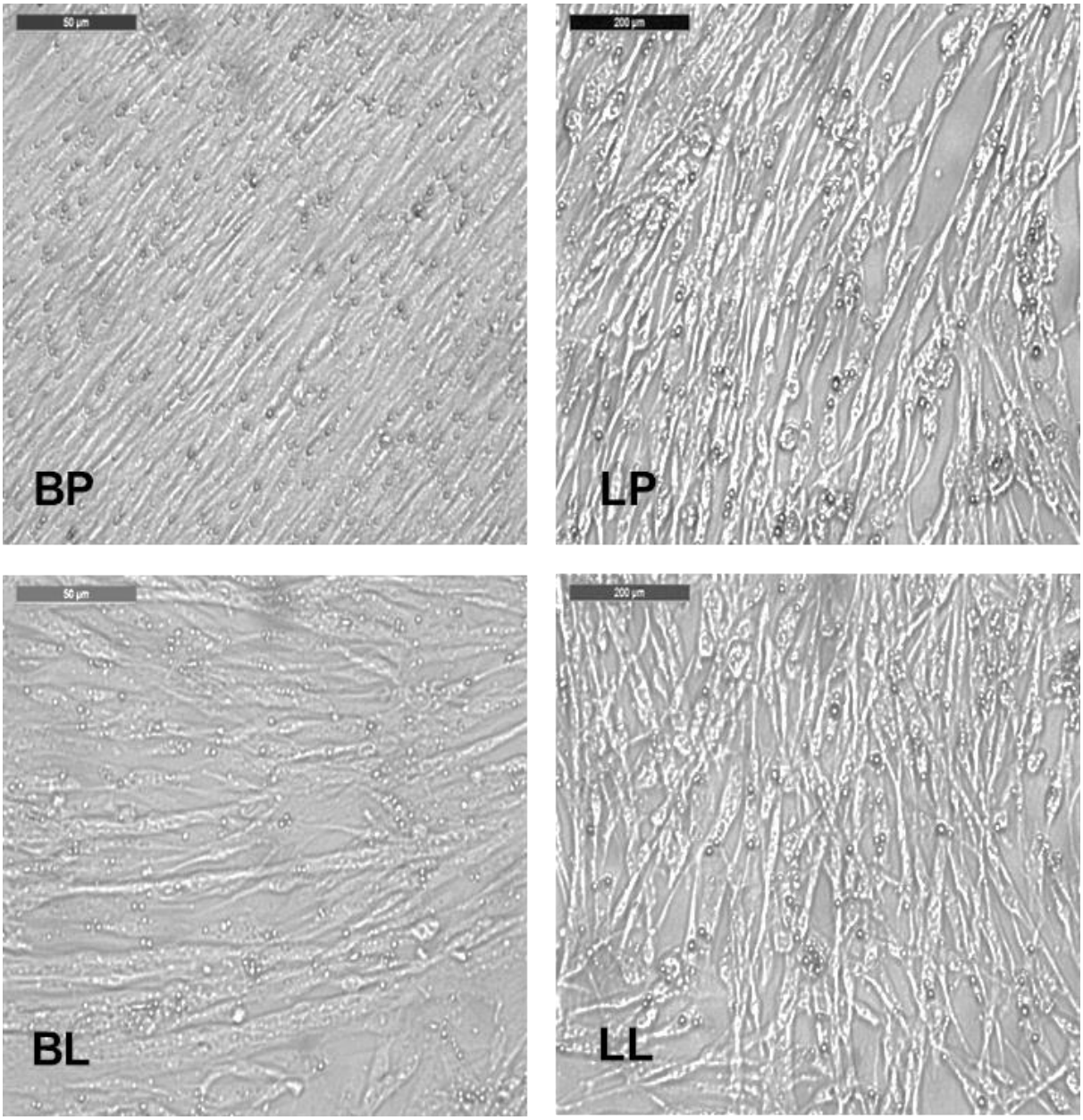


Figure 3.11. Phase-contrast micrographs depicting the morphology of chicken satellite cells following 4 days in culture. SCs were isolated from the breast (pectoralis major) and leg (peroneus longus) muscles of broiler and layer chickens and maintained in GM. Micrographs were taken on the 4th day of incubation. Abbreviations: BP = broiler pectoralis major muscle; LP = layer pectoralis major muscle; BL = broiler peroneus longus muscle; LL = layer peroneus longus muscle; µm = micrometre.

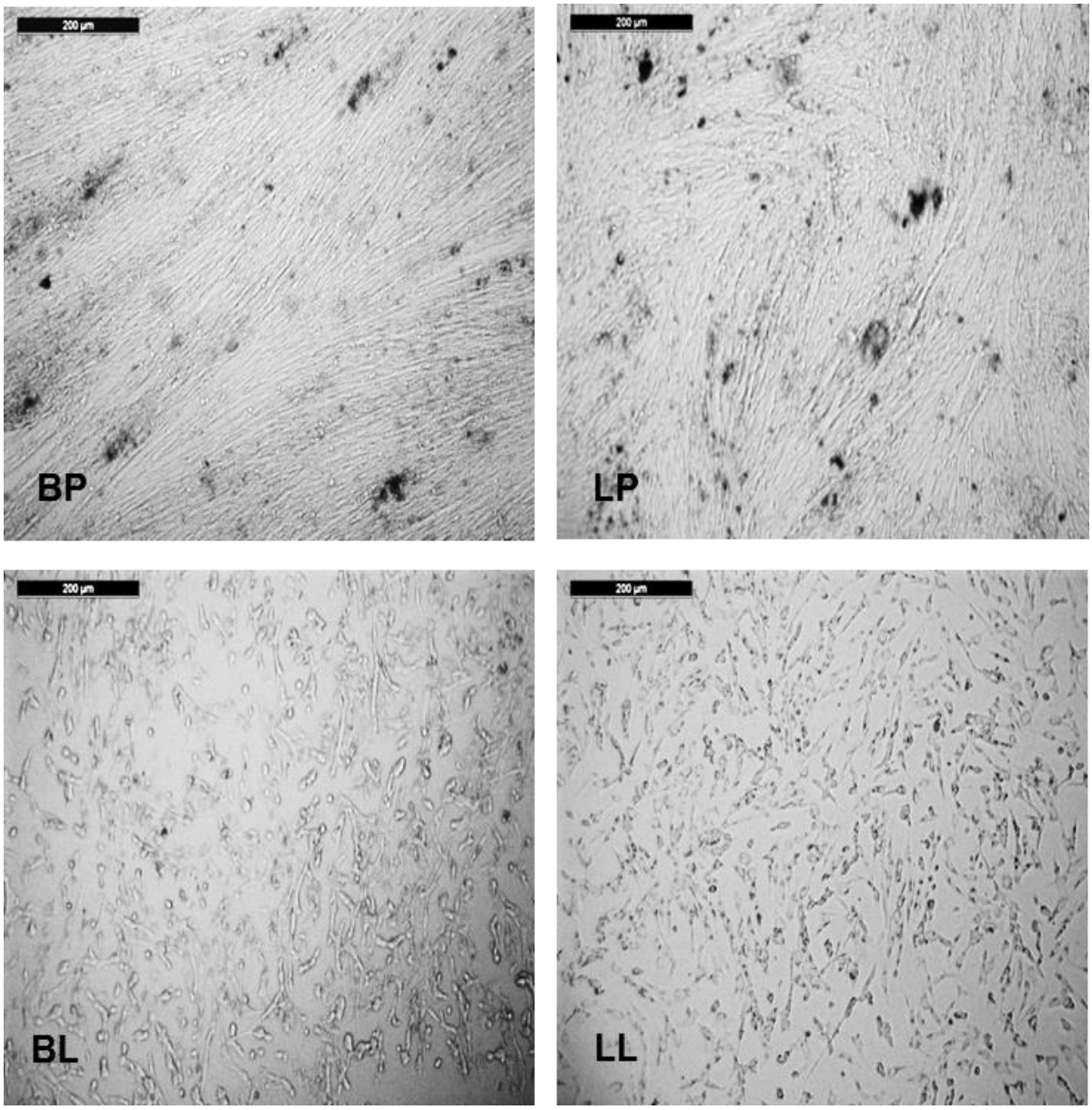


Figure 3.12. Phase-contrast micrographs showing the morphology of chicken satellite cells following 5 days in culture. SCs were isolated from the breast (pectoralis major) and leg (peroneus longus) muscles of broiler and layer chickens and maintained in GM. Micrographs were taken on the 5th day of incubation. Abbreviations: BP = broiler pectoralis major muscle; LP = layer pectoralis major muscle; BL = broiler peroneus longus muscle; LL = layer peroneus longus muscle; µm = micrometre.

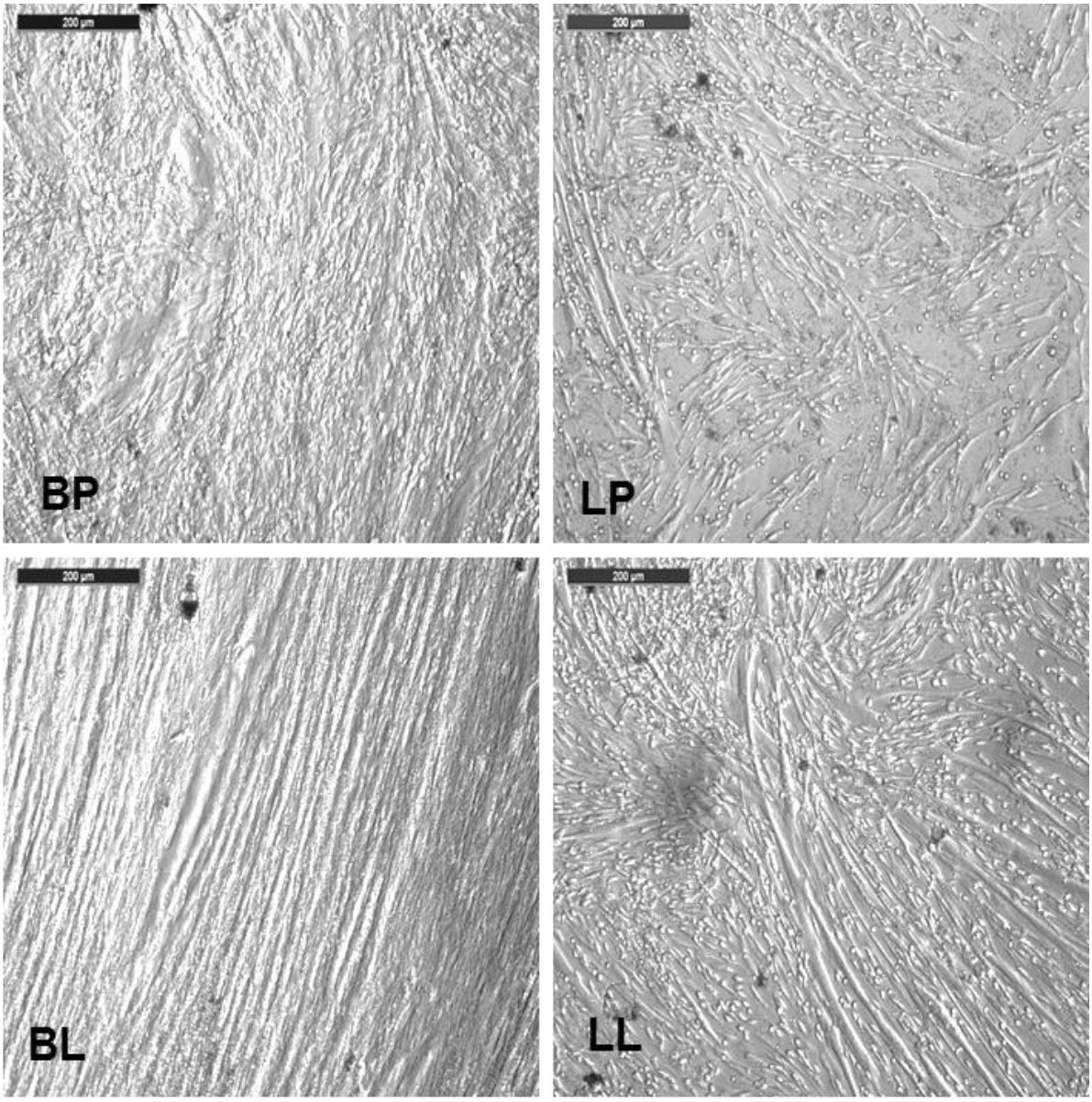


Figure 3.13. Phase-contrast micrographs showing the morphology of chicken satellite cells following 8 days in culture. SCs were isolated from the breast (pectoralis major) and leg (peroneus longus) muscles of broiler and layer chickens and maintained in GM. Micrographs were taken on the 8th day of incubation. Abbreviations: BP = broiler pectoralis major muscle; LP = layer pectoralis major muscle; BL = broiler peroneus longus muscle; LL = layer peroneus longus muscle; µm = micrometre.

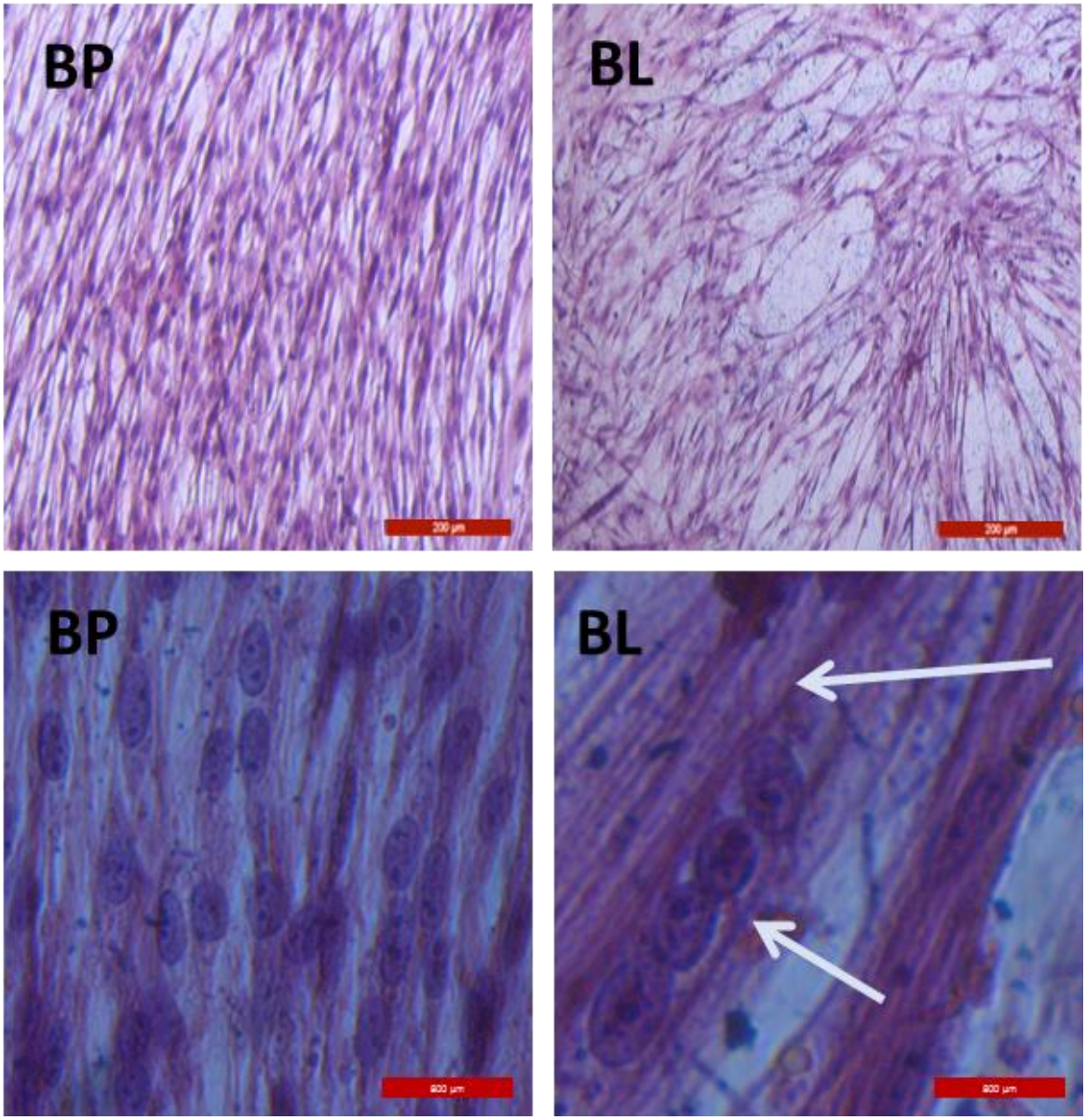


Figure 3.14. Phase-contrast micrographs of H&E-stained chicken satellite cells following 8 days in culture. SCs were isolated from the breast (pectoralis major) and leg (peroneus longus) muscles of broiler chickens and maintained in GM for 8 days. Arrows indicate the alignment of myonuclei into myotubes. Abbreviations: BP = broiler pectoralis major muscle; BL= broiler peroneus longus muscle; H&E = haematoxylin and eosin stain; µm = micrometre.

3.4.10. Assessing the expression of myogenic markers in chicken satellite cell cultures

We assessed the differentiation capacities of breast- and leg-derived SCs of broiler and layer chickens. The differentiation of SCs derived from the PM and PL muscles of both chicken breeds, was examined using the expression of three differentiation markers desmin, MyoD and MyHC-*fast*. Results indicated that the expression of these markers was density-dependent, as cultures seeded at a high density expressed more desmin when compared with cultures seeded at a medium density (Figure 3.15).

Results also revealed that MyoD was expressed to a greater extent in cultures derived from the leg (PL) muscles, when compared with breast (PM) derived ones (Figure 3.16). Desmin was also expressed to a greater extent in cultures derived from the leg muscles, when compared with breast-derived ones (Figure 3.17); and in cultures derived from the leg muscle of layer chickens when compared with cultures derived from the same muscle of broilers (Figure 3.18). Finally, MyHC-*fast* was highly expressed in cultures derived from the leg muscle of layer chickens, when compared with cultures derived from the breast muscle of the same breed and cultures derived from the breast and leg muscles of broilers (Figure 3.19).

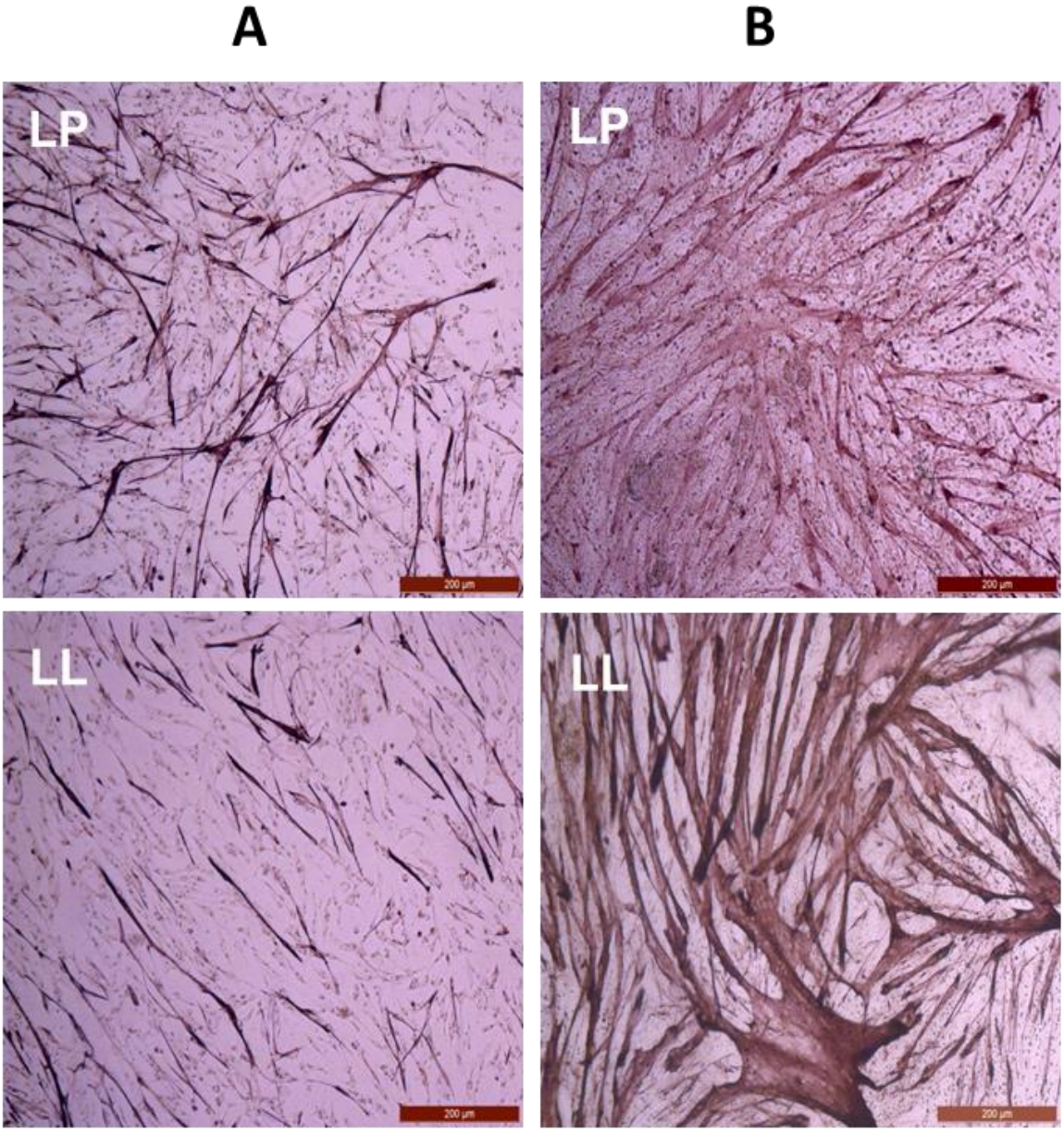


Figure 3.15. Expression of desmin in chicken satellite cultures seeded at different densities; assessed using indirect immunocytochemistry. SCs derived from the breast (pectoralis major) and leg (peroneus longus) muscles of layer chickens, were plated at medium (A) or high (B) densities and maintained in GM. Micrographs were taken on the 8th day of incubation. Abbreviations: LP = layer pectoralis major muscle; LL = layer peroneus longus muscle; µm = micrometre.

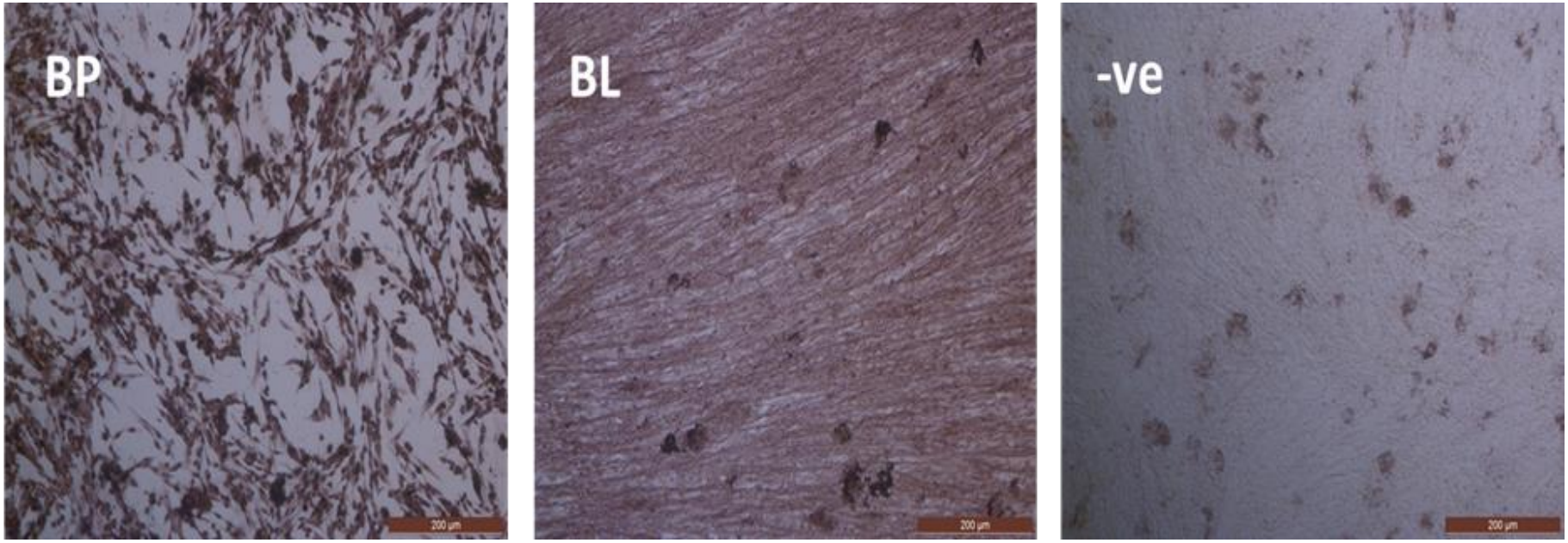


Figure 3.16. Expression of MyoD in chicken satellite cultures following 3 days in culture; assessed using indirect immunocytochemistry. SCs derived from the breast (pectoralis major) and leg (peroneus longus) muscles of broiler chickens were plated at a medium density and maintained in GM. Micrographs were taken on the 3rd day of incubation. Abbreviations: BP = broiler pectoralis major muscle; BL = broiler peroneus longus muscle; MyoD = myoblast determination protein 1; -ve = negative (no primary antibody) control; μm = micrometre.

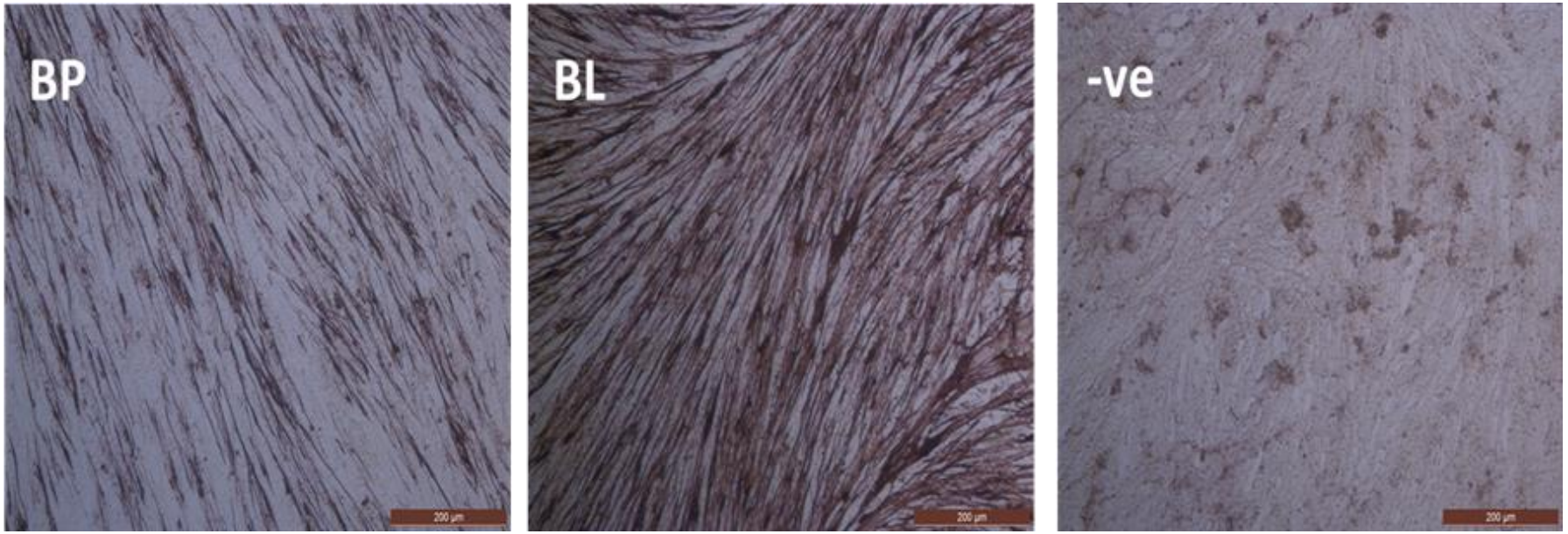


Figure 3.17. Expression of desmin in chicken satellite cultures following 3 days in culture; assessed using indirect immunocytochemistry. SCs derived from the breast (pectoralis major) and leg (peroneus longus) muscles of broiler chickens were plated at a medium density and maintained in GM. Micrographs were taken on the 3rd day of incubation. Abbreviations: BP = broiler pectoralis major muscle; BL = broiler peroneus longus muscle; -ve = negative (no primary antibody) control; µm = micrometre.

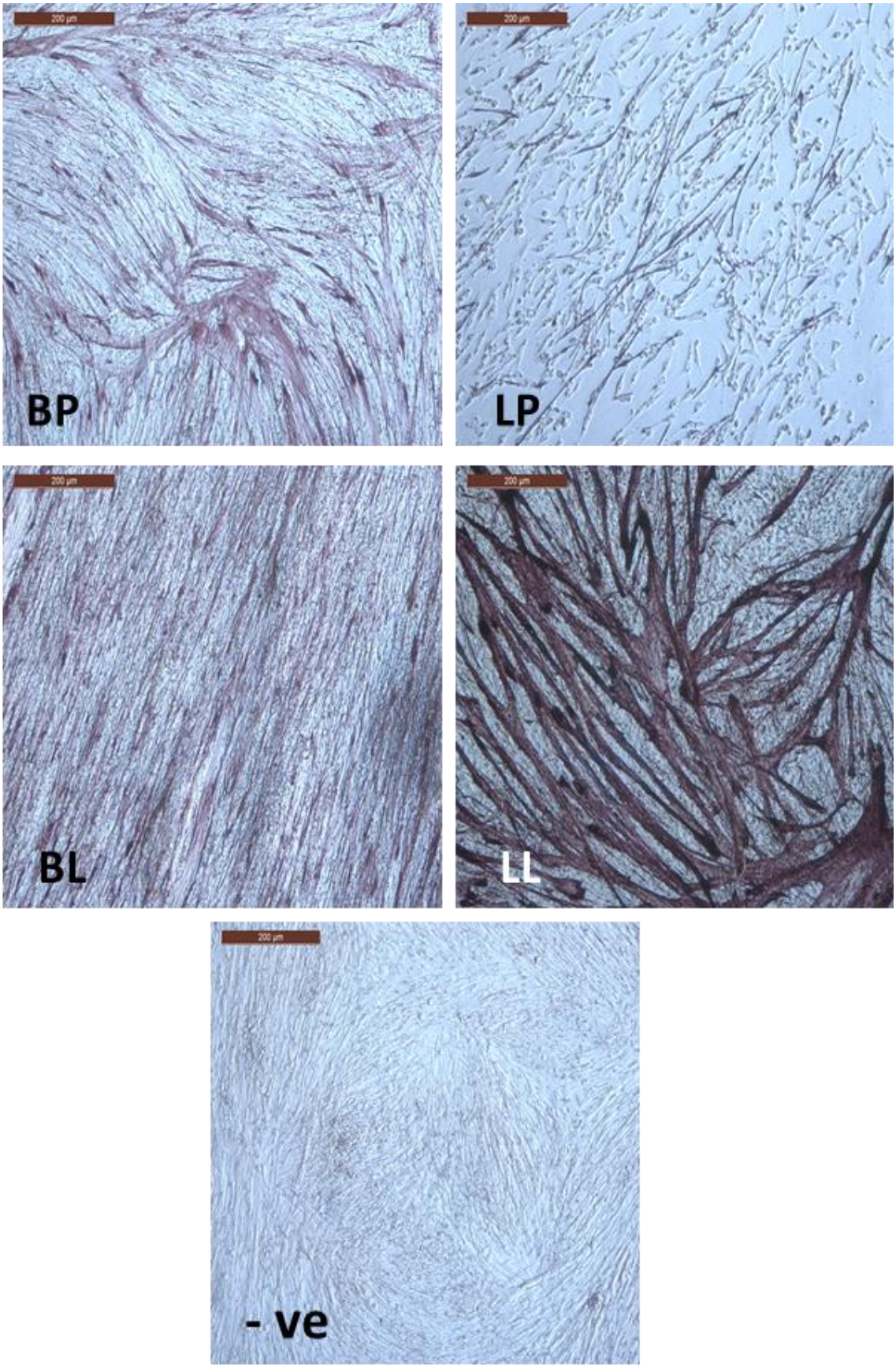

200 µm
BP
200 µm
LP
200 µm
BL
200 µm
LL
200 µm
- ve

Figure 3.18. Expression of desmin in chicken satellite cultures following 5 days in culture; assessed using indirect immunocytochemistry. SCs derived from the breast (pectoralis major) and leg (peroneus longus) muscles of broiler and layer chickens, were plated at a medium density and maintained in GM. Micrographs were taken on the 5th day of incubation. Abbreviations: BP = broiler pectoralis major muscle; LP = layer pectoralis major muscle; BL = broiler peroneus longus muscle; LL = layer peroneus longus muscle; -ve = negative (no primary antibody) control; μm = micrometre.

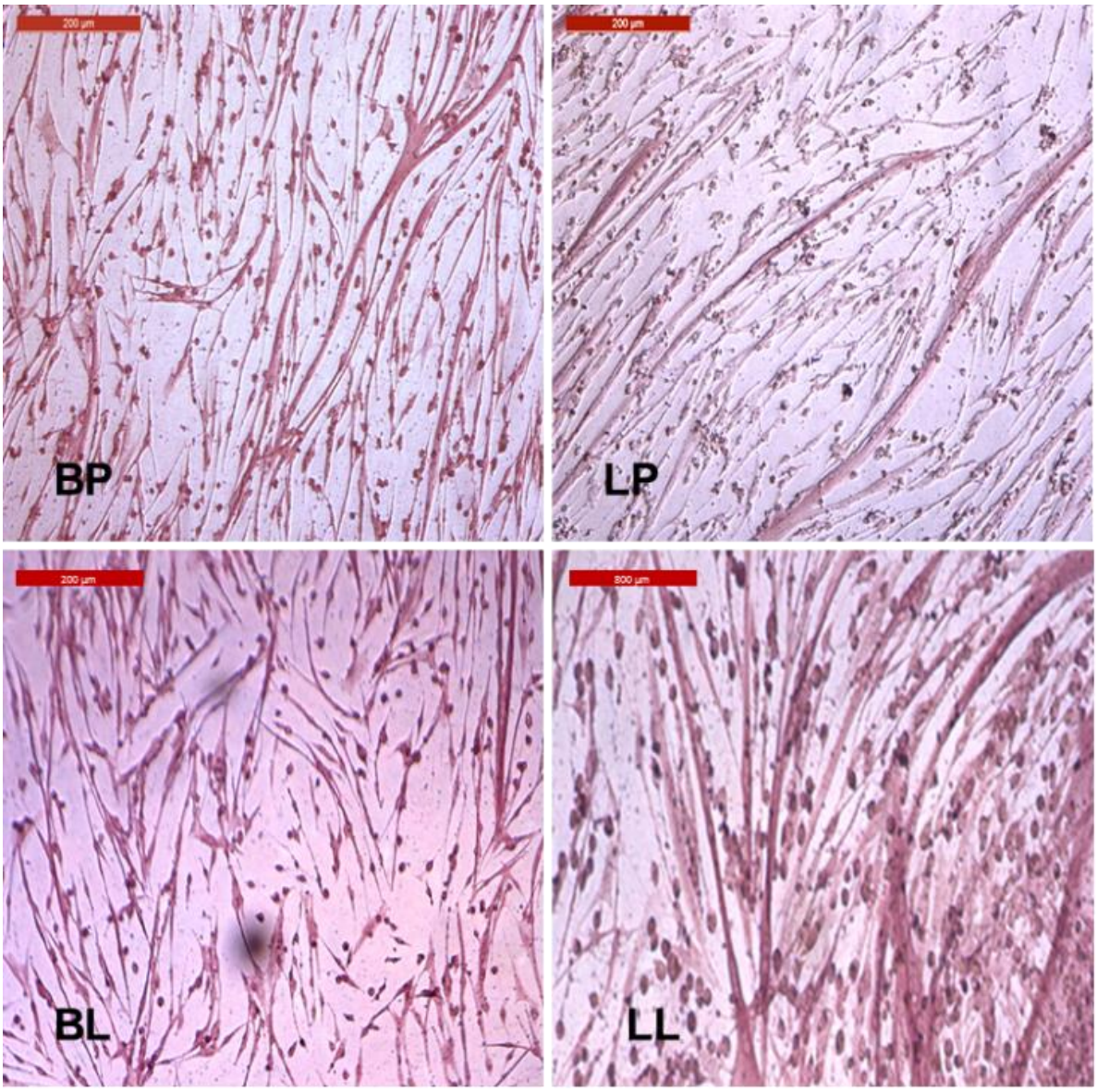


Figure 3.19. Expression of MyHC-*fast* in chicken satellite cultures following 8 days in culture; assessed using indirect immunocytochemistry. SCs derived from the breast (pectoralis major) and leg (peroneus longus) muscles of broiler and layer chickens were plated at a medium density and maintained in GM. Micrographs were taken on the 8th day of incubation. Abbreviations: BP = broiler pectoralis major muscle; LP = layer pectoralis major muscle; BL = broiler peroneus longus muscle; LL = layer peroneus longus muscle; µm = micrometre.

3.4.11. The impact of serum-restricted medium on chicken satellite cells in

We examined the impact of shifting SCCs derived from the breast (PM) and leg (PL) muscles of broiler chickens into serum-restricted DM. Results showed that shifting the leg-derived SCCs into DM resulted in myotube formation (Figure 3.20). A concomitant increase in the expression of myosin heavy chain-*fast* in these cultures was also observed (Figure 3.21). Conversely, cell loss and poor expression of myosin heavy chain-*fast* were the outcomes of shifting breast-derived SCCs into DM (Figures 3.20 and 3.21 respectively).

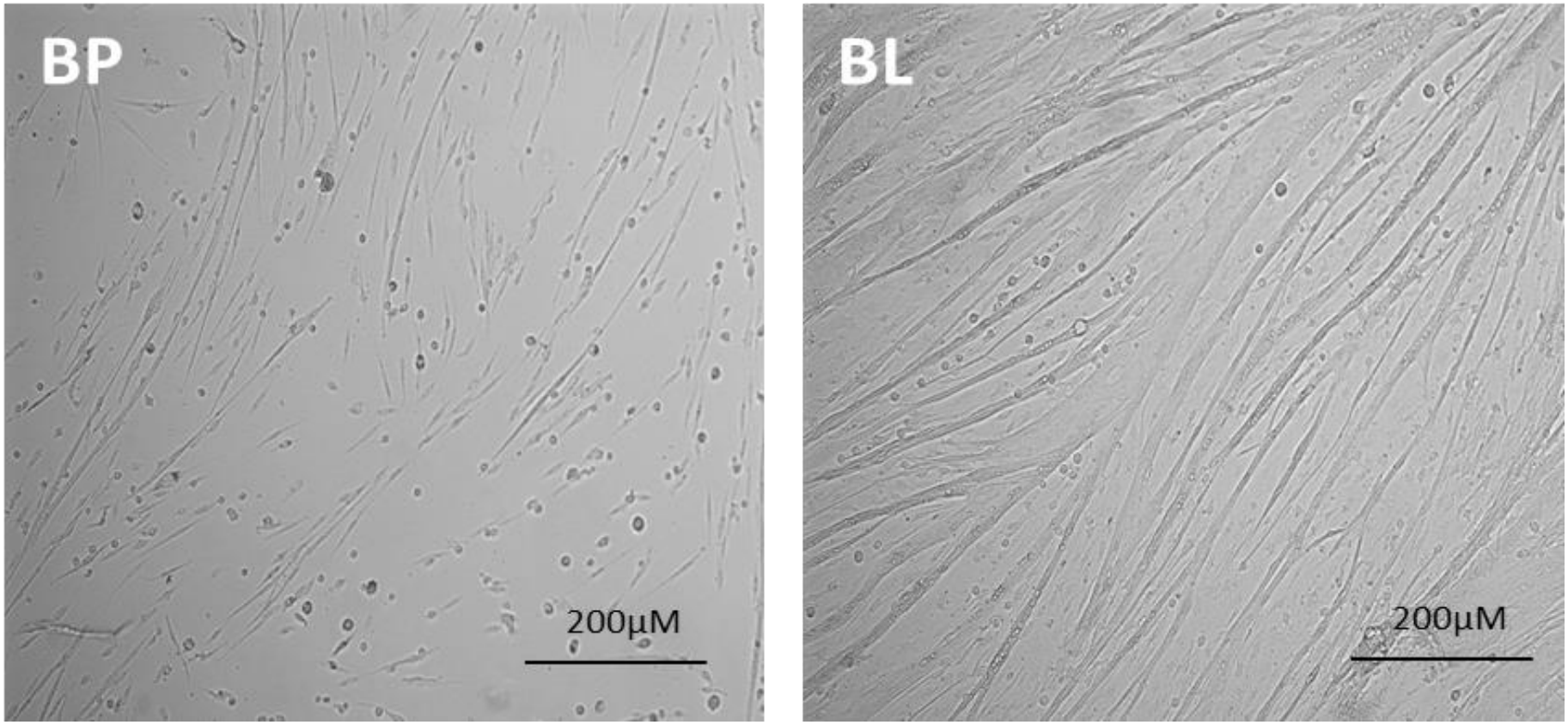


Figure 3.20. Phase-contrast micrographs depicting the morphology of chicken satellite cultures shifted into serum-restricted medium. SCs isolated from the breast (pectoralis major) and leg (peroneus longus) muscles of broiler chickens were expanded in GM until they reached ~70% confluence; then shifted into DM for 48 h. Abbreviations: BP = broiler pectoralis major muscle; BL = broiler peroneus longus muscle; µM = micrometre.

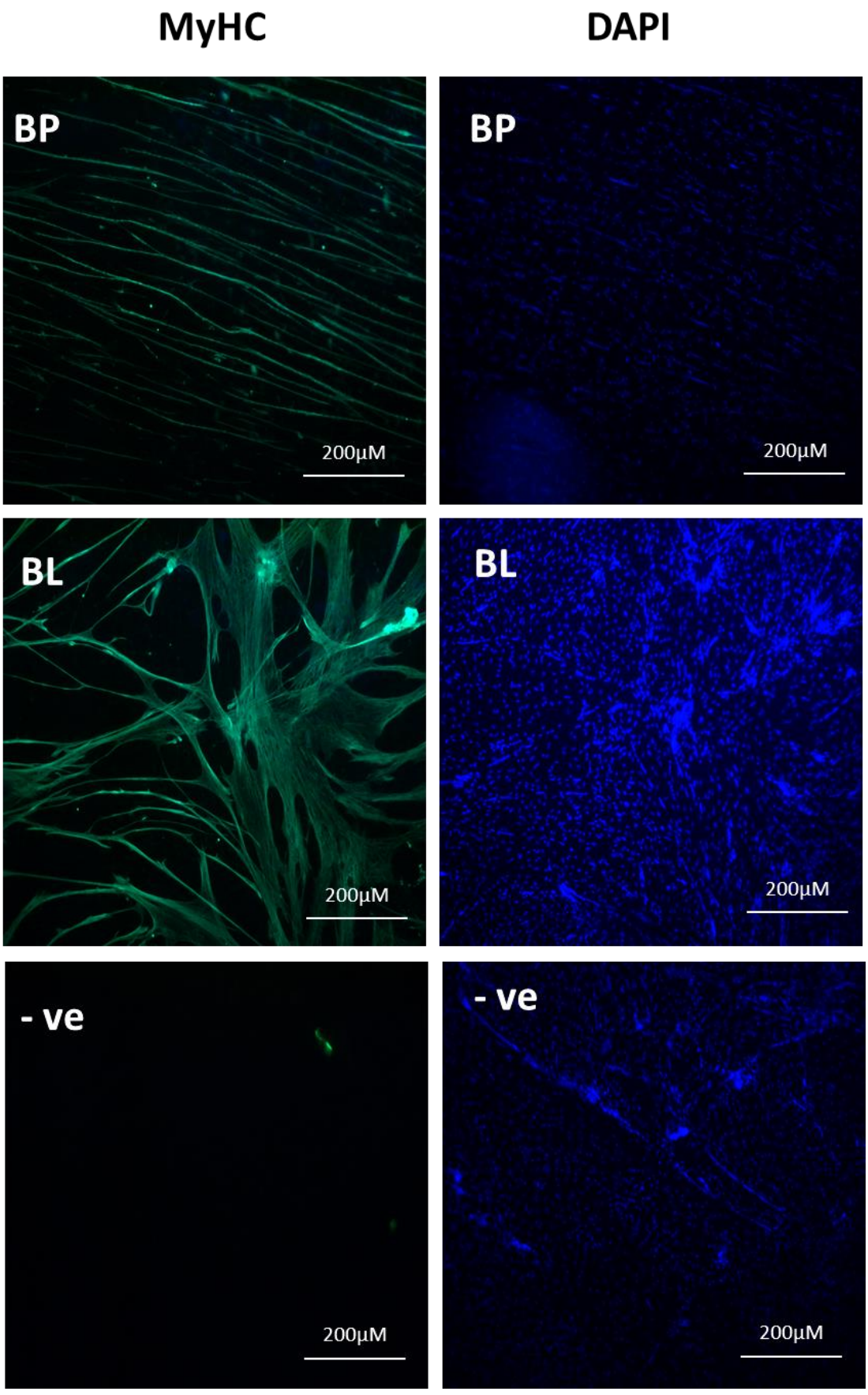

MyHC
DAPI
BP
200µM
BP
200µM
BL
200µM
BL
200µM
- ve
200µM
- ve
200µM

Figure 3.21. Expression of MyHC-*fast* in chicken satellite cultures shifted into serum-restricted medium; assessed using indirect immunofluorescence. SCs isolated from the breast (pectoralis major) and leg (peroneus longus) muscles of broiler chickens were expanded in GM until they reached ~ 70% confluence; then shifted into serum-restricted DM for 48 h. Abbreviations: BP = broiler pectoralis major muscle; BL = broiler peroneus longus muscle; MyHC = myosin heavy chain; -ve = negative (no primary antibody) control; µM = micrometre.

3.4.12. Assessing the effect of leucine on chicken satellite cells

The aim of this study was to assess the response of SCs, derived from selected (breast) and unselected (leg) muscles, to increasing concentrations of leucine. Results indicated that long-term exposure of leg-derived SCs to different concentrations of leucine enhanced the proliferation and differentiation of cells (Figure 3.22). Similarly, exposing SCs derived from the breast muscle to low concentrations (1 mM and 2 mM) of leucine enhanced their differentiation (Figure 3.23 B and C). However, long-term exposure of these cells to higher concentrations of leucine (3-, 4-and 5 mM) resulted in cell loss (Figure 3.23 D-F).

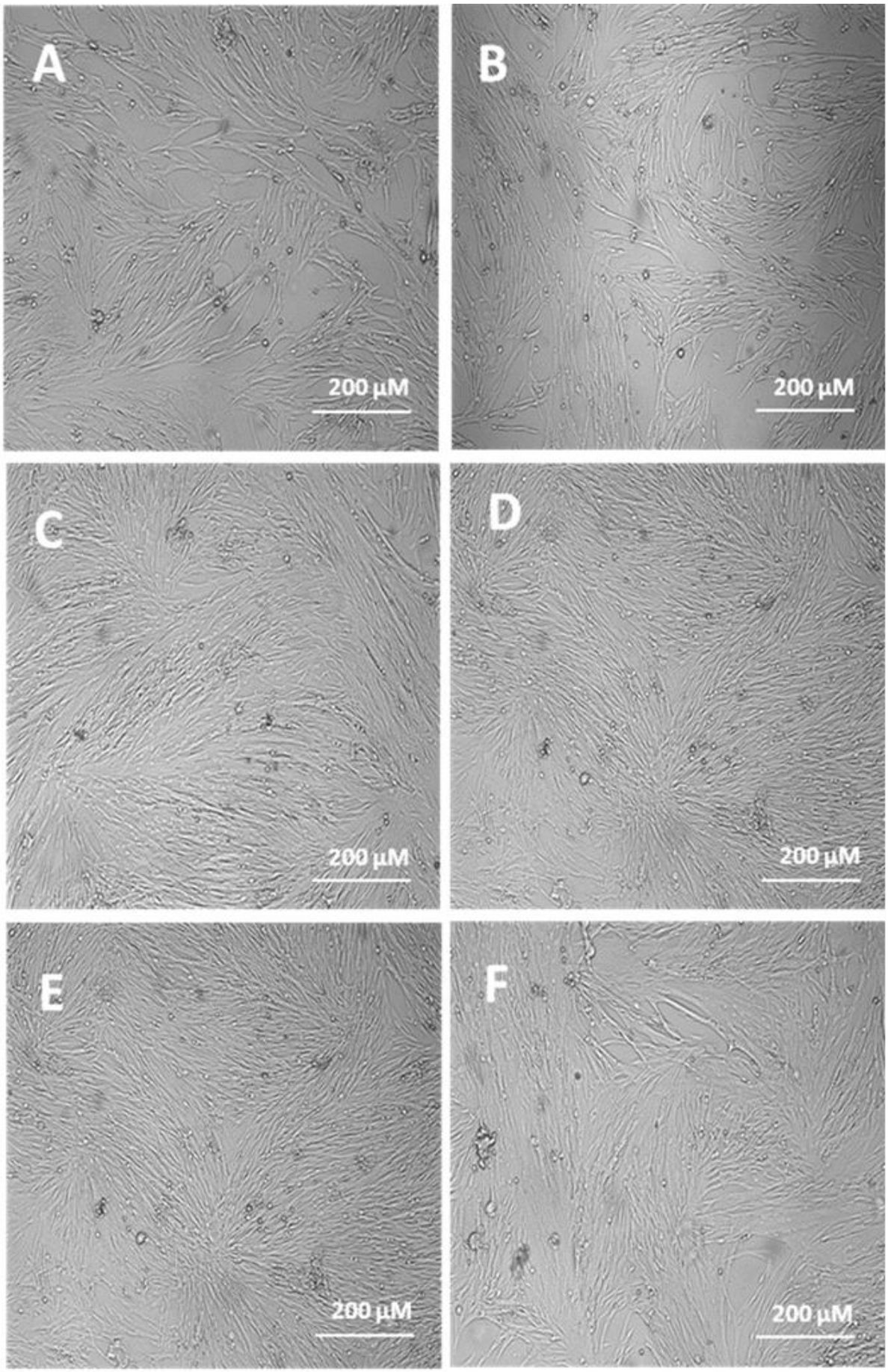

A
200 µM
B
200 µM
C
200 µM
D
200 µM
E
200 µM
F
200 µM

Figure 3.22. Phase-contrast micrographs of satellite cells derived from the leg muscle of broiler chicken after long-term exposure to increasing concentrations of leucine. SCs cells isolated from the leg (peroneus longus) muscles of broiler chickens were expanded in GM to ~60% confluence, then shifted into GM devoid of leucine (A), or supplemented with 1 mM (B), 2 mM (C), 3 mM (D), 4 mM (E) or 5 mM (F) of L-leucine for 24 h. Abbreviation: µM = micrometre.

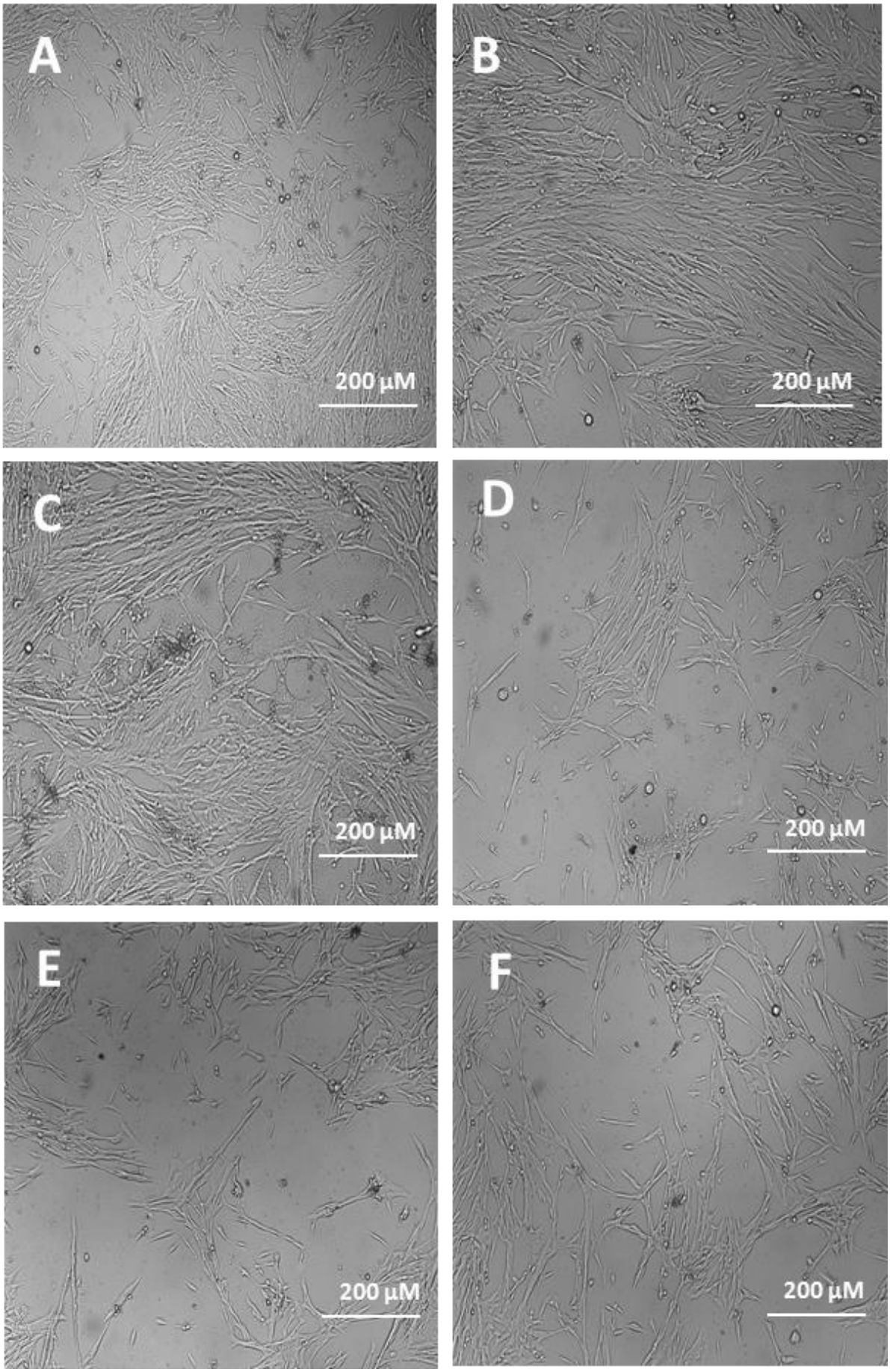

A
200 µM
B
200 µM
C
200 µM
D
200 µM
E
200 µM
F
200 µM

Figure 3.23. Phase-contrast micrographs of satellite cells derived from the breast muscle of broiler chicken after long-term exposure to increasing concentrations of leucine. SCs isolated from the breast (pectoralis major) muscles of broiler chickens were incubated in GM to ~ 60% confluent; then shifted into GM devoid of leucine (A), or supplemented with 1 mM (B), 2 mM (C), 3 mM (D), 4 mM (E) or 5 mM (F) of L-leucine for 24 h. Abbreviation: µM = micrometre.

3.5. Discussion

The divergent growth rate between broiler and layer chickens has been a subject of research in animal science for more than three decades. The fact that most muscle mass gain is postnatal (Mott and Ivarie, 2002; White *et al*., 2010), along with the key role of SCs during postnatal muscle growth (reviewed by Yablonka-Reuveni, 2011), suggests that this divergent muscle growth may be due to different proliferating and/or differentiating capacities of broilers and layers SCs.

The present work was undertaken to isolate and establish a sustainable culture system in order to evaluate and compare the proliferative and differentiation capacities of SCs isolated from the breast and leg muscles of young broiler and layer chickens. Published literature suggested that the highest growth phase of broiler chickens takes place from the second to the sixth week of age. It has also been reported that the weight of broilers was twice that of layers by the age of two weeks (Jones *et al*., 1986; Zheng *et al*., 2009). Additionally, a decline in the number of SCs with advancing age has been reported in chickens (Jones *et al*., 1986; Zheng *et al*., 2009).

For these reasons, SCs were isolated at the youngest age of maximum weight difference between broiler and layer chickens, which is two weeks. Also, SCs were isolated from male broiler and layer chickens that had been raised under the same housing conditions in order to minimise the influence of gender and environmental factors. Additionally, a leg muscle with the highest content of fast-twitch fibres (PL) was selected to be compared with the fast-twitch breast (PM) muscle, in order to minimise differences due to fibre type. Results of this work showed that the hatching weight of broiler chicken was higher than layers. This concurs with reports of a high hatching weight of broiler chicks, when compared with layers (Jones *et al*., 1986; Ohta *et al*., 2004; Sato *et al*., 2006; Druyan, 2010). Also, the weight of broiler chickens was higher than layers at the end of the first and second post-hatching weeks, which confirms reports of two-week-old broilers being double the weight of layers (Jones *et al*., 1986; Zheng *et al*., 2009). Modern broiler chickens feed on a diet that contains higher levels of protein than layers, the dietary requirements for broiler and layer chickens are listed in Appendix 12. Herein, both chicken breeds were fed on the same ration. Therefore, the remarkable differences in growth and body weight between broilers and layers cannot be attributed to diet. It has been suggested that even under optimum growth conditions, the post-hatching weight of layers will be lower than broilers, due to the genetic differences between the two breeds (Buzala and Janicki, 2016). Notably, selection for rapid growth has increased the feeding efficiency and

altered the systemic metabolism of broiler chickens (Julian, 2005; Buzała *et al.*, 2015). Therefore, it is plausible to propose that broilers were more efficient of utilising food for growth, and this could be the reason for the weight difference between broiler and layer chickens in the present study.

3.5.1. Optimising the isolation and cultivation of chicken satellite cells

SCs were isolated to a protocol previously described by Baquero-Perez and colleagues (Baquero-Perez *et al*., 2012). The efficiency of the isolation protocol was assessed using the expression of muscle-specific proteins. Results revealed that ~ 85% of isolated cells were muscle cells, indicating the efficiency of the isolation technique. However, the applied isolation technique had several limitations that needed to be addressed.

The first of these limitations was to determine the proper duration of enzymatic digestion of muscle samples. The isolation protocol of Baquero-Perez and colleagues recommended that chicken muscles need to be digested in Pronase (a powerful proteolytic agent) for 60 min. However, that protocol employed older chicken, whereas muscles in the present chapter were collected from young chicks, which contain less connective tissue. There was some concern that 60 min of enzymatic digestion may result in over digestion of muscles and negatively affect the viability of isolated cells. Therefore, muscle fragments were incubated with Pronase for 30, 45 or 60 min in order to evaluate the most efficient digestion time. Results indicated that 30 min of incubation in Pronase was insufficient for digestion of connective tissues and interfered with proper dissociation of muscle fibres. In contrast, both 45- and 60-min incubation in Pronase yielded viable cells that propagated well in culture.

The second limitation of this isolation protocol was the time-consuming and labour-intensive manual dissociation of muscle fibres to liberate their cellular contents. This prolonged process shifted the pH of the media and, consequently, undermined the viability and survival of isolated cells. To resolve this limitation, dissociation of muscle fibres by multiple passages through pipettes of different sizes, was replaced by the use of a gentleMACS homogeniser. Dissociating muscle fragments using the gentleMACS homogeniser yielded viable cells and was ten times faster than manual dissociation. However, the cell yield was much less than the manual method.

The third limitation of the published isolation protocol was the heterogeneous nature of isolated cells, which included, in addition to SCs, blood cells, fibroblasts, immune cells and myotube

fragments. This heavy contamination of isolated cells made cell counting a challenge and hampered the application of sensitive proliferation and differentiation assays. To overcome this, differential plating was undertaken for the purpose of enriching isolated SCs. It has been proposed that plating freshly isolated cell suspensions on uncoated culture vessels results in the attachment of non-myogenic cells, mostly fibroblasts, to the culture vessel's surface, consequently yielding a purer myogenic cell population (Rando and Blau, 1994; Qu-Petersen *et al*., 2002). However, in the present work, SCs retrieved from differential plating failed to grow properly in culture.

A further limitation was how to determine the optimal seeding density of isolated cells, as failure to grow and early differentiation were the outcomes of seeding cells at low and high densities, respectively. In the present work, accurately counting SCs was unfeasible due to the heavy contamination of freshly isolated cells with tissue debris and other cell contaminants. It is worth noting that enrichment of SCs by FACS was also unfeasible as the process relies on antibodies that react with surface antigens, whereas Pax7, a hallmark for CSCs, is intracellular. Despite this challenge, SCs numbers were approximated and seeded at three different (low, medium and high) densities and the growth of cultures was assessed morphologically. Our results indicated that cultures seeded at low density either failed to grow or failed to form a monolayer, while high density cultures differentiated very early. On the other hand, the best results have been obtained with cultures seeded at a medium density. These observations indicate that monitoring the proliferation and differentiation of SCs is best achieved in cultures seeded at medium densities.

Inducing the differentiation of cultured muscle cells is usually achieved by shifting cells to low/restricted-serum media (Blau *et al*., 1985). Here, shifting SCs from the leg muscles of broiler chicken into serum-restricted DM resulted in myotube formation and an increase in the expression of MyHC-*fast*. However, cell loss, poor myotube formation and weak expression of MyHC-*fast* were the outcomes of incubating breast-derived SCs in DM. This observation could be explained by previous reports indicating that SCs of animals selected for high growth are more prone to proliferation (Feldman and Stockdale, 1991; Dusterhoft and Pette, 1993; Molnar and Dodson, 1993; Merly *et al*., 1998). It is well known that selection of broiler chickens for high growth affects the breast, but not the leg, muscles of these birds. In the present work, SCs derived from the breast muscles of broiler chickens were shown to have an accelerated proliferation rate when compared with SCs derived from the leg muscles. This high

proliferation rate requires a serum rich environment, and this may be the reason behind the death of those cells when shifted into serum-restricted medium.

This observation also indicates the differences that exist among SCs of selected (breast) and non-selected (leg) muscles within the same chicken breed. Due to this unforeseen response of breast-derived SCs to DM, GM was used to support the proliferation and differentiation of cells. Differentiation of SCs in the same GM has previously been recommended (Shefer, *et al*., 2006). The differentiation of SCs in serum-rich GM occurs via contact inhibition (Tanaka *et al*., 2011).

Finally, sub-culturing and long-term storage of SCs were essential to maintain a continuous supply of these primary cells. Information on the freezing conditions and passaging of CSCs was lacking in the published literature. Therefore, different concentrations of Trypsin-EDTA were tested on broiler-derived SCs. Results indicated that both 1X and 0.5X trypsin reduced the viability of SCs, indicated by the failure of harvested cells to grow in culture. However, a low trypsin concentration (0.05X Trypsin-EDTA) was effective for sub-culturing these primary cells and preserving their viability. The unfeasibility of multiple passaging of CSCs was another drawback of these primary cultures. Here, sub-culturing SCs yielded unviable cells that did not grow in culture. This finding concurs with reports that multiple sub-culturing of SCs yields non-myogenic, mainly fibroblast cells (Yablonka-Reuveni *et al*., 1987).

Long-term storage of SCs was crucial, a fresh supply of chicken muscles for each experiment is both expensive and time-consuming. Long-term storage of SCs in liquid nitrogen was attempted using traditional high-serum FM with DMSO, which has been previously used to preserve other primary cells (Smith, 1967). However, resuscitation and growth of SCs derived from the leg muscles failed. On the other hand, breast-derived SCs frozen in that same medium grew in culture, but only after a lag phase of 4 days after retrieving them from liquid nitrogen; however, their proliferation rate was also reduced. Therefore, a commercial FM, specific for primary stem cells, was used. This specific medium preserved the viability of both breast- and leg-derived SCs.

3.5.2. Comparing the proliferative and differentiation capacities of chicken satellite cells

After addressing the aforementioned challenges, the next step was to evaluate and compare the proliferative capacities of SCs isolated from the breast and leg muscles of broiler and layer

chickens. Unfortunately, using quantitative assays to determine the proliferation of SCs was not applicable, as the heterogeneous nature of isolated cells (cell debris, myofibrillar fragments, fibroblasts, blood and immune cells) hampered the accurate counting of SCs. The difficulty in counting SCs from a given muscle sample has been previously reported (Young *et al*., 1978; Blau and Webster, 1981; Matsuda *et al*., 1983). This also impaired the assessment of various physical and pharmacological treatments of primary SCs (Yablonka-Reuveni *et al*., 1987).

Therefore, the proliferation of SCs in the current study was assessed morphologically. Our results indicated that SCs derived from the breast muscle (PM) of broiler chickens were more proliferative, when compared with SCs isolated from the breast muscles of layer chickens and the leg muscles of both chicken breeds. This observation agrees with our hypotheses that the proliferative capacities of SCs derived from chickens selected for high growth (broilers) and chickens with normal growth rate (layers), and SCs derived from selected (breast) and unselected (leg) muscles of the same bird have been altered. It is also consistent with reports of enhanced proliferation capacities of SCs derived from animals selected for high growth, when compared with SCs of unselected animals (Knizetova *et al*., 1972; Penney *et al*., 1983; Brown and Stickland, 1994; Rehfeldt *et al*., 2002). Contrarily, SCs isolated from the leg (PL) muscles of both chicken breeds fused and differentiated at higher rates than breast-derived cells. Also, the fusion rate among leg-derived SCs was higher in layer cultures than in broiler ones. These observations support our initial hypothesis that selection for high growth has altered the differentiation capacities of broiler and layer SCs. They also agree with previous findings of high fusion rates, concomitant with more nuclei per myotube, in primary cultures derived from the skeletal muscles of layer embryos, when compared with broilers (Orcutt and Young, 1982).

In the present work, the temporal expression of desmin, MyoD and MyHC proteins was investigated to evaluate the differentiation of CSCs. Interestingly, both desmin and MyoD were expressed in proliferative myoblasts, but they are required for the early commitment of myoblasts to differentiation. The role of desmin, an intermediate filament, in muscle differentiation is not fully defined. However, it is known to promote differentiation of muscle cells by enhancing the fusion of myoblasts (Smythe *et al.,* 2001). In support of this, inhibition of desmin in differentiating C2C12 cultures halted myoblast fusion and myotube formation (Li *et al*., 1994). Similarly, the inhibition of MyoD in C2C12 cultures prevented myoblasts from exiting the cell cycle following the withdrawal of serum, and halted their differentiation into myotubes (Langley *et al*., 2002). MyHC is a late differentiation marker whose expression is a

hallmark of the differentiation of post-mitotic myocytes to form multinucleated myotubes (Andres and Walsh, 1996). Results from the present work showed that desmin, MyoD and MyHC-*fast* were highly expressed in leg-derived cultures, when compared with breast-derived ones and in layers when compared with broilers. The high expression of these markers in leg-derived cultures is essential for a normal function of leg muscles, while the low expression of these markers in broilers is consistent with increased incidence of leg deformities and lameness of these chickens when compared with layers (Julian, 1998; Julian, 2005, Jones *et al*., 2005). This finding is a further support to our hypothesis that selection for high growth has altered the differentiation capacities of broiler and layer SCs. It also supports our second hypothesis about how selection for high growth has altered the differentiation capacities of breast- and leg-derived SCs of the same bird.

3.5.3. Evaluating the response of breast- and leg-derived chicken satellite cells to leucine supplementation

Broiler chickens feed on a diet that contains higher levels of leucine than layers. Leucine is a BCAA that has been reported to increase the proliferation of, and protein synthesis in, muscle cells (Chen *et al*., 2013). Previously published literature suggested that SCs of chickens selected for high growth are more sensitive to mitogens than those of non-selected chickens (Duclos *et al*., 1996). A role for muscle fibre type in mediating the response of SCs to leucine has been also suggested in which leucine increased the rate of MPS in slow-twitch (soleus and diaphragm) muscles of rats (Buse, 1981). However, it did not alter the rate of MPS in a fast-twitch (gastrocnemius) muscle (Buse, 1981).

In the current study, long-term incubation with all leucine concentrations stimulated the proliferation and differentiation of leg-derived SCs. A finding that agrees with those of a previous study where leucine increased the proliferation and differentiation of preterm rat SCs (Dai *et al*., 2015). However, only low concentrations of leucine (1 mM and 2 mM) accelerated the proliferation of breast-derived SCs. Notably, higher concentrations of leucine were not tolerated by breast-derived SCs in which long-term incubation in 3-, 4- and 5 mM leucine resulted in cell loss. These results agree with our third hypothesis that breast- and leg-derived SCs respond differently to nutritional stimuli. They also indicate that the proliferation of breast-derived SCs was maximally stimulated at short-term exposure to leucine and long-term

exposure of these cells to higher concentrations of leucine resulted in cell death. This observation emphasises the differential response of SCs derived from selected (i.e., breast) and unselected (i.e., leg) muscles to the anabolic properties of leucine; and further work is warranted to determine the mechanism(s) that underlie this differential response. Unfortunately, due to the repeated contamination of SCCs with mycoplasma, which failed to clear even with the use of MycoZap^{TM} antibiotic, further work on primary chicken cells was terminated.

3.6. Conclusion

In summary, this chapter optimised the culture conditions required for the cultivation of SCs isolated from the breast and leg muscles of young broiler and layer chickens. This chapter also compared the proliferation and differentiation capacities of these different populations of primary cells. Results showed that cells derived from the breast muscle of broiler chickens were more proliferative, compared to the other populations of SCs. On the other hand, cells derived from the leg muscle of layers had the highest capacity of differentiation. Most importantly, the findings of this chapter have shown the differences between SCs derived from the breast and leg muscles of the same bird. Here, the effect of long-term exposure of broiler breast- and leg-derived SCs to increasing concentrations of leucine was evaluated. Results showed that high concentrations of leucine increased the differentiation of leg- but not breast-derived SCs.

4. Optimising experimental protocols to evaluate the effects of leucine on C2C12 cells

4.1. Introduction

Leucine is a BCAA with a well-documented role in enhancing protein synthesis within muscle cells, both *in vivo* and *in vitro* (Anthony *et al*., 2001; Kimball and Jefferson, 2001; Layman, 2003; Lynch *et al*., 2003; Lang and Frost, 2005; Norton and Layman, 2006; Schmelzle and Hall, 2000; Hara *et al*., 2002). However, most studies that have investigated the effect of leucine on primary and established muscle cell lines tended to evaluate its effect on differentiated myotubes rather than proliferating myoblasts. Additionally, starving cells of serum prior to leucine treatment and assessing the effects of leucine under serum-free or reduced-serum conditions were common regimens in most leucine studies (Mao *et al*., 2015; Zhang *et al*., 2014, Kim *et al*., 2009; Huang *et al*., 2013). Nevertheless, the effect of serum restriction on the viability and protein content of muscle cells has been understudied. The effect of leucine on proliferating myoblasts also remains unclear, as does the role of serum in potentiating the anabolic effects of leucine on muscle cells.

Serum is rich in nutrients, growth factors, hormones, attachment factors, binding proteins, cytokines, enzymes and protease inhibitors that are essential for supporting the growth, proliferation, differentiation and metabolism of cultured cells (Ham and McKeehan, 1979; Bettger and McKeehan, 1986; Butler and Jenkins, 1989; Brunner *et al*., 2010). However, serum starvation has been a valuable experimental procedure in many biological studies. Current opinion is that serum withdrawal decreases the basal metabolic activities of cells (Codeluppi *et al*., 2011) and generates a homogenous population of rapidly dividing cells (Pirkmajer and Chibalin, 2011) by synchronizing the mitosis of all cells at the G0/G1 phase (Khammanit *et al*., 2008; Pontarin *et al*., 2011). Serum withdrawal is a popular procedure especially in molecular biology and metabolic research because of the less defined nature of serum, which may confound the interpretation of results (Aghababazadeh and Kerachian, 2014).

Many studies have reported on the catabolic outcomes of serum withdrawal on cells (Smith *et al*., 1999; Khalyfa *et al*., 2005; Goyeneche *et al*., 2006; Maldonado and Muñoz-Pinedo; 2011). However, starving cells of serum prior to, and during, leucine treatment remains a prerequisite

in most studies. With this in mind, the short- and long-term effects of serum deprivation on proliferating and differentiated C2C12 cells were investigated in the present study.

Dialysed FCS has been proposed as an alternative to normal FCS in many biological studies. Many studies have tested the effect of leucine on proliferating muscle cells in media supplemented with dialysed FCS. The removal of small molecules such as glucose, salts, cytokines, AAs and hormones during the dialysis of FCS undermines its ability to support the growth and proliferation of metabolically demanding myoblast cells. Hence, it was important to examine the effect of dialysed FCS on the viability, proliferation, survival and protein content of cultured C2C12 myoblasts in the present study.

The protein anabolic properties of leucine have been suggested to function only under optimal conditions. This was inferred from the failure of leucine to induce protein synthesis in cultured C2C12 myotubes under serum-free and amino acid-free conditions (Areta *et al*., 2014). Based on this fact, assessing protein accumulation in C2C12 cells following exposure to leucine in serum-free, serum-restricted and serum-enriched media, was investigated in the present study.

Finally, despite glutamine being a non-essential AA, it remains crucial for culturing many eukaryotic cells (Ehrensvard *et al*., 1949; Eagle *et al*., 1956). Glutamine supports the synthesis of DNA, RNA and protein within cells (Ko *et al*., 1993) and most culture media are supplemented with glutamine at higher levels than other AAs (Newsholme, 2001). The interdependence between glutamine and leucine metabolism is well-established (Holeck, 2002). However, many studies chose to evaluate the effect of leucine in the absence of glutamine. Therefore, the present study aimed to examine the short- and long-term impact of glutamine deprivation on the viability of C2C12 cells.

The overall aim of this chapter was to optimise the experimental conditions to attain the anabolic effects of leucine on C2C12 cells. This included identification of the best starvation strategy and the most efficient media, to preserve cell viability and support protein accumulation in proliferating (myoblasts) and differentiated (myotubes) C2C12 cells during leucine treatment. To achieve this, the viability, vitality and the total protein content of C2C12 cells were examined in response to different starvation protocols, incubation in media with variant serum contents and exposure to different concentrations of leucine added to media with different serum compositions.

4.2. Research hypotheses

Serum has an essential role in maintaining the growth and function of many primary and continuous cell lines *in vitro* (Ham and McKeehan, 1979; Bettger and McKeehan, 1986; Butler and Jenkins, 1989; Brunner *et al*., 2010). Several reports associated serum restrictions with cell necrosis, protein loss and apoptosis (Smith *et al*., 1999; Khalyfa *et al*., 2005; Goyeneche *et al*., 2006; Pirkmajer and Chibalin, 2011; Maldonado and Muñoz-Pinedo; 2011). The impaired ability of leucine to induce and/or sustain protein synthesis under restricted serum conditions has also been documented (Wilson *et al*., 2010; Areta *et al*., 2014). Therefore, it is reasonable to hypothesise that short-term serum starvation, prior to leucine exposure and long-term incubation of C2C12 cells in serum-free media, during exposure to leucine, will adversely affect the viability, survival and protein content of these cells, incubation of C2C12 cells in reduced-serum medium will adversely affect the viability, survival and protein content of these cells and serum restriction will disrupt the anabolic effects of leucine on C2C12 cells.

4.3. The aims of this chapter

1. To evaluate the impact of different starvation regimens on the viability and protein content of C2C12 cells.

2. To evaluate the impact of short- and long-term incubation in reduced-serum and serum-free media on the viability and total protein content of C2C12 cells.

3. To evaluate the viability and total protein content of C2C12 cells post-exposure to leucine added to media with different serum contents.

4.4. Results

4.4.1. Long-term glutamine deprivation enhances the viability of C2C12 myoblasts

The aim of this experiment was to establish the optimal conditions for testing the anabolic effect of leucine on proliferating C2C12 cells. To this purpose, the impact of short- and long-term

glutamine deprivation on the viability of proliferating C2C12 cells was evaluated and compared with the viability of cells that had not been deprived of glutamine (control). Results indicated that cell viability was not altered by short-term glutamine deprivation (Figure 4.1 A and B). However, a significant increase ($P < 0.05$) in the viability of C2C12 myoblasts was recorded following long-term glutamine deprivation (Figure 4.1 C).

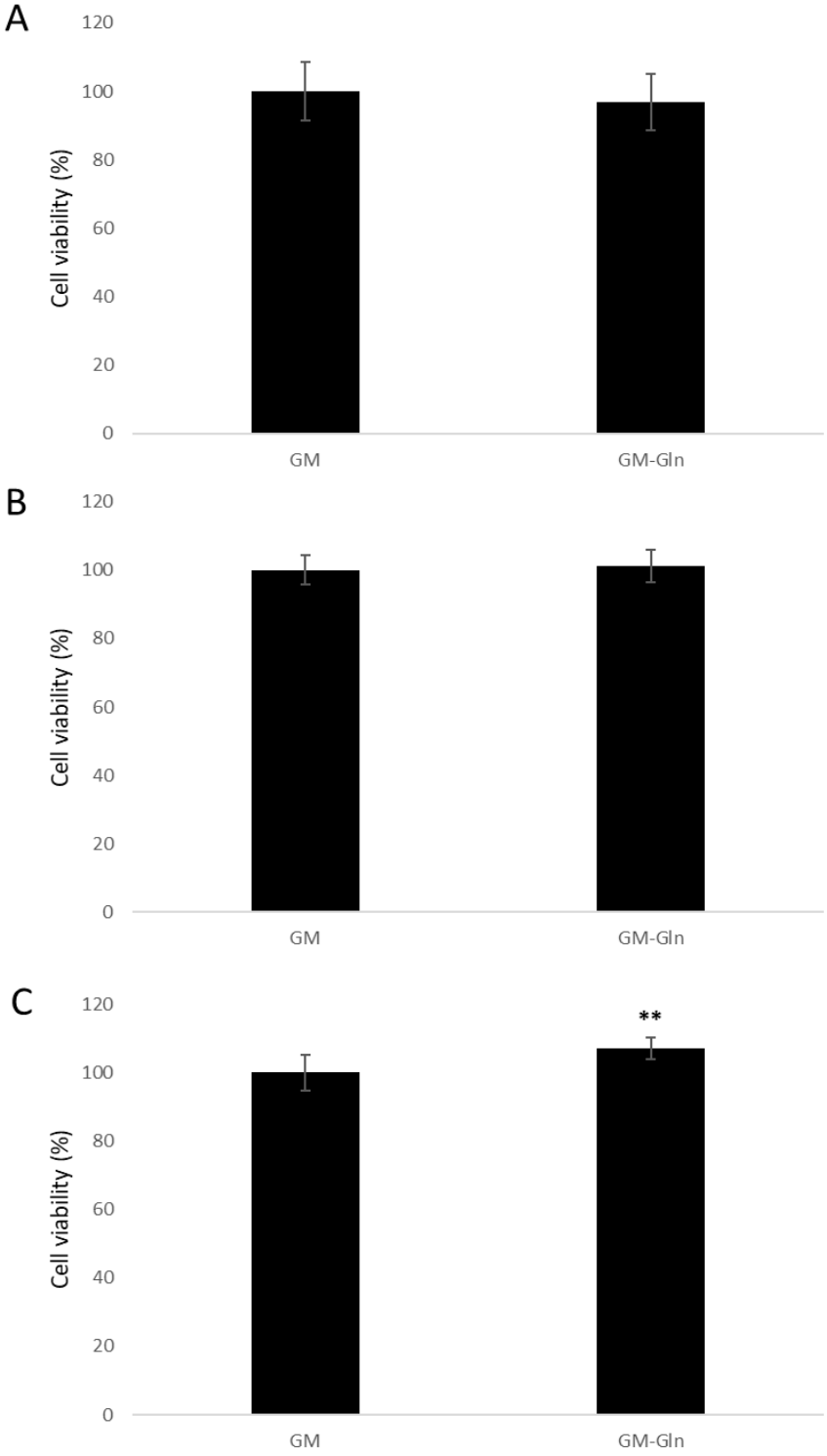


Figure 4.1. The viability of C2C12 myoblasts following short- and long-term glutamine starvation (x-axis); assessed using an AlamarBlue viability assay. C2C12 myoblasts were incubated in GM or GM-Gln for 1- (A), 4- (B) or 24- (C) h. Data are presented as means ± SD of three biological replica (n = 3). Asterisks indicate significant differences compared to the control (GM) $^{**}P < 0.01$. Analysed by a paired two-tailed Student's t-test using Microsoft Excel. Abbreviations: GM = growth medium supplemented with glutamine; GM-Gln = growth medium devoid of glutamine, Gln = L-glutamine.

4.4.2. Long-term incubation in serum-free medium undermines the viability of C2C12 myoblasts

The aim was to determine a feasible starvation regimen that does not negatively impact the viability of proliferating C2C12 cells. The viability of proliferating C2C12 cells was evaluated after short- and long-term incubation in SM. There was no change in viability following 1 h of serum withdrawal (Figure 4.2 A). On the other hand, a statistically significant increase in viability was recorded after 4 h of serum starvation ($P < 0.001$) (Figure 4.2 B). However, long-term serum starvation resulted in a significant decrease in cell viability ($P < 0.05$) (Figure 4.2 C).

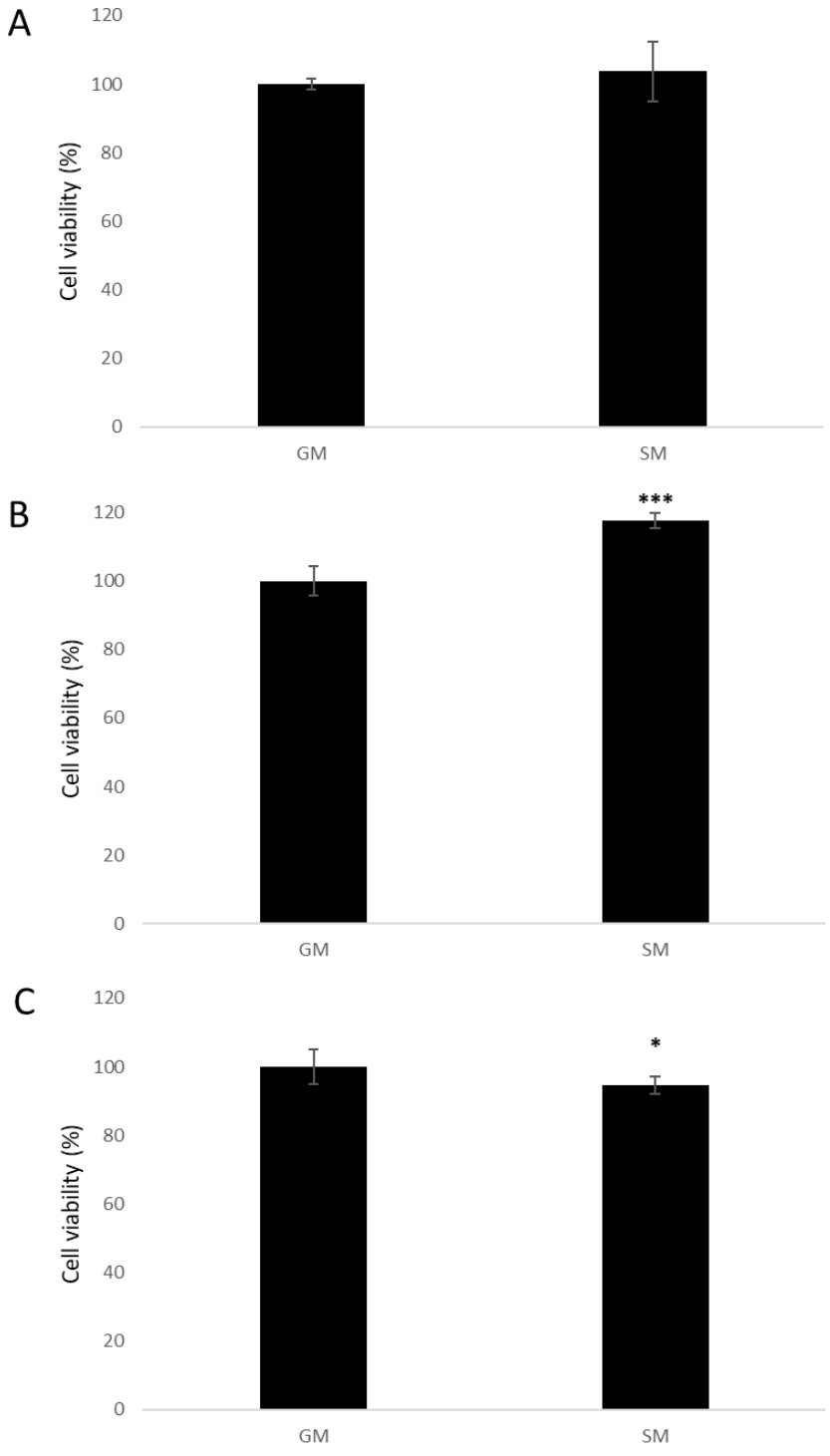


Figure 4.2. The viability of C2C12 myoblasts following short- and long-term serum starvation (x-axis); assessed using an AlamarBlue viability assay. C2C12 myoblasts were incubated in GM or SM for 1- (A), 4- (B) or 24 h (C). Data are presented as means ± SD from three biological replica (n = 3). Asterisks indicate significant differences compared to the control (GM) $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$. Analysed by a paired two-tailed Student's t-test using Microsoft Excel. Abbreviations: GM = growth medium supplemented with FCS serum; SM = serum-free medium; FCS = foetal calf serum.

4.4.3. Dialysed foetal calf serum reduces the viability of C2C12 myoblasts

We investigated the most suitable medium to support the viability of proliferating C2C12 cells. The impact of short- and long-term incubation of proliferating C2C12 cells in dialysed FCS on viability was assessed and compared to the viability of cells incubated in FCS (control). Results revealed a significant decline in cell viability following 1- ($P < 0.05$), 4- ($P < 0.01$) and 24- ($P < 0.05$) h of incubation in dGM from the control (Figure 4.3).

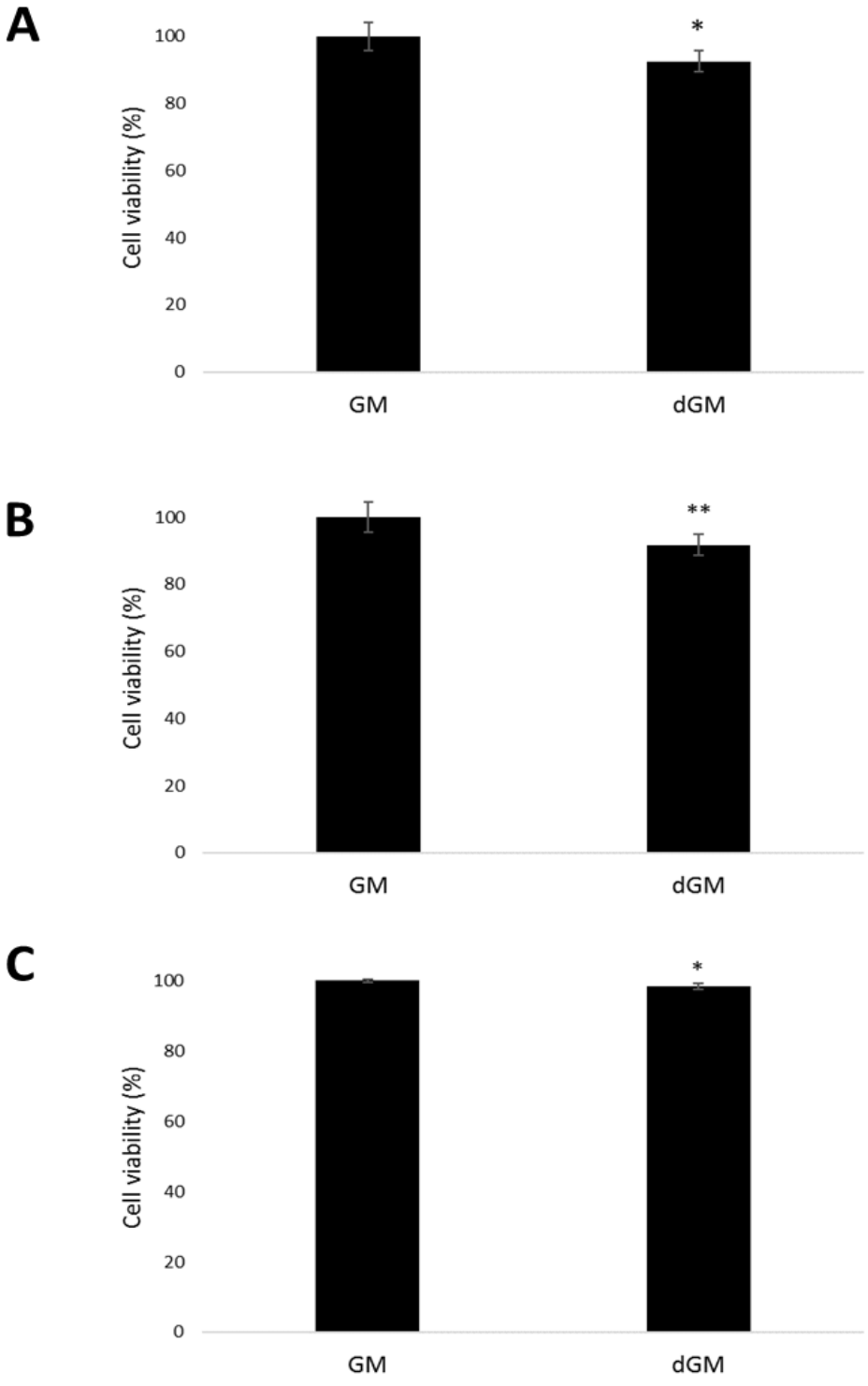


Figure 4.3. The viability of C2C12 myoblasts following short- and long-term incubation in dialysed foetal calf serum (x-axis); assessed using an AlamarBlue viability assay. C2C12 myoblasts were incubated in GM or dGM for 1- (A), 4- (B) or 24- (C) h. Data are presented as means ± SD from three biological replica (n = 3). Asterisks indicate significant differences compared to the control (GM) $^{*}P < 0.05$ and $^{**}P < 0.01$. Analysed by a paired two-tailed Student's t-test using Microsoft Excel. Abbreviations: GM = growth medium supplemented with FCS; dGM = growth medium supplemented with dialysed FCS; FCS = foetal calf serum.

4.4.4. Serum is essential for preserving the viability of C2C12 myoblasts over time

We examined the most suitable medium to support the viability of proliferating C2C12 cells, by comparing the impact of short- and long-term incubation of C2C12 myoblasts in media with different serum compositions. Results indicated a significant decline in the viability of cells maintained in dialysed FCS, when compared to cells maintained in FCS, at all tested time points (Figure 4.4). A transient increase in viability occurred following 4 h of serum withdrawal ($P < 0.05$) (Figure 4.4 B). However, long-term incubation (24 h) in SM significantly decreased the viability of C2C12 myoblasts compared to cells maintained in GM ($P < 0.001$) (Figure 4.4 C and D).

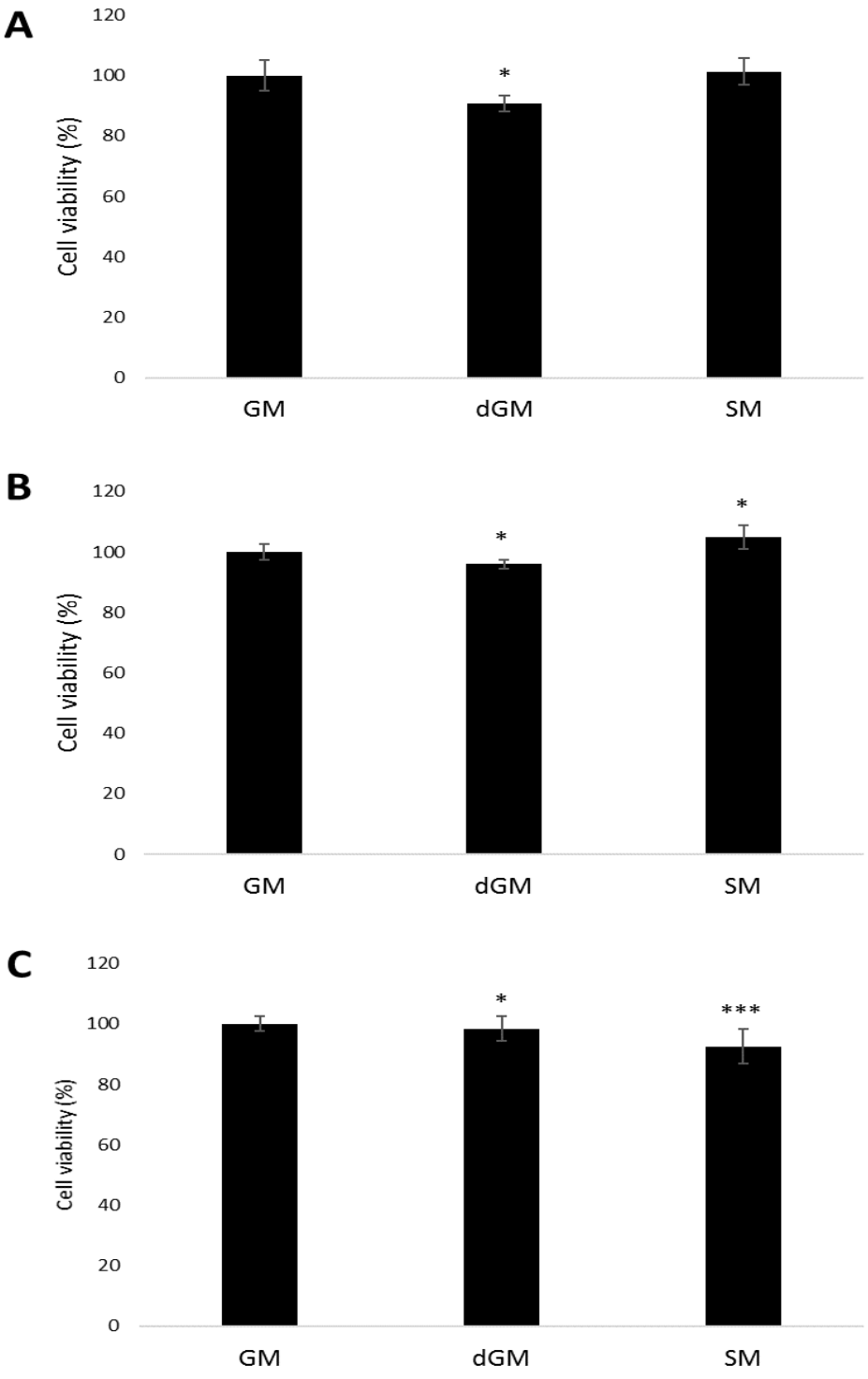


Figure 4.4. The viability of C2C12 myoblasts following short- and long-term incubation in media with different serum compositions (x-axis); assessed using an AlamarBlue viability assay. C2C12 myoblasts were incubated in GM, dGM or SM for 1- (A), 4- (B) or 24- (C) h. Data are presented as means ± SD from three biological replica ($n = 3$). Asterisks indicate significant differences compared to the control (GM) $^{*}P < 0.05$ and $^{***}P < 0.001$. Analysed by one-way ANOVA using Microsoft Excel followed by Tukey's HSD *post hoc* test using SPSS Statistics 20. Abbreviations: GM = growth medium supplemented with FCS; dGM = growth medium supplemented with dialysed FCS; SM = serum-free medium; FCS = foetal calf serum.

4.4.5. The vitality of C2C12 myoblasts was reduced by short-term serum restriction

We evaluated the short-term impact of reduced-serum and serum-free conditions on the vitality of proliferating C2C12 cells. Results showed that short-term incubation in the absence of serum or in the presence of dialysed FCS significantly decreased the vitality of C2C12 myoblasts ($P < 0.05$ and < 0.01, respectively) compared with FCS (control) (Figures 4.5 and 4.6). The absence of serum significantly decreased ($P < 0.05$) cell vitality compared with dialysed FCS (Figures 4.5 and 4.6).

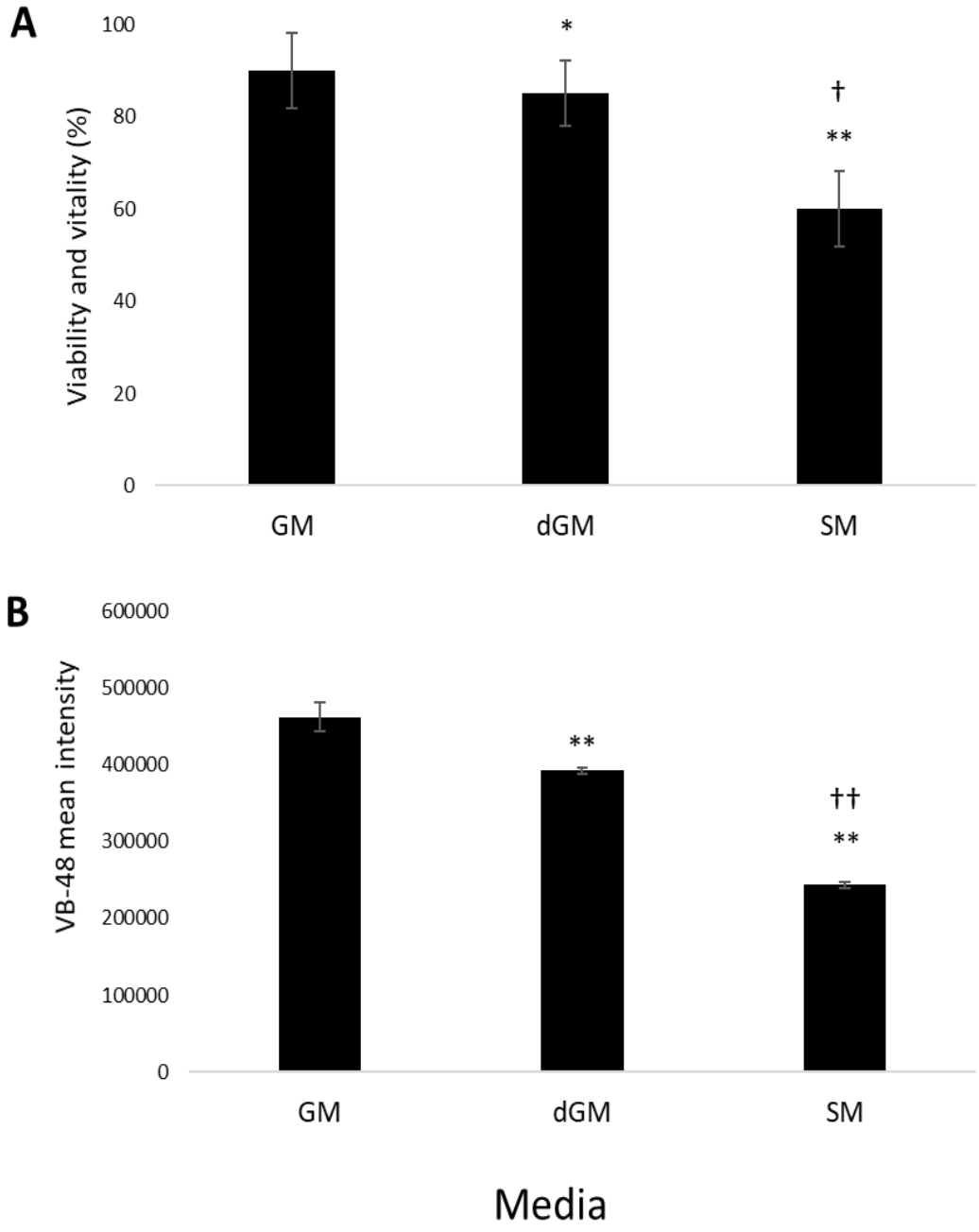


Figure 4.5. The vitality of C2C12 myoblasts following short-term incubation in media with different serum compositions; assessed using a NucleoCounter® NC-3000™ Vitabright-48™ assay. C2C12 myoblasts were incubated in GM, dGM or SM for 4 h. Data are presented as means ± SD from three biological replica (n = 3). Asterisks indicate significant differences from the control (GM) $^{*}P < 0.05$ and $^{**}P < 0.01$. Crosses indicate significant differences compared to dGM $^{+}P < 0.05$ and $^{++}P < 0.01$. Analysed by one-way ANOVA using Microsoft Excel followed by Tukey's HSD *post hoc* test using SPSS Statistics 20. Abbreviations: GM = growth medium supplemented with FCS; dGM = growth medium supplemented with dialysed FCS; SM = serum-free medium; FCS = foetal calf serum; VB-48 = Vitabright-48 stain.

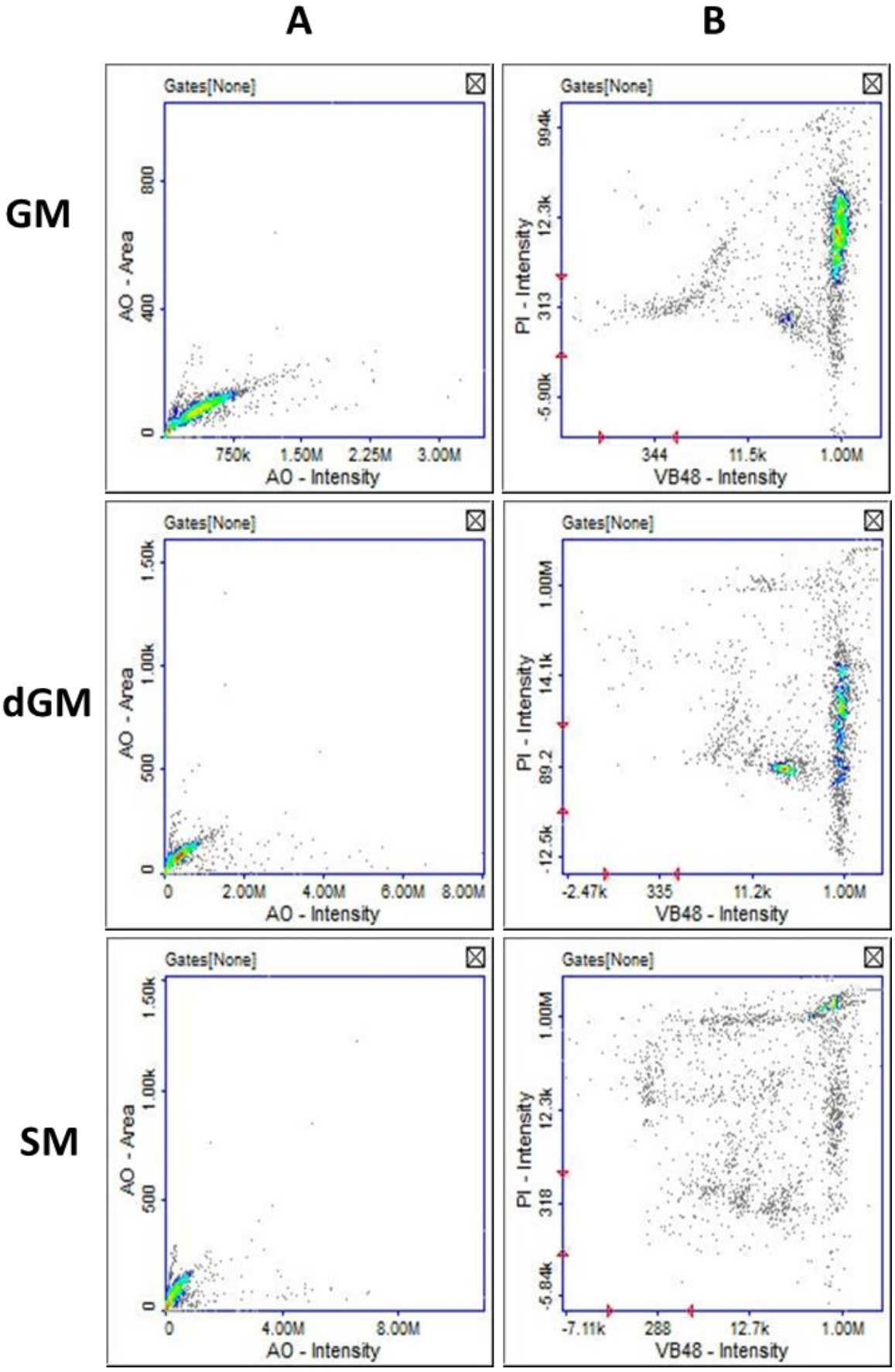

A
B
GM
dGM
SM
Gates[None]
AO - Area
AO - Intensity
PI - Intensity
VB48 - Intensity
0
400
800
750k
1.50M
2.25M
3.00M
313
12.3k
994k
-5.90k
344
11.5k
1.00M
500
1.00k
1.50k
2.00M
4.00M
6.00M
8.00M
89.2
14.1k
1.00M
-12.5k
-2.47k
335
11.2k
318
-5.84k
-7.11k
288
12.7k

Figure 4.6. Scatterplots showing the vitality of C2C12 myoblasts following short-term incubation in media with different serum compositions; assessed using a NucleoCounter® NC-3000™ Vitabright-48™ assay. C2C12 myoblasts were incubated in GM, dGM or SM for 4 h. Analysed by NucleoView™ software (n = 3). Panel A shows the intensity of AO-stained cells. Panel B shows the intensity of VB48-stained cells versus the intensity of PI-stained cells. Abbreviations: GM = growth medium supplemented with FCS; dGM = growth medium supplemented with dialysed FCS; SM = serum-free medium; FCS = foetal calf serum; VB48 = Vitabright-48 stain; AO = Acridine orange stain; PI = propidium iodide stain.

4.4.6. The proliferation and survival of C2C12 myoblasts decreased following short-term serum restriction

The aim of this experiment was to evaluate the short-term influence of reduced-serum and serum-free conditions on the proliferation and survival of C2C12 cells. Results showed that short-term incubation in SM significantly compromised the viability ($P < 0.05$) and decreased the total cell count ($P < 0.001$) compared with GM (i.e., control) (Figures 4.7 A&D and 4.8). A decrease in the count of live cells, compared with GM and dGM ($P < 0.05$ and < 0.01, respectively), was also recorded (Figures 4.7 B&C and 4.8). Short-term incubation in dGM significantly decreased both the viability and total cell count ($P < 0.05$) compared with GM (Figures 4.7 A&D and 4.8) and increased the number of dead cells ($P < 0.01$) compared with both GM and SM (Figures 4.7 C and 4.8).

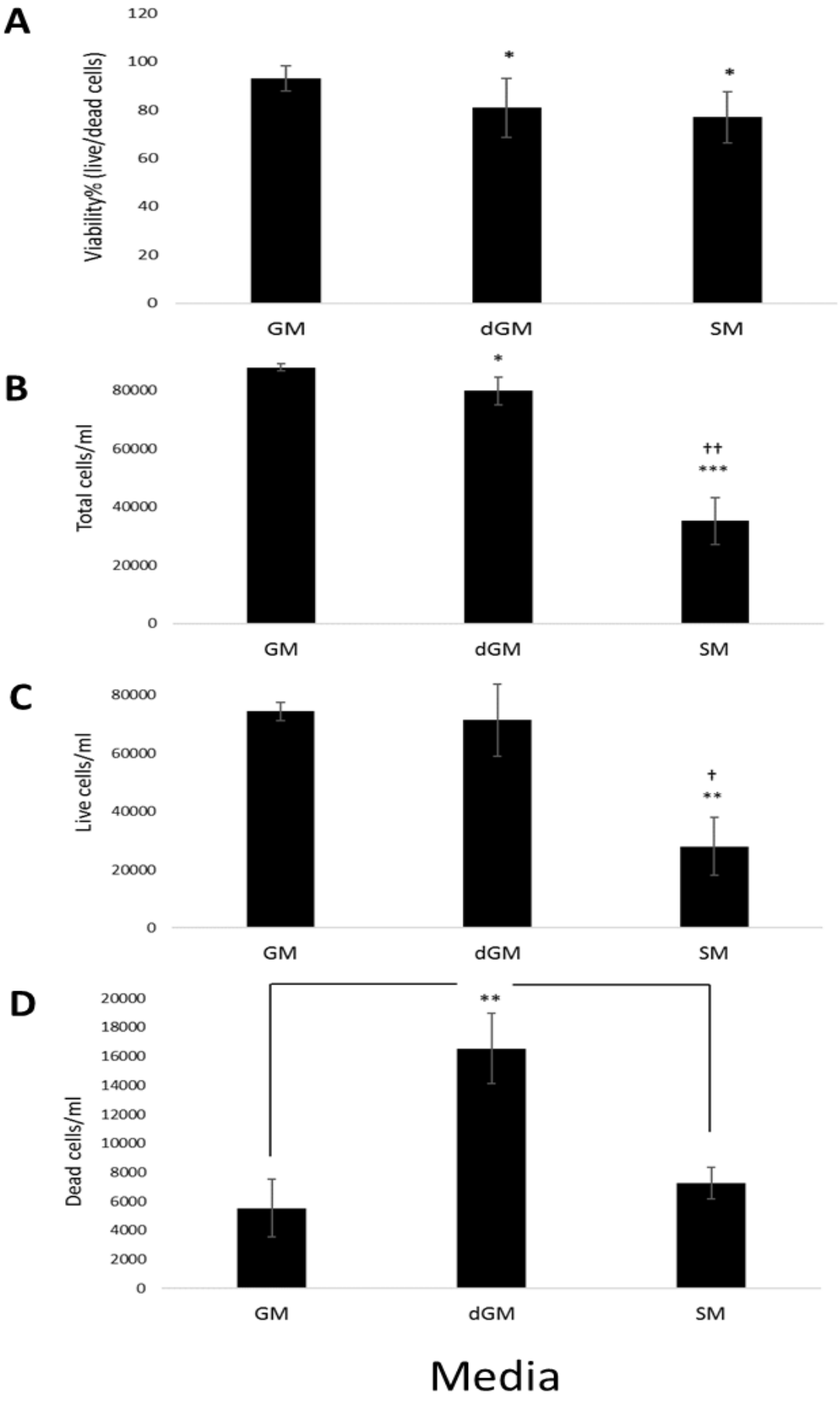

A
Viability% (live/dead cells)
120
100
80
60
40
20
0
*
*
GM
dGM
SM
B
Total cells/ml
80000
60000
40000
20000
0
*
††
***
GM
dGM
SM
C
Live cells/ml
80000
60000
40000
20000
0
†
**
GM
dGM
SM
D
Dead cells/ml
20000
18000
16000
14000
12000
10000
8000
6000
4000
2000
0
**
GM
dGM
SM
Media

Figure 4.7. The viability and differential cell count of C2C12 myoblasts following short-term incubation in media with different serum compositions; assessed using a NucleoCounter® NC-3000™ Viability and Cell Count assay. C2C12 myoblasts were incubated in GM, dGM or SM for 4 h**.** Panel A shows the viability of cells, panel B shows the total cell count per ml, panel C shows the count of live cells per ml and panel D shows the count of dead cells per ml. Data are presented as means ± SD from three biological replica (n = 3). Asterisks indicate significant Data are presented as means ± SD from three biological replica (n = 3). Asterisks indicate significant differences compared to the control (GM) $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$. Crosses indicate significant differences compared with dGM $^{+}P < 0.05$ and $^{++}P < 0.01$. Analysed by one-way ANOVA using Microsoft Excel followed by Tukey's HSD *post hoc* test using SPSS Statistics 20. Abbreviations: GM = growth medium supplemented with FCS; dGM = growth medium supplemented with dialysed FCS; SM = serum-free medium; FCS = foetal calf serum.

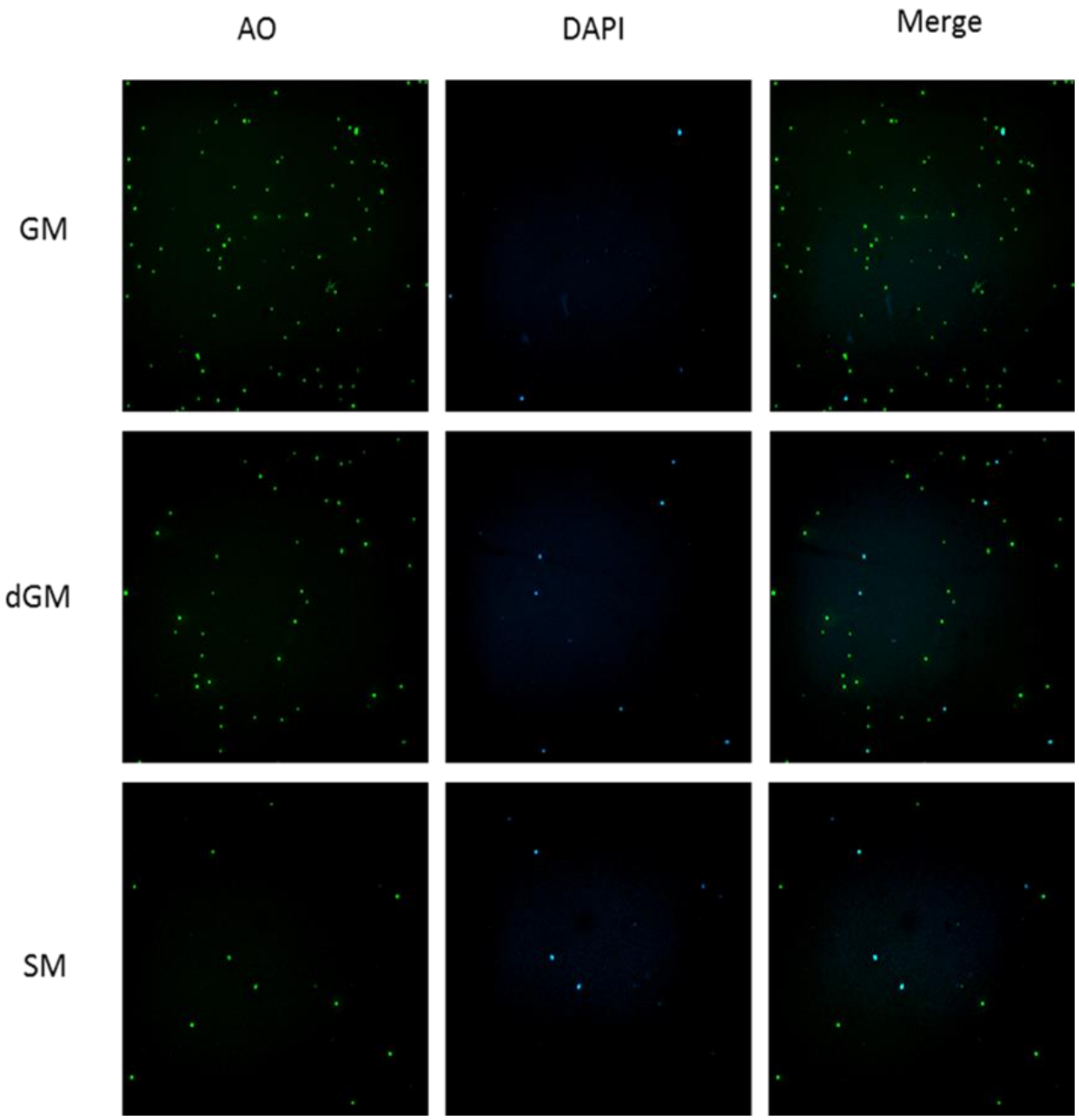


Figure 4.8. Representative images of C2C12 myoblasts following short-term incubation in media with different serum contents; obtained using a NucleoCounter® NC-3000™ Advanced Image Cytometer. C2C12 myoblasts were incubated in GM, dGM or SM for 4 h. (n = 3). The left panel shows cells stained with AO (green). The middle panel shows cells stained with DAPI (blue). The right panel is a merge of AO- and DAPI-stained cells. Abbreviations: GM = growth medium supplemented with FCS; dGM = growth medium supplemented with dialysed FCS; SM = serum-free medium; FCS = foetal calf serum; AO = Acridine orange stain.

4.4.7. The vitality of C2C12 myoblasts was reduced by long-term serum restriction

The long-term effect of reduced-serum and serum-free conditions on the vitality of proliferating C2C12 cells was examined. Results indicated that long-term incubation in SM and dGM significantly decreased ($P < 0.001$) the vitality of C2C12 myoblasts when compared with GM (Figures 4.9 and 4.10).

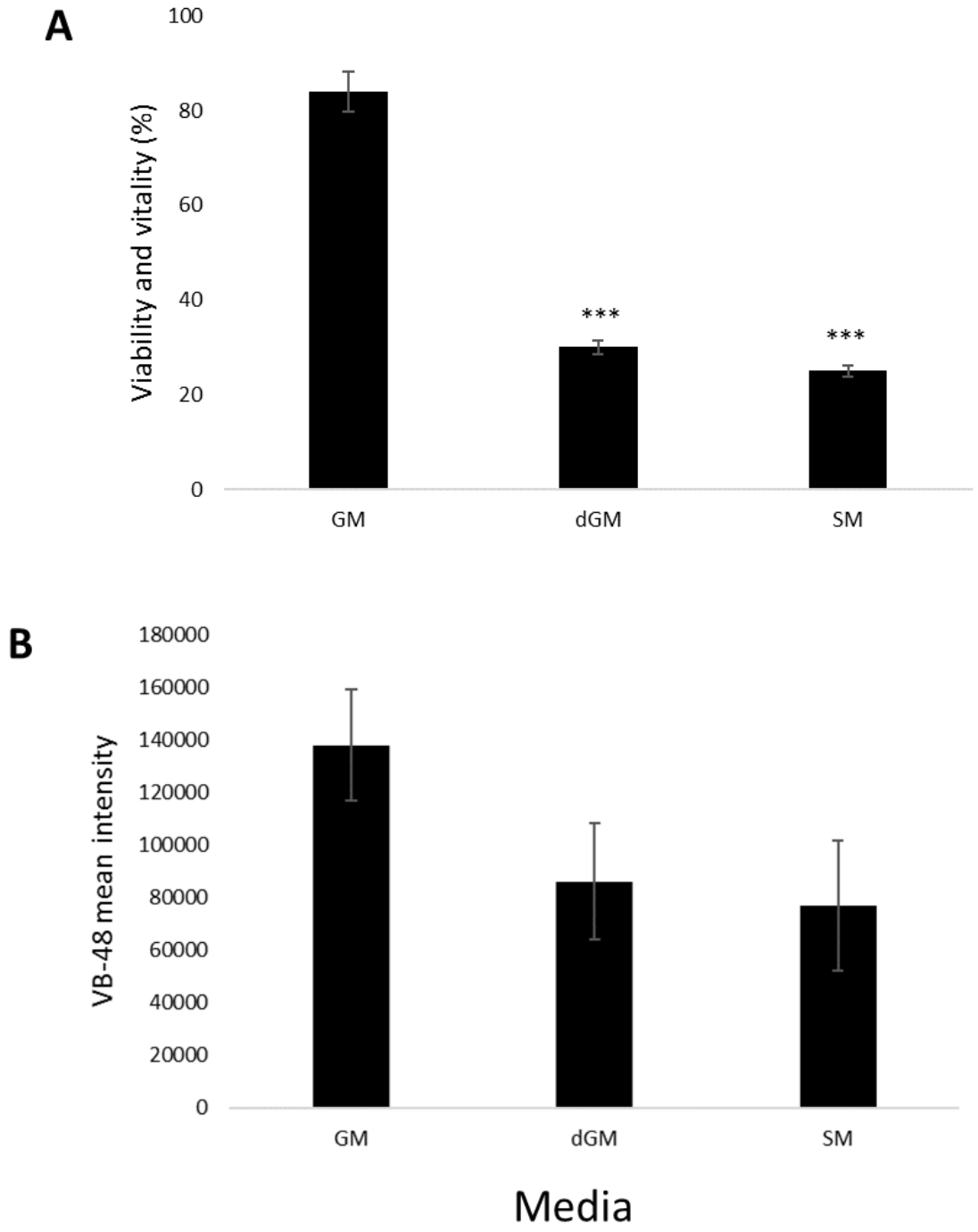


Figure 4.9. The vitality of C2C12 myoblasts following long-term incubation in media with different serum compositions; assessed using a NucleoCounter® NC-3000™ Vitabright-48™ assay. C2C12 myoblasts were incubated in GM, dGM or SM for 24 h. Data are presented as means ± SD from three biological replica (n = 3). Asterisks indicate significant differences compared to the control (GM) $^{***}P < 0.001$. Analysed by one-way ANOVA using Microsoft Excel followed by Tukey's HSD *post hoc* test using SPSS Statistics 20. Abbreviations: GM = growth medium supplemented with FCS; dGM = growth medium supplemented with dialysed FCS; SM = serum-free medium; FCS = foetal calf serum; VB-48 = Vitabright-48 stain.

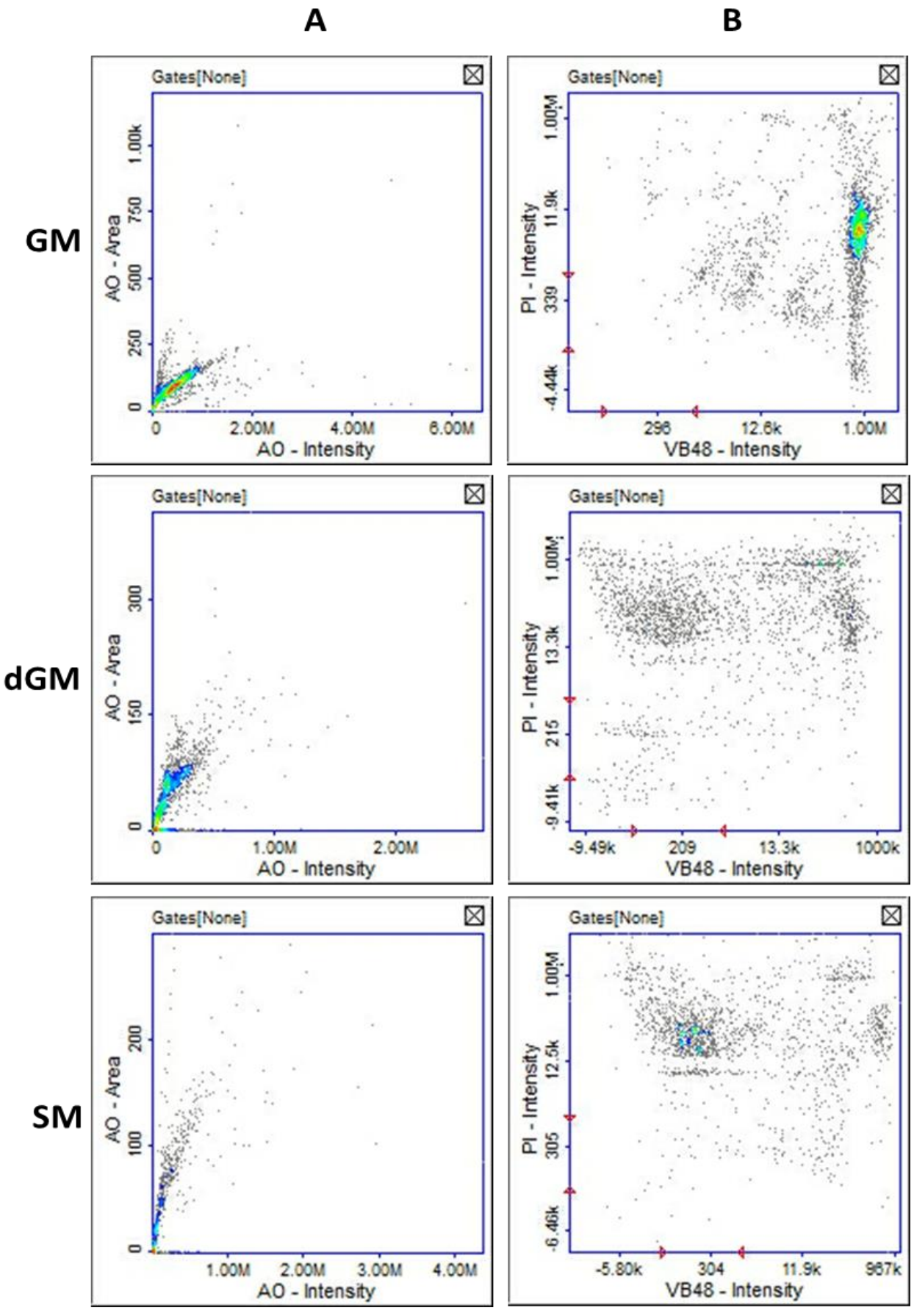

A
B
GM
dGM
SM
Gates[None]
AO - Area
AO - Intensity
PI - Intensity
VB48 - Intensity
0
250
500
750
1.00k
2.00M
4.00M
6.00M
339
11.9k
1.00M
-4.44k
296
12.6k
150
300
1.00M
2.00M
13.3k
215
-9.41k
-9.49k
209
1000k
100
200
3.00M
4.00M
12.5k
305
-6.46k
-5.80k
304
967k

Figure 4.10. Scatterplots showing the vitality of C2C12 myoblasts following long-term incubation in media with different serum contents; assessed using a NucleoCounter® NC-3000™ Vitabright-48™ assay. C2C12 myoblasts were incubated in GM, dGM or SM for 4 h. Analysed by NucleoView™ software (n = 3). Panel A shows the fluorescence intensity of AO-stained cells. Panel B shows the fluorescence intensity of VB-48-stained cells versus the fluorescence intensity of PI-stained cells. Abbreviations: GM = growth medium supplemented with FCS; dGM = growth medium supplemented with dialysed FCS; SM = serum-free medium; FCS = foetal calf serum; VB-48 = Vitabright-48 stain; AO = Acridine orange stain; PI = propidium iodide.

4.4.8. The cellular turnover of C2C12 myoblasts was reduced by long-term serum restriction

We evaluated the long-term impact of reduced-serum and serum-free conditions on the proliferation and survival of C2C12 cells. The viability, live, dead and total cell count of proliferating C2C12 cells after 24 h incubation in media with different serum contents was assessed. Results indicated that long-term incubation in SM and dGM significantly decreased cell viability ($P < 0.05$ and < 0.01, respectively) compared with GM (Figures 4.11 D and 4.12). A significant decrease ($P < 0.001$) in the number of total, live and dead cells, compared with GM, occurred in cultures incubated in SM and dGM (Figures 4.11 A-C and 4.12).

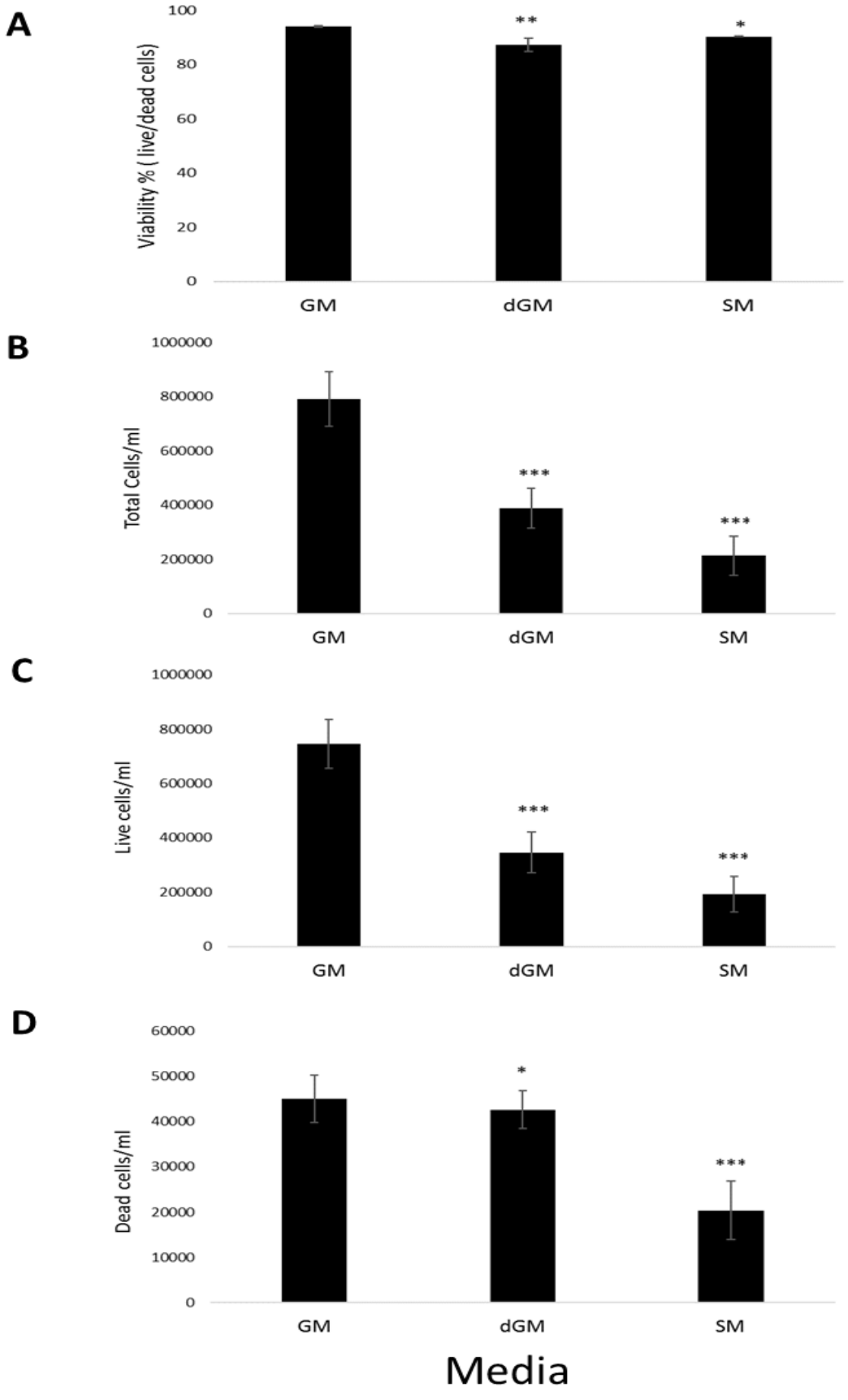

A
Viability % (live/dead cells)
100
80
60
40
20
0
**
*
GM
dGM
SM
B
Total Cells/ml
1000000
800000
600000
400000
200000
0
***
***
GM
dGM
SM
C
Live cells/ml
1000000
800000
600000
400000
200000
0
***
***
GM
dGM
SM
D
Dead cells/ml
60000
50000
40000
30000
20000
10000
0
*
***
GM
dGM
SM
Media

Figure 4.11. The viability and differential cell count of C2C12 myoblasts following long-term incubation in media with different serum contents; assessed using a NucleoCounter® NC-3000™ Viability and Cell Count assay. C2C12 myoblasts were incubated in GM, dGM or SM for 24 h. Panel A shows the viability of cells, panel B shows the total cell count per ml, panel C shows the count of live cells per ml and panel D shows the count of dead cells per ml. Data are presented as means ± SD from three biological replica (n = 3). Asterisks indicate significant Data are presented as means ± SD from three biological replica (n = 3). Asterisks indicate significant differences compared to the control (GM) $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$. Analysed by one-way ANOVA using Microsoft Excel followed by Tukey's HSD *post hoc* test using SPSS Statistics 20. Abbreviations: GM = growth medium supplemented with FCS; dGM = growth medium supplemented with dialysed FCS; SM = serum-free medium; FCS = foetal calf serum.

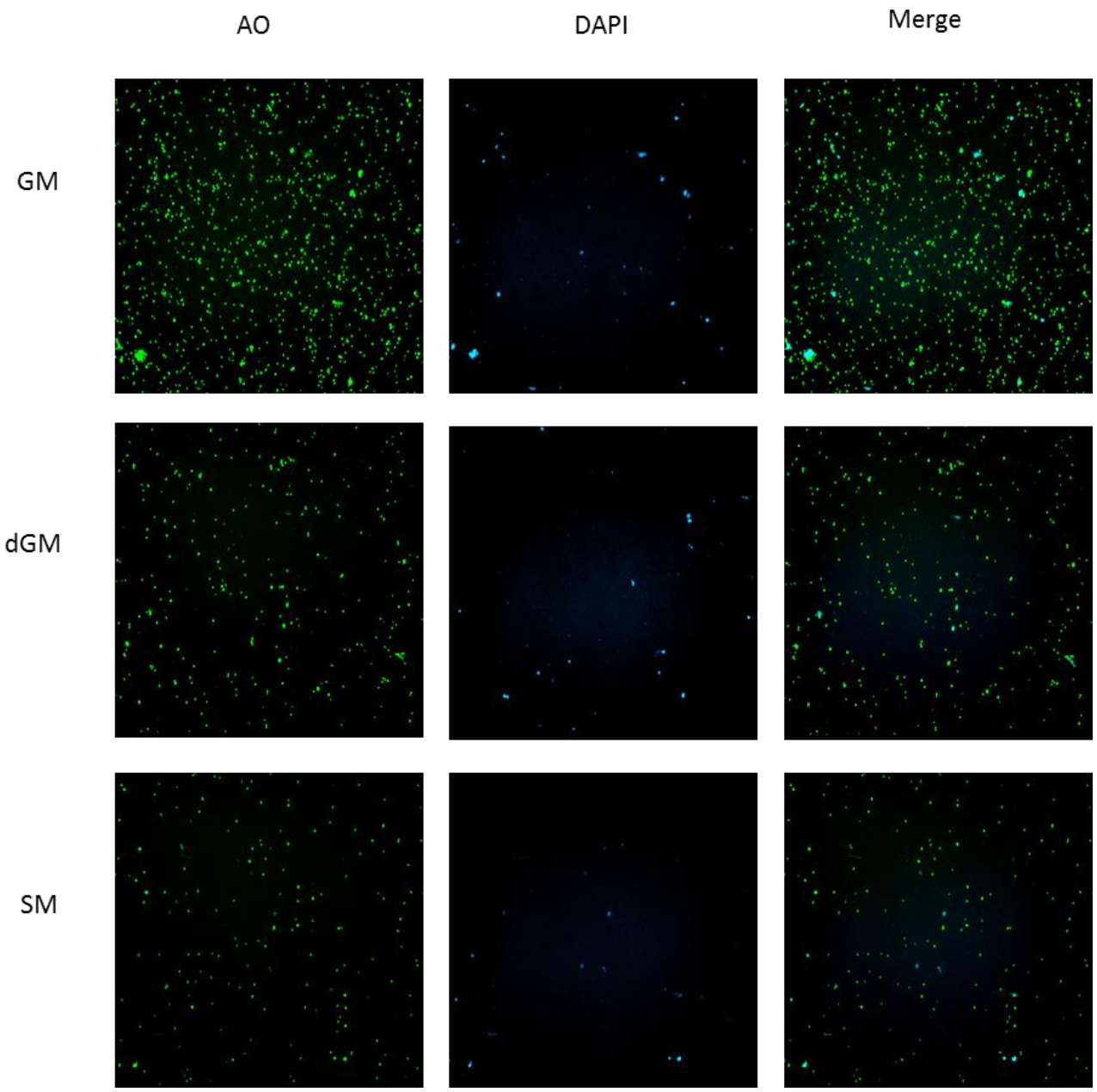


Figure 4.12. Representative images of C2C12 myoblasts following long-term incubation in media with different serum contents; obtained using a NucleoCounter® NC-3000™ Advanced Image Cytometer. C2C12 myoblasts were incubated in GM, dGM or SM for 24 h. (n = 3). The left panel shows cells stained with AO (green). The middle panel shows cells stained with DAPI (blue). The right panel is a merge of AO-stained cells and DAPI-stained cells. Abbreviations: GM = growth medium supplemented with FCS; dGM = growth medium supplemented with dialysed FCS; SM = serum-free medium; FCS = foetal calf serum; AO = Acridine orange stain.

4.4.9. Long-term serum restriction induced cell loss in proliferating C2C12 cultures

We examined the growth of C2C12 myoblasts following incubation in media with different serum contents. Morphological images of C2C12 cultures revealed a high rate of cell death following long-term incubation in dGM (Figure 4.13). This was also observed in cultures incubated in SM (Figure 4.13).

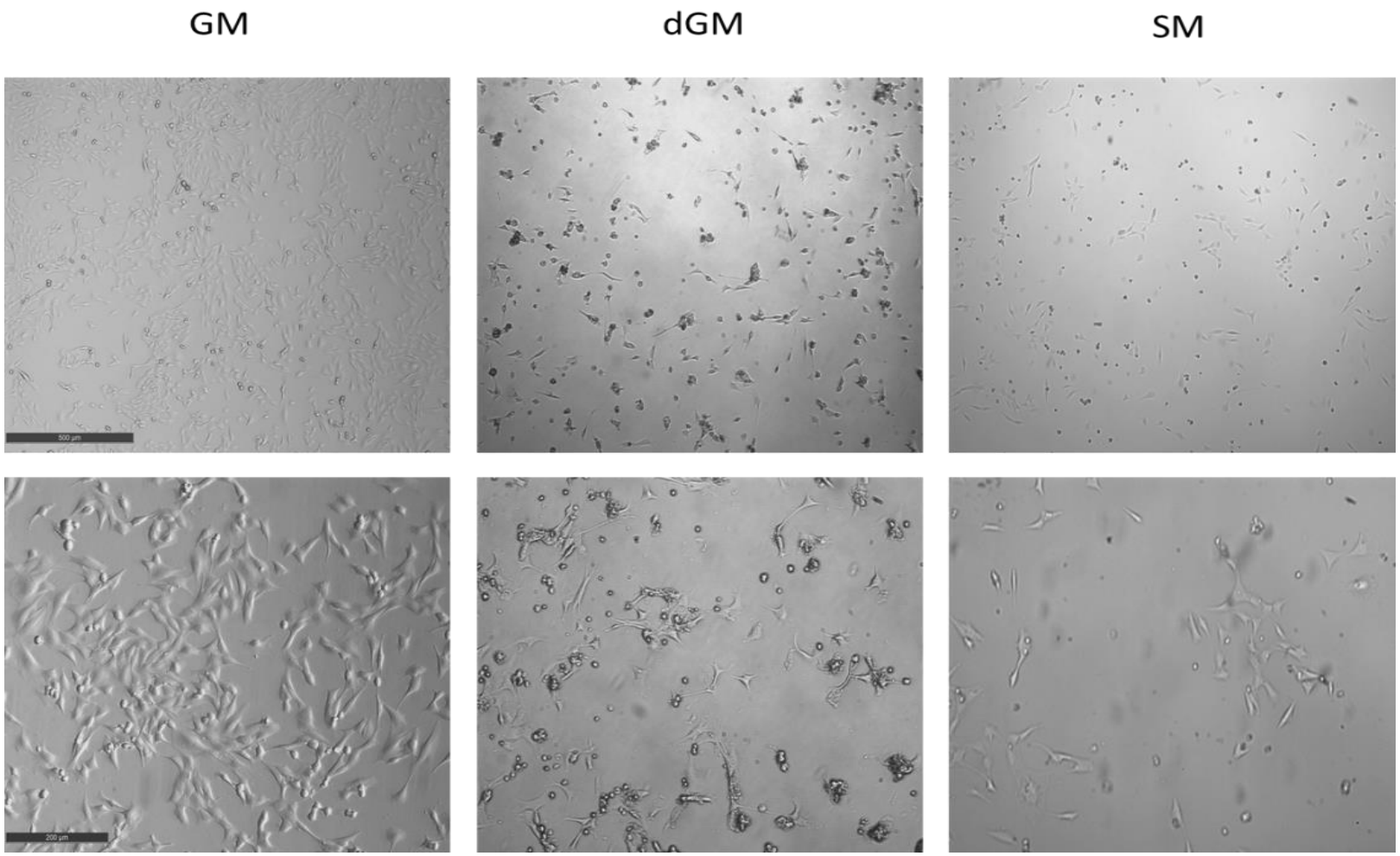


Figure 4.13. Phase-contrast images depicting the morphology of C2C12 myoblasts following long-term incubation in media with different serum contents. C2C12 myoblasts were incubated in GM, dGM or SM for 24 h. Numerous cell deaths are evident in cultures incubated in dGM and SM. (n = 3) Abbreviations: GM = growth medium supplemented with foetal calf serum; dGM = growth medium supplemented with dialysed foetal calf serum; SM = serum-free medium; FCS = foetal calf serum; µm = micrometre.

4.4.10. Serum restriction does not affect the diameter of C2C12 myoblasts

We evaluated the impact of short- and long-term reduced-serum and serum-free conditions on the size of proliferating C2C12 cells. Results indicated that short- and long-term serum deprivation did not affect the diameter of C2C12 myoblasts (Figures 4.14-17). However, short-term incubation with dialysed FCS resulted in a transient decrease in cell diameter ($P < 0.05$) compared with FCS (Figures 4.14 and 4.15).

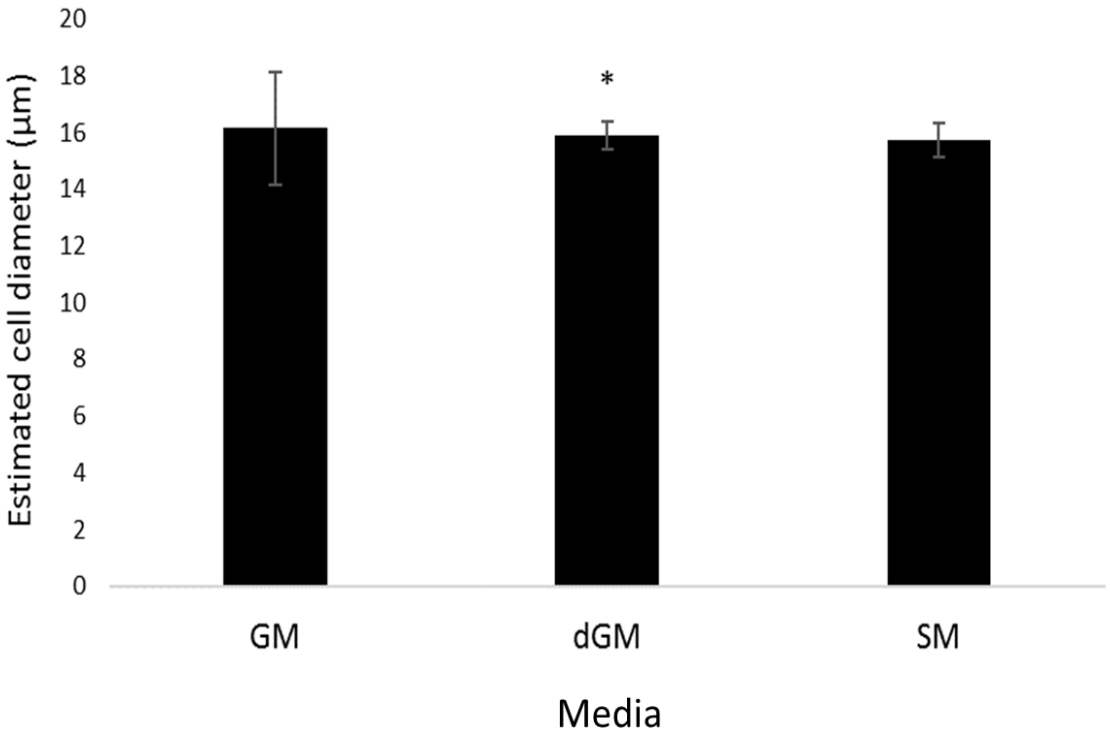


Figure 4.14. Cell diameter of C2C12 myoblasts following short-term incubation in media with different serum contents; assessed using a NucleoCounter® NC-3000™ Viability and Cell Count assay. C2C12 myoblasts were incubated in GM, dGM or SM for 4 h. Data are presented as means ± SD from three biological replica (n = 3). Asterisks indicate significant differences compared to the control (GM) $^{*}P < 0.05$. Analysed by one-way ANOVA using Microsoft Excel followed by Tukey's HSD *post hoc* test using SPSS Statistics 20. Abbreviations: GM = growth medium supplemented with FCS; dGM = growth medium supplemented with dialysed FCS; SM = serum-free medium; FCS = foetal calf serum; AO = Acridine orange; µm = micrometre.

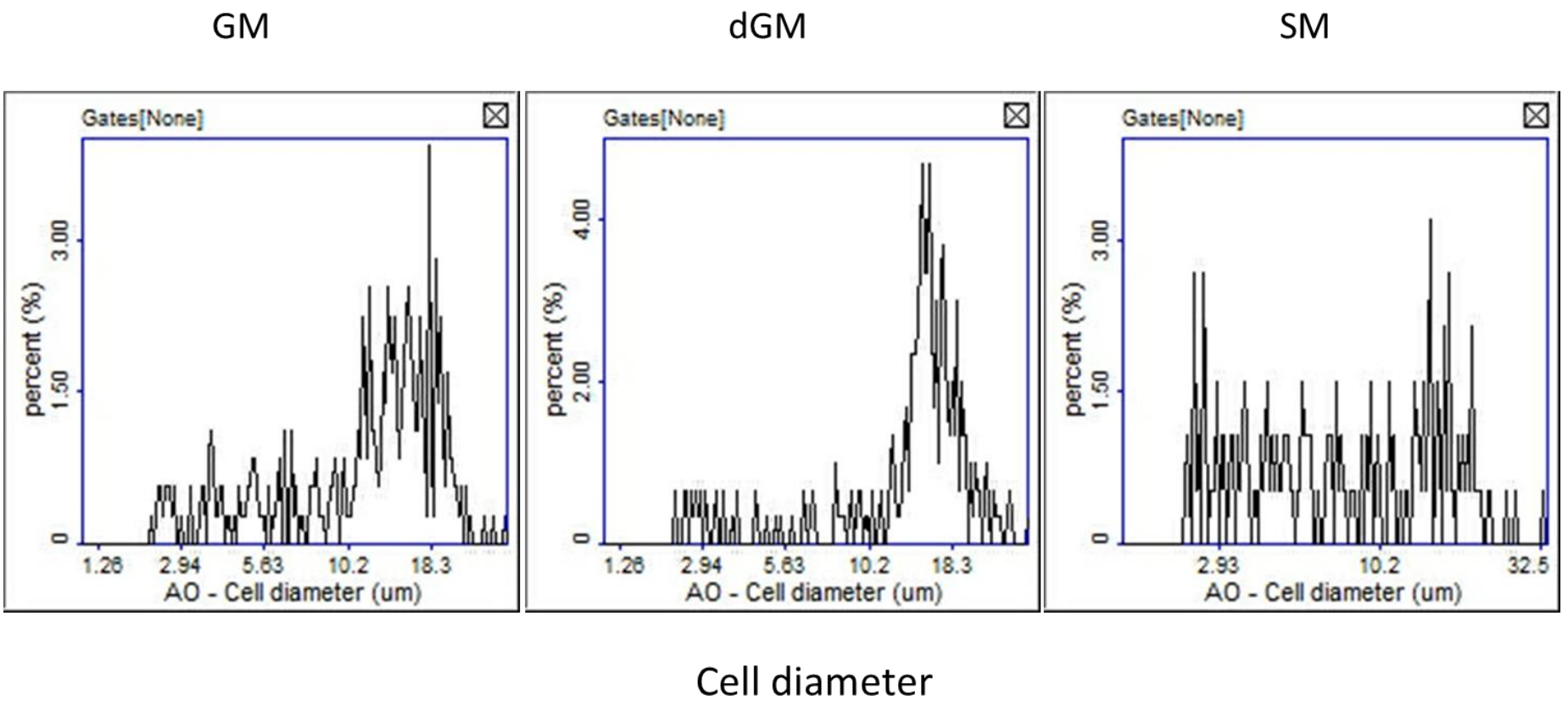


Figure 4.15. Histograms showing the diameter of C2C12 myoblasts following short-term incubation in media with different serum contents; assessed using a NucleoCounter® NC-3000™ Viability and Cell Count assay. C2C12 myoblasts were incubated in GM, dGM or SM for 4 h. Analysed by NucleoView™ software ($n = 3$). Abbreviations: GM = growth medium supplemented with FCS; dGM = growth medium supplemented with dialysed FCS; SM = serum-free medium; FCS = foetal calf serum; AO = Acridine orange stain; µm = micrometre.

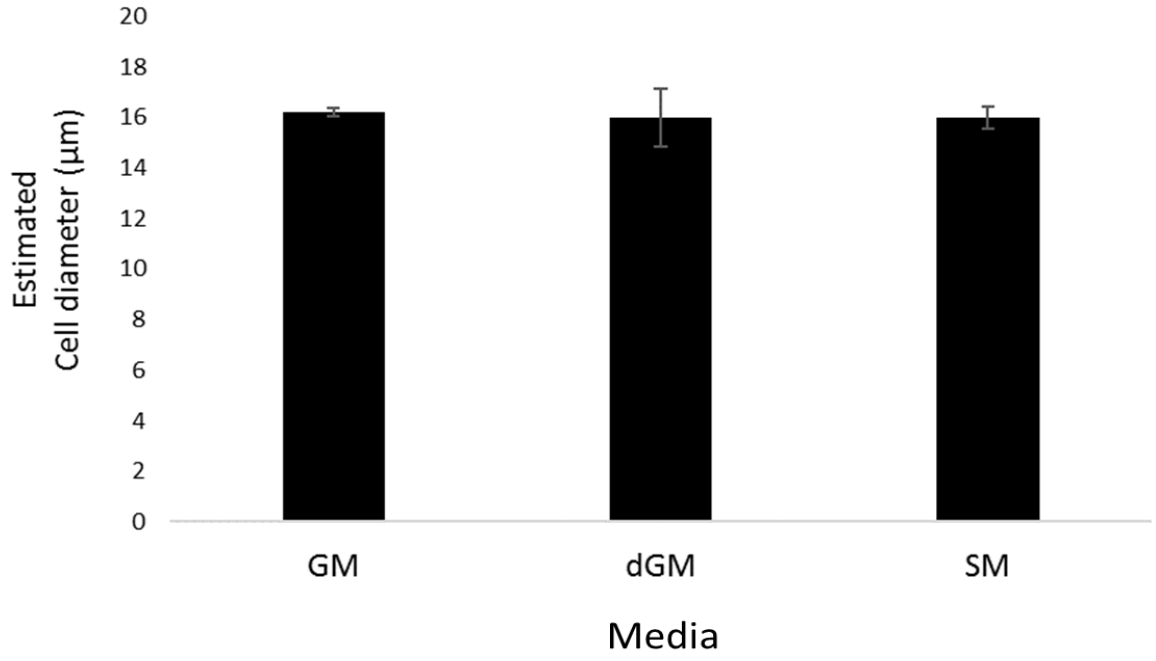


Figure 4.16. Cell diameter of C2C12 myoblasts following long-term incubation in media with different serum contents; assessed using a NucleoCounter® NC-3000™ Viability and Cell Count assay. C2C12 myoblasts were incubated in GM, dGM or SM for 24 h. Data are presented as means ± SD from three biological replica (*n* = 3). Analysed by one-way ANOVA using Microsoft Excel. Abbreviations: GM = growth medium supplemented with FCS; dGM = growth medium supplemented with dialysed FCS; SM = serum-free medium; FCS = foetal calf serum; µm = micrometre.

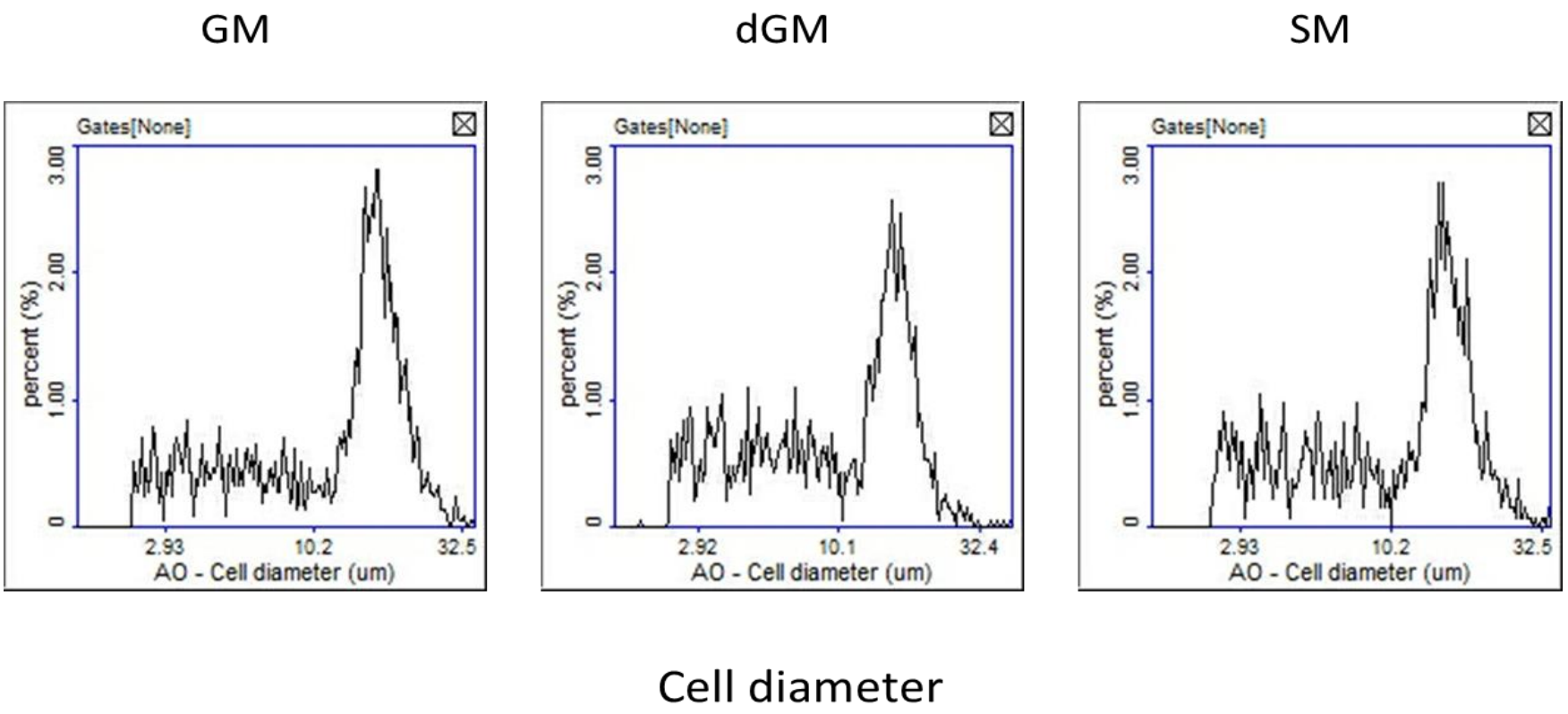


Figure 4.17. Histograms showing the diameter of C2C12 myoblasts following long-term incubation in media with different serum contents; assessed using a NucleoCounter® NC-3000™ Viability and Cell Count assay. C2C12 myoblasts were incubated in GM, dGM or SM for 24 h. Analysed by NucleoView™ software (n = 3). Abbreviations: GM = growth medium supplemented with FCS; dGM = growth medium supplemented with dialysed FCS; SM = serum-free medium; FCS = foetal calf serum; AO = Acridine orange stain; µm = micrometre.

4.4.11. Serum restriction weakens the mitochondrial transmembrane membrane potential of C2C12 myoblasts

We evaluated the impact of serum restriction on the mitochondrial transmembrane potential of C2C12 myoblasts. Results showed that serum-free conditions reduced the integrity of mitochondrial membranes, when compared with FCS (Figures 4.18 and 4.19). However, the mitochondrial transmembrane potential was not affected by long-term incubation with dialysed FCS (Figures 4.18 and 4.19).

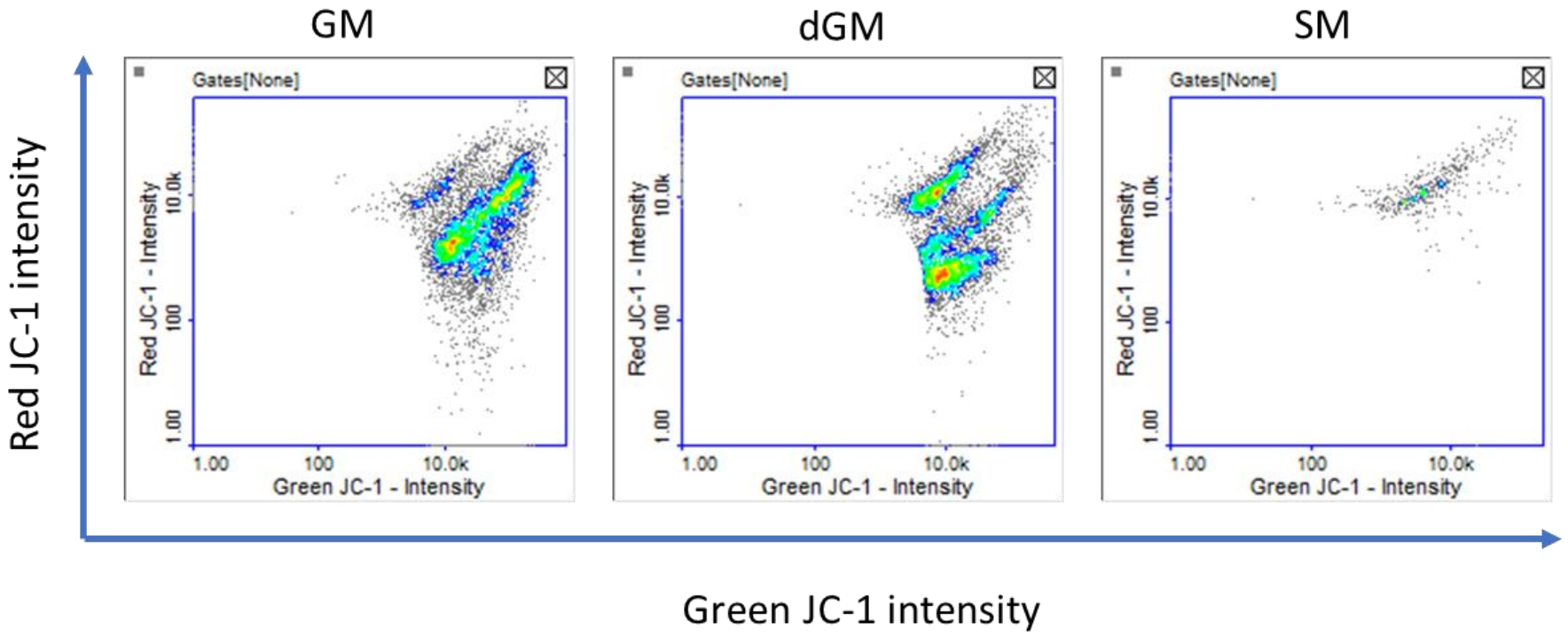


Figure 4.18. Scatterplots showing the mitochondrial membrane potential of C2C12 myoblasts following long-term incubation in media with different serum compositions; assessed using a NucleoCounter® NC-3000 Mitochondrial Potential assay. C2C12 myoblasts were incubated in GM, dGM or SM for 24 h. Analysed by NucleoView™ software (n = 3). Abbreviations: GM = growth medium supplemented with FCS; dGM = growth medium supplemented with dialysed FCS; SM = serum free medium; FCS = foetal calf serum.

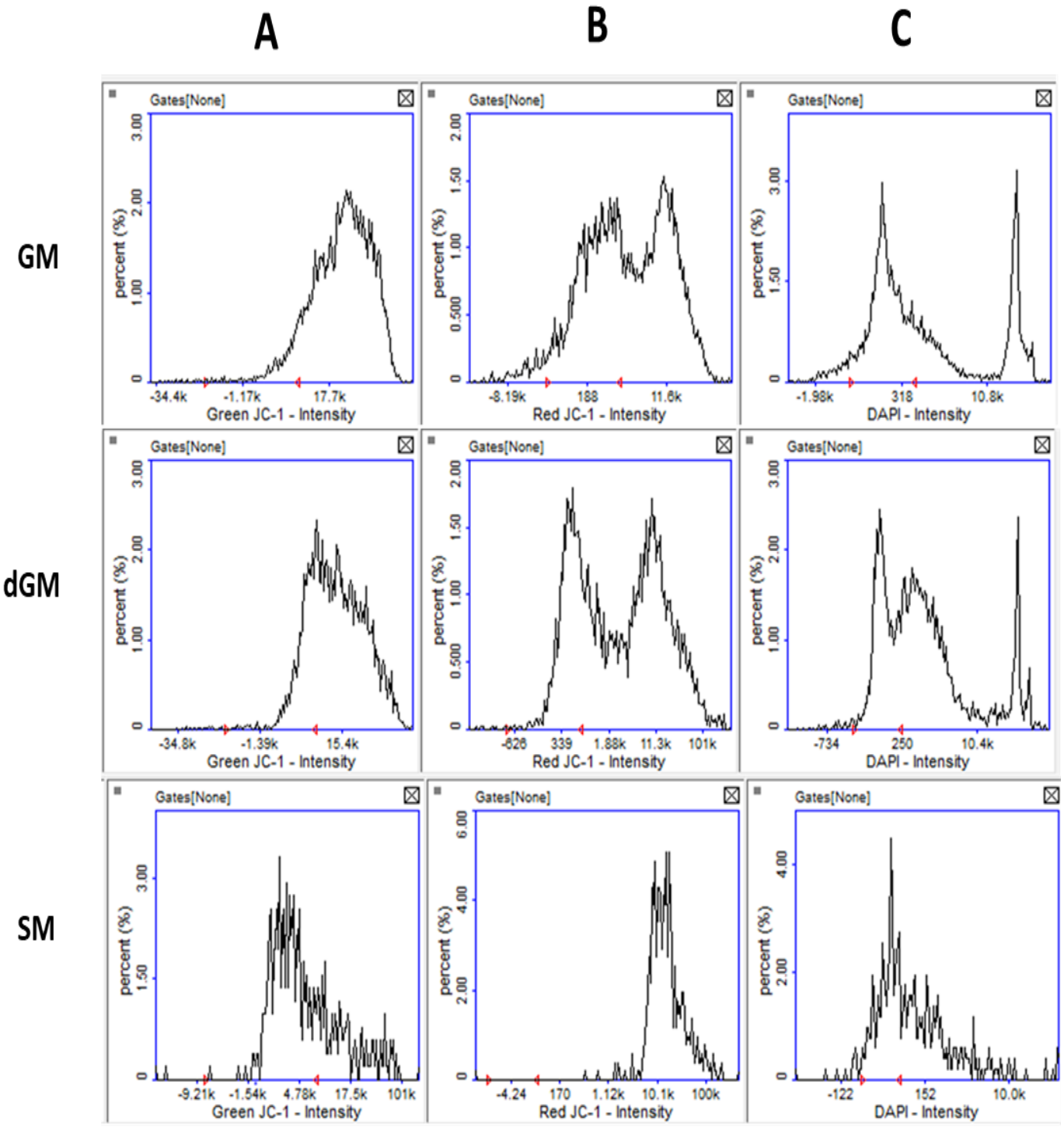


Figure 4.19. Histograms showing the mitochondrial membrane potential of C2C12 myoblasts following long-term incubation in media with different serum compositions; assessed using a NucleoCounter® NC-3000 Mitochondrial Potential assay. C2C12 myoblasts were incubated in GM, dGM or SM for 24 h. Analysed by NucleoView™ software ($n = 3$). Panel A shows the fluorescence intensity of green JC-1. Panel B shows the fluorescence intensity of red JC-1. Panel C shows the fluorescence intensity of blue DAPI. Abbreviations: GM = growth medium supplemented with FCS; dGM = growth medium supplemented with dialysed FCS; SM = serum-free medium; FCS = foetal calf serum.

4.4.12. Short-term serum starvation decreased the total cell count of C2C12 myoblasts

Here, we investigated the impact of different starvation protocols on the total protein content of proliferating C2C12 cells. The total cell count was assessed in cells maintained in GM; control and following short-term serum starvation. Results showed a significant decrease ($P < 0.001$) in the total cell count of C2C12 myoblasts after 1 and 4 h of serum withdrawal (Figure 4.20).

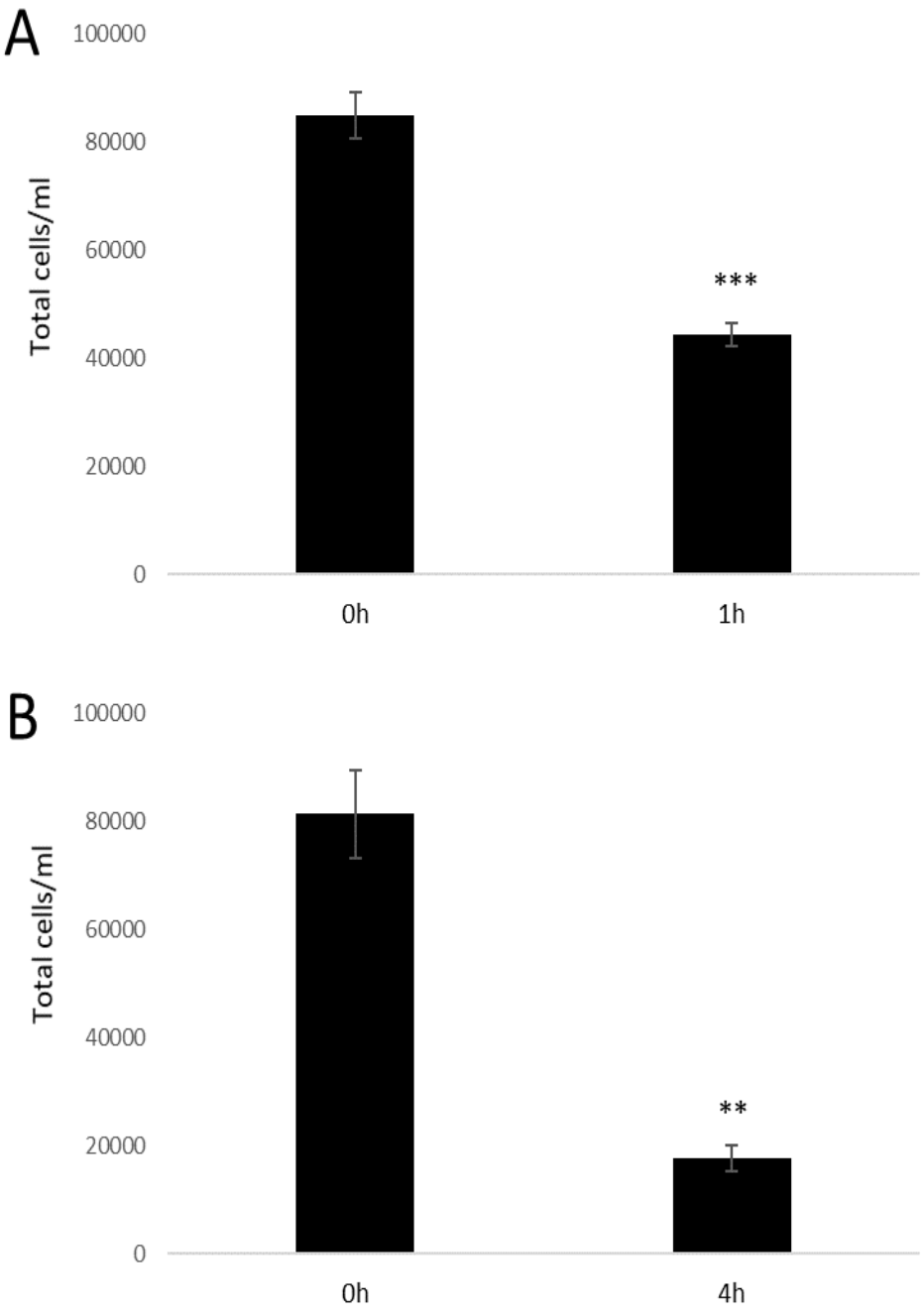


Figure 4.20. Total cell count of C2C12 myoblasts prior to and following short-term serum starvation; assessed using a NucleoCounter® NC-3000™ Viability and Cell Count assay. C2C12 myoblasts were kept in GM (0h) or shifted into SM for 1 (A) or 4 (B) h. Data are presented as means ± SD from three biological replica (n = 3). Asterisks indicate significant differences compared to the control (0h) $^{**}P < 0.01$, $^{***}P < 0.001$. Analysed by a paired two-tailed Student's t-test using Microsoft Excel. Abbreviations: 0h = cells prior to serum starvation; 1h = cells after 1 hour of serum starvation; 4h = cells after 4 hours of serum starvation.

4.4.13. Short-term serum starvation decreased the total protein content of C2C12 myoblasts

The impact of different starvation protocols on the total protein content of proliferating C2C12 cells was examined. Total protein content was assessed in cells maintained in GM (i.e., before serum starvation); control, and following short-term serum starvation. Results showed a significant decrease ($P < 0.001$) in the total protein content of C2C12 myoblasts following 1 h and 4 h of serum withdrawal (Figure 4.21).

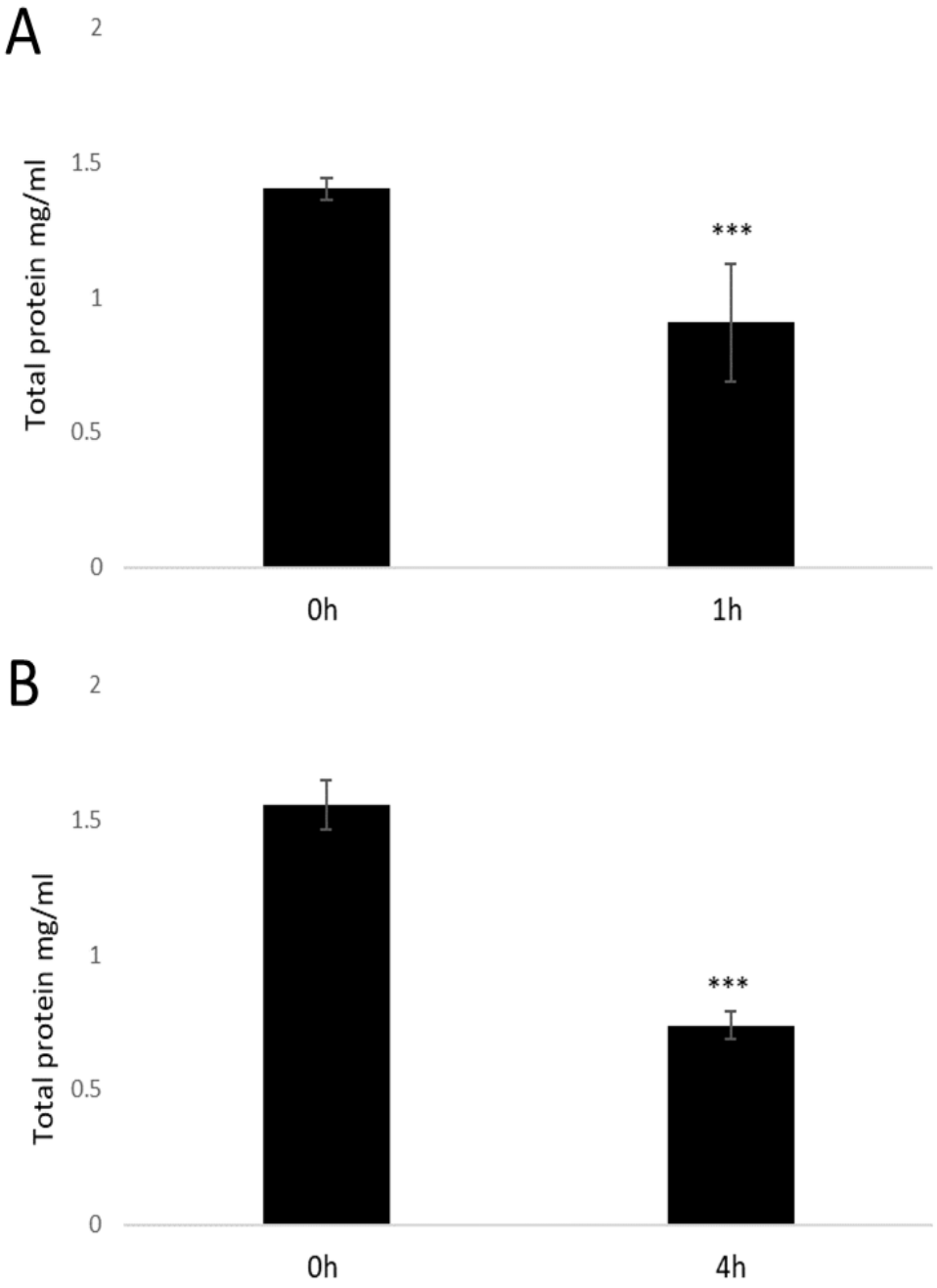


Figure 4.21. Total protein content of C2C12 myoblasts prior to and following short-term serum starvation; assessed using a BCA protein assay. C2C12 myoblasts were kept in GM (0h) or shifted into SM for 1 (A) or 4 (B) h. Data are presented as means ± SD from three biological replica ($n = 3$). Asterisks indicate significant differences compared to the control (0h) $^{***}P < 0.001$. Analysed by a paired two-tailed Student's *t*-test using Microsoft Excel. Abbreviations: 0h = cells prior to serum starvation; 1h = cells after 1 hour of serum starvation; 4h = cells after 4 hours of serum starvation; mg = milligrams; ml = millilitre.

4.4.14. Long-term serum restriction decreased the total protein content of C2C12 myoblasts

The aim was to identify the optimal medium for testing the protein anabolic effect of leucine on C2C12 cells. The impacts of long-term incubation under reduced-serum and serum-free conditions on the total protein content of proliferating C2C12 cells were assessed. A significant decrease in the total protein content of C2C12 myoblasts, when compared with control (GM), was evident following 24 h of incubation in dGM and SM ($P < 0.001$) (Figure 4.22).

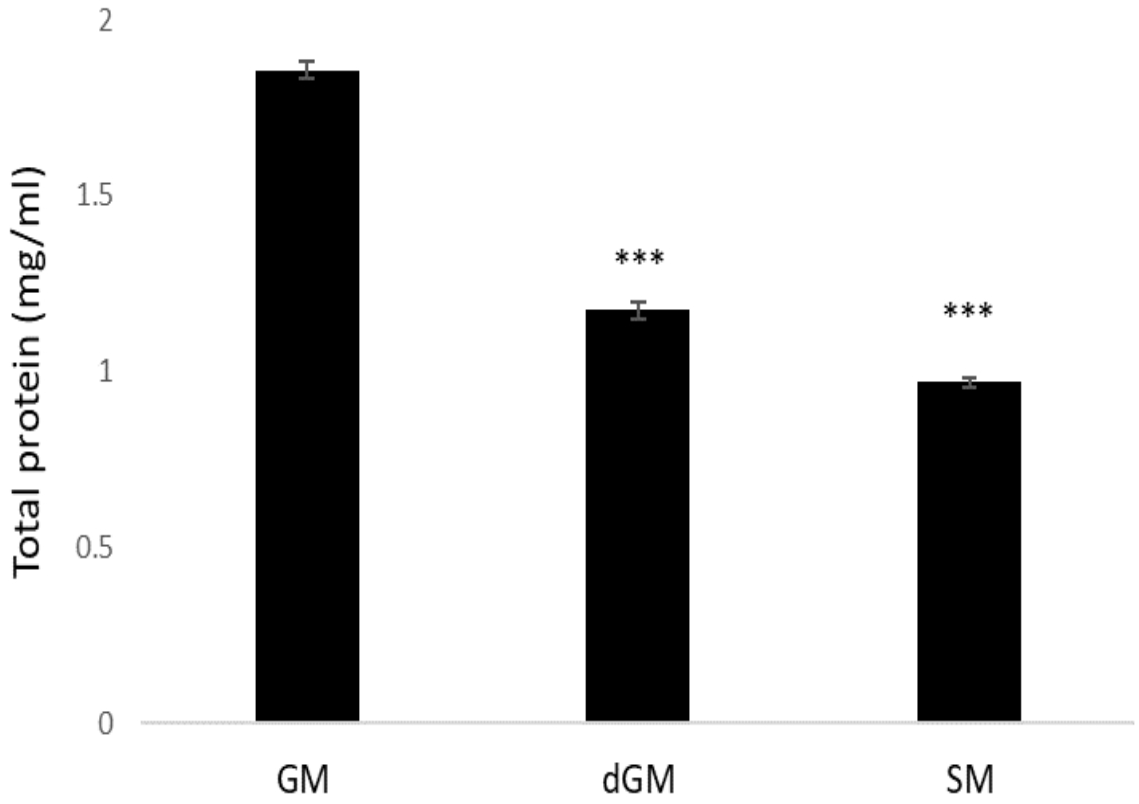


Figure 4.22. Total protein content of C2C12 myoblasts following long-term incubation in media with different serum compositions (x-axis); assessed using a BCA protein assay. C2C12 myoblasts were incubated in GM, dGM or SM for 24 h. Data are presented as means ± SD from three biological replica ($n = 3$). Asterisks indicate significant differences compared to the control (GM) $^{***}P < 0.001$. Analysed by one-way ANOVA using Microsoft Excel followed by Tukey's HSD *post hoc* test using SPSS Statistics 20. Abbreviations: GM = growth medium supplemented with FCS; dGM = growth medium supplemented with dialysed FCS; SM = serum-free medium; FCS = foetal calf serum; mg = milligrams; ml = millilitre.

4.4.15. Short-term serum starvation does not induce the differentiation of C2C12 myoblasts

Here, we investigated whether a short-term serum starvation induced the differentiation of C2C12 myoblasts. The expression of desmin (an early differentiation marker) was evaluated in C2C12 myoblast cultures, prior to, and following 1 h of serum starvation. The expression of desmin was evident at both time points, with no difference between serum starved and non-starved cells (Figure 4.23). Desmin was also expressed in these cultures after 24 h, with no difference between control and serum starved groups (Figure 4.24). Finally, the expression of MyHC-*fast* (a late differentiation marker) was not observed in either control or serum starved cultures (Figure 4.25).

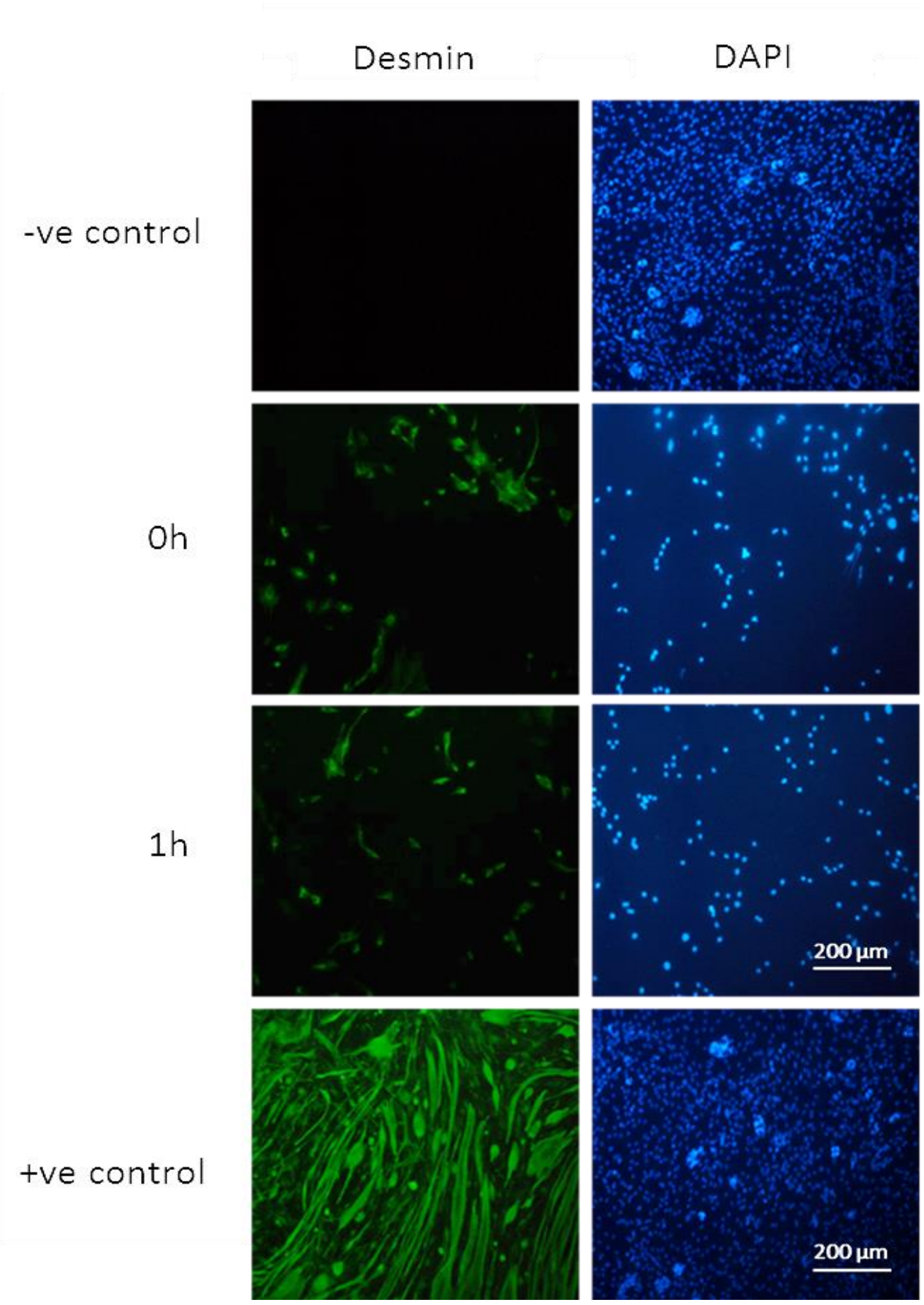

Desmin
DAPI
-ve control
0h
1h
+ve control
200 µm
200 µm

Figure 4.23. Expression of desmin in 40% confluent C2C12 myoblast cultures prior to and following short-term serum starvation; assessed by indirect immunofluorescence. The expression of desmin was examined in ~40-50% confluent C2C12 cultures before (0h) and after 1 h (1h) of serum starvation. The left panel shows the expression of desmin (green). The right panel shows DAPI-stained nuclei (blue). (*n* = 3). Abbreviations: -ve = negative control (i.e., no primary antibody); +ve = positive control (i.e., differentiated C2C12 myotubes); 0h = cells prior to serum starvation; 1h = cells after 1 hour of serum starvation; μm = micrometre.

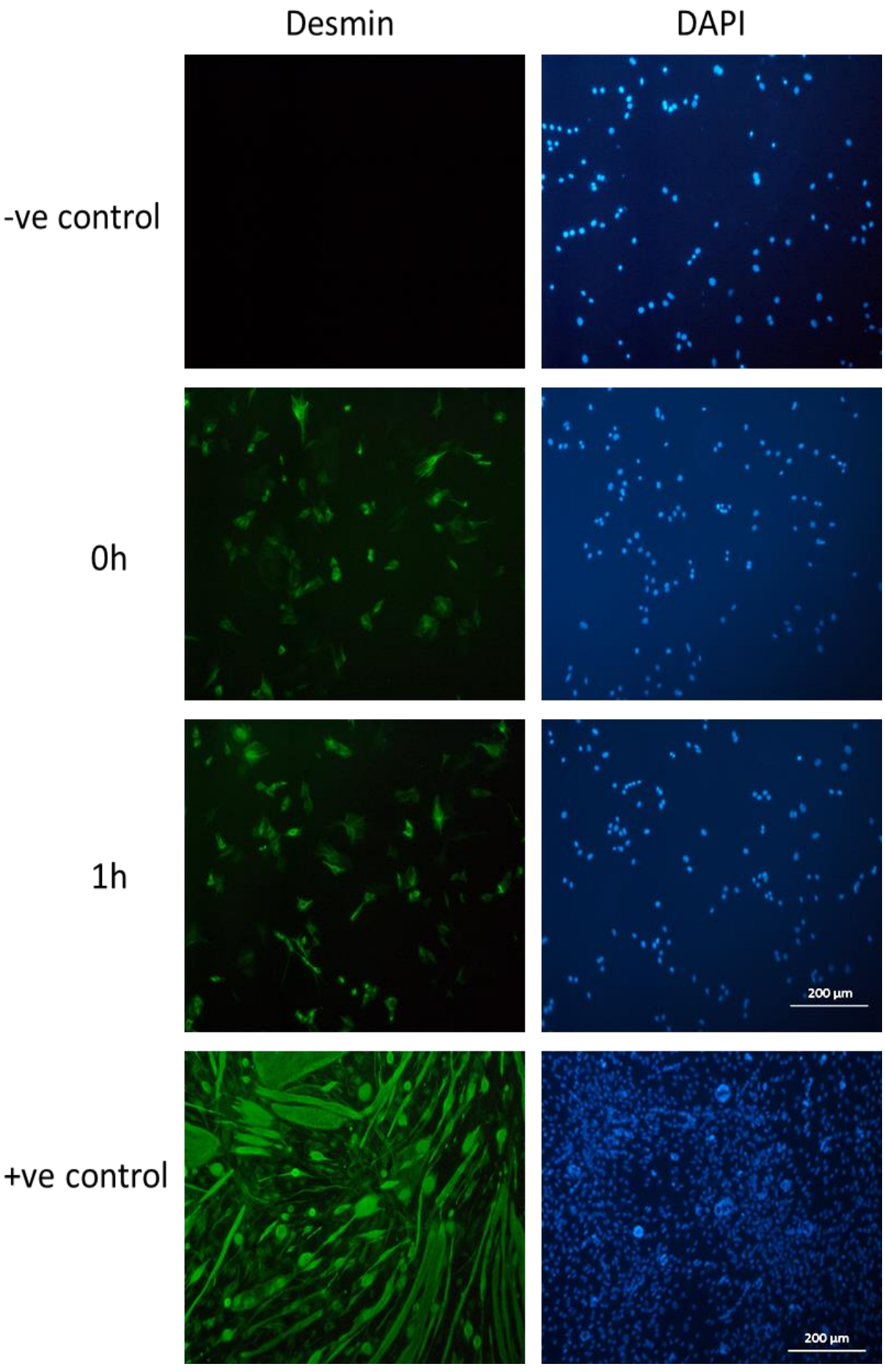

Desmin
DAPI
-ve control
0h
1h
+ve control
200 µm
200 µm

Figure 4.24. The expression of desmin in 50-60% confluent C2C12 myoblast cultures prior to and following short-term serum starvation; assessed by indirect immunofluorescence. Non-starved (0h) or serum starved (1h) C2C12 cells were incubated in GM for 24 h. The left panel shows the expression of desmin (green). The right panel shows DAPI-stained nuclei (blue). (*n* = 3). Abbreviations: -ve = negative control (i.e., no primary antibody); +ve = positive control (i.e., differentiated C2C12 myotubes); 0h = cells prior to serum starvation; 1h = cells after 1 hour of serum starvation; μm= micrometre.

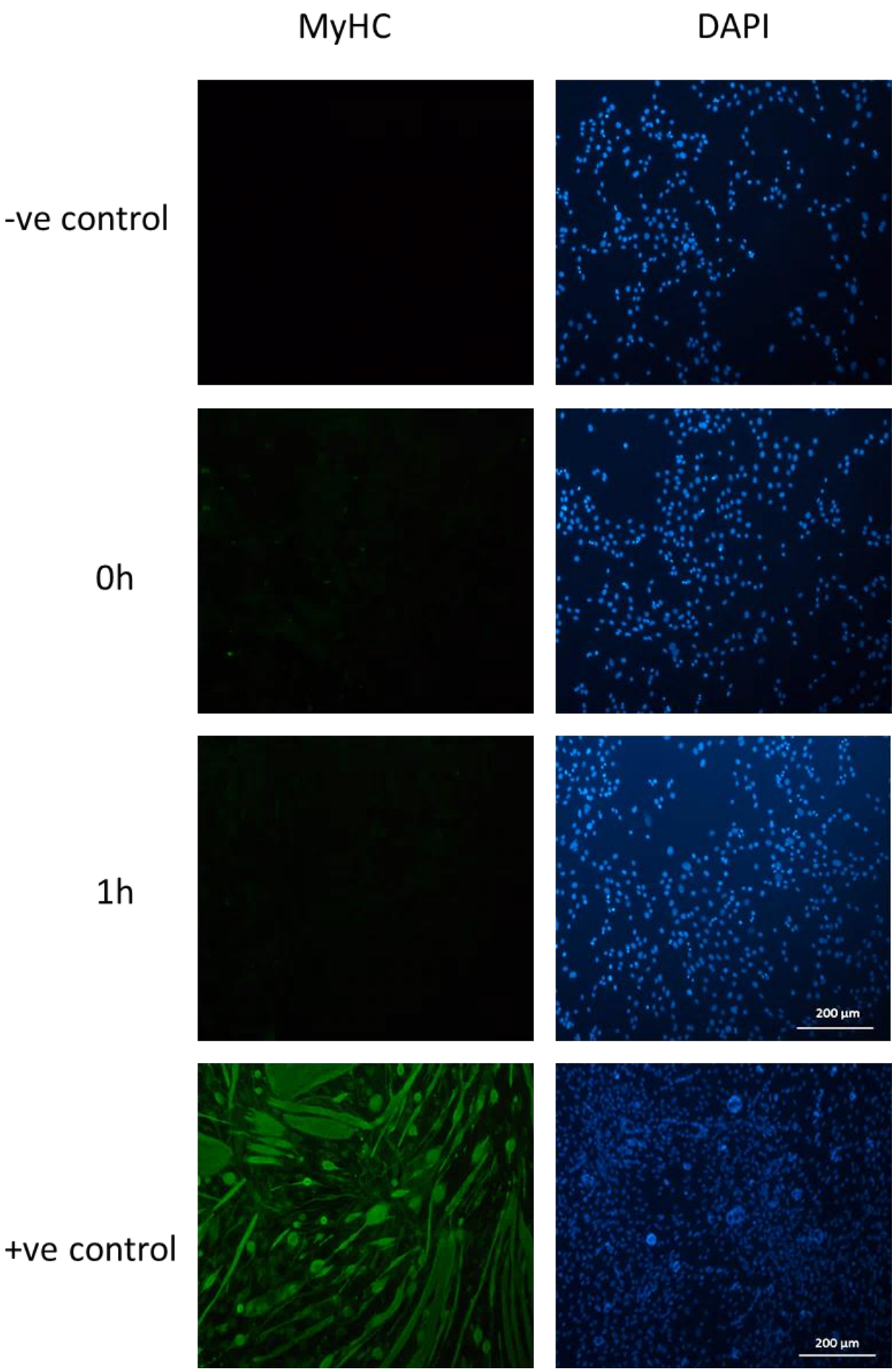

MyHC
DAPI
-ve control
0h
1h
+ve control
200 µm
200 µm

Figure 4.25. Expression of MyHC-*fast* in 50-60% confluent C2C12 myoblast cultures prior to and following short-term serum starvation, assessed by indirect immunofluorescence. Non-starved (0h) or serum starved (1h) C2C12 cells were incubated in GM for 24 h. The left panel shows the expression of MyHC-*fast* (green). The right panel shows DAPI-stained nuclei (blue). Abbreviations: -ve = negative control (i.e., no primary antibody); +ve = positive control (i.e., differentiated C2C12 cells); MyHC = myosin heavy chain; 0h = cells prior to serum starvation; 1h = cells after 1 hour of serum starvation; μm = micrometre.

4.4.16. Leucine alters the viability of C2C12 myoblasts

The aim of this experiment was to determine the optimal medium to support the anabolic effect of leucine on proliferating C2C12 cells. The viability of C2C12 myoblasts was assessed following long-term incubation in different concentrations of leucine introduced to media with different serum contents. Results showed that leucine failed to alter the viability of C2C12 myoblasts following its addition to cells in dGM (Figure 4.26 B). However, the viability of C2C12 myoblasts was significantly altered when leucine was added to GM or SM (Figure 4.26 A&C). A significant decline in cell viability, from control, was observed following exposure to a high concentration of leucine (10 mM) in GM and SM ($P < 0.05$ and < 0.01, respectively) (Figure 4.26 A&C). Notably, exposure to all leucine concentrations (2 mM, 5 mM and 10 mM) failed to increase the viability of C2C12 myoblasts above the control level (0 mM). However, exposure to 5 mM leucine significantly increased the viability of C2C12 ($P < 0.05$) from other leucine (2 mM and 10 mM) groups (Figure 4.26 A&C).

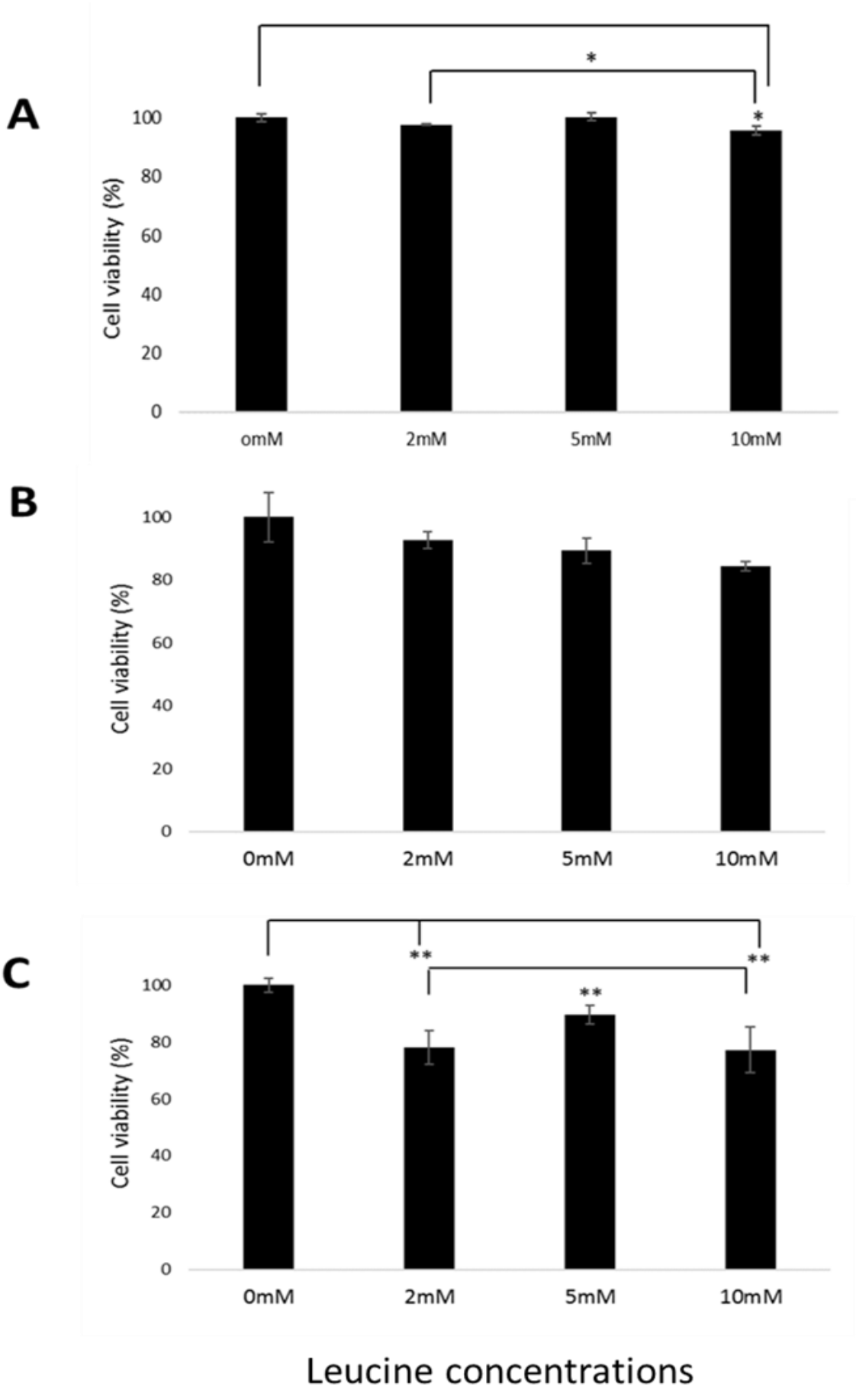

A
Cell viability (%)
100
80
60
40
20
0
omM
2mM
5mM
10mM
*
*
B
Cell viability (%)
100
80
60
40
20
0
0mM
2mM
5mM
10mM
C
Cell viability (%)
100
80
60
40
20
0
0mM
2mM
5mM
10mM
**
**
**
Leucine concentrations

Figure 4.26. The viability of C2C12 myoblasts following long-term exposure to leucine in media with different serum compositions; assessed using an AlamarBlue viability assay. C2C12 myoblasts were exposed to different concentrations of leucine added to GM (A), dGM (B) or SM (C) for 24 h. Data are presented as means ± SD from three biological replica ($n = 3$). Asterisks indicate significant differences compared to the control (0 mM) and indicate significant differences among leucine treated groups $^{*}P < 0.05$ and $^{**}P < 0.01$. Analysed by one-way ANOVA using Microsoft Excel followed by Tukey's HSD *post hoc* test using SPSS Statistics 20. Abbreviations: GM = growth medium supplemented with FCS; dGM = growth medium supplemented with dialysed FCS; SM = serum-free medium; FCS = foetal calf serum; mM = millimolar.

4.4.17. Leucine changes the total protein content of C2C12 myoblasts when introduced in serum-rich medium

In this experiment, we determined the optimal medium to boost the protein anabolic effect of leucine on proliferating C2C12 cells. The total protein content of C2C12 myoblasts was assessed following 24 h of exposure to different concentrations of leucine, introduced in media with various serum compositions. Results revealed that 24 h of incubation with leucine in dGM and SM failed to induce protein accumulation, compared with the control (0 mM) (Figure 4.27 B and C, respectively). However, exposure to 5 mM in GM significantly increased the total protein content of C2C12 myoblasts when compared with the control ($P < 0.001$) (Figure 4.27 A).

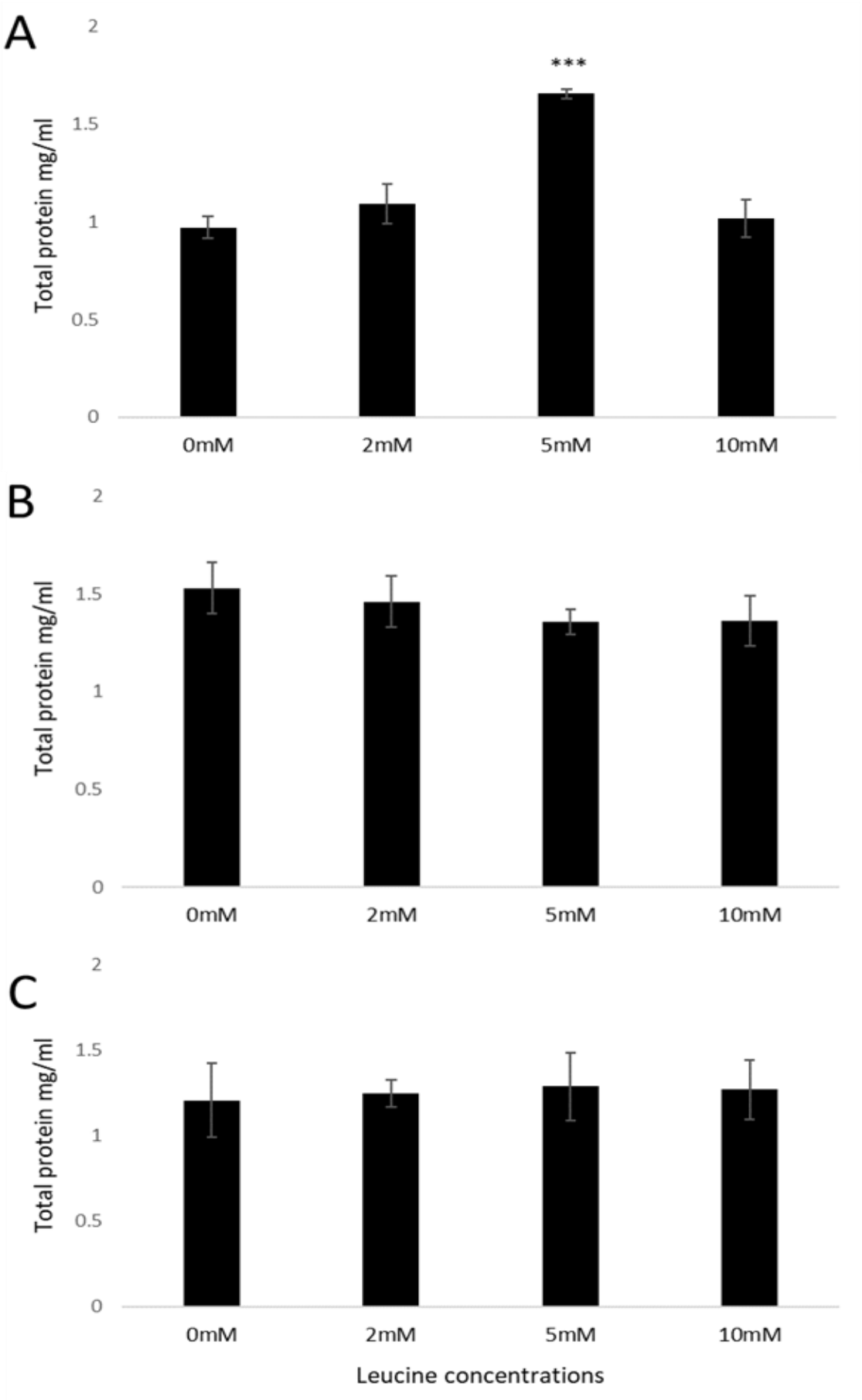

A
Total protein mg/ml
2
1.5
1
0.5
0
***
0mM
2mM
5mM
10mM
B
Total protein mg/ml
2
1.5
1
0.5
0
0mM
2mM
5mM
10mM
C
Total protein mg/ml
2
1.5
1
0.5
0
0mM
2mM
5mM
10mM
Leucine concentrations

Figure 4.27. Total protein content of C2C12 myoblasts following long-term incubation with increasing concentrations of leucine added to media with different serum compositions; assessed using a BCA protein assay. C2C12 myoblasts were exposed to different concentrations of leucine added to GM (A), dGM (B) or SM (C) for 24 h. Data are presented as means ± SD from three biological replica (n = 3). Asterisks indicate significant difference compared to the control (0 mM) $^{***}P < 0.001$. Analysed by one-way ANOVA using Microsoft Excel followed by Tukey's HSD *post hoc* test using SPSS Statistics 20. Abbreviations: GM = growth medium supplemented with FCS; dGM = growth medium supplemented with dialysed FCS; SM = serum-free medium; FCS = foetal calf serum; mM = millimolar; mg = milligrams; ml = millilitre.

4.4.18. Short-term exposure to leucine does not alter the viability of C2C12 myoblasts

Based on the previous results, which suggested that leucine alters the viability of proliferating C2C12 cells when introduced in GM or SM but not dGM, the effect of short-term exposure to different concentrations of leucine on the viability of C2C12 myoblasts was assessed in GM and SM only. Results showed that short-term exposure to different concentrations of leucine did not affect the viability of C2C12 myoblasts. However, a gradual decline in viability, that was inversely proportional to leucine concentrations, was noticed in cultures exposed to leucine in SM (Figure 4.28 A), while a gradual increase in viability, directly proportional to leucine concentrations, was recorded in cells exposed to leucine in GM (Figure 4.28 B).

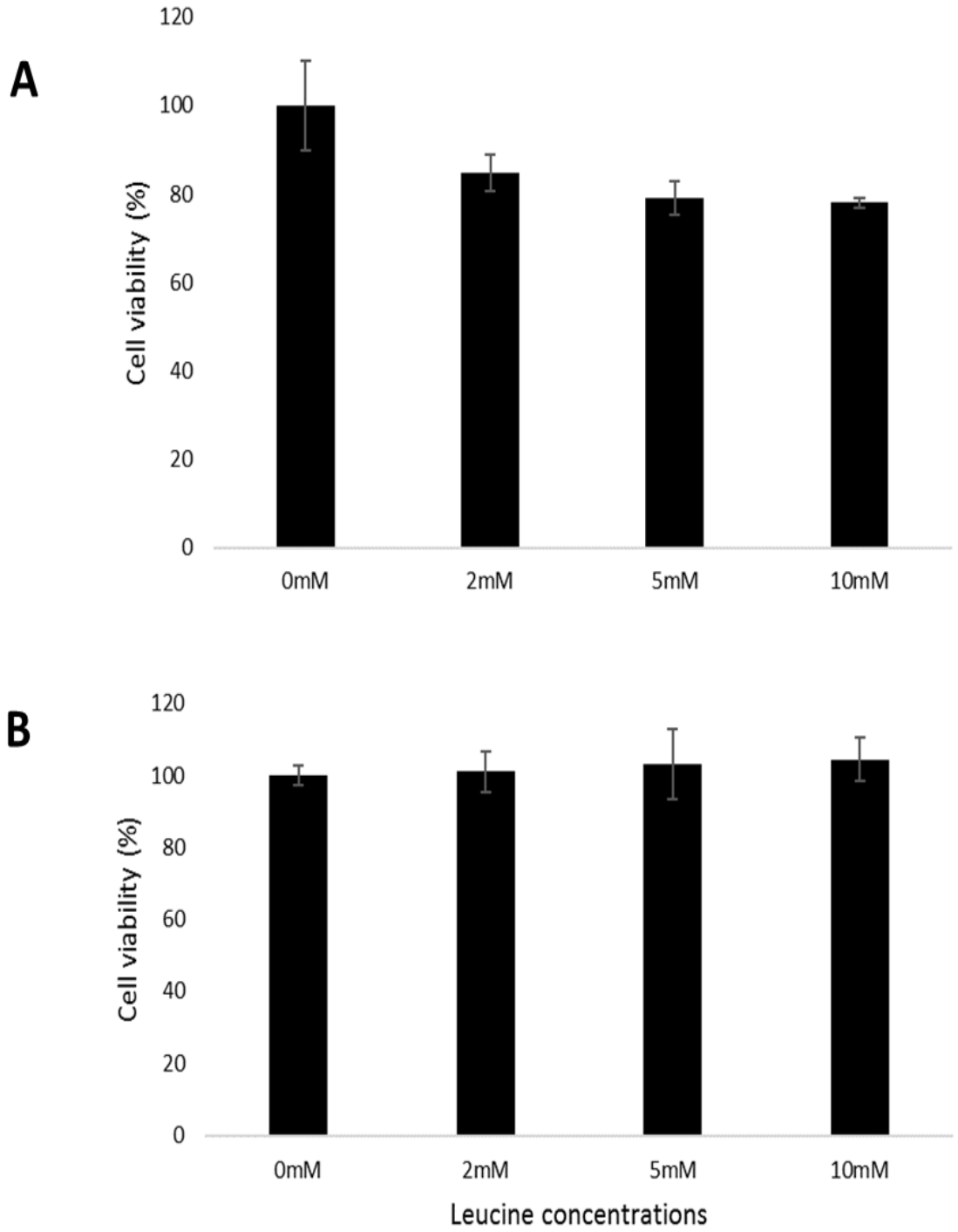


Figure 4.28. The viability of C2C12 myoblasts following short-term exposure to different concentrations of leucine in the absence and in the presence of serum; assessed using an AlamarBlue viability assay. C2C12 myoblasts were exposed to different concentrations of leucine added to SM (A) or GM (B) for 1 h. 0 mM is a negative control. Data are presented as means ± SD from three biological replica (n = 3). Analysed by one-way ANOVA using Microsoft Excel. Abbreviations: GM = growth medium with FCS; SM = serum-free medium; FCS = foetal calf serum; mM = millimolar.

4.4.19. Short-term exposure to leucine changes the total protein content of C2C12 myoblasts

In this experiment, we examined the total protein content of C2C12 myoblasts following short-term incubation with different leucine concentrations added to GM or SM. Results revealed that exposure to all leucine concentrations in GM or SM failed to induce any significant difference in total protein from control (Figure 4.29). Nevertheless, a significant difference within leucine groups was recorded. Exposure of C2C12 myoblasts to different concentrations of leucine in SM induced a stepwise decrease in total protein content, which was inversely correlated to the concentration of leucine. The protein content of C2C12 myoblasts exposed to 5 mM and 10 mM leucine in SM was significantly lower than the protein content of cells exposed to 2 mM leucine ($P < 0.05$) (Figure 4.29 A). In contrast, the protein content of C2C12 myoblasts exposed to different leucine concentrations in GM was directly proportional to the concentrations of leucine. The protein content of C2C12 myoblasts exposed to 10 mM leucine in GM was significantly higher than the protein content of cells exposed to 2 mM and 5 mM leucine ($P < 0.01$ and < 0.05, respectively) (Figure 4.29 B).

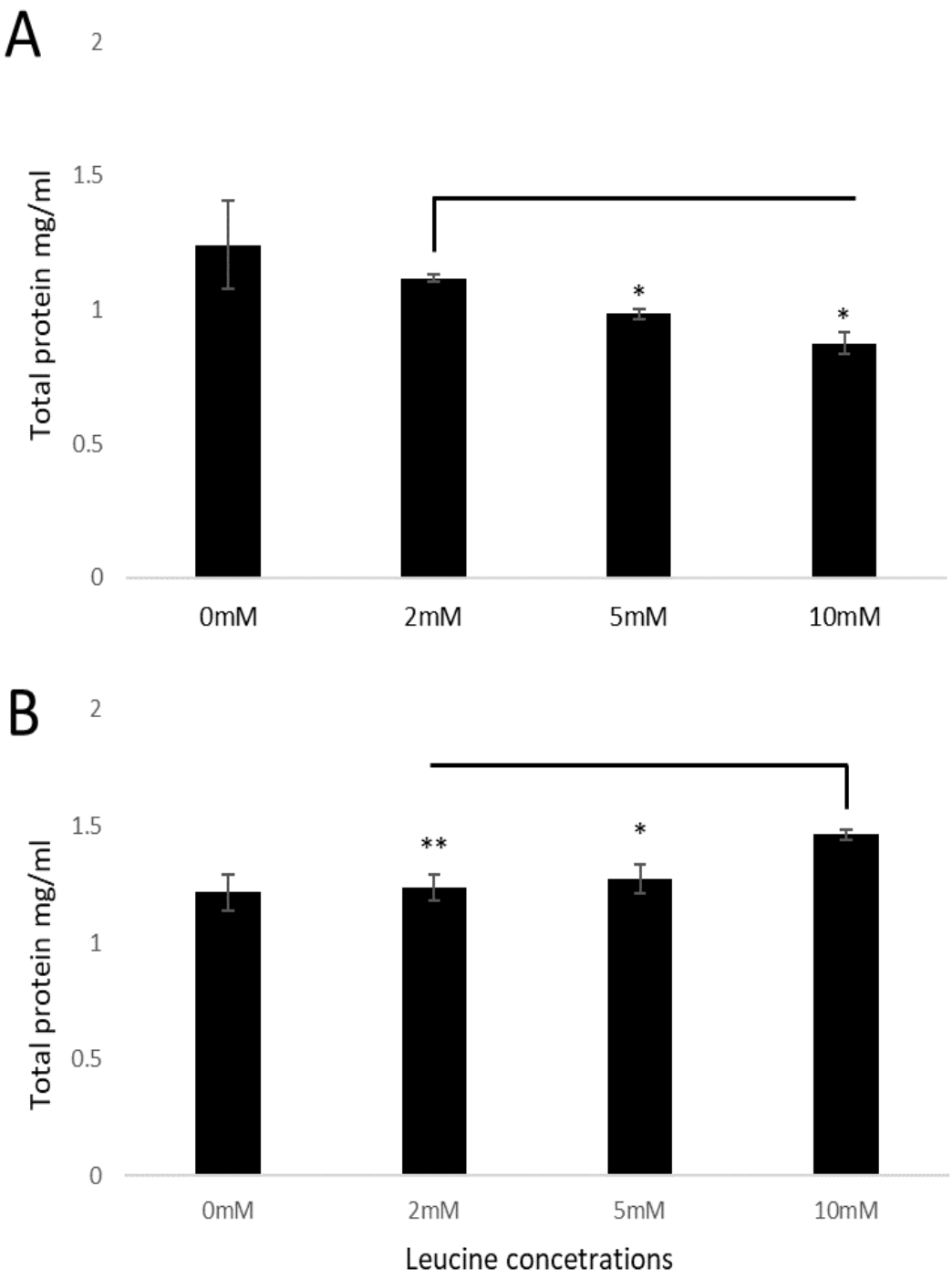


Figure 4.29. Total protein content of proliferating C2C12 cells following short-term exposure to different concentrations of leucine in the absence and in the presence of serum; assessed using a BCA protein assay. C2C12 myoblasts were exposed to different concentrations of leucine added to SM (A) or GM (B) for 1 h. 0 mM is the control. Data are presented as means ± SD from three biological replica (n = 3). Asterisks indicate significant differences among groups $^{*}P < 0.05$ and $^{**}P < 0.01$. Analysed by one-way ANOVA using Microsoft Excel followed by Tukey's HSD *post hoc* test using SPSS Statistics 20. Abbreviations: GM = growth medium with FCS; SM = serum-free medium; FCS = foetal calf serum; mM = millimolar; mg = milligrams; ml = millilitre.

4.4.20. Serum starvation decreases the total protein content of C2C12 myotubes

The aim of this experiment was to investigate the impact of different starvation protocols on differentiated C2C12 cells. The total protein content of C2C12 myotubes was evaluated before and following serum withdrawal. Results indicated that brief serum starvation for 1 h did not result in a significant decrease in total protein content (Figure 4.30 A). However, both 4 h and 16 h of serum starvation significantly decreased the total protein content of C2C12 myotubes ($P < 0.01$) when compared with the control (Figure 4.30 B and C, respectively).

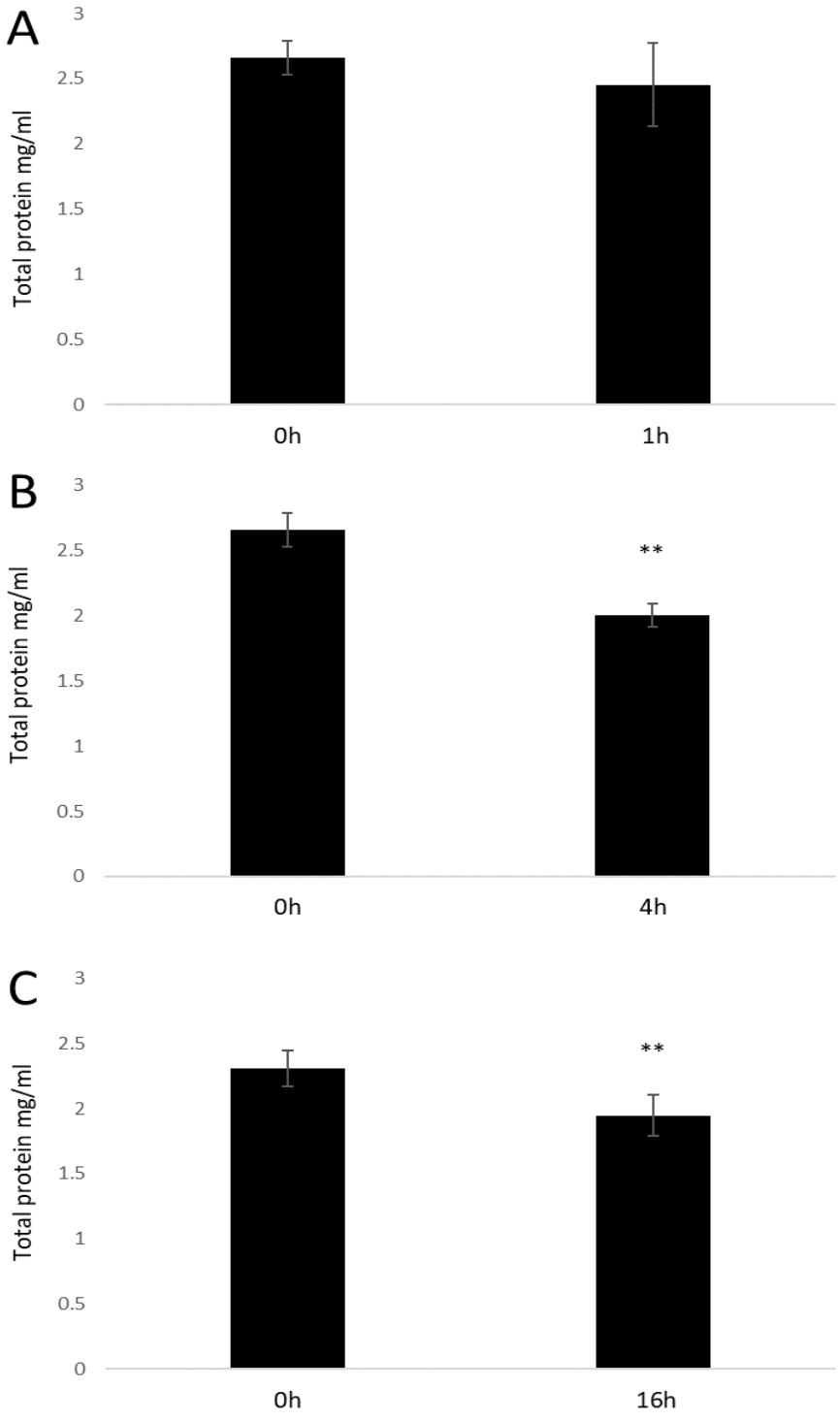


Figure 4.30. Total protein content of C2C12 myotubes prior to and following short- and long-term serum starvation; assessed using a BCA protein assay. C2C12 myotubes were kept in GM (0h) or starved of serum for 1- (A), 4- (B) or 16- (C) h. Data are presented as means ± SD from three biological replica (n = 3). Asterisks indicate significant difference compared to the control (cells prior to serum starvation; 0h) $^{**}P < 0.01$. Analysed by a paired two-tailed Student's t-test using Microsoft Excel. Abbreviations: 0h = cells prior to serum starvation; 1h = cells after 1 hour of serum starvation; 4h = cells after 4 hours of serum starvation; 16h = cells after 16 hours of serum starvation; mg = milligrams; ml = millilitre.

4.4.21. Long-term incubation in serum-free medium decreases the total protein content of C2C12 myotubes

The aim was to identify the optimal medium for testing the protein anabolic effects of leucine on differentiated C2C12 cells. Results showed that 24 h incubation in SM significantly decreased ($P < 0.05$) the protein content of C2C12 myotubes, compared with the control; DM (Figure 4.29).

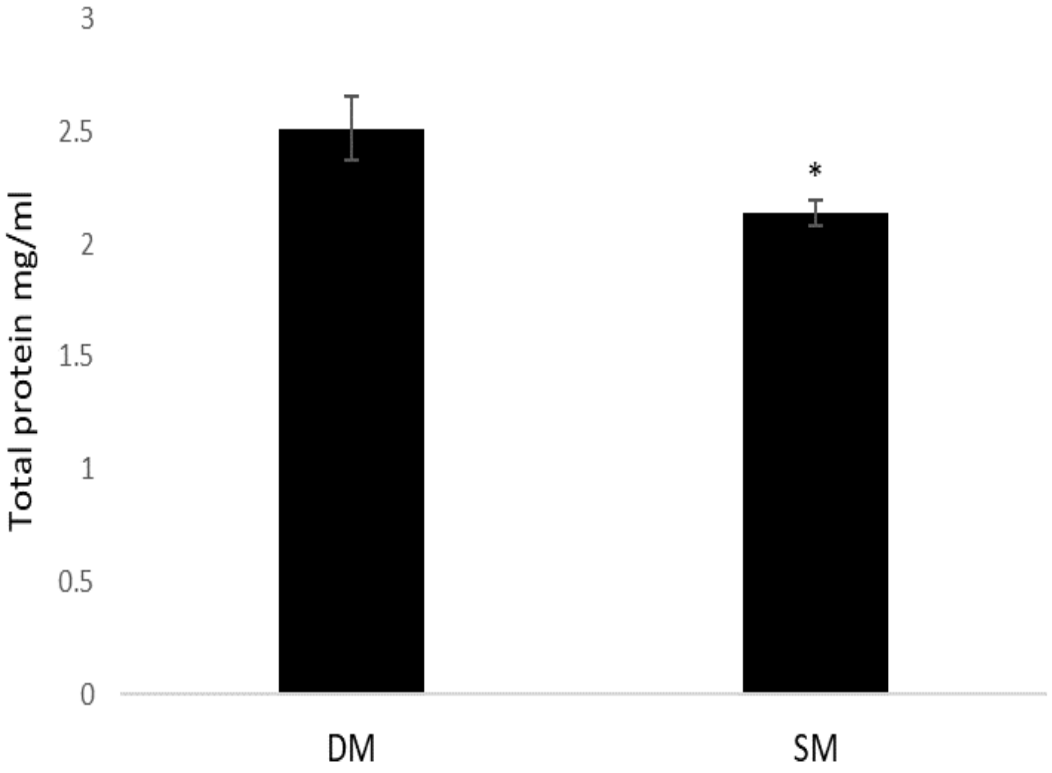


Figure 4.31. Total protein content of C2C12 myotubes following long-term incubation in serum-free medium; assessed using a BCA protein assay. C2C12 myotubes were incubated in DM or SM for 24 h. Data are presented as means ± SD from three biological replica ($n = 3$). Asterisks indicate significant differences compared to the control (cells incubated in DM) $^{*}P < 0.05$. Analysed by a paired two-tailed Student's *t*-test using Microsoft Excel. Abbreviations: DM = differentiation medium supplemented with HS; SM = serum-free medium; HS = horse serum; mg = milligrams; ml = millilitre.

4.4.22. Serum changes the morphology of C2C12 myotubes

We examined the impact of serum deprivation on differentiated C2C12 cells. A morphological examination of differentiated C2C12 cultures revealed an increased number of hypertrophic myotubes in cultures maintained in serum (DM) compared with cultures deprived of serum (SM) (Figure 4.32).

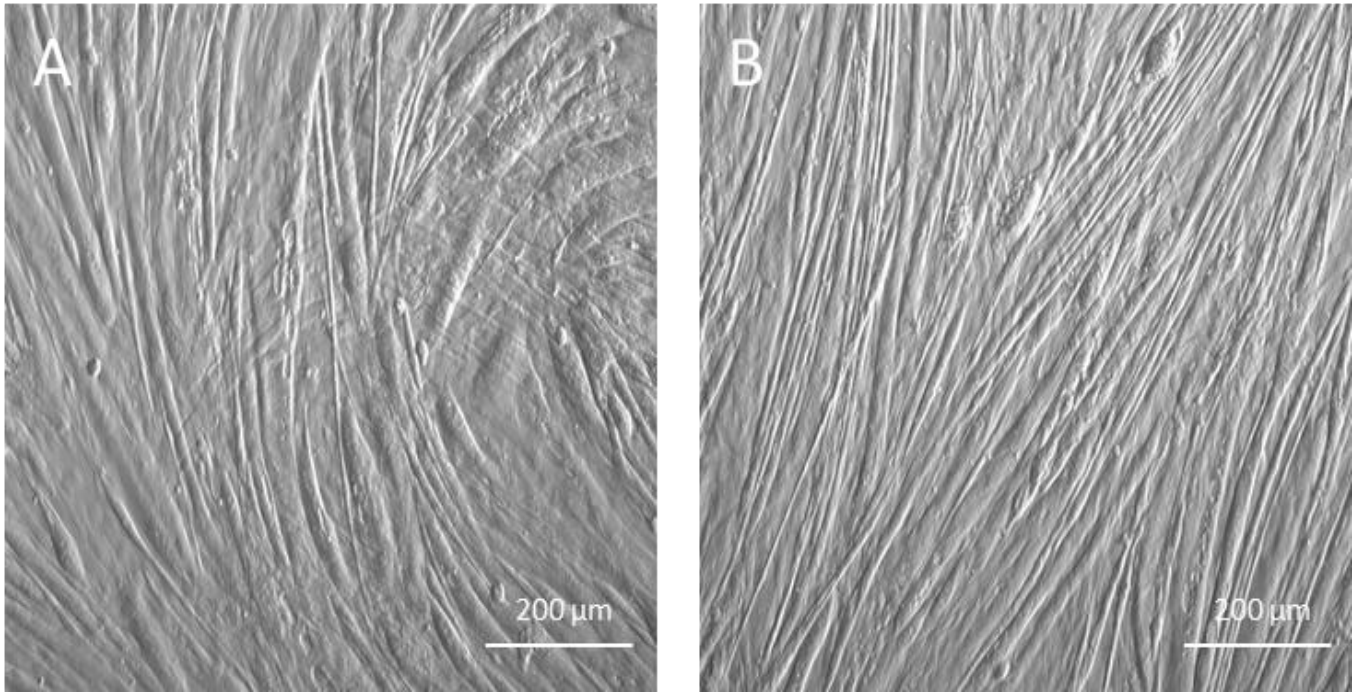


Figure 4.32. Phase-contrast images revealing the morphology of C2C12 myotubes following long-term incubation in the presence and in the absence of serum. C2C12 myotubes were incubated in DM (A) or SM (B) for 24 h. (*n* = 3). Abbreviations: DM = differentiation medium supplemented with HS; SM = serum-free medium; HS = horse serum; µm = micrometre.

4.4.23. Leucine alters the total protein content of C2C12 myotubes when introduced in serum-containing medium

We determined the optimal medium to boost the protein anabolic effect of leucine on differentiated C2C12 cells. The total protein content of C2C12 myotubes was assessed following long-term exposure to different concentrations of leucine added to DM or SM. Results showed that 24 h of exposure to leucine in SM did not alter the total protein content of C2C12 myotubes (Figure 4.33 A). However, a significant increase in the total protein content, compared with the control (0 mM), occurred following incubation of C2C12 myotubes with 2- and 5 mM leucine added to DM ($P < 0.05$) (Figure 4.33 B). Notably, a significant decrease in total protein occurred post-incubation of C2C12 myotubes with 10 mM leucine added to DM ($P < 0.05$) (Figure 4.33 B).

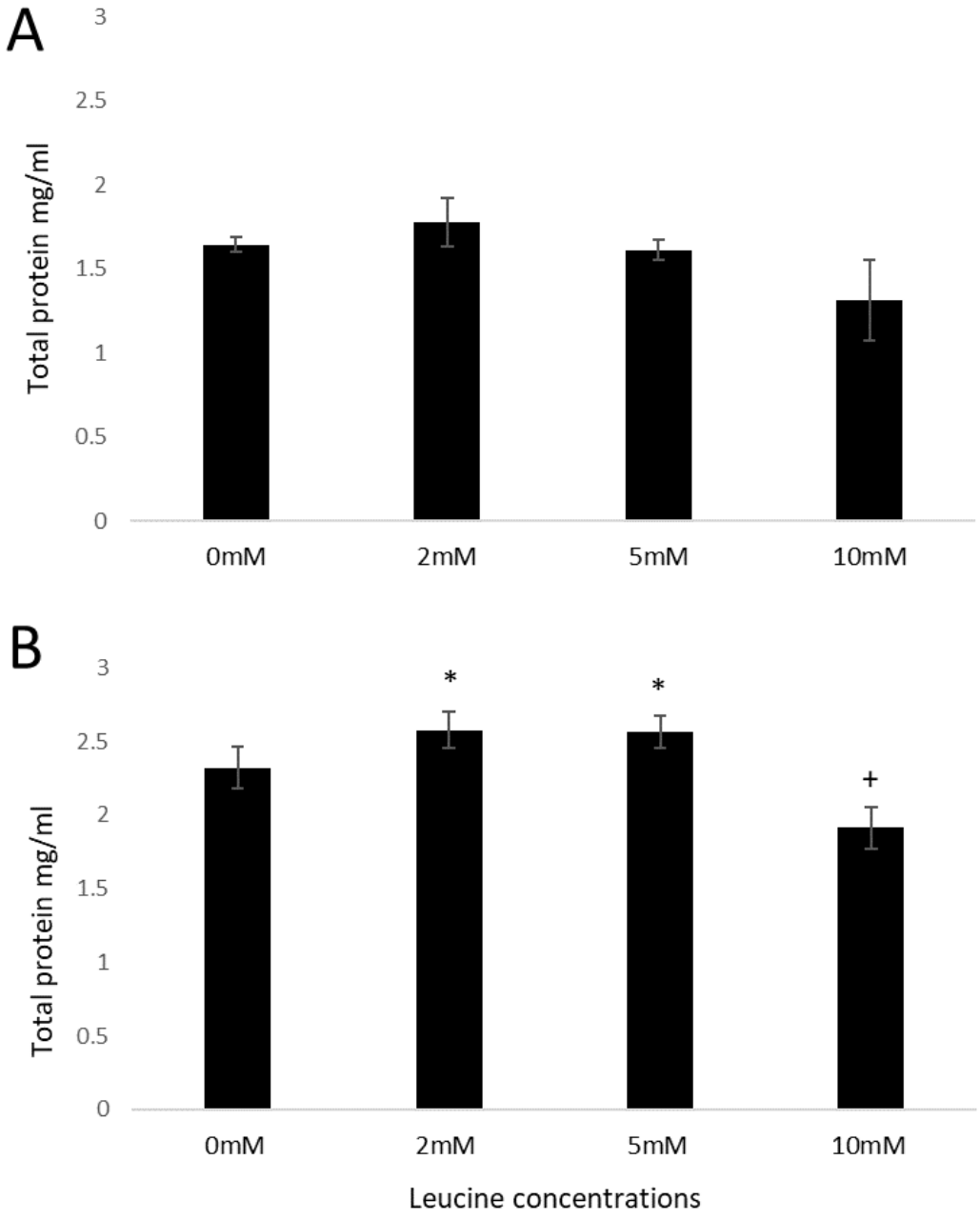


Figure 4.33. Total protein content of C2C12 myotubes following long-term exposure to different concentrations of leucine in the absence and in the presence of serum; assessed using a BCA protein assay. C2C12 myotubes were exposed to 0-, 2-, 5- and 10 mM leucine in SM (A) or DM (B) for 24 h**.** Data are presented as means ± SD from three biological replica (*n* = 3). Asterisks indicate significant increase compared to the control (0 mM) $^{*}P < 0.05$ and $^{**}P < 0.01$. Crosses indicate significant decrease from control $^{+}P < 0.05$. Analysed by one-way ANOVA using Microsoft Excel followed by Tukey's HSD *post hoc* test using SPSS Statistics 20. Abbreviations: DM = differentiation medium supplemented with HS; SM = serum-free medium; HS = horse serum; mM = millimolar; mg = milligrams; ml = millilitre.

4.4.24. Prolonged serum starvation increases the sensitivity of C2C12 myotubes to lower concentrations of leucine

Here, we determined the optimal starvation protocol to boost the protein anabolic effect of leucine on differentiated C2C12 cells. Results showed that exposure to 2 mM leucine significantly increased ($P < 0.01$) the total protein content, compared with control, in myotubes starved of serum for 16 h prior to leucine treatment (Figure 4.34 A). However, exposure to 5 mM leucine significantly ($P < 0.05$) increased the total protein content of myotubes starved of serum for 4 h (Figure 4.34 B). Interestingly, 24 h exposure to 10 mM leucine decreased ($P < 0.05$) the total protein in both groups (Figure 4.34).

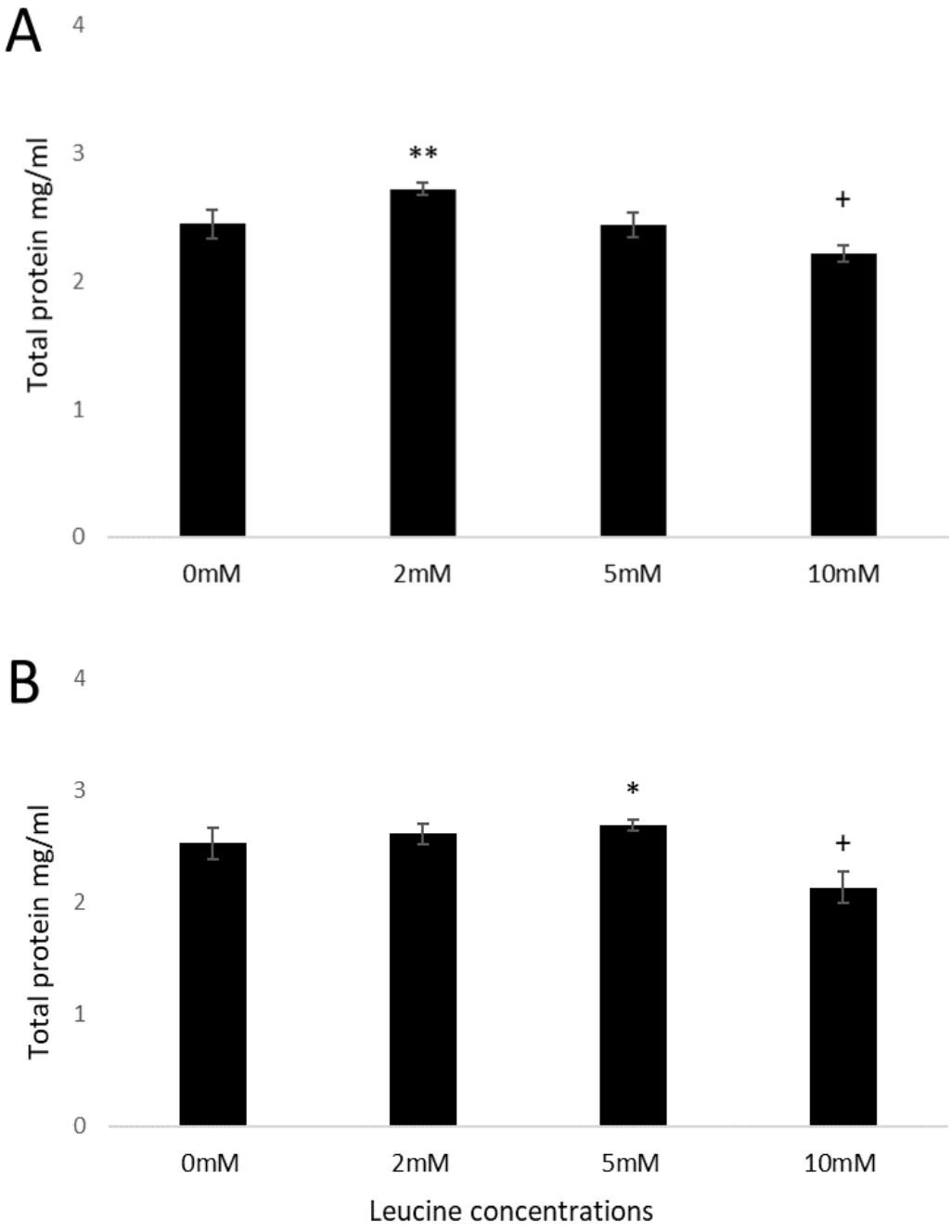


Figure 4.34. Total protein content of C2C12 myotubes following exposure to leucine after short- and long-term serum starvation; assessed using a BCA protein assay. C2C12 myotubes were starved of serum for 4- (B) or 16- (A) h before 24 h incubation with different concentrations of leucine added to DM. Data are presented as means ± SD from three biological replica ($n = 3$). Asterisks indicate significant increase compared to the control (0 mM) $^{*}P < 0.05$ and $^{**}P < 0.01$. Crosses indicate significant decrease from the control $^{+}P < 0.05$. Analysed by one-way ANOVA using Microsoft Excel followed by Tukey's HSD *post hoc* test using SPSS Statistics 20. Abbreviations: mM = millimolar; mg = milligram; ml = millilitre.

4.5. Discussion

The overall goal of this chapter was to assess the effect of increasing concentrations of leucine on proliferating and differentiated C2C12 cells. To achieve this, it was essential to identify the optimal culture conditions (starvation regimens and incubation media) that would preserve the viability and protein content of cells prior to, and during, leucine treatment.

4.5.1. Impact of glutamine deprivation on the viability of C2C12 myoblast cells

Glutamine is essential for cell growth and mitochondrial function (Eagle., 1955; Ahmad *et al*., 2001; Yang *et al*., 2014; Jeong *et al*., 2016). However, many previous studies examined the effect of leucine in the absence of glutamine- despite the catabolic effects of glutamine deprivation on a wide range of cells (Shanware *et al*., 2014; Zhu *et al*., 2015; Chen *et al*., 2015). Therefore, in the present study it was essential to examine the short- and long-term effects of glutamine deprivation on the viability of C2C12 myoblasts. Results showed that short-term glutamine deprivation did not affect the viability of C2C12 myoblasts. However, long-term deprivation of glutamine induced a significant increase in the viability of C2C12 myoblasts. This surprising result may be an adaptive response of proliferating C2C12 cells to glutamine shortage. In support to this postulation, two reports exist of an adaptive mechanism undertaken by glutamine-utilising cells (i.e., monocytes) to compensate for long-term glutamine shortage in which neither the viability nor the vitality of cells were altered (Spittler *et al*., 1995; Spittler *et al*., 1997).

4.5.2. Impact of serum restriction on the viability of C2C12 myoblast cells

Serum is very rich in compounds that are crucial for supporting the growth and functions of a broad range of eukaryotic cells *in vitro*. However, serum withdrawal results in synchronization of the cell cycle, and has become an essential approach in many scientific studies (reviewed by Pirkmajer and Chibalin, 2011). It has been common to examine the effect of leucine in the absence of serum. To date, the effect of serum-deprivation prior to leucine treatments has not been thoroughly investigated in myogenic cells. Results of the present study revealed that the viability of C2C12 myoblasts was unaffected by 1 h of serum deprivation. However, a

significant increase in cell viability took place following 4 h of serum withdrawal.

This boost in viability following 4 h of serum withdrawal was unexpected, and contradicts our hypothesis that serum starvation, prior to leucine exposure, adversely affects the viability of C2C12 cells. Taking into consideration that the AB viability assay reflects the metabolic status of cells, it is possible that short-term serum deprivation made C2C12 myoblasts metabolically active and consequently more viable. It is also possible that short-term serum starvation increased the flow of energy from the culture medium to the cells, in order to mitigate the effect of serum limitation – a measure that cannot be sustained for long periods, which explains why, in this study, a significant decrease in viability occurred following long-term incubation of C2C12 cells in SM. Herein, long-term serum starvation resulted in a decline in cell viability concomitant with low cell density and multiple cell deaths. This finding agrees with our hypothesis that long-term incubation in serum-free media will adversely affect the viability and survival of C2C12 cells and concurs with several reports of cell death and apoptosis in different cell types following long-term serum starvation (Smith *et al*., 1999; Khalyfa *et al*., 2005; Goyeneche *et al*., 2006; Maldonado and Muñoz-Pinedo; 2011).

Dialysed FCS was proposed as an alternative to FCS for testing the effect of many compounds on proliferating cells *in vitro*. The process of dialysis reduces the concentrations of low molecular weight compounds (the cut-off molecular weight is 10,000 dalton) such as cytokines, hormones, nucleotides and AAs, which results in weaker growth promotion properties of dialysed FCS, when compared with normal FCS (Palm *et al*., 2015). Herein, short- and long-term incubation of C2C12 myoblasts in dialysed FCS resulted in a substantial decline in cell viability. This finding agrees with our hypothesis in which, we proposed that incubating C2C12 cells in reduced-serum conditions will adversely affect the viability, survival and protein content of cells. It is unclear whether this decreased viability was the result of low metabolic activities of cells or was due to cell loss. However, the morphological appearance of myoblast cultures revealed low cell populations and numerous cell deaths following long-term incubation in dGM. Since the AB assay, used to assess viability, is indirectly correlated with cell number, the low cell viability caused by dialysed FCS may have resulted from decreased cell population. A conclusion that agrees with reports of cell proliferation inhibition, decreased protein synthesis and induction of autophagy in cells cultured in media containing dialysed FCS (Palm *et al*., 2015; Manifava *et al*., 2016). It is also unclear which of the compounds that have been removed during the process of dialysis that caused this reduced proliferation and increased the death rate

of C2C12 myoblasts. However, it has been suggested that low proliferation rate of cells cultured in dialysed FCS, is the outcome of low AAs levels (Palm *et al*., 2015; Manifava *et al*., 2016). It is well-established that mTORC1 is sensitive to the availability of AAs (Wang and Proud, 2009; Goberdhan *et al*., 2016). During periods of AAs scarcity, when FCS is replaced by dialysed FCS, mTORC1 deactivation takes place. This prevents the utilisation of extracellular AAs, especially EAAs, by cultured cells resulting in reduced cell survival and proliferation, and impaired protein synthesis (Palm *et al*., 2015; Manifava *et al*., 2016).

Because of the rather unexpected results obtained from the AB assay, there was a need to assess cell viability by other means. Therefore, the VB-48^{TM} and the VCC assays were used to assess the viability of C2C12 cells. Unlike AB, the VCC assay assesses viability by calculating the percentage ratio of live to dead cells, which is particularly useful in identifying proliferating C2C12 myoblasts. The VB-48^{TM} assay measures viability by assessing intracellular levels of free thiol (reduced glutathione), making it suitable for metabolically active muscle cells. Results of the VB-48^{TM} and the VCC assays revealed a significant reduction in the vitality of C2C12 myoblasts following short-term incubation in SM and dGM– when compared with GM. This low viability was concomitant with low counts of total and live cells, and high numbers of dead cells. In the same line, results of the VB-48^{TM} and the VCC assays indicated a low viability following long-term incubation of C2C12 myoblasts in dGM and SM media, compared with the viability of cells maintained in GM. This low viability was also associated with low counts of total and live cells. These findings agree with our hypothesis and reinforce the earlier conclusion on how dGM and SM media fail to support the proliferation and the survival of C2C12 myoblasts. Surprisingly, long-term incubation of C2C12 myoblasts in GM increased the number of dead cells. This finding could be attributed to a higher rate of cell turnover in cultures supplemented with FCS. If this is the case, then the availability of FCS increased the proliferation of C2C12 myoblasts, and consequently resulted in a higher number of dead cells compared to cells incubated in dGM and SM.

Estimating the diameter of C2C12 myoblasts following short- and long-term incubation in GM, dGM and SM indicated no correlation between serum availability and cell size. Nevertheless, the C2C12 myoblasts that were used to estimate cell diameter were suspended in solution. Suspended myoblasts do not retain their morphology in culture, when they are elongated spindle-shaped, and this may have been the reason underlying this result. Finally, our results indicated that long-term incubation in SM and dGM decreased the mitochondrial membrane

potential of C2C12 myoblasts. A decreased mitochondrial transmembrane potential is known to precede cell apoptosis. Cell apoptosis following long-term serum restriction has been previously reported in many scientific studies (Smith *et al*., 1999; Khalyfa *et al*., 2005; Goyeneche *et al*., 2006; Maldonado and Muñoz-Pinedo; 2011). Although, a statistical conclusion could not be obtained, the findings of this experiment support our hypotheses and reflected how long-term incubation under reduced-serum, and serum-free conditions stressed C2C12 myoblasts.

4.5.3. Serum restriction decreases the total protein content of C2C12 myoblast cells

Here, we examined the impact of serum-free and reduced-serum conditions on the total protein content of proliferating C2C12 cells. We first examined the impact of short-term (1 h and 4 h) serum starvation, prior to leucine treatment, on the protein content of C2C12 myoblasts. The choice of those two time-points was determined from the published literature. Our results revealed a significant reduction in the total protein content of cells following short-term serum withdrawal. Next, we examined the impact of long-term incubation of C2C12 cells in SM and dGM. Results indicated that long-term incubation, in both media, significantly decreased the total protein content. This agrees with our hypotheses that short-term serum starvation, prior to leucine exposure, and long-term incubation of C2C12 cells in serum-free and reduced-serum media, during leucine exposure, will adversely affect the total protein content of cells. Until this stage of work, the reason behind this decline in total protein was unclear. The loss in total protein, following short-term serum starvation of muscle cells, can be attributed to a decreased rate of MPS and/or an increased rate of MPB (Epstein *et al*., 1975; Essén *et al*., 1992; Talvas *et al*., 2006). Herein, the decrease in total protein of C2C12 myoblasts following short- and long-term serum starvation may be related to a lower cell population due to decreased proliferation, and increased cell death as inferred from the results of the VCC Assay.

Overall results indicated that short- and long-term incubation in SM and dGM decreased the viability, the vitality, the cellular turnover, the survival and the total protein content of C2C12 myoblasts. At this stage it became clear that neither SM nor dGM media are suitable for testing the anabolic effects of leucine on C2C12 myoblasts. To further support this conclusion, the effect of increasing concentrations of leucine added to SM, dGM and GM on the viability and

total protein content of C2C12 myoblasts was investigated.

4.5.4. The effect of leucine on the viability and total protein contents of the C2C12 myoblast cells is mediated by the serum compositions of the culture medium

Our results showed that long-term exposure to leucine in dGM did not induce any changes in cell viability. However, cell viability was significantly improved following exposure of C2C12 myoblasts to a physiological leucine concentration (5 mM) in both SM and GM. The increased viability of C2C12 myoblasts exposed to 5 mM leucine in GM agrees with our third hypothesis. However, the increased viability of C2C12 myoblasts incubated with this leucine concentration in SM was unexpected. Nevertheless, knowing that the viability of cells was assessed using the AB assay, suggests that this increase might be due to a compensatory mechanism undertaken by serum-deprived C2C12 myoblasts as discussed earlier. On the other hand, exposure to a high concentration of leucine (10 mM) decreased the viability of C2C12 myoblasts. The reduction in cell viability following long-term exposure to 10 mM leucine may have resulted from reaching an intracellular supraphysiological concentrations of this BCAA, which can suppress cell viability; a suggestion that is supported by previous findings in C2C12 myotubes (Haegens *et al*., 2012). Interestingly, long-term leucine deprivation enhanced the viability of C2C12 myoblasts. This finding has not been previously reported and contradicts reports of autophagy in C2C12 cells following leucine withdrawal (Mordier *et al*., 2000; Mordier *et al*., 2006). It is feasible that leucine deprivation induced a cell cycle arrest, a conclusion that agrees with the findings of a previous study (Everhart and Prescott, 1972). This conclusion also explains why leucine deprivation, in present work, was associated with increased numbers of live cells.

Results also showed that long-term exposure to leucine in dGM and SM failed to alter the protein content of C2C12 myoblasts; although 5 mM leucine did promote protein accumulation when it was introduced to C2C12 myoblasts in GM. These findings agree with our third hypothesis where we proposed that serum restriction will disrupt the anabolic effects of leucine on C2C12 cells. These findings are also consistent with a previous report of impaired ability of leucine to induce protein synthesis in C2C12 cells when introduced in saline (Areta *et al*., 2014) and indicate a key role for serum in attaining the protein effects of leucine on muscle cells.

Altogether, the results of viability and protein accumulation following long-term incubation of proliferating C2C12 cells with leucine, emphasised the essential role of serum in mediating the anabolic properties of leucine. The results of this work also strongly suggest that GM is the best medium to evaluate the long-term effect of leucine on C2C12 myoblasts, without sacrificing either their viability or their protein content.

4.5.5. The effects of serum restriction on the total protein content of C2C12 myotubes

The second part of this chapter was devoted to identifying the optimal conditions – starvation protocols and incubation media – for preserving the viability and protein content of differentiated C2C12 cells prior to, and during, leucine treatment. As C2C12 myotubes' requirement for serum is low, the choice of incubation media was limited to either DM or SM. One limitation of note was that the viability of differentiated C2C12 myotubes could not be assessed by either the AB viability assay, which is not recommended for differentiated muscle cells or the VB-48^{TM} assay, which requires cells to be in suspension. Alternatively, assessing the total protein content was the approach used to evaluate the effect of serum withdrawal on differentiated C2C12 myotubes.

Using information from previously published literature, C2C12 myotubes were starved of serum for 1-, 4- or 16 h. Results showed that serum starvation for 1 h did not affect the protein content of C2C12 myotubes. However, serum starvation for 4 and 16 h resulted in a significant decrease in total protein. Similarly, long-term incubation of C2C12 myotubes in SM also resulted in a significant decline in protein content alongside a decrease in myotube size, when compared with DM. These findings suggest that protein degradation occurs within C2C12 myotubes following serum starvation and agree with our first hypothesis.

4.5.6. The effect of leucine on the total protein contents of the C2C12 myotubes is mediated by the serum compositions of the culture medium

The results of incubating C2C12 myotubes with different concentrations of leucine in DM and SM revealed that the total protein content of C2C12 myotubes was not altered by leucine introduced in SM. However, long-term exposure to physiological concentrations of leucine (2

mM and 5 mM) in DM significantly increased the total protein content of cells. These findings indicate that leucine-induced protein accumulation in differentiated C2C12 myotubes is serum-dependent. They also agree with our third hypothesis and are consistent with a previous study, which showed that exposure to leucine in saline failed to induce protein synthesis in C2C12 myotubes (Areta *et al*., 2014).

4.6. Conclusion

Taken together, this chapter optimised the experimental conditions (starvation regimens and incubation media) required for testing the effect of leucine on proliferating and differentiated C2C12 cells. This study also evaluated the effect of long-term exposure of proliferating and differentiated C2C12 cells to increasing concentrations of leucine added to media with different serum contents. Results showed that serum starvation, prior to leucine treatment, reduced the viability, vitality and protein content of C2C12 myoblasts. Additionally, incubation of C2C12 myoblasts in serum-free and reduced-serum media reduced the viability, vitality, proliferation, survival and the total protein content of C2C12 myoblasts. In common with myoblasts, prolonged serum starvation and incubation of differentiated C2C12 myotubes in serum-free media significantly decreased the protein content of these cells. Most importantly, the findings of this chapter have shown that serum mediates the anabolic effects of leucine on C2C12 myoblasts and myotubes.

5. Characterising the long-term effects of leucine and/or glutamine on C2C12 cells

5.1. Introduction

BCAAs are a group of three EAAs, leucine, valine and isoleucine, which are well-known for their protein-enhancing properties. Notably, all of the protein anabolic properties of BCAAs are mainly attributed to leucine (Escobar *et al*., 2006). Although a large body of literature illustrating the protein anabolic effect of leucine on muscle cells exists, little is known about leucine's effect on cell viability. One study reported an inverse relationship between the viability of C2C12 myotubes and leucine concentrations (Kim *et al*., 2015). However, this study tested low concentrations of leucine (< 1 mM) and it has been reported that only high concentrations of leucine are capable of stimulating MPS (Fox *et al*., 1998; Garlick, 2005; Kobayashi *et al*., 2006; Areta *et al*., 2014). Therefore, it is essential to assess the impact of higher concentrations of leucine (≥ 2 mM) on the viability of C2C12 cells.

Additionally, limited attention has been paid towards assessing protein accumulation post-leucine treatment. Most studies focused on reporting alterations in the rate of MPS following exposure to leucine and failed to examine the total protein content. One study suggested that leucine does not alter the total protein content of differentiated C2C12 myotubes (Haegens *et al*., 2012). However, in that study, C2C12 myotubes were exposed to low concentrations of leucine in nutrient-defined medium (Haegens *et al*., 2012). These experimental conditions may have hampered the protein anabolic effects of leucine.

Also, most studies investigated the effect of leucine on the rate of MPS, following short-term (≤ 60 min) exposure to leucine (Anthony *et al*., 2000, Anthony *et al*., 2002; Crozier *et al*. 2005). However, assessing the short-term effect of leucine is not informative, as it has been demonstrated in both cardiac and skeletal muscle cells that any increase in MPS rates following leucine infusion cannot be sustained beyond 2 h (Escobar *et al*., 2005; Wilson *et al*., 2010).

Finally, the interdependence between glutamine metabolism and leucine metabolism has been well-established (Holeck, 2002), with glutamine also facilitating the delivery of leucine into cells (Nicklin *et al*., 2009). However, the effect of treating myogenic cells with a combination

of leucine and glutamine has not been well-defined, as a recent study demonstrated that leucine and glutamine exhibit antagonistic effects on the activation of mTOR in C2C12 myotubes (Deldicque *et al*., 2008). Also, data on the combined effects of leucine and glutamine on the viability and proliferation of muscle cells are lacking, as only studies using intestinal and neoplastic cells have been performed in which glutamine and leucine were shown to increase cell proliferation (Ko *et al*., 1993; Chen *et al*., 2013). Whether a combination of both AAs would exert an additive effect on the proliferation of C2C12 myoblasts remains unknown.

Taken together, the aforementioned studies indicate a need to assess the viability and total protein content of muscle cells following long-term exposure to higher concentrations of leucine (≥ 2 mM) in nutrient-rich media. Previous studies also highlight the need to investigate the combined effects of leucine and glutamine on the viability, proliferation and protein content of muscle cells.

5.2. Research hypotheses

A growing number of studies suggest that high concentrations of leucine are required to induce protein synthesis within muscle cells. However, the impact of high leucine concentrations on muscle cell viability is poorly understood. Therefore, we examined the impact of different leucine concentrations on the viability of C2C12 cells by testing the hypothesis that exposure to supraphysiological concentration of leucine will decrease the viability of C2C12 cells.

Despite numerous reports of increased protein synthesis following leucine infusion; such increases were transitory. Therefore, we investigated the long-term effect of leucine on protein accumulation within C2C12 cells by testing the hypothesis that exposure to leucine in nutrient-rich media will induce a sustained increase in the total protein content of C2C12 cells.

Finally, it has been reported that leucine and glutamine exert antagonistic effects on mTOR signalling. Hence, we examined the effects of leucine and/or glutamine on muscle cells by testing the hypothesis that exposure to leucine and glutamine, combined, will enhance the proliferation, viability and total protein content of C2C12 cells.

5.3. The aims of this chapter

The present study was performed in order to define the optimal experimental conditions, specifically leucine concentrations and exposure times required to elicit the protein anabolic properties of leucine on C2C12 cells, to assess the effect of leucine on the viability of C2C12 cells and to assess the combined effect of glutamine and leucine on C2C12 cells.

This was achieved through the following aims:

1. Evaluating the effects of increasing leucine concentrations on the viability of C2C12 cells.
2. Evaluating the effects of increasing leucine concentrations on the total protein content of C2C12 cells.
3. Evaluating the effects of increasing leucine concentrations on the expression of desmin and MyHC-*fast* in C2C12 cells
4. Evaluating the effect of combining different concentrations of leucine with a fixed concentration of glutamine on the viability of C2C12 cells.

5.4. Results

5.4.1. Long-term exposure to leucine changes the viability of C2C12 myoblasts

The aim of this experiment was to determine the optimal leucine concentration capable of increasing the viability of proliferating C2C12 cells. Here, the effect of ascending concentrations of leucine on the viability of C2C12 myoblasts was evaluated. Results demonstrated that none of the leucine concentrations increased cell viability above the negative control. Nonetheless, exposure to 5 mM of leucine induced a significant increase in cell viability ($P < 0.05$) compared with other leucine concentrations (2 mM and 10 mM). A further finding was that long-term exposure to 10 mM leucine resulted in a significant reduction ($P < 0.05$) in cell viability compared with the control (Figure 5.1).

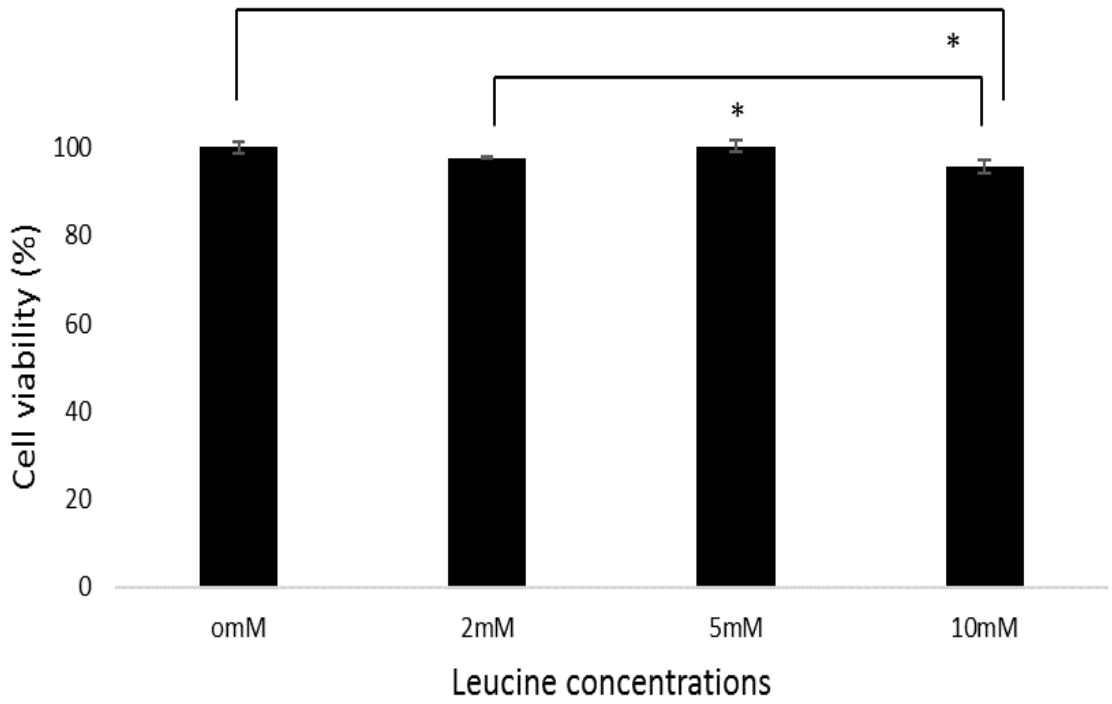
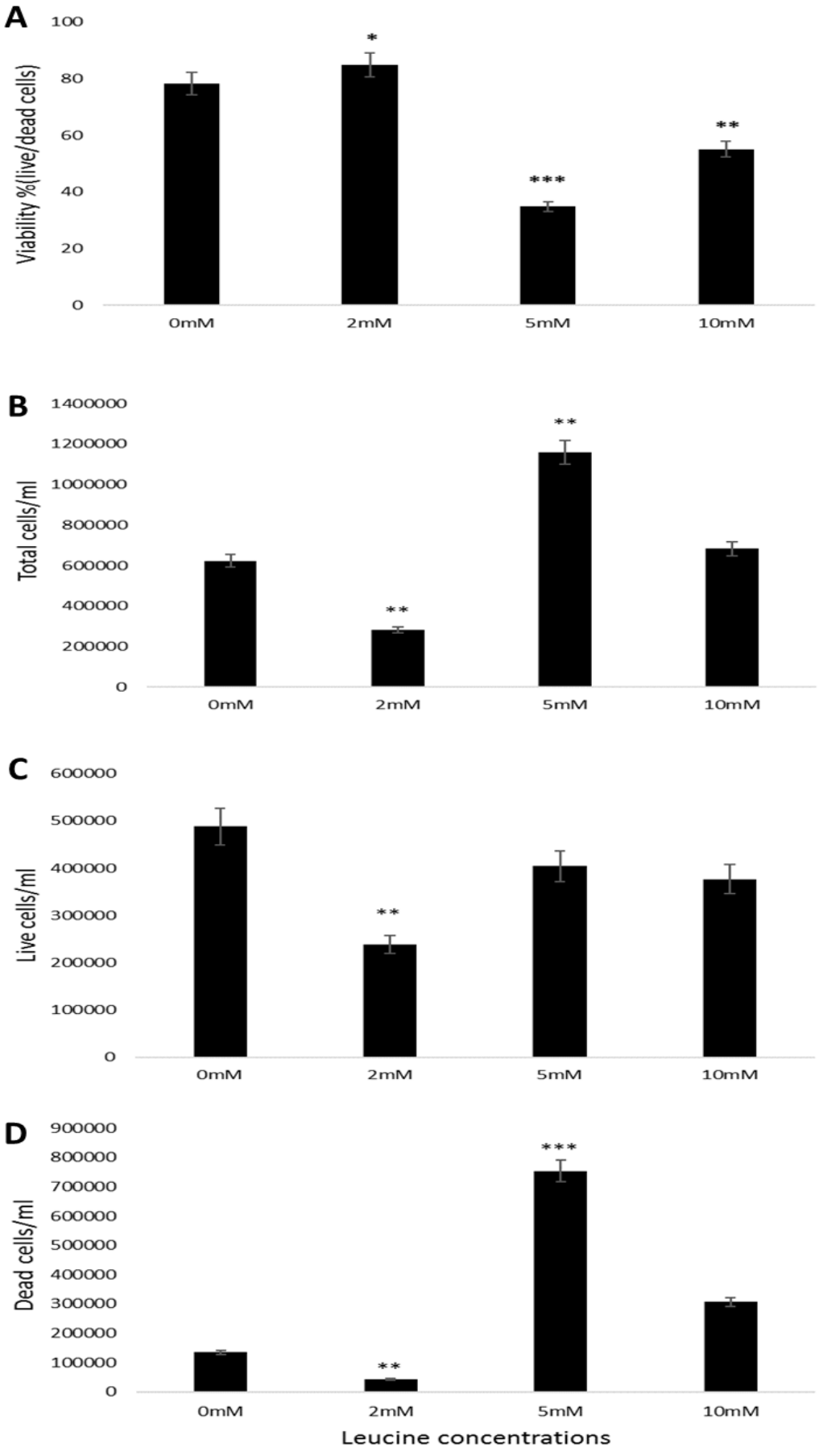


Figure 5.1. The viability of C2C12 myoblasts following long-term incubation with different concentrations of leucine; assessed using an AlamarBlue viability assay. C2C12 myoblasts were exposed for 24 h to different concentrations of leucine added to GM. Data are presented as means ± SD from three biological replica (n = 3). Asterisks indicate significant differences from the control (0 mM) and indicate significant differences among leucine groups $^{*}P < 0.05$. Analysed by one-way ANOVA using Microsoft Excel followed by Tukey's HSD *post hoc* test using SPSS Statistics 20. Abbreviation: mM = millimolar.

5.4.2. Leucine alters the proliferation and turnover rate of C2C12 myoblasts

The aim of this experiment was to detect the optimal leucine concentration to increase the proliferation and survival of C2C12 myoblasts. Results showed that cultures exposed to 5 mM leucine had the highest number of total and dead cells ($P < 0.01$ and < 0.001, respectively) compared with the control (Figures 5.2 B&D and 5.3). Exposure to 2 mM leucine resulted in the lowest number of total, live and dead cell counts, when compared with the control ($P < 0.01$) (Figures 5.2 and 5.3). Similarly, morphological examination of C2C12 myoblast cultures suggested an enhanced rate of proliferation when incubated with 5 mM leucine, as opposed to cell loss when 10 mM leucine was used (Figure 5.4). In addition, results from the Click-iT EdU proliferation assay suggested an increase in the proliferation of C2C12 myoblasts incubated with 5 mM leucine (Figure 5.5).


A
Viability %(live/dead cells)
100
80
60
40
20
0
*
***
**
0mM
2mM
5mM
10mM
B
Total cells/ml
1400000
1200000
1000000
800000
600000
400000
200000
0
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0mM
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C
Live cells/ml
600000
500000
400000
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0mM
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0mM
2mM
5mM
10mM
Leucine concentrations

Figure 5.2. The viability and differential cell count of C2C12 myoblasts following long-term-incubation with different concentrations of leucine; assessed using a NucleoCounter® NC-3000™ Viability and Cell Count Assay. C2C12 myoblasts were exposed for 24 h to increasing concentrations of leucine added to GM. Panel A shows the viability of cells, panel B shows the total cell count per ml, panel C shows the count of live cells per ml and panel D shows the count of dead cells per ml. Data are presented as means ± SD from three biological replica (n = 3). Asterisks indicate significant Data are presented as means ± SD from three biological replica (n = 3). Asterisks indicate significant differences from the control (0 mM) $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$. Analysed by one-way ANOVA using Microsoft Excel followed by Tukey's HSD *post hoc* test using SPSS Statistics 20. Abbreviation: mM = millimolar.

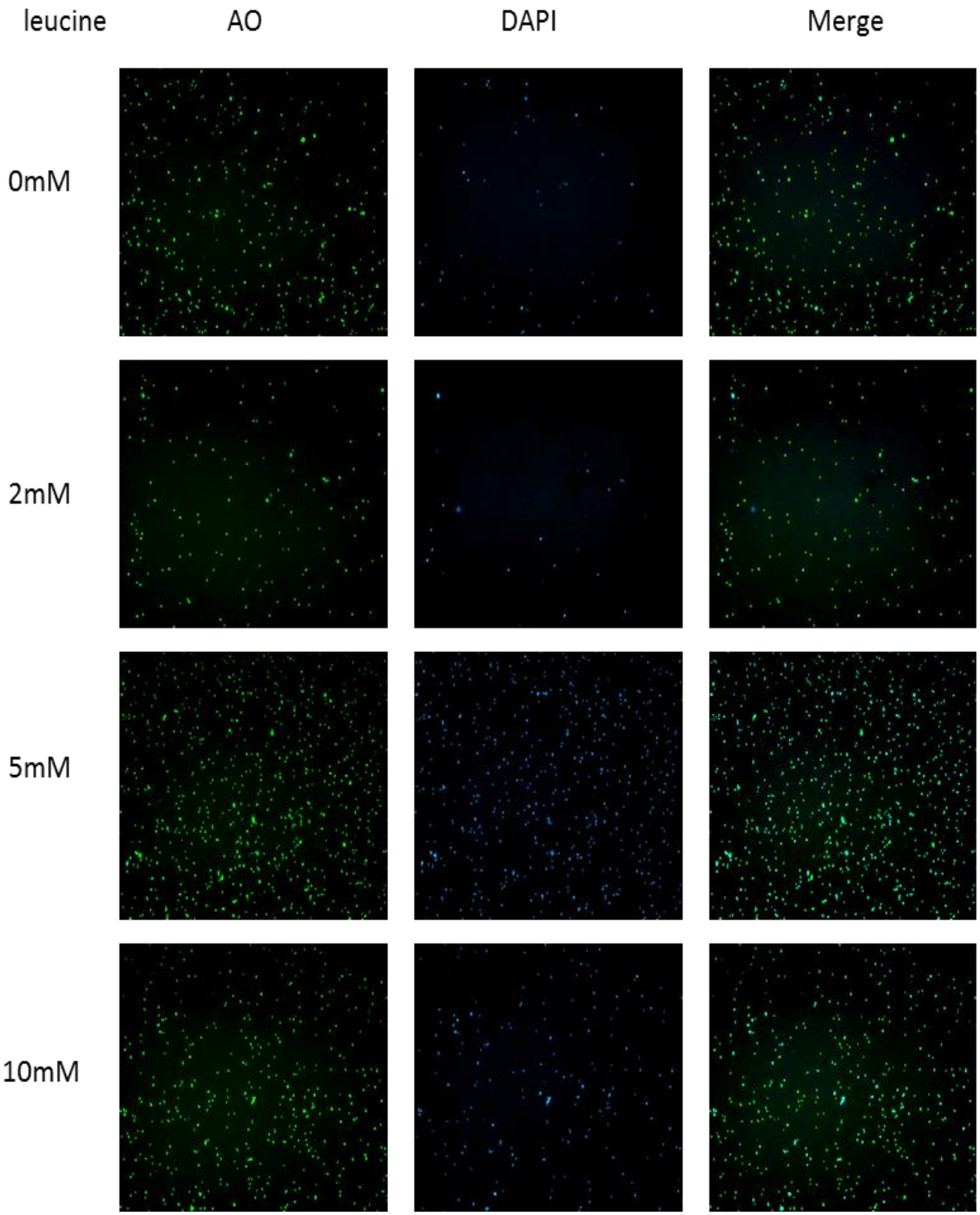


Figure 5.3. Representative images of C2C12 myoblasts following long-term incubation with different concentrations of leucine; obtained using a NucleoCounter® NC-3000™ Advanced Image Cytometer. C2C12 myoblasts were exposed for 24 h to increasing concentrations of leucine added to GM. (n = 3). The left panel shows cells stained with AO (green). The middle panel shows cells stained with DAPI (blue). The right panel is a merge of AO and DAPI stained cells. Abbreviations: AO = Acridine orange stain; mM= millimolar.

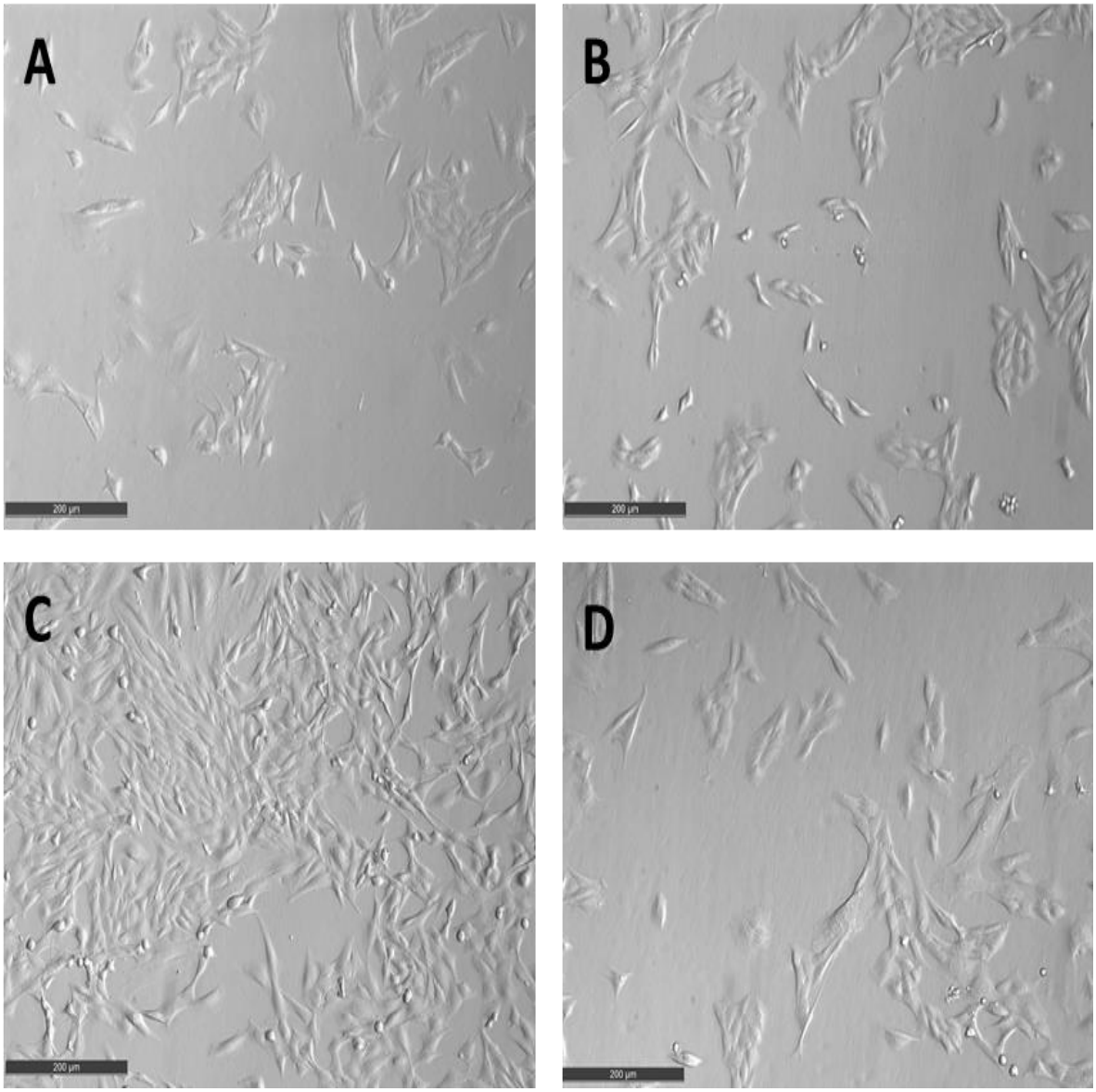


Figure 5.4. Phase-contrast images depicting the morphology of C2C12 myoblast cells following long-term incubation with different concentrations of leucine. C2C12 myoblasts were exposed for 24 h to 0- (A), 2- (B), 5- (C) and 10- (D) mM L-leucine added to GM. (*n* = 3). Abbreviation: µm = micrometre.

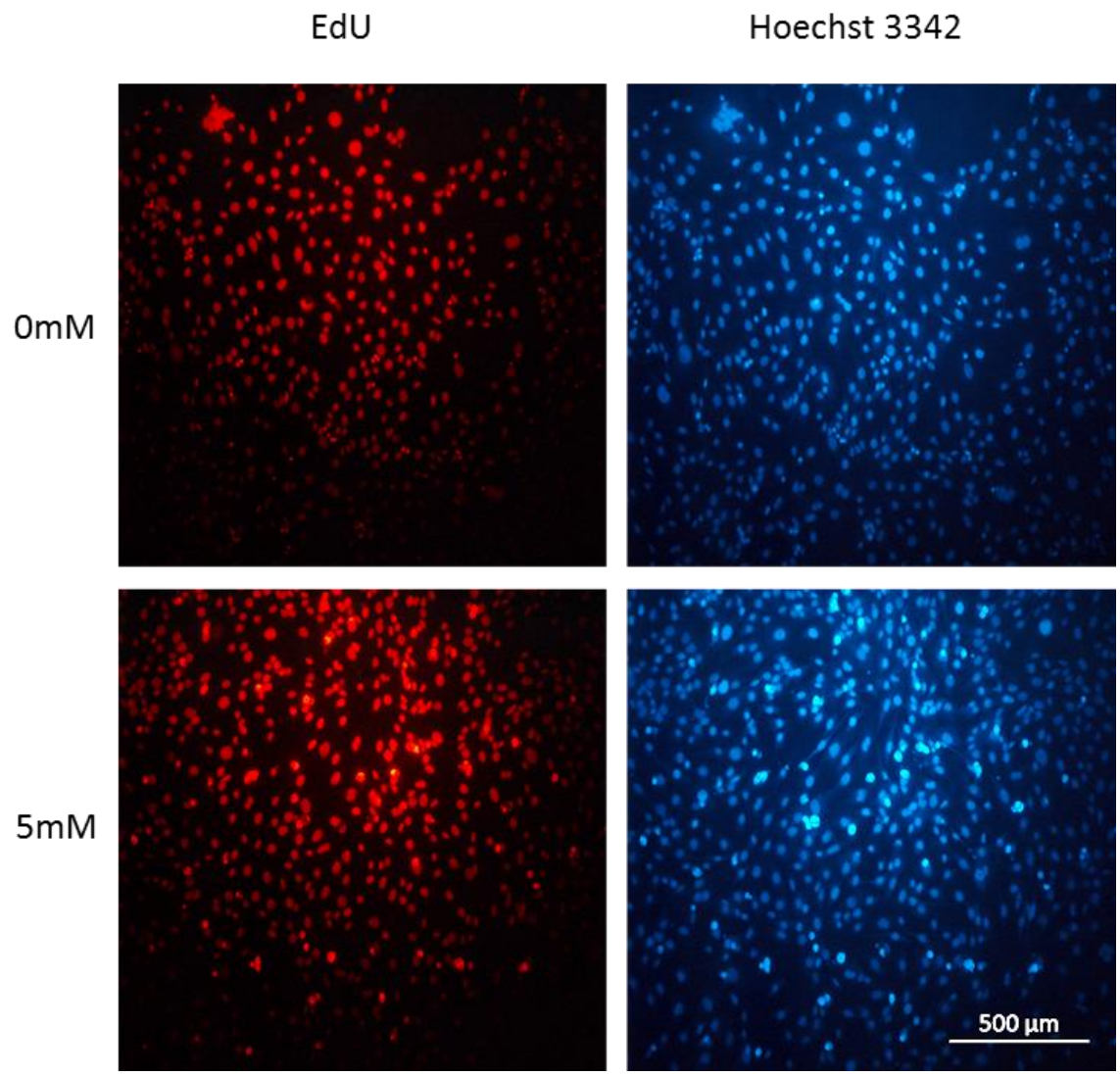


Figure 5.5. Proliferation of C2C12 myoblast cells after long-term exposure to leucine; assessed using a Click-iT EdU proliferation assay. C2C12 myoblasts were exposed for 24 h to 0 mM or 5 mM L-leucine added to GM. (*n* = 1). The left panel shows cells stained with EdU (red). The right panel shows cells stained with Hoechst 3342 (blue). Abbreviations: EdU = 5-Ethynyl-2′-deoxyuridine; mM= millimolar; µm = micrometre.

5.4.3. Exposure to physiological concentrations of leucine increased the expression of desmin in proliferating C2C12 cultures

The aim was to test whether a long-term exposure to leucine would induce the differentiation of proliferating C2C12 cells. Results indicated an increase in the expression of desmin in myoblast cultures exposed to physiological concentrations of leucine (2 mM and 5 mM), when compared with cultures exposed to a supraphysiological leucine concentration (10 mM) and cultures deprived of leucine (Figure 5.6).

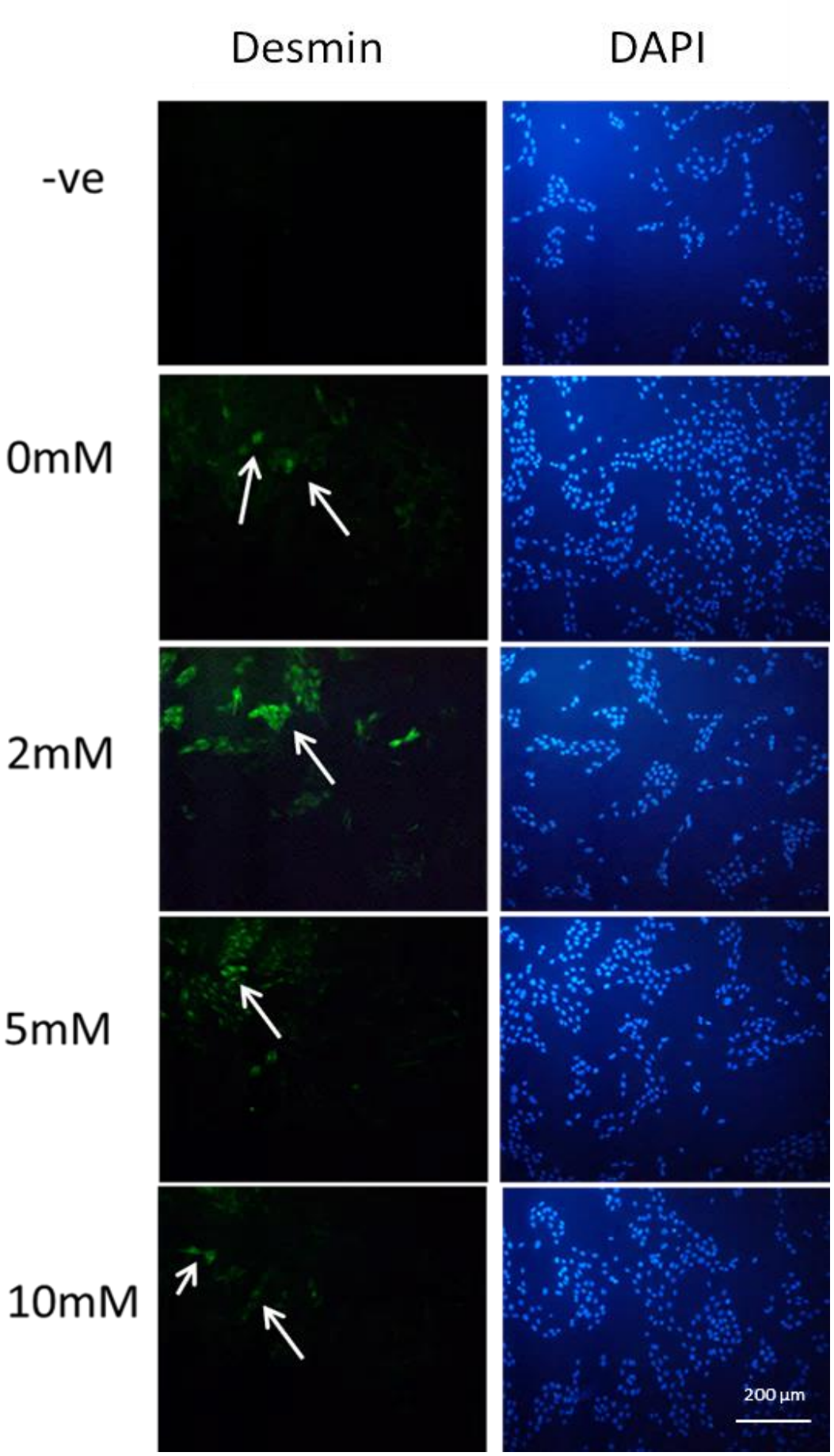

Desmin
DAPI
-ve
0mM
2mM
5mM
10mM
200 µm

Figure 5.6. Expression of desmin in C2C12 myoblast cultures following long-term incubation with different concentrations of leucine; assessed by indirect immunofluorescence. C2C12 myoblasts were exposed for 24 h to increasing concentrations of leucine added to GM. (n = 3). The left panel shows the expression of desmin (green). The right panel shows DAPI stained nuclei (blue). Arrows indicate cells that express desmin. Abbreviations: -ve = negative control (i.e., no primary antibody); mM = millimolar; µm = micrometre.

5.4.4. Long-term exposure to leucine alters the total protein content of C2C12 myoblasts

We examined intracellular protein accumulation following long-term incubation of C2C12 myoblasts with different concentrations of leucine. Results showed a significant increase in total protein, compared with 0 mM, following 24 h of exposure to 5 mM leucine ($P < 0.01$) (Figure 5.7 A). A significant increase in total protein content also occurred following 48 h of incubation with 2 mM and 5 mM leucine ($P < 0.05$ and < 0.01, respectively). However, a significant decrease in the total protein content of cells was detected following incubation with 10 mM leucine ($P < 0.05$) (Figure 5.7 B). Interestingly, exposure to different concentrations of leucine for 72 h failed to change the total protein content of C2C12 myoblasts (Figure 5.7 C).

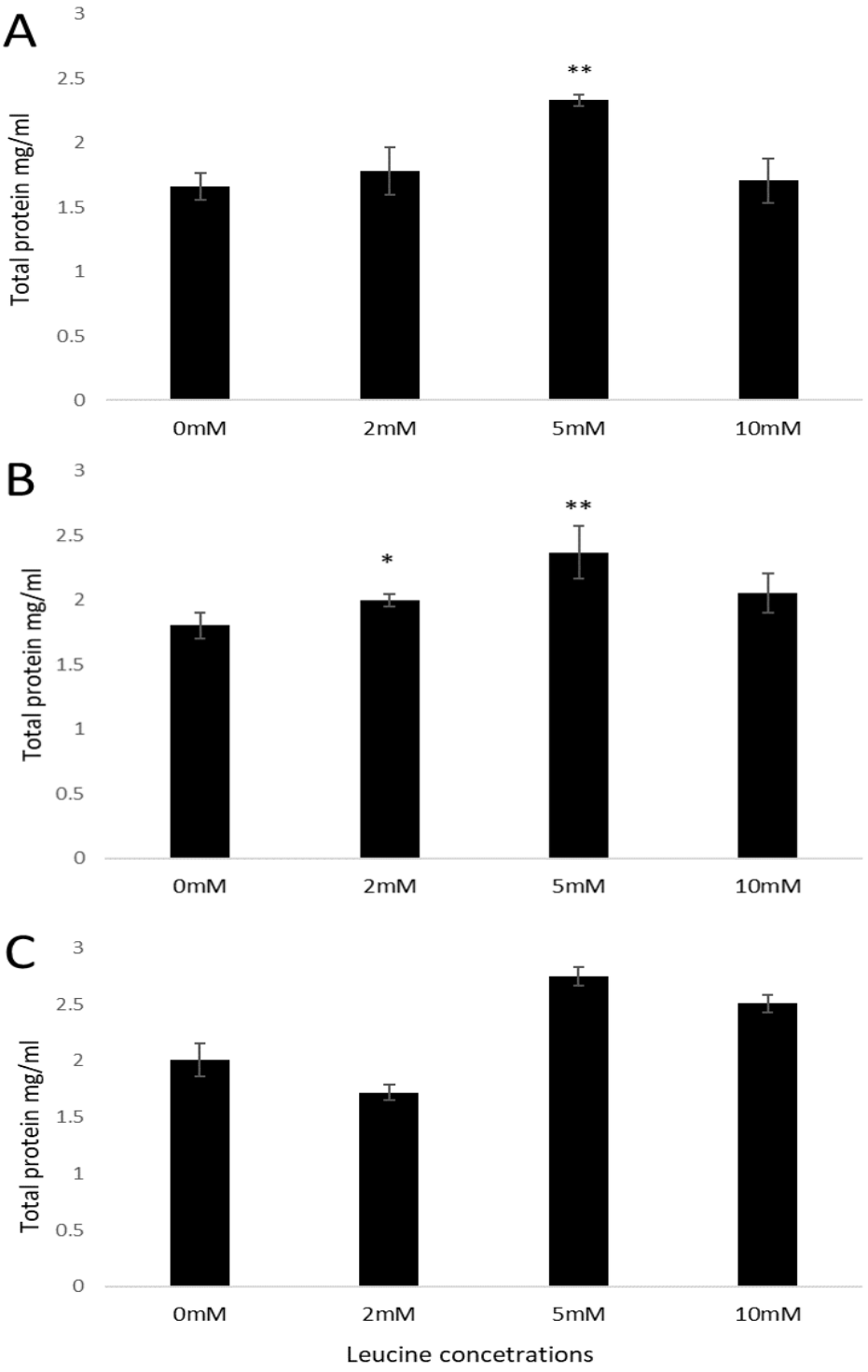


Figure 5.7. Total protein content of C2C12 myoblasts following long-term incubation with increasing concentrations of leucine; assessed using a BCA protein assay. C2C12 myoblasts were exposed for 24- (A), 48- (B) and 72- (C) h to 0-, 2-, 5- and 10 mM L-leucine added to GM. Data are presented as means ± SD from three biological replica ($n = 3$). Asterisks indicate significant differences from the control (0 mM) $^{*}P < 0.05$ and $^{**}P < 0.01$. Analysed by one-way ANOVA using Microsoft Excel followed by Tukey's HSD *post hoc* test using SPSS Statistics 20. Abbreviation: mM = millimolar.

5.4.5. Combining leucine and glutamine enhances the proliferation of C2C12 myoblasts

We investigated whether the administration of glutamine and leucine combined, exerts an additive effect on the proliferation of C2C12 myoblasts. The morphological appearance of C2C12 myoblast cultures suggested an enhanced rate of cell proliferation following 24 h of incubation with leucine and glutamine combined, when compared with leucine alone (Figure 5.8). Results of the VCC assay indicated that combining leucine with glutamine significantly increased the number of total and live cells compared with leucine alone (Figures 5.9 B & C, 5.10 and 5.11). Notably, glutamine decreased the number of dead cells when it was combined with 5 mM leucine but increased the number of dead cells when it was combined with 10 mM leucine (Figures 5.9 D, 5.10 and 5.11).

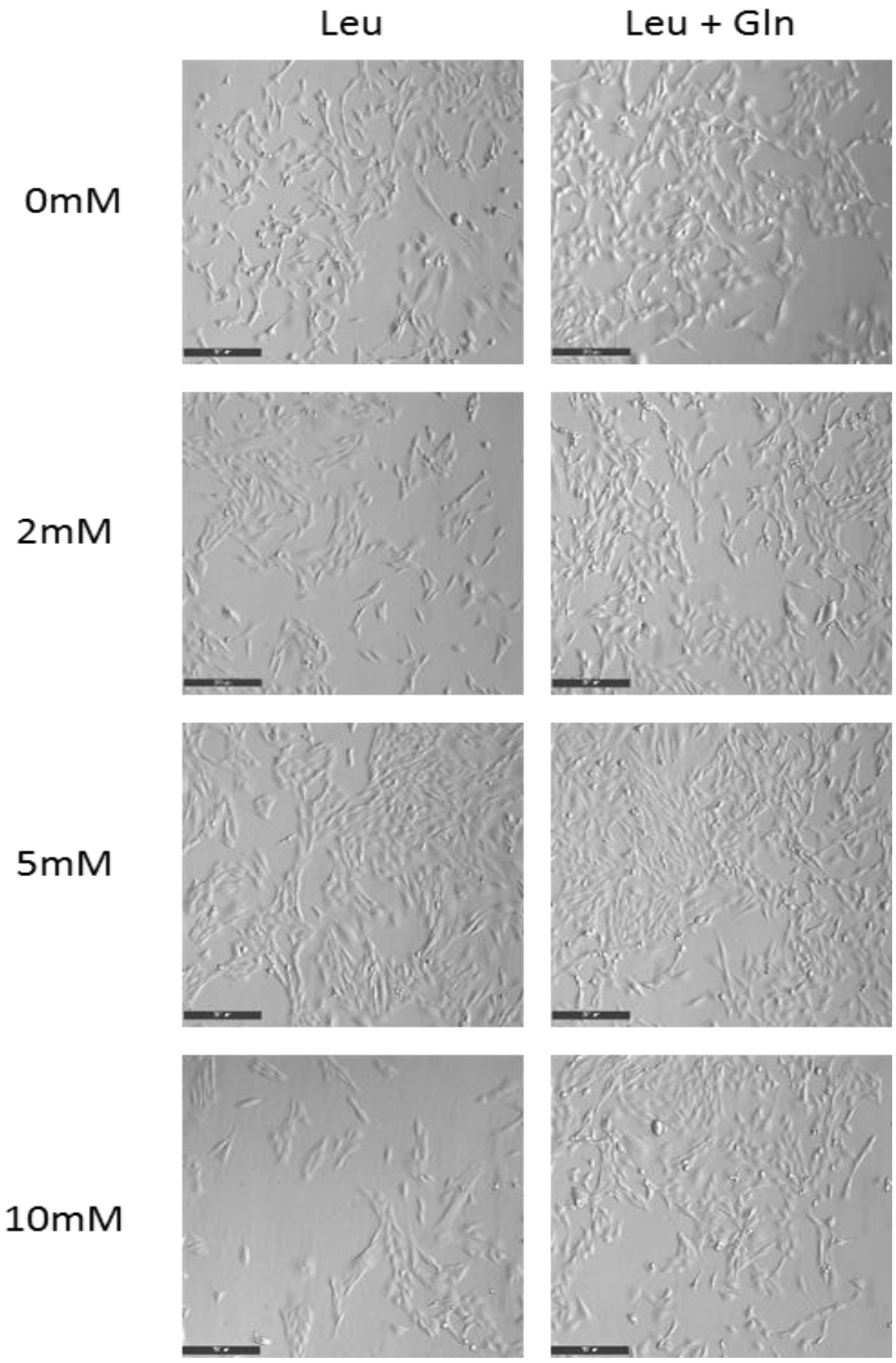

Leu
Leu + Gln
0mM
2mM
5mM
10mM

Figure 5.8. Phase-contrast images depicting the morphology of C2C12 myoblasts following long-term incubation with increasing concentrations of leucine alone, or leucine combined with glutamine. C2C12 myoblasts were exposed for 24 h to 0-, 2-, 5- or 10 mM L-leucine alone (Leu) or combined with glutamine (Leu + Gln), added to GM. (*n* = 3) Abbreviations: Leu = L-leucine; Gln = L-glutamine; mM = millimolar; μm = micrometre.

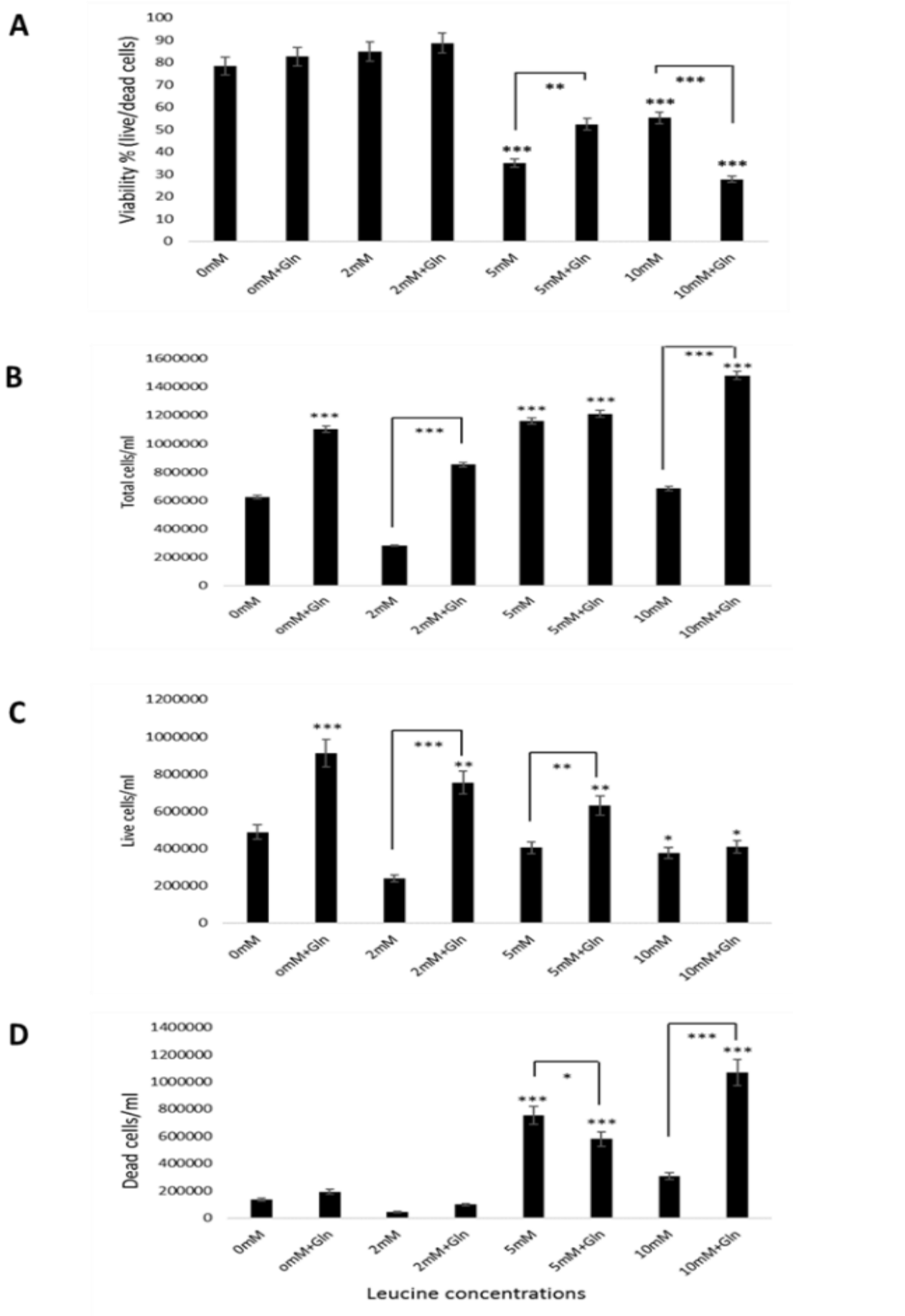

A
Viability % (live/dead cells)
100
90
80
70
60
50
40
30
20
10
0
**
***
***
***
***
0mM
0mM+Gln
2mM
2mM+Gln
5mM
5mM+Gln
10mM
10mM+Gln
B
Total cells/ml
1600000
1400000
1200000
1000000
800000
600000
400000
200000
0
***
***
***
***
***
***
0mM
0mM+Gln
2mM
2mM+Gln
5mM
5mM+Gln
10mM
10mM+Gln
C
Live cells/ml
1200000
1000000
800000
600000
400000
200000
0
***
***
**
**
**
*
*
0mM
0mM+Gln
2mM
2mM+Gln
5mM
5mM+Gln
10mM
10mM+Gln
D
Dead cells/ml
1400000
1200000
1000000
800000
600000
400000
200000
0
***
*
***
***
***
0mM
0mM+Gln
2mM
2mM+Gln
5mM
5mM+Gln
10mM
10mM+Gln
Leucine concentrations

Figure 5.9. The viability and differential cell count of C2C12 myoblasts following long-term incubation with increasing concentrations of leucine alone or leucine combined with glutamine; assessed using a NucleoCounter® NC-3000™ Viability and Cell Count Assay. C2C12 myoblasts were exposed for 24 h to 0-, 2-, 5- or 10 mM L-leucine alone or combined with L-glutamine, added to GM. Panel A shows the viability of cells, panel B shows the total cell count per ml, panel C shows the count of live cells per ml and panel D shows the count of dead cells per ml. Data are presented as means ± SD from three biological replica (n = 3). Asterisks indicate significant differences from the control (0 mM) and indicate significant differences between leucine and leucine + glutamine groups $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$. Analysed by one-way ANOVA using Microsoft Excel followed by Tukey's HSD *post hoc* test using SPSS Statistics 20. Abbreviations: Gln = L-glutamine; mM = millimolar.

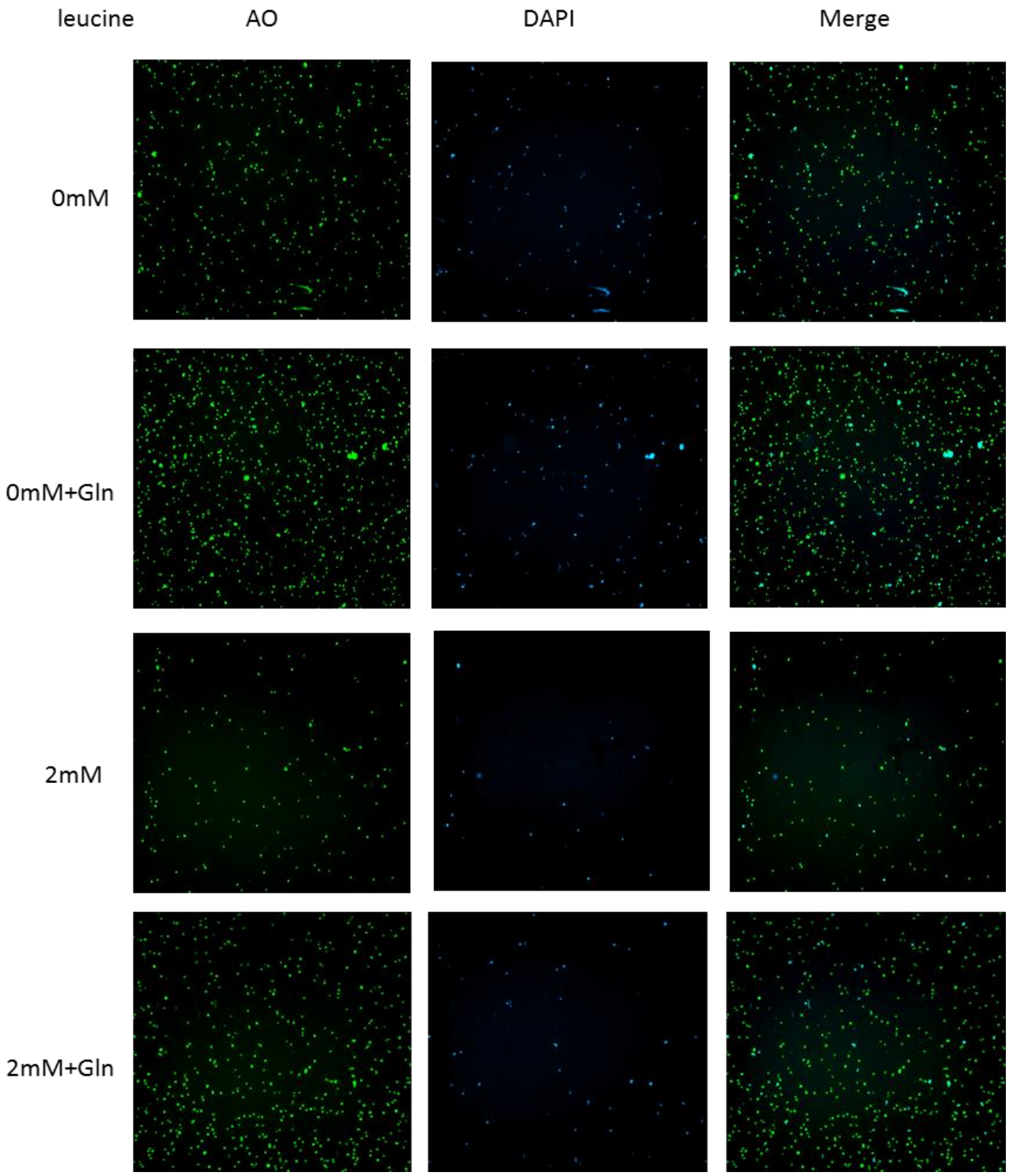


Figure 5.10. Representative images of C2C12 myoblasts following long-term incubation with increasing concentrations of leucine alone, or leucine combined with glutamine; obtained using a NucleoCounter® NC-3000™ Advanced Image Cytometer. C2C12 myoblasts were exposed for 24 h to 0 mM and 2 mM L-leucine alone or combined with L-glutamine, added to GM. (n = 3). The left panel shows cells stained with AO (green). The middle panel shows cells stained with DAPI (blue). The right panel is a merge of AO and DAPI stained cells. Abbreviations: AO = Acridine Orange stain; Gln = L-glutamine; mM = millimolar.

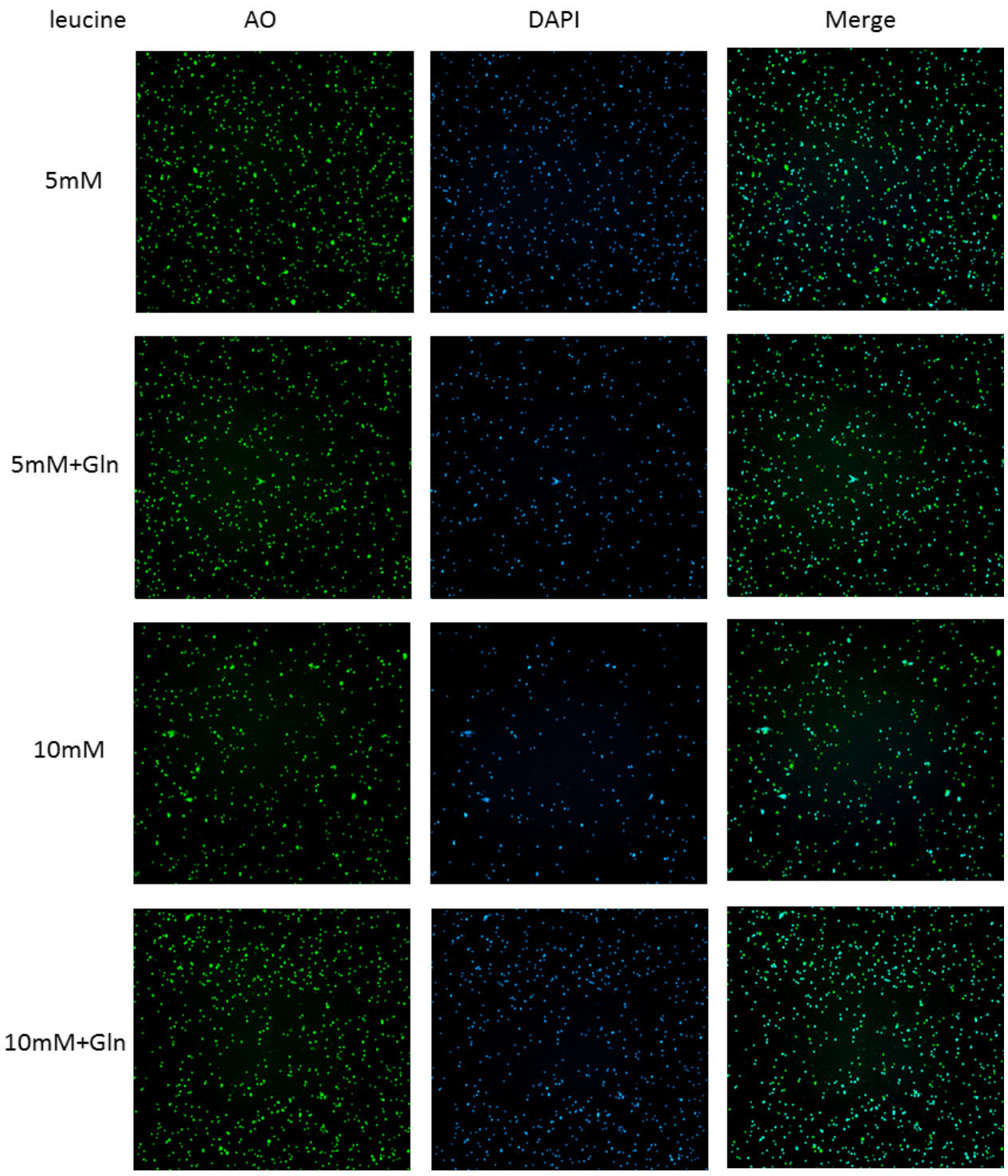
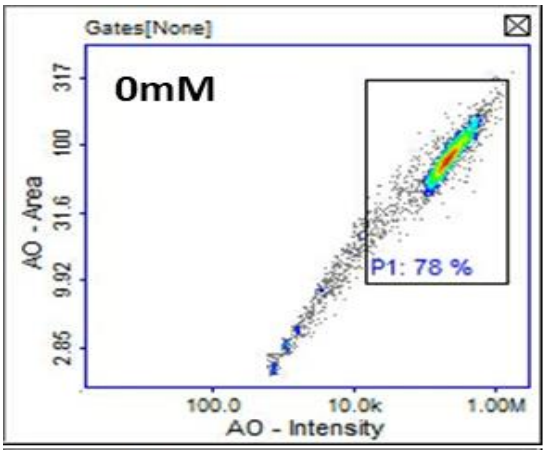
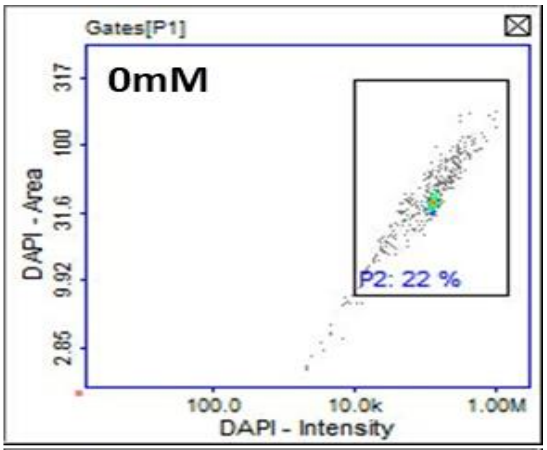
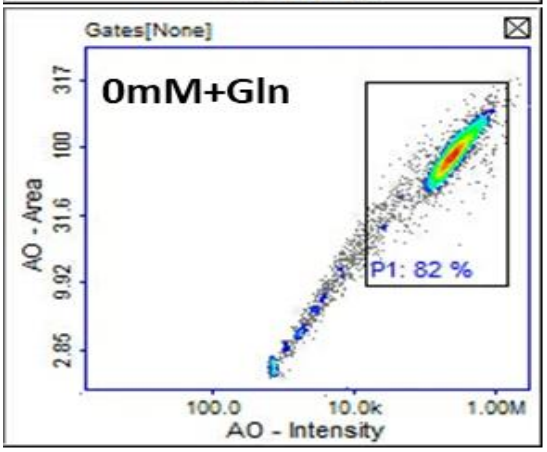
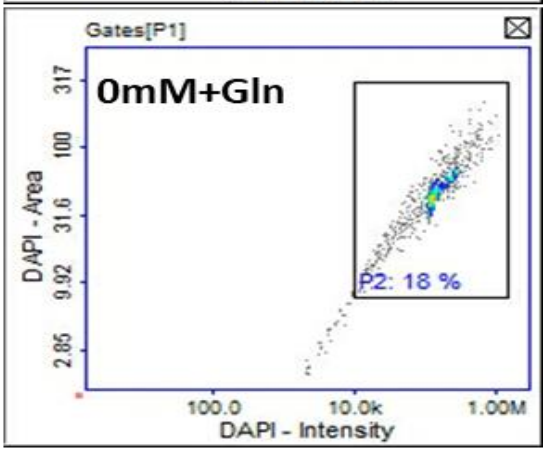


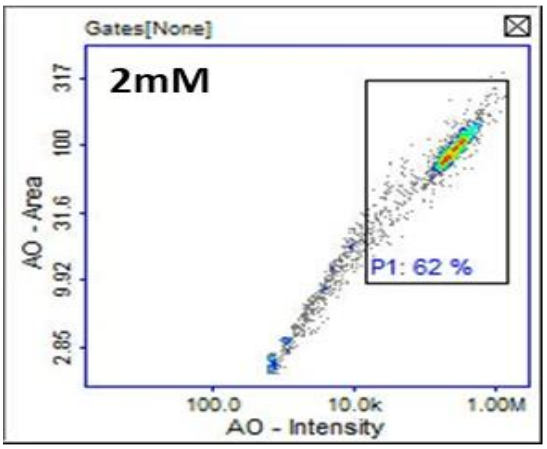
Figure 5.11. Representative images of C2C12 myoblasts following long-term incubation with increasing concentrations of leucine alone, or leucine combined with glutamine; obtained using a NucleoCounter® NC-3000™ Advanced Image Cytometer. C2C12 myoblasts were exposed for 24 h to 5 mM and 10 mM L-leucine alone or combined with L-glutamine, added to GM. ($n = 3$). The left panel shows cells stained with AO (green). The middle panel shows cells stained with DAPI (blue). The right panel is a merge of AO and DAPI stained cells. Abbreviations: AO = Acridine Orange stain; Gln = L-glutamine; mM = millimolar.

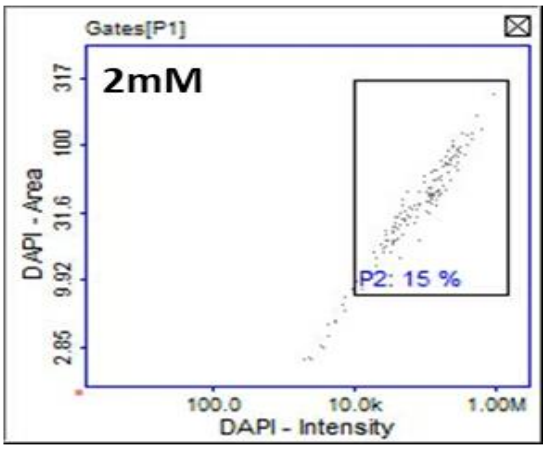

Gates[None]
0mM
P1: 78 %
AO - Area
AO - Intensity
317
100
31.6
9.92
2.85
100.0
10.0k
1.00M

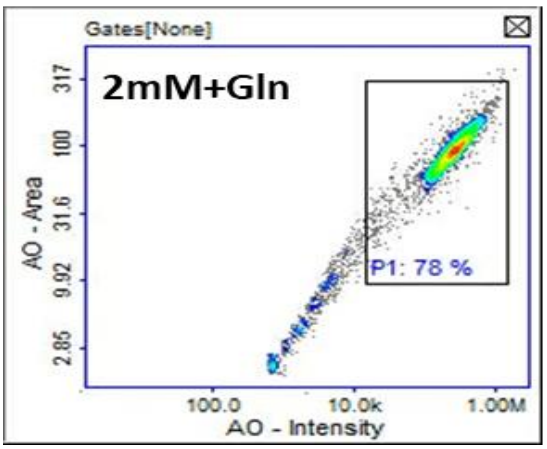

Gates[P1]
0mM
P2: 22 %
DAPI - Area
DAPI - Intensity
317
100
31.6
9.92
2.85
100.0
10.0k
1.00M


Gates[None]
0mM+Gln
P1: 82 %
AO - Area
AO - Intensity
317
100
31.6
9.92
2.85
100.0
10.0k
1.00M


Gates[P1]
0mM+Gln
P2: 18 %
DAPI - Area
DAPI - Intensity
317
100
31.6
9.92
2.85
100.0
10.0k
1.00M


Gates[None]
2mM
P1: 62 %
AO - Area
AO - Intensity
317
100
31.6
9.92
2.85
100.0
10.0k
1.00M


Gates[P1]
2mM
P2: 15 %
DAPI - Area
DAPI - Intensity
317
100
31.6
9.92
2.85
100.0
10.0k
1.00M


Gates[None]
2mM+Gln
P1: 78 %
AO - Area
AO - Intensity
317
100
31.6
9.92
2.85
100.0
10.0k
1.00M

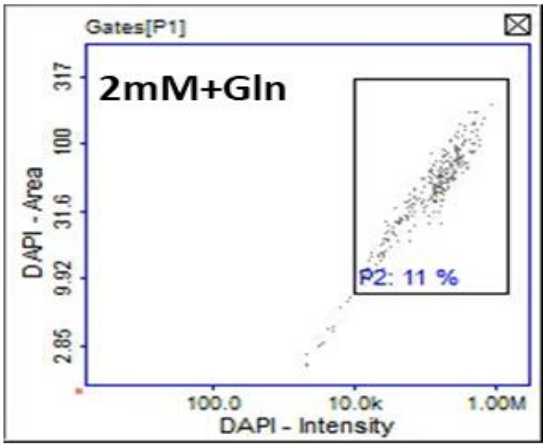

Gates[P1]
2mM+Gln
P2: 11 %
DAPI - Area
DAPI - Intensity
317
100
31.6
9.92
2.85
100.0
10.0k
1.00M

Figure 5.12. Scatter plots showing the fluorescence intensity of AO and DAPI stained C2C12 myoblasts after long-term exposure to increasing concentrations of leucine alone or leucine combined with glutamine; obtained using a NucleoCounter® NC-3000™ Advanced Image Cytometer. C2C12 myoblasts were exposed for 24 h to 0 mM and 2 mM L-leucine alone or combined with L-glutamine, added to GM. Analysed by NucleoView™ software (n = 3). The left panel shows the fluorescence intensity of AO-stained cells. The right panel shows the fluorescence intensity of DAPI stained cells. Abbreviations: AO = Acridine Orange stain; Gln = L-glutamine; mM = millimolar.

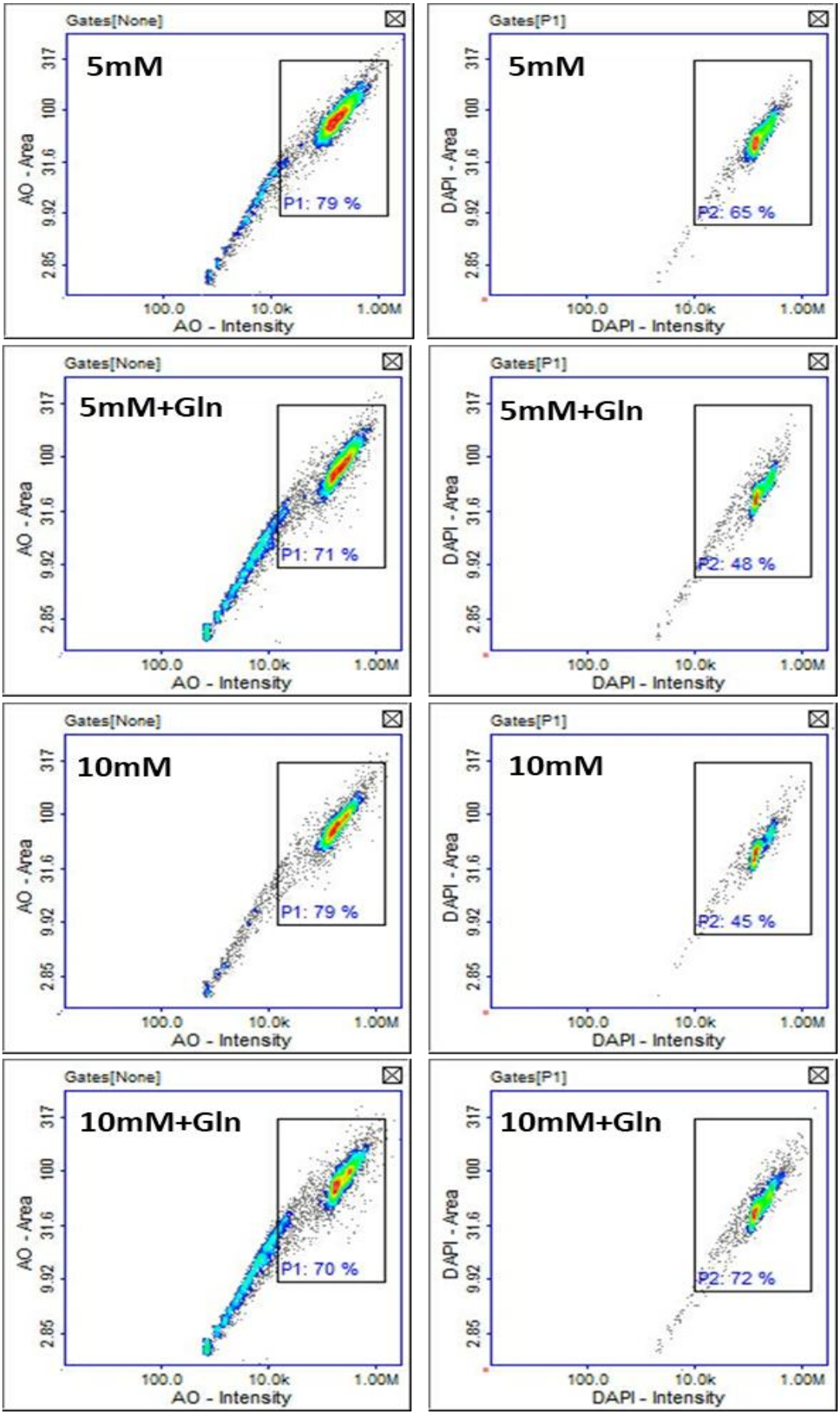

Gates[None]
5mM
AO - Area
AO - Intensity
P1: 79 %
Gates[P1]
5mM
DAPI - Area
DAPI - Intensity
P2: 65 %
Gates[None]
5mM+Gln
P1: 71 %
Gates[P1]
5mM+Gln
P2: 48 %
Gates[None]
10mM
P1: 79 %
Gates[P1]
10mM
P2: 45 %
Gates[None]
10mM+Gln
P1: 70 %
Gates[P1]
10mM+Gln
P2: 72 %
317
100
31.6
9.92
2.85
100.0
10.0k
1.00M

Figure 5.13. Scatter plots showing the fluorescence intensity of AO and DAPI stained C2C12 myoblasts after long-term exposure to increasing concentrations of leucine alone or leucine combined with glutamine; obtained using a NucleoCounter® NC-3000™ Advanced Image Cytometer. C2C12 myoblasts were exposed for 24 h to 5 mM and 10 mM L-leucine alone or combined with L-glutamine, added to GM. Analysed by NucleoView™ software (n = 3). The left panel shows the fluorescence intensity of AO-stained cells. The right panel shows the fluorescence intensity of DAPI stained cells. Abbreviations: AO = Acridine Orange stain; Gln = L-glutamine; mM = millimolar.

5.4.6. Leucine alone, and leucine combined with glutamine, does not alter the diameter of C2C12 myoblasts

We examined the long-term effect of exposure to leucine, glutamine and both AAs combined on the size of proliferating C2C12 cells. Results indicated that the treatments did not affect the diameter of C2C12 myoblasts (Figures 5.14 and 5.15).

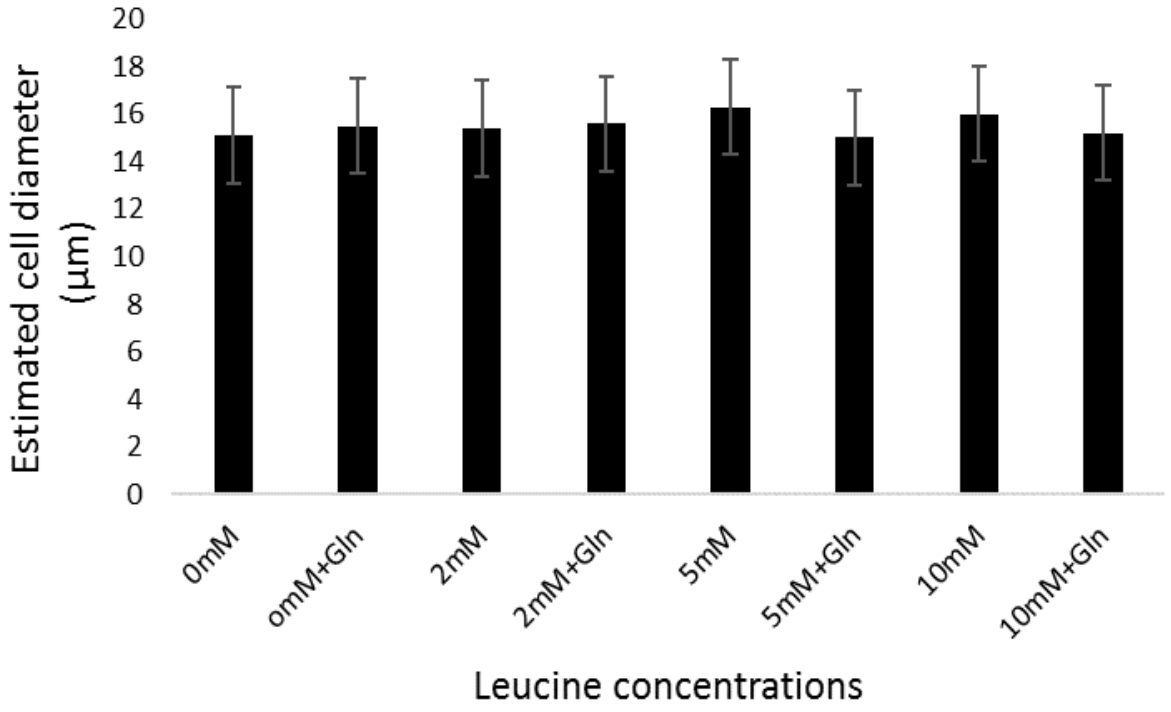
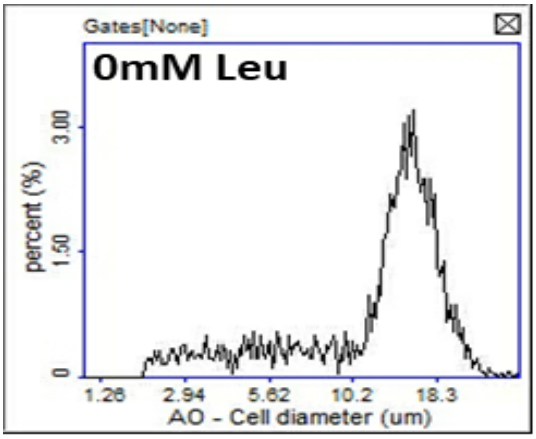
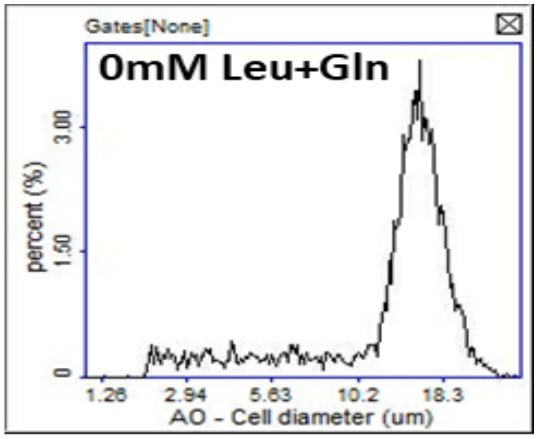
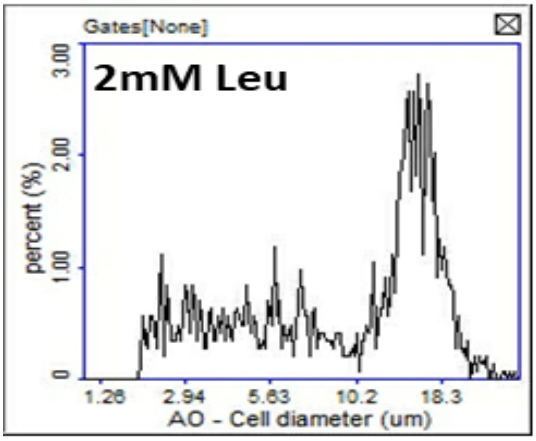
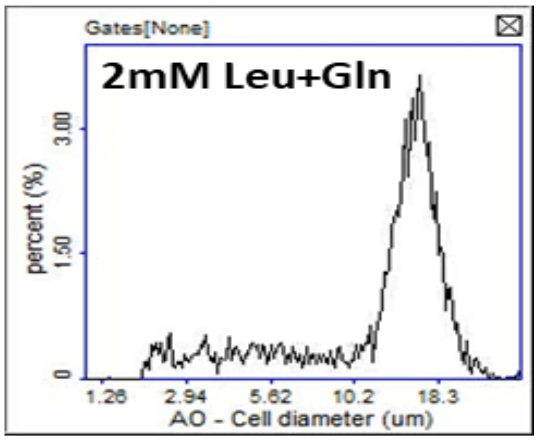


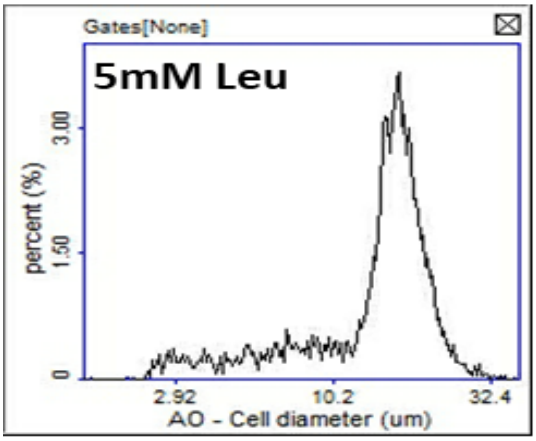
Figure 5.14. Cell diameter of C2C12 myoblasts following long-term incubation with increasing concentrations of leucine alone, or leucine combined with glutamine; assessed using a NucleoCounter® NC-3000™ Viability and Cell Count Assay. C2C12 myoblasts were exposed for 24 h to 0-, 2-, 5- or 10 mM L-leucine alone or combined with L-glutamine, added to GM. Data are presented as means ± SD from three biological replica (n = 3). Analysed by one-way ANOVA using Microsoft Excel. 0mM is the control. Abbreviations: Gln = L-glutamine; mM = millimolar; µm = micrometre.

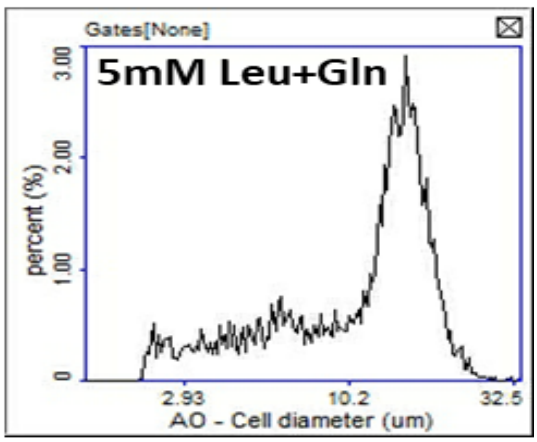

Gates[None]
0mM Leu
percent (%)
0
1.50
3.00
1.26
2.94
5.62
10.2
18.3
AO - Cell diameter (um)

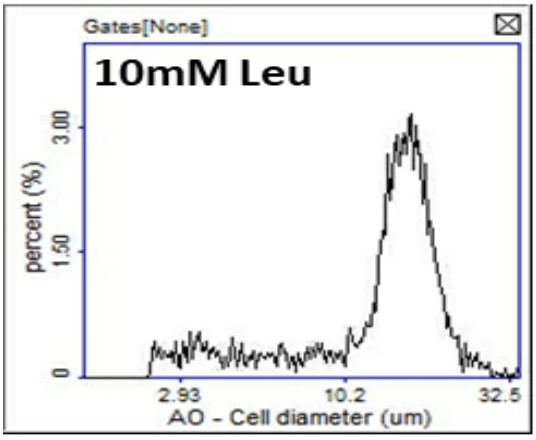

Gates[None]
0mM Leu+Gln
percent (%)
0
1.50
3.00
1.26
2.94
5.63
10.2
18.3
AO - Cell diameter (um)


Gates[None]
2mM Leu
percent (%)
0
1.00
2.00
3.00
1.26
2.94
5.63
10.2
18.3
AO - Cell diameter (um)


Gates[None]
2mM Leu+Gln
percent (%)
0
1.50
3.00
1.26
2.94
5.62
10.2
18.3
AO - Cell diameter (um)


Gates[None]
5mM Leu
percent (%)
0
1.50
3.00
2.92
10.2
32.4
AO - Cell diameter (um)


Gates[None]
5mM Leu+Gln
percent (%)
0
1.00
2.00
3.00
2.93
10.2
32.5
AO - Cell diameter (um)


Gates[None]
10mM Leu
percent (%)
0
1.50
3.00
2.93
10.2
32.5
AO - Cell diameter (um)

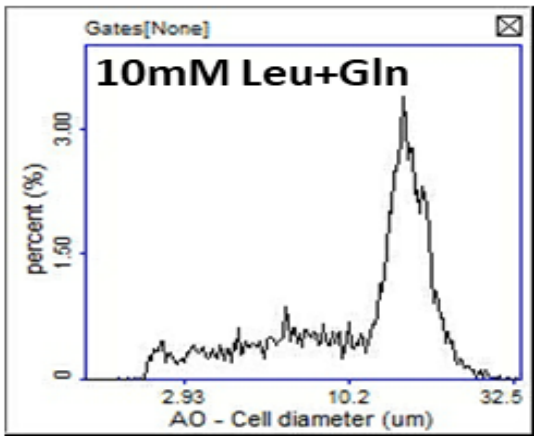

Gates[None]
10mM Leu+Gln
percent (%)
0
1.50
3.00
2.93
10.2
32.5
AO - Cell diameter (um)

Figure 5.15. Histograms showing the cell diameter of C2C12 myoblasts incubated with increasing concentrations of leucine alone, or leucine combined with glutamine; obtained using a NucleoCounter® NC-3000™ Advanced Image Cytometer. C2C12 myoblasts were incubated for 24 h with 0-, 2-, 5- or 10 mM L-leucine alone or combined with L-glutamine, added to GM. Analysed by NucleoView™ software ($n = 3$). Abbreviations: Gln = L-glutamine; Leu = L-leucine; mM = millimolar; um = micrometre.

5.4.7. Leucine does not alter the morphology of C2C12 myotubes

Here, the effect of different leucine concentrations on differentiated C2C12 cells was investigated. Morphological examination of differentiated C2C12 cultures did not reveal any difference between leucine treated cultures (2 mM, 5 mM and 10 mM) and the control (0 mM) (Figure 5.16).

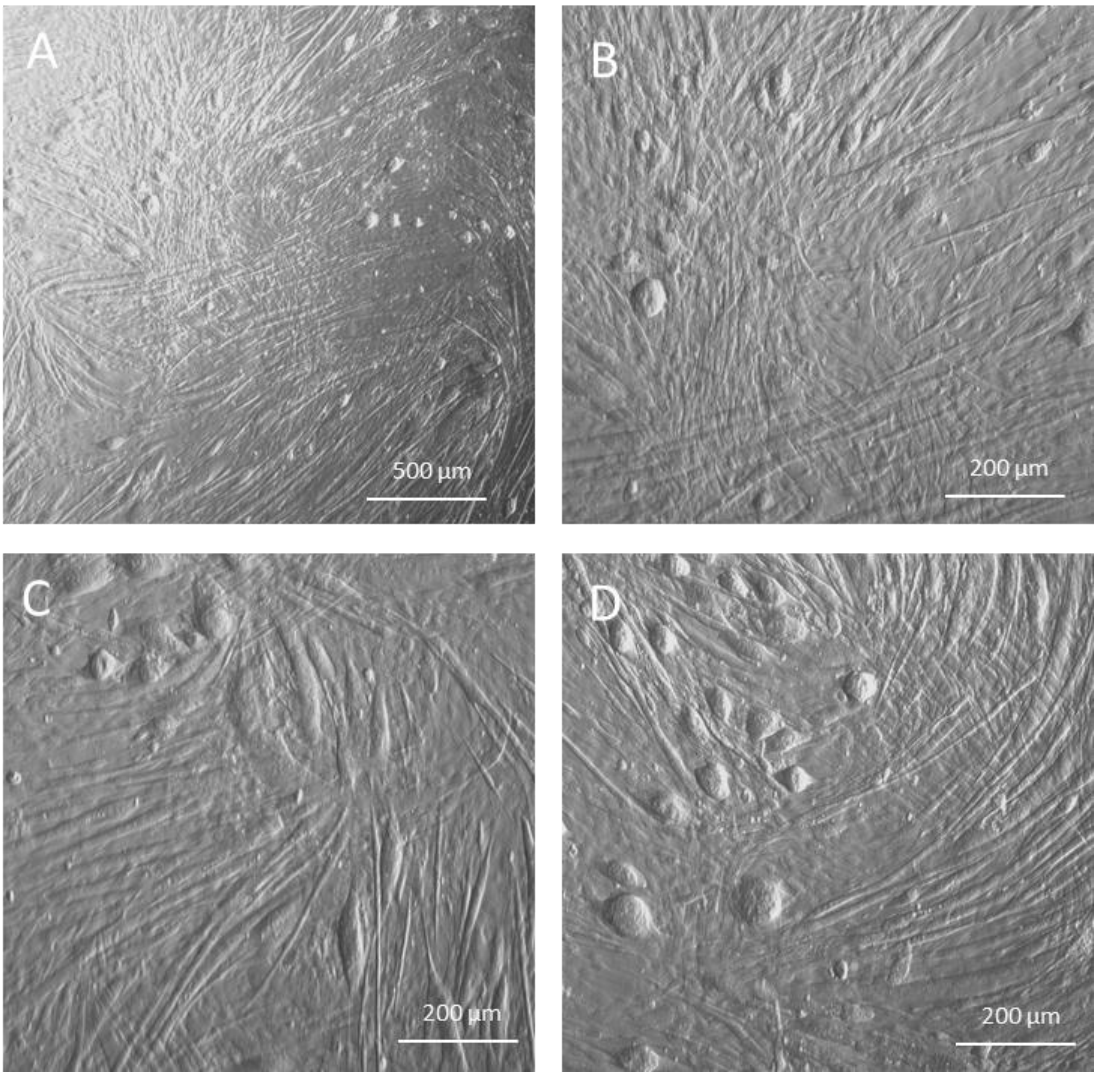


Figure 5.16. Phase-contrast images depicting the morphology of C2C12 myotubes following long-term incubation with different leucine concentrations. C2C12 myotubes were incubated for 24 h with 0- (A), 2- (B), 5- (C) or 10- (D) mM L-leucine, added to DM. (*n* = 3). Abbreviation: µm = micrometre.

5.4.8. Leucine does not alter the expression of MyHC-*fast* in C2C12 myotubes

The aim of this experiment was to determine if long-term exposure to leucine would increase the expression of MyHC in differentiated C2C12 cells. Results indicated no difference in the expression of MyHC-*fast* among leucine treated and the control groups (Figure 5.17).

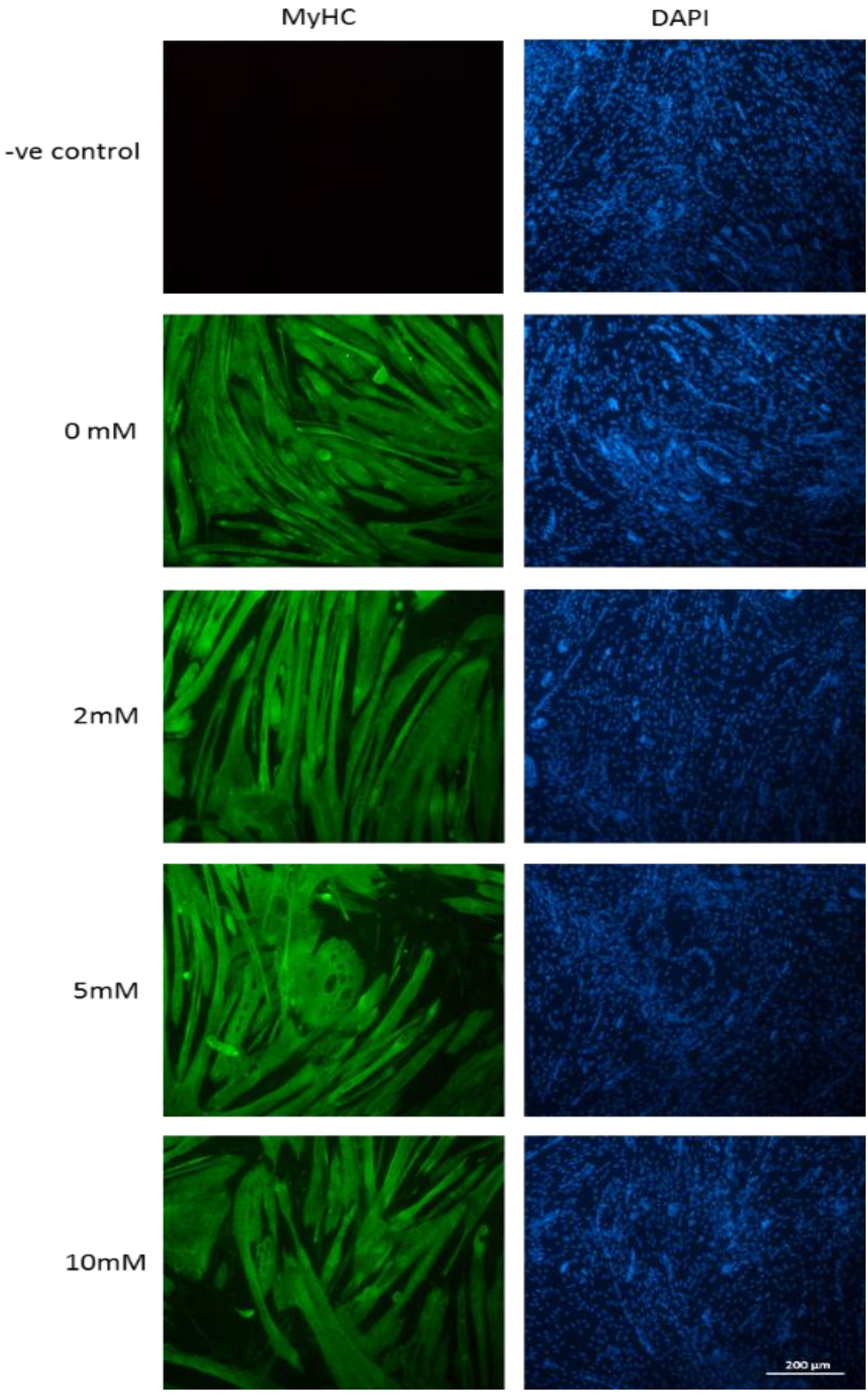

MyHC
DAPI
-ve control
0 mM
2mM
5mM
10mM
200 µm

Figure 5.17. Expression of MyHC-*fast* in differentiated C2C12 cultures following long-term incubation with increasing concentrations of leucine. C2C12 myotubes were incubated for 24 h with 0-, 2-, 5- or 10 mM L-leucine, added to DM. (*n* = 3). The left panel shows the expression of MyHC- *fast* (green). (*n* = 3). The right panel shows DAPI stained nuclei (blue). Abbreviations: -ve = negative control (i.e., no primary antibody); MyHC = myosin heavy chain; mM = millimolar; µm = micrometre.

5.4.9. Leucine alters the total protein content of C2C12 myotubes

We evaluated protein accumulation following long-term incubation of differentiated C2C12 cells with leucine. The results revealed a significant increase in total protein content, when compared with control (0mM), following 24 h incubation with 5 mM leucine ($P < 0.05$) (Figure 5.18 A). This was concomitant with a significant decrease in total protein following incubation with 10 mM leucine (Figure 5.18 A) ($P < 0.05$). Similarly, exposure to leucine for 48 h indicated that 5 mM leucine was capable of increasing protein accumulation within C2C12 myotubes compared with the control (0 mM) ($P < 0.05$) (Figure 5.18 B). Finally, 72 h of exposure to leucine led to a significant increase in total protein content in response to 2 mM and 5 mM leucine ($P < 0.01$ and < 0.001, respectively) (Figure 5.18 C).

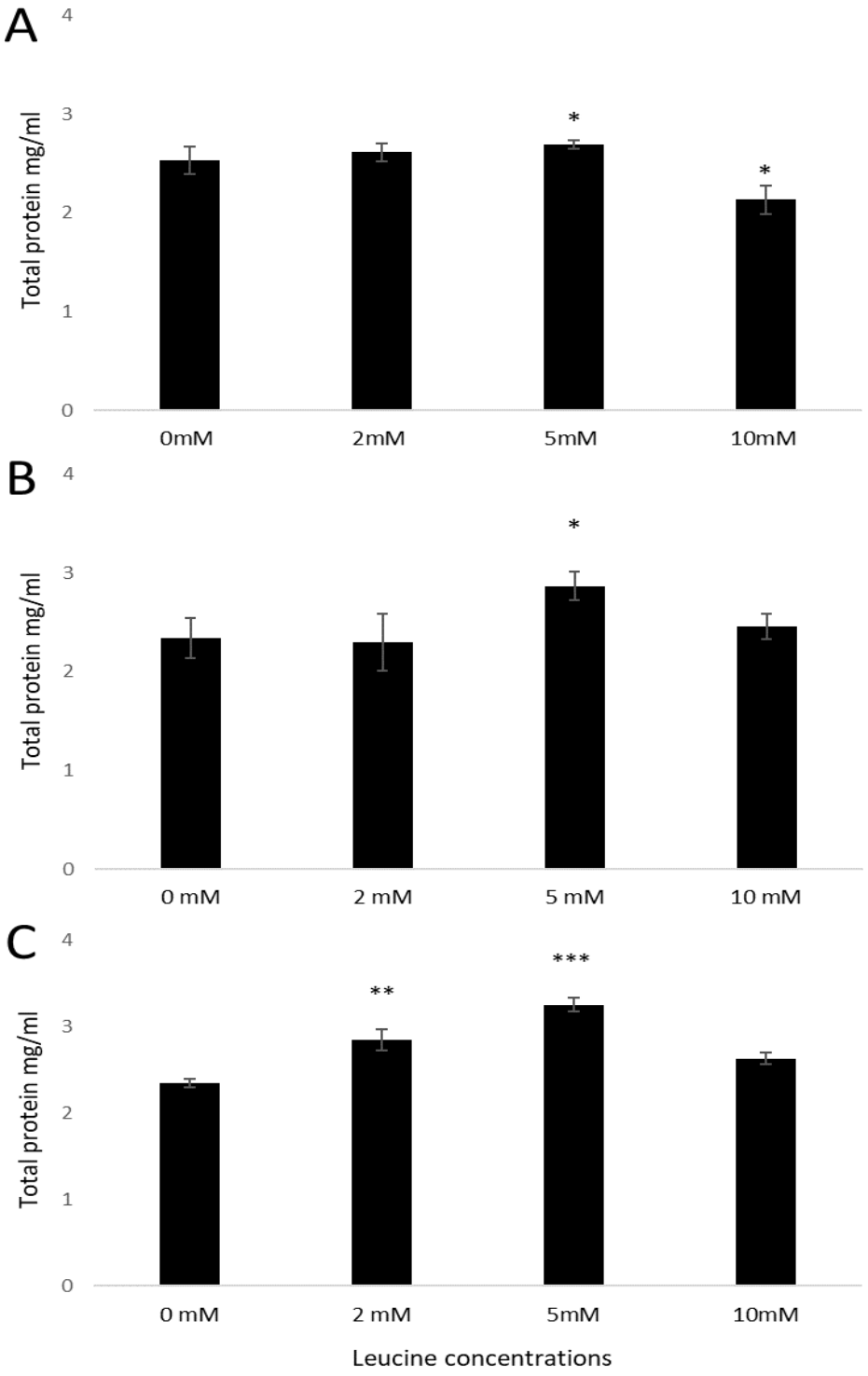


Figure 5.18. Total protein content of C2C12 myotubes following incubation with different concentrations of leucine; assessed using a BCA protein assay. C2C12 myotubes were incubated for 24- (A), 48- (B) and 72- (C) h with 0-, 2-, 5- or 10 mM L-leucine, added to DM. Data are presented as means ± SD from three biological replica (n = 3). Asterisks indicate significant differences compared to the control (0 mM) $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$. Analysed by one-way ANOVA using Microsoft Excel followed by Tukey's HSD *post hoc* test using SPSS Statistics 20. Abbreviations: mM = millimolar, mg = milligram; ml = millilitre.

5.4.10. Glutamine, when combined with leucine, improves the survival of C2C12 myotubes

The aim of this experiment was to examine if glutamine combined with leucine exerts an additive effect on the survival of differentiated C2C12 cells. The effect of combining different concentrations of leucine with glutamine were examined by assessing the total, live and dead cell counts of C2C12 myotubes after 24 h of treatment. The results revealed that the number of total, live and dead cells was inversely proportional to the concentration of leucine (Figures 5.19-21). The results also suggested that combining glutamine with all the concentrations of leucine used, resulted in fewer total, live and dead cells, when compared with leucine alone (Figures 5.19-21).

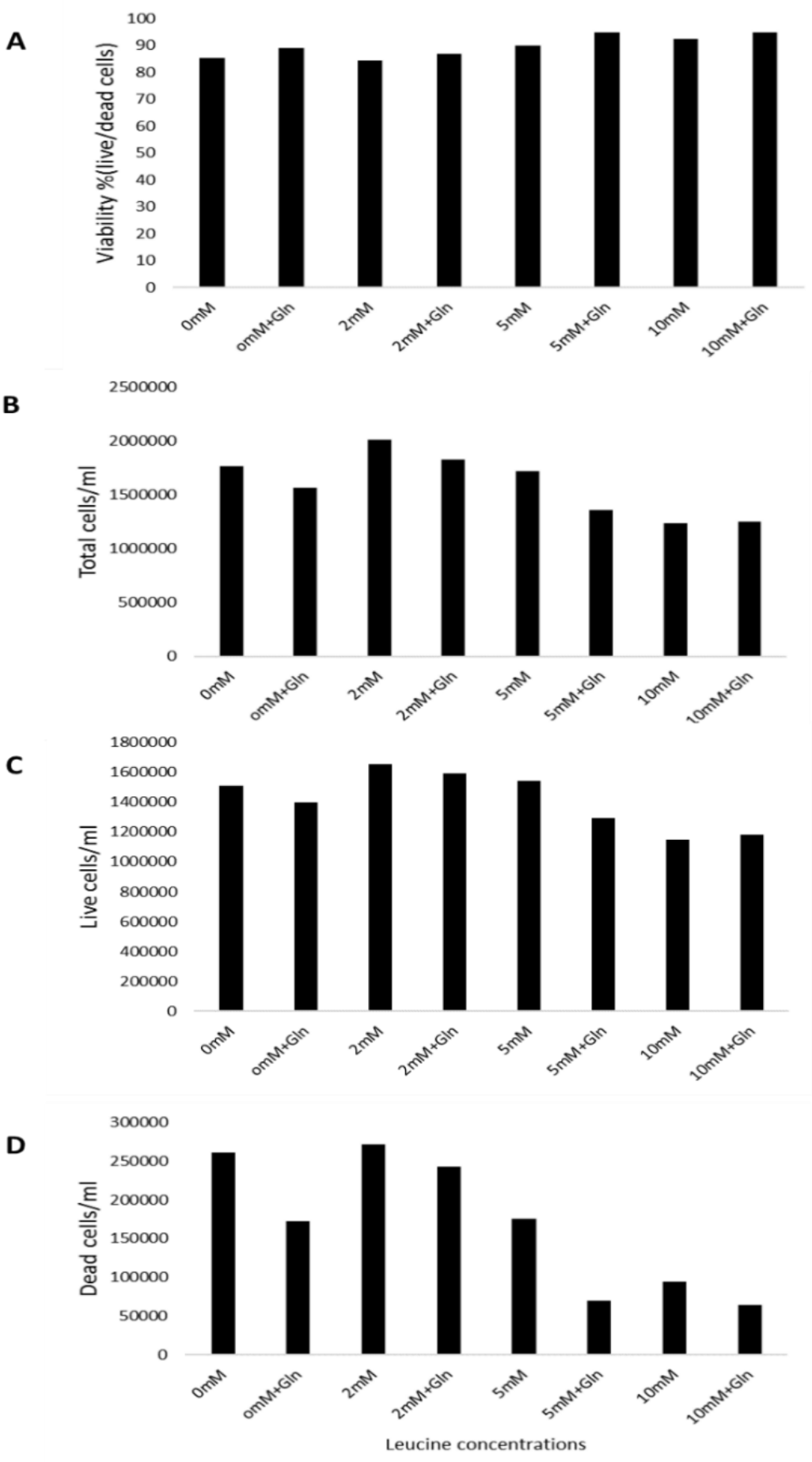

A
Viability %(live/dead cells)
100
90
80
70
60
50
40
30
20
10
0
0mM
0mM+Gln
2mM
2mM+Gln
5mM
5mM+Gln
10mM
10mM+Gln
B
Total cells/ml
2500000
2000000
1500000
1000000
500000
0
C
Live cells/ml
1800000
1600000
1400000
1200000
1000000
800000
600000
400000
200000
0
D
Dead cells/ml
300000
250000
200000
150000
100000
50000
0
Leucine concentrations

Figure 5.19. The viability, and differential cell count of C2C12 myotubes following long-term incubation with increasing concentrations of leucine alone, or leucine combined with glutamine; assessed using a NucleoCounter® NC-3000™ Viability and Cell Count assay for aggregates. C2C12 myotubes were incubated for 24 h with 0-, 2-, 5- or 10 mM L-leucine alone or combined with L-glutamine, added to DM. Data represent the numerical values of one experiment (n = 1). Analysed by NucleoView™ software. Abbreviations: Gln = L-glutamine; mM = millimolar; ml = millilitre.

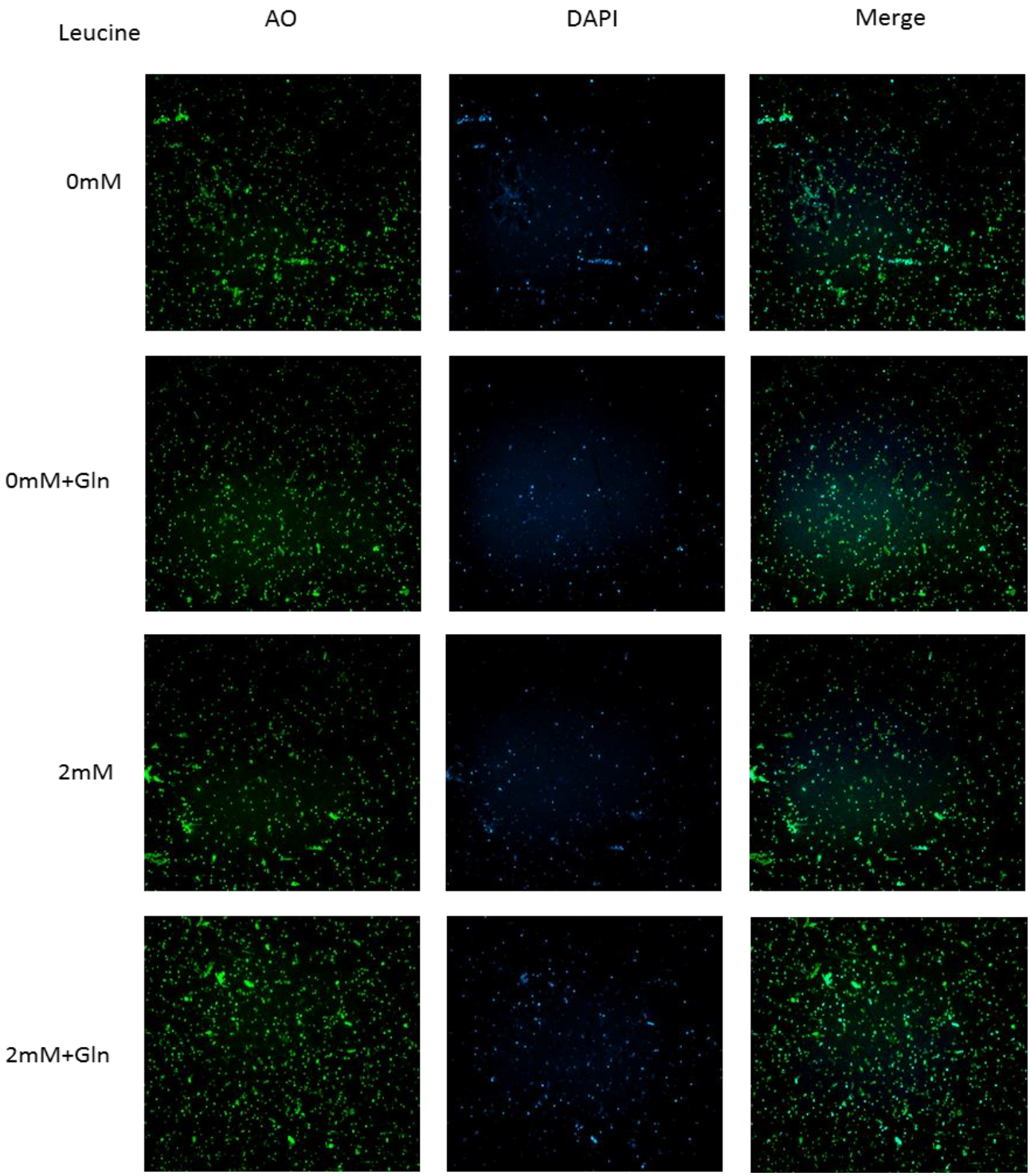


Figure 5.20. Representative images of C2C12 myotubes following long-term incubation with increasing concentrations of leucine alone, or leucine combined with glutamine; obtained using a NucleoCounter® NC-3000™ Advanced Image Cytometer. C2C12 myotubes were exposed for 24 h to 0 mM and 2 mM L-leucine alone or combined with L-glutamine, added to DM. (n = 1). The left panel shows cells stained with AO (green). The middle panel shows cells stained with DAPI (blue). The right panel is a merge of AO and DAPI stained cells. Abbreviations: AO = Acridine orange stain; Gln = L-glutamine; mM = millimolar.

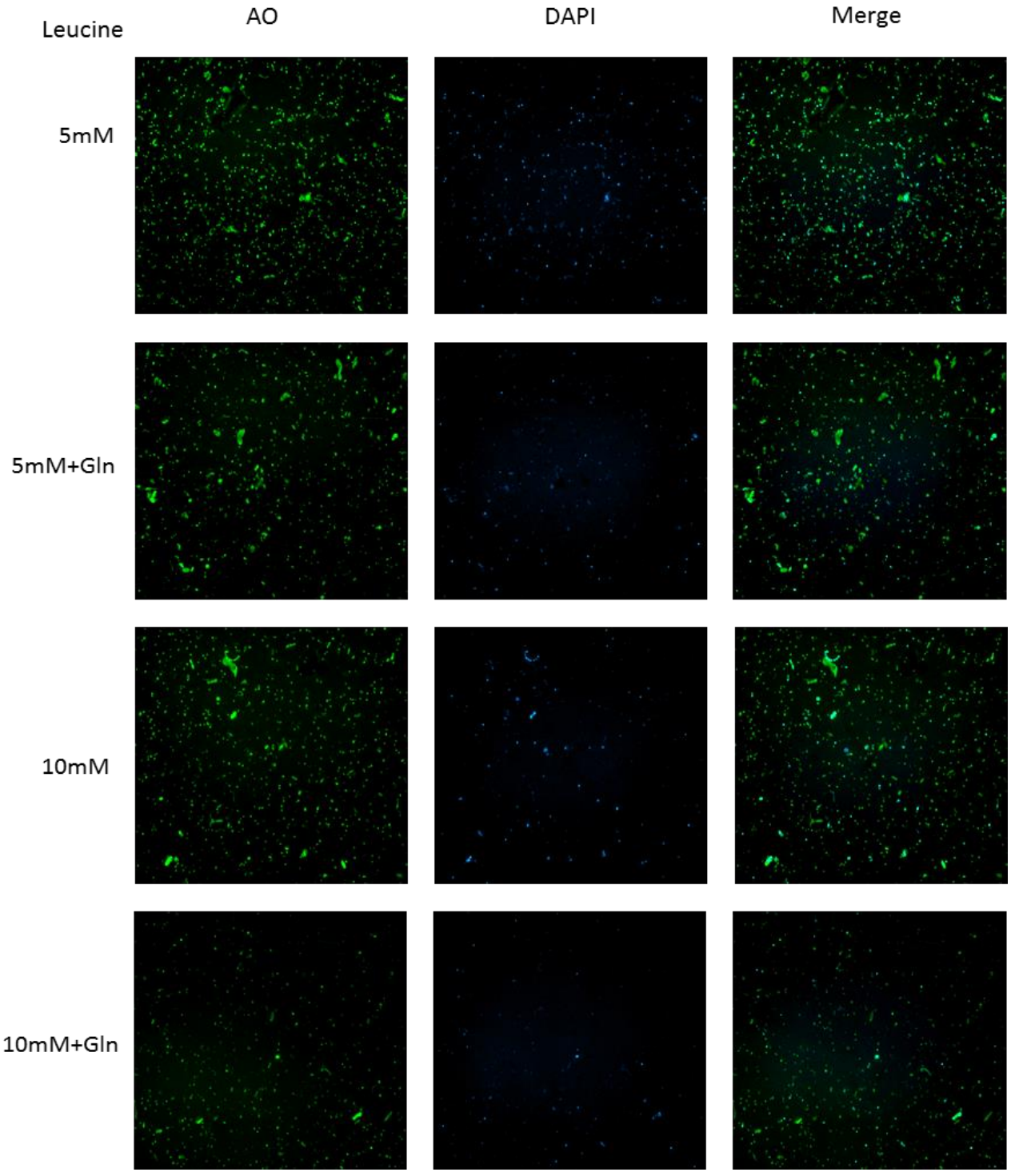


Figure 5.21. Representative images of C2C12 myotubes following long-term incubation with increasing concentrations of leucine alone, or leucine combined with glutamine; obtained using a NucleoCounter® NC-3000™ Advanced Image Cytometer. C2C12 myotubes were exposed for 24 h to 5 mM and 10 mM L-leucine alone or combined with L-glutamine, added to DM. (n = 1). The left panel shows cells stained with AO (green). The middle panel shows cells stained with DAPI (blue). The right panel is a merge of AO and DAPI stained cells. Abbreviations: AO = Acridine orange stain; Gln = L-glutamine; mM = millimolar.

5.4.11. Leucine alters the diameter of C2C12 myotubes

We examined the long-term effect of exposure to leucine, glutamine and both AAs combined, on the size of differentiated C2C12 cells. The results suggested that only a high concentration (10 mM) of leucine increased the diameter of C2C12 myotubes, when compared with control (0 mM). Glutamine, on its own, failed to increase the diameter of C2C12 above the control. Combining glutamine with physiological concentrations (2 mM and 5 mM) of leucine increased the diameter of C2C12 myotubes; however, combining glutamine with a supraphysiological concertation of leucine (10 mM) decreased the diameter of C2C12 myotubes, when compared with leucine alone (Figure 5.22).

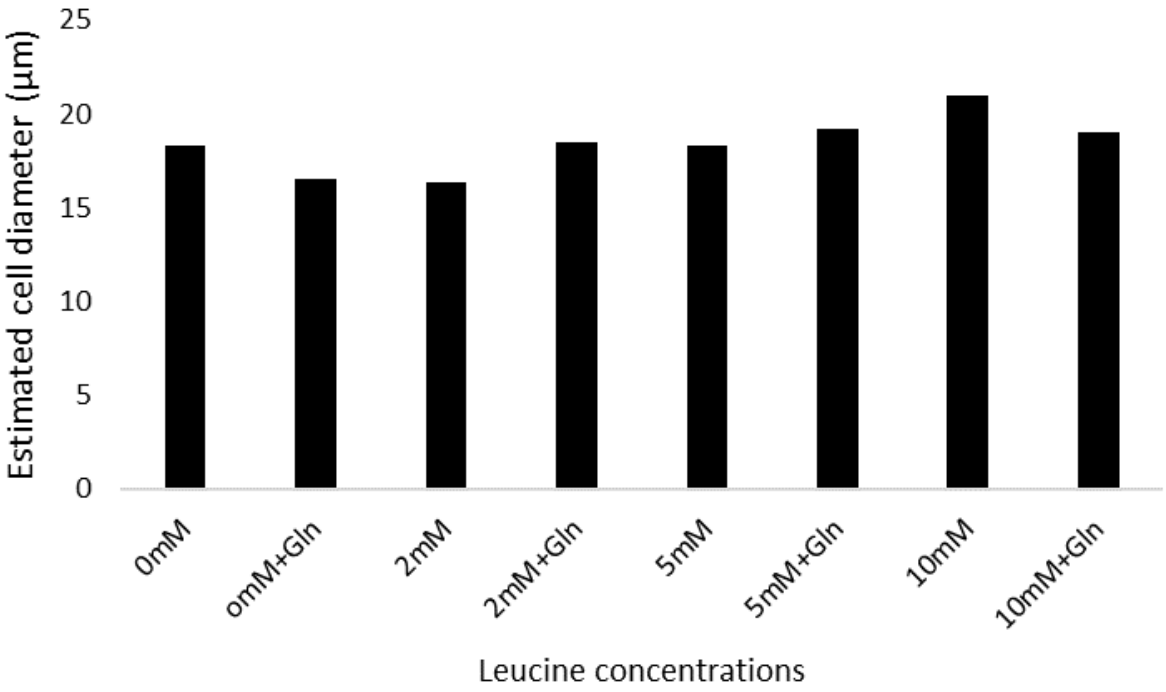


Figure 5.22. The diameter of C2C12 myotubes following long-term incubation with increasing concentrations of leucine alone or leucine combined with glutamine; assessed using a NucleoCounter® NC-3000™ Viability and Cell Count assay for aggregates. C2C12 myotubes were incubated for 24 h with 0-, 2-, 5- or 10 mM L-leucine alone or combined with L-glutamine, added to DM. Data represent the numerical values of a single experiment (n = 1). Analysed by NucleoView™ software. Abbreviations: Gln = L-glutamine; mM = millimolar.

5.5. Discussion

The aim of this chapter was to assess the effect of long-term exposure to increasing concentrations of leucine and/or glutamine on proliferating, and differentiated, C2C12 cells. Previous studies have mostly focused on assessing the rate of MPS post-stimulation of muscle cells with leucine. However, it is equally important to assess the effect of leucine on cell viability – given the fact that high/supra-physiological concentrations of leucine were required to achieve a significant increase in protein synthesis within muscle cells, both *in vivo* and *in vitro* (Fox *et al*., 1998; Garlick, 2005; Kobayashi *et al*., 2006; Areta *et al*., 2014). Also, long-term treatment with leucine is required for the management of many muscular disorders, such as low body weight in neonates, sarcopenia in the elderly and cachexia that occurs in conjunction with several pathological conditions such as cancer. Thus, it is important to define the optimal exposure times needed to elicit the protein anabolic properties of leucine on muscle cells. Additionally, this study addressed the question of whether glutamine in combination with different concentrations of leucine exerts an additive effect.

5.5.1. Physiological concentrations of leucine increase the proliferation, viability and cell turnover of C2C12 myoblasts

Data obtained in chapter 4 strongly suggest that examining the effect of leucine on proliferating C2C12 myoblasts should be carried out in FCS-enriched GM, in order to avoid unnecessary cell death and protein loss. Therefore, in the present study, assessment of the effect of long-term incubation of C2C12 myoblasts with increasing concentrations of leucine were conducted in GM. Results showed a significant increase in the viability of C2C12 myoblasts following long-term incubation with 5 mM L-leucine, when compared with other leucine concentrations. A further assessment indicated that long-term exposure to 5 mM leucine significantly increased cell proliferation and the number of total, live and dead cells. These results support our hypothesis that exposure to physiological concentrations of leucine will boost the viability and survival of C2C12 cells.

However, long-term exposure to 10 mM significantly reduced the viability of C2C12 myoblasts, which agrees with our first hypothesis and with previous findings in C2C12 myotubes (Haegens *et al*., 2012). This reduction in viability suggests that such high leucine

concentration may have impacted proliferating C2C12 cells; however, the high number of live cells, and the low number of dead cells, reported following long-term incubation of C2C12 myoblasts with 10 mM leucine, does not support the assumption that this leucine concentration is toxic to cells. Were this the case, a higher number of dead cells would have been recorded. Therefore, it is likely that exposure to 10 mM leucine decreased the proliferation of C2C12 myoblasts (i.e., a cytostatic rather than a cytotoxic effect). Because the AB viability assay is also indicative of cell number, a decrease in cell proliferation may underlie this low cell viability. Intriguingly, short-term exposure to 10 mM leucine did not negatively impact the viability of C2C12 myoblasts, leading to the conclusion that the response of these cells to leucine is both concentration- and time-dependent.

Another noteworthy finding was the relatively high viability recorded in proliferating C2C12 cultures, following long-term leucine deprivation. A high number of live cells, and a low dead cell count, were also recorded in these cultures. Such observations were consistent with the morphological appearance of C2C12 myoblast cultures incubated in GM devoid of leucine. These findings suggest that leucine deprivation may have induced a cell cycle arrest in which little proliferation, if any, occurred, resulting in low cell death and increased live cell count. This assumption concurs with reports of halted proliferation in chondrocytes and human breast cancer cells following leucine restriction (Kim *et al*., 2009; Xiao *et al*., 2016), and a cell cycle arrest after depriving Chinese hamster cells of leucine (Everhart and Prescott, 1972).

5.5.2. Long-term exposure to physiological concentrations of leucine induces the differentiation of C2C12 myoblasts

Assessing the expression of desmin, an early differentiation marker in C2C12 myoblast cultures, indicated that 2 mM and 5 mM leucine increased the expression of desmin compared with both the control (0 mM) and 10 mM leucine groups. This increased expression of desmin may be linked to enhanced proliferation of C2C12 cells when exposed to physiological leucine concentrations. Such enhanced proliferation may have induced spontaneous differentiation of C2C12 myoblast via contact inhibition (Tanaka *et al*., 2011).

5.5.3. Long-term exposure to a supraphysiological concentration of leucine decreased the total protein content of C2C12 myoblasts

Assessing the total protein content of cells has been employed by several studies evaluating the response of various cultured cells to several experimental conditions (Lou *et al*., 2001; Li and Reid, 2000; Plaisance *et al*., 2008; Holloway and Reid, 1998). In the present study, quantifying the total protein content was not as sensitive as measuring the rates of MPS and MPB. However, assessing the total protein content is useful, as it reflects the overall outcome of treating C2C12 cells with leucine. Assessing protein accumulation is also a valid method since an enhanced rate of MPS after leucine treatment is usually unsustainable (Escobar *et al*., 2005; Wilson *et al*., 2010).

Here, long-term incubation of C2C12 myoblasts with 2 mM and 5 mM leucine induced a significant increase in protein accumulation, when compared with control (0 mM). However, long-term incubation with 10 mM leucine resulted in a significant decrease in the total protein content. The correlation between the total cell count and the total protein content is well recognised. Therefore, it is likely that the protein accumulation, which occurred following exposure of C2C12 myoblasts to physiological leucine concentrations, was a direct result of enhanced proliferation of C2C12 cells (i.e., hyperplasia). However, this conclusion needs further confirmation.

5.5.4. Glutamine increases the viability, proliferation and total protein content of C2C12 myoblasts

After evaluating the independent effect of different concentrations of leucine on proliferating C2C12 cells, C2C12 myoblasts were co-treated with a combination of leucine and glutamine. Results indicated an increase in cell proliferation in response to both AAs, when compared with leucine alone. This was further supported by the results of the cell count assay in which treatment with a combination of leucine and glutamine, increased the total cell count of C2C12 myoblasts when compared with leucine alone. Also, glutamine combined with physiological concentrations (2 mM and 5 mM) of leucine, significantly increased the number of live cells when compared with leucine alone. These results indicate that glutamine on its own, or combined with leucine, stimulated the proliferation, and increased the survival, of C2C12

myoblasts. This agrees with our third hypothesis and is consistent with several previous studies reporting the ability of glutamine to stimulate the proliferation of different cell types (Ko *et al*., 1993; Turowski *et al*., 1994 Yuan *et al*., 2015). However, when glutamine was combined with a supraphysiological concentration (10 mM) of leucine, it failed to increase the live cell count compared with 10 mM leucine. In addition, glutamine combined with this high concentration of leucine significantly increased the number of dead cells, when compared with leucine alone (10 mM leucine). The high count of dead cells indicated that combining glutamine with this high concentration of leucine overstimulated the proliferation of C2C12 cells, eventually resulting in a high number of dead cells.

In this study, glutamine on its own, or combined with different concentrations of leucine, did not alter the diameter of C2C12 myoblasts. This result lies in opposition to a previous report of increased size of cardiac myocytes in response to glutamine treatment (Xia *et al.*, 2003). This contradiction may be explained by the nature of the assay used to estimate cell diameter. The VCC assay estimates the diameter of suspended cells, which do not reflect the actual size of C2C12 myoblasts in monolayer cultures.

5.5.5. Long-term exposure to leucine increases the total protein contents of C2C12 myotubes

The long-term effect of leucine on differentiated C2C12 myotubes was also investigated in this study. A morphological examination of C2C12 myotube cultures did not reveal any significant difference between leucine and the control (no leucine) groups. Similarly, the expression of MyHC-*fast*, a late differentiation marker, was the same in the leucine and the control groups. However, long-term exposure to 5 mM leucine significantly altered the total protein content of C2C12 myotubes. Unlike proliferating C2C12 myoblasts, protein augmentation in differentiated C2C12 myotubes could not be achieved through hyperplasia. Therefore, the increased protein content in differentiated C2C12 cells must have been attained through *de novo* protein synthesis (i.e., hypertrophy). This result agrees with our second hypothesis that long-term exposure to physiological concentrations of leucine will increase the total protein contents of C2C12 cells. However, this result contradicts a previous report in which long-term incubation of C2C12 myotubes with leucine did not affect the total protein content of cells (Haegens *et al*., 2012). However, this contradiction may well be due to different AAs contents in the culture

media used in each study.

In this study, the viability of C2C12 myotubes was assessed following treatment with different concentrations of leucine and/or glutamine. However, assessing the viability of differentiated C2C12 cells is not an easy task, as the AB viability assay is not recommended for differentiated muscle cells. Alternatively, assessing the total, live and dead cells, another indication of cell viability, was attempted in C2C12 myotubes using the NucleoCounter® NC-3000™ flow-cytometer. C2C12 myotubes are multinucleated syncytia, and pose great challenges when cell counting. Therefore, cytofluorometric analysis of single-cell suspension is not the optimal method for assessing the viability of this cell type. In order to move further toward this goal, an Aggregated Cells Assay was used. However, due to a technical limitation, this assay was run only once. Although, no statistics could be generated from this work, results gained from this single experiment may suggest that the total cell count was inversely proportional to the concentration of leucine.

The obvious reduction in the number of dead cells in differentiated C2C12 cultures treated with a combination of leucine and glutamine was another important finding in the present study. It points to the ability of glutamine to increase the survival of C2C12 myotubes and supports our third hypothesis. Finally, the diameter of differentiated C2C12 cells appeared to be directly proportional to leucine concentrations. However, no effect on cell diameter was observed when different concentrations of leucine were combined with glutamine. Yet again, the VCC assay do not reflect the actual size of C2C12 myotubes in monolayer cultures.

5.6. Conclusion

The results of the present study suggest that long-term exposure to physiological concentrations of leucine (2 mM and 5 mM) increased the viability, proliferation and, consequently, desmin expression in C2C12 myoblasts. Importantly, long-term exposure to 5 mM leucine increased the total protein content of C2C12 myoblasts. However, long-term exposure to a high concentration of leucine (10 mM) decreased the viability, proliferation and protein content of C2C12 myoblasts. Combining glutamine and leucine increased the proliferation of C2C12 myoblasts when compared with leucine alone. Long-term incubation of differentiated C2C12 myotubes with physiological concentrations of leucine increased protein accumulation within these cells. However, exposure to a supraphysiological (10 mM) concentration of leucine

decreased the total protein content of C2C12 myotubes. Finally, combined treatment with both glutamine and leucine appeared to increase the survival and decreased the death of differentiated C2C12 myotubes.

6. Characterising the signalling pathways that mediate the effects of leucine and glutamine on C2C12 cells

6.1. Introduction

Leucine has the unique ability to stimulate protein synthesis within muscles (Anthony *et al*., 2001; Lynch *et al*., 2003; Lang and Frost, 2005). The protein anabolic properties of leucine have been linked to its ability to initiate the translation process for protein synthesis (Layman, 2003) via the activation of the mTOR signalling pathway (Lynch *et al*., 2003; Tokunaga *et al*., 2004; Stipanuk, 2007), and the subsequent phosphorylation of mTOR's two downstream targets the eukaryotic initiation factor 4E binding protein 1 (4E-BP1) and the p70 S6K1 (Schmelzle and Hall, 2000; Hara *et al*., 2002; Shen *et al*., 2005; Suryawan *et al*., 2010; Areta *et al*., 2014).

The phosphorylation of p70 S6K positively controls protein synthesis by promoting mRNA translation. The phosphorylation of 4E-BP1 represses the cap-binding protein eukaryotic translation initiation factor (eIF) 4E (eIF4E), thus allowing the formation of the eIF4F complex and the initiation of protein translation (Hara *et al*., 1998). The serine/threonine protein kinase mTOR has recently been recognised as a powerful signalling pathway mediating protein synthesis and muscle hypertrophy, in response to nutritional (leucine) and hormonal (insulin) stimuli (Hay and Sonenberg, 2004; Teleman *et al*., 2008). The link between the protein enhancing effects of leucine and the activation of mTOR signalling has been further supported by the finding that blocking the mTOR pathway abrogated leucine-mediated protein synthesis in the skeletal muscles of neonatal pigs (Suryawan *et al*. 2008). It has also been suggested that leucine can enhance MPS, in part by interfering with the AMPK signalling, a well-known regulator of mTOR (Du *et al*., 2007).

MAPK is an anabolic signalling pathway that has been shown to be altered in response to provision of BCAAs. The ERK1/2 is the most recognised member of the MAPK pathway. The activation of ERK1/2 occurs in response to the availability of nutrients, growth factors and hormones (Cargnello and Roux, 2011). ERK1/2 activation leads to a number of biological outcomes, such as accelerating cell proliferation, promoting cell differentiation, increasing cell survival and facilitating cell migration (Zhang and Liu, 2002). ERK1/2 has been shown to

contribute to the activation of mTOR signalling (Carriere *et al*., 2011). This finding suggests that leucine-induced protein synthesis may be mediated by the activation of ERK1/2 signalling. However, information about the contribution of ERK1/2 to leucine-mediated activation of mTOR is limited.

Glutamine is a conditional EAA with the ability to induce protein synthesis, and decrease protein breakdown, within a wide variety of cells (MacLennan *et al*., 1987; Yoshida *et al*., 1995; Lambertucci *et al*., 2012; Murakami *et al*., 2013). Growing evidence suggests that glutamine exerts this favourable protein effect via the activation of mTOR and MAPK signalling pathways. In support of this are reports of increased phosphorylation of mTOR and ERK1/2 in IPEC-1 cells (Zhu *et al*., 2015), an increased phosphorylation of S6K (a downstream target of mTOR) and ERK1/2 within ovarian cancer cell lines (Yuan *et al*., 2015), and an activation of the MAPK signalling pathway in HDPCs cells (Kim *et al*., 2014) after exposure to glutamine. However, the role of glutamine in mediating protein synthesis through the activation of mTOR and MAPK pathways is controversial. Glutamine failed to induce the phosphorylation of mTOR within muscle (C2C12) cells (Deldicque *et al*., 2008). It also deactivated both mTOR and MAPK pathways in rat small intestinal epithelial (IEC18) and human colon carcinoma (Caco-2) cell lines (Sakiyama *et al*., 2009).

Leucine and glutamine are usually supplemented together, and the published literature suggests that glutamine is needed for full activation of the mTOR signalling following leucine treatment, by inhibiting AMPK signalling (Gleason *et al*., 2007; Xu *et al*., 2001; Nicklin *et al*., 2009). However, the combined effect of leucine and glutamine is controversial and remains undefined. Glutamine has been shown to antagonise leucine-induced phosphorylation of mTOR and its downstream elements in enterocytes (Nakajo *et al.*, 2005), but it failed to alter leucine-induced mTOR phosphorylation in C2C12 myotubes (Deldicque *et al.*, 2008).

6.2. Research hypotheses

A large number of studies have demonstrated the ability of leucine to activate the mTOR signalling pathway in muscle cells, without exploring the dose-response relationship. Therefore, we explored whether leucine-mediated phosphorylation of mTOR downstream substrates (p70 S6K and 4E-BP1) in C2C12 cells is dose-dependent, by testing the hypothesis

that exposure to increasing concentrations of leucine will induce a dose-dependent increase in protein accumulation and phosphorylation of p70 S6K and 4E-BP1 in C2C12 myotubes.

It has also been shown that MAPK signalling contributes to the activation of mTOR signalling, but the role of MAPK in mediating leucine activation of mTOR remains ill-defined. Therefore, we examined whether ERK1/2 (members of the MAPK signalling pathway) is essential for leucine-induced phosphorylation of mTOR signalling downstream substrates (p70 S6K and 4E-BP1) by testing the hypothesis that blocking ERK1/2 activity will have a negative effect on the activation of mTOR substrates in C2C12 cells following exposure to leucine.

In addition, studies have shown the ability of leucine to induce the phosphorylation of PI3K/Akt in muscle cells, however the role of PI3K/Akt in mediating leucine activation of mTOR is unclear. Therefore, we investigated whether PI3K/Akt is essential for leucine-induced phosphorylation of p70 S6K and 4E-BP1 by testing the hypothesis that blocking PI3K/Akt signalling will have a negative effect on the activation mTOR substrates in C2C12 cells following exposure to leucine.

Finally, glutamine has been shown to activate mTORC1, however, data on the combined effect of leucine and glutamine on the activation of mTOR signalling in muscle cells are both limited and controversial. Therefore, we investigated whether glutamine augments the effect of leucine-induced phosphorylation of mTOR (p70 S6K and 4E-BP1) and MAPK (ERK1/2) signalling pathways in C2C12 cells by testing the hypothesis that glutamine will increase leucine-mediated phosphorylation of p70 S6K, 4E-BP1 and ERK1/2.

6.3. The aims of this chapter

The aims of the present study were to

1. Examine the concentration- and time-related effect of leucine on the phosphorylation of mTOR signalling in C2C12 myotubes.

2. Examine the concentration- and time-related effect of leucine on the phosphorylation of MAPK signalling in C2C12 myoblasts and myotubes.

3. Examine the combined effect of leucine and glutamine on the phosphorylation of mTOR and MAPK pathways in C2C12 cells.

4. Identify the role of MAPK signalling in leucine-mediated activation of mTOR in C2C12 myotubes.

5. Identify the role of PI3K signalling in mediating leucine-activation of mTOR in C2C12 myotubes.

6. Identify potential signalling pathways that mediates the anabolic effects of leucine on muscle cells.

6.4. Results

6.4.1. Determination of protein accumulation in C2C12 myotubes following exposure to leucine and/or glutamine

The aim of this experiment was to evaluate the independent and combined effects of leucine and glutamine on the total protein content of differentiated C2C12 cells. Results showed that the protein content of C2C12 myotubes was directly proportional to leucine concentrations following short- and medium-term exposure (Figure 6.1 A-C). On the other hand, protein accumulation became inversely proportional to leucine concentrations following long-term exposure (Figure 6.1 D). Notably, all leucine concentrations failed to increase the total protein content of C2C12 myotubes, above the levels of positive control, at any time point (Figure 6.1). In contrast, short-term exposure to glutamine alone transiently increased the total protein content, when compared with positive control ($P < 0.05$) (Figure 6.1 A). However, medium-term exposure to glutamine significantly decreased the protein content of C2C12 myotubes, when compared with both positive and negative controls ($P < 0.01$ and < 0.05, respectively) (Figure 6.1 C&D). Similarly, long-term exposure to glutamine significantly decreased the protein content of C2C12 myotubes, when compared with positive control ($P < 0.05$) Finally, short-term exposure to leucine and glutamine, combined significantly increased the total protein content of C2C12 myotubes, when compared with leucine alone groups (Figure 6.1 A&B).

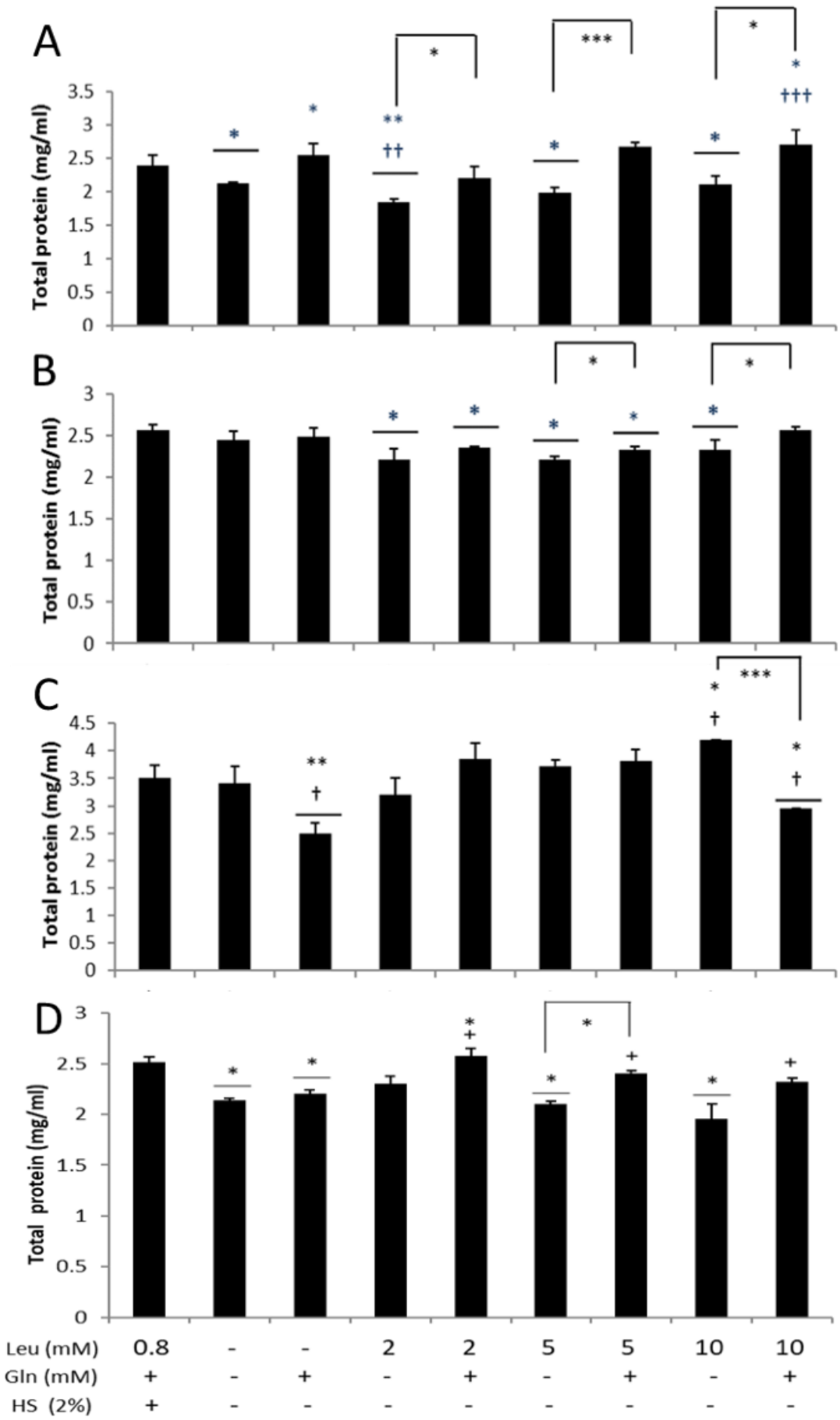

A
B
C
D
Total protein (mg/ml)
Total protein (mg/ml)
Total protein (mg/ml)
Total protein (mg/ml)
Leu (mM) 0.8 - - 2 2 5 5 10 10
Gln (mM) + - + - + - + - +
HS (2%) + - - - - - - - -

Figure 6.1. Total protein content of C2C12 myotubes following exposure to leucine and/or glutamine; assessed using a BCA protein assay. C2C12 myotubes were maintained in DM or exposed to leucine, glutamine or leucine combined with glutamine, added to StM, for 10 min (A), 30 min (B), 60 min (C) and 24 h (D). Data are presented as means ± SD from three biological replica (n = 3). Asterisks indicate significant increase compared with the positive control (first column to the left) and indicate significant differences between leucine and leucine + glutamine groups $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$. Crosses indicate significant increase compared with the negative control (second column to the left) $^{+}P < 0.05$ and $^{+++}P < 0.001$. Asterisks with bars indicate significant decrease compared with the positive control $\overset{*}{-}P < 0.05$ and $\overset{**}{-}P < 0.01$. Crosses with bars indicate significant decrease compared with the negative control $^{\pm}P < 0.05$ and $^{\pm\pm}P < 0.01$. Analysed by one-way ANOVA using Microsoft Excel followed by Tukey's HSD *post hoc* test using SPSS Statistics 20. Abbreviations: Leu = L-leucine; Gln = L-glutamine; HS = horse serum; mM = millimolar; mg = milligrams; ml = millilitre.

6.4.2. Examining the phosphorylation of p70 S6K in C2C12 myotubes following exposure to leucine and/or glutamine

The phosphorylation of p70 S6K was determined following exposure of C2C12 myotubes to glutamine, different concentrations of leucine, or different concentrations of leucine combined with glutamine. Results indicated that short-term exposure to leucine substantially stimulated the phosphorylation of p70 S6K in a dose-dependent manner (Figures 6.2 A&B and 6.3). However, the phosphorylation of p70 S6K became inversely proportional to leucine concentration after medium-term exposure (Figures 6.2 C and 6.3). In contrast, short-term exposure to glutamine caused a significant decrease in the phosphorylation of p70 S6K, when compared with positive and negative controls ($P < 0.001$ and < 0.01, respectively) (Figures 6.2 A and 6.3). However, further exposure to glutamine for 30 and 60 min significantly increased the phosphorylation of p70 S6K ($P < 0.05$) compared with the controls (Figures 6.2 B&C and 6.3). No difference was detected in the phosphorylation of p70 S6K after short-term exposure to leucine and glutamine combined (Figures 6.2 A&B and 6.3). However, following medium-term exposure, glutamine exerted antagonistic effects. On the one hand, glutamine decreased the phosphorylation of p70 S6K when combined with a low concentration (2 mM) of leucine, compared to leucine alone ($P < 0.05$) (Figures 6.2 C and 6.3). On the other hand, glutamine increased the phosphorylation of p70 S6K when combined with a high (10 mM) concentration of leucine, compared with leucine alone ($P < 0.001$) (Figures 6.2 C and 6.3). Finally, long-term exposure to leucine and glutamine combined, significantly increased the phosphorylation of p70 S6K, when compared with leucine groups ($P < 0.01$) (Figures 6.2 D and 6.3).

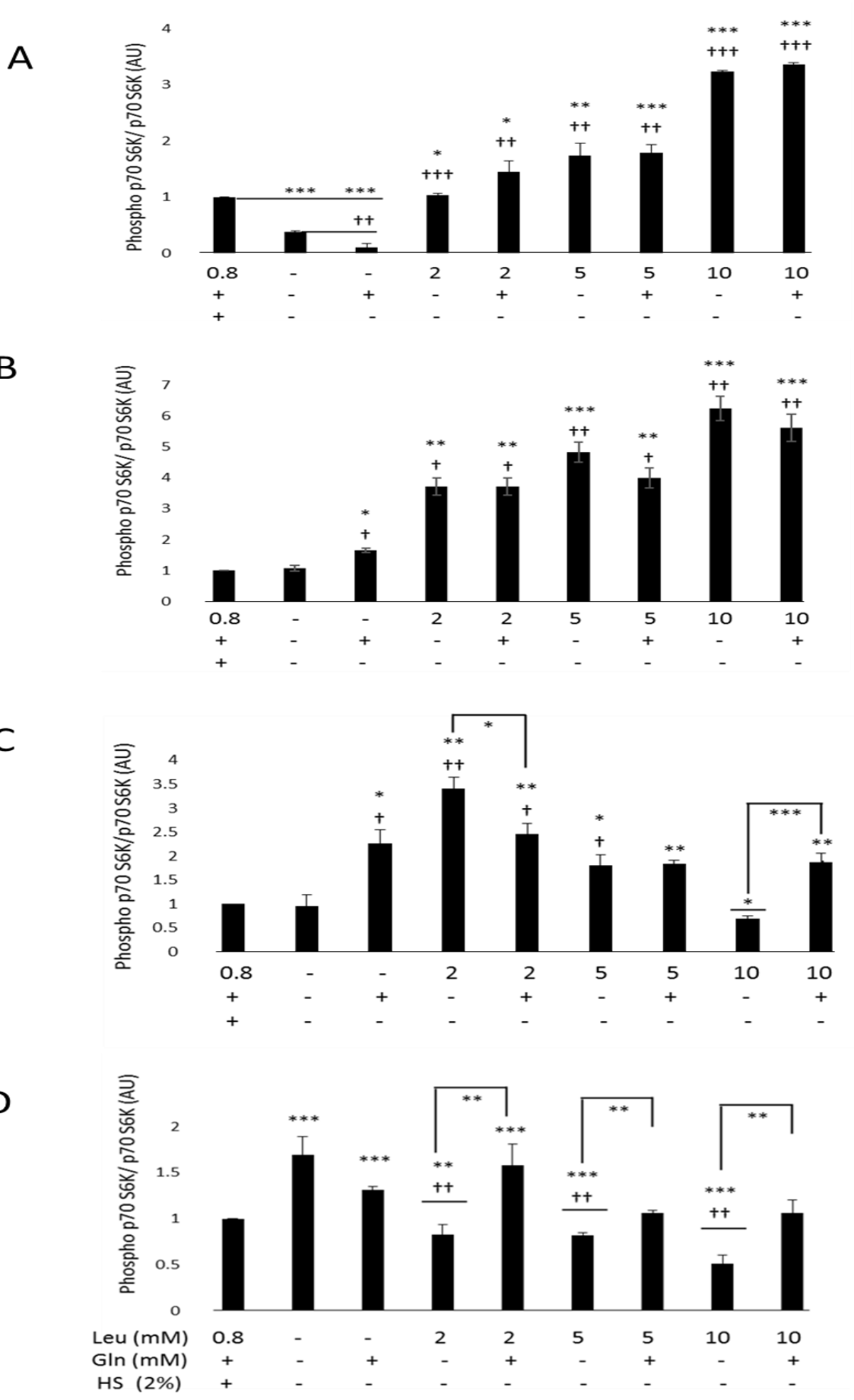

A
Phospho p70 S6K/ p70 S6K (AU)
B
Phospho p70 S6K/ p70 S6K (AU)
C
Phospho p70 S6K/p70 S6K (AU)
D
Phospho p70 S6K/ p70 S6K (AU)
Leu (mM) 0.8 - - 2 2 5 5 10 10
Gln (mM) + - + - + - + - +
HS (2%) + - - - - - - - -

Figure 6.2. The phosphorylation of p70 S6K following exposure of C2C12 myotubes to leucine and/or glutamine. C2C12 myotubes were maintained in DM or exposed to leucine, glutamine or leucine combined with glutamine, added to StM, for 10 min (A), 30 min (B), 60 min (C) and 24 h (D). Data are presented as means ± SD of three biological replica (n = 3). Asterisks indicate significant increase compared with the positive control (first column to the left) and significant difference between leucine and leucine + glutamine groups $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$. Crosses indicate significant increase compared with the negative control (second column to the left) $^{+}P < 0.05$, $^{++}P < 0.01$ and $^{+++}P < 0.001$. Asterisks with bars indicate significant decrease compared with the positive control $^{*}P < 0.05$, $\overline{^{**}}P < 0.01$ and $\overline{^{***}}P < 0.001$. Crosses with bars indicate significant decrease compared with the negative control $^{\pm\pm}P < 0.01$. Analysed by one-way ANOVA using Microsoft Excel followed by Tukey's HSD *post hoc* test using SPSS Statistics 20. Abbreviations: Leu = L-leucine; Gln = L-glutamine; p70 S6K = ribosomal protein S6 kinase; Phospho = phosphorylated; HS = horse serum; mM = millimolar.

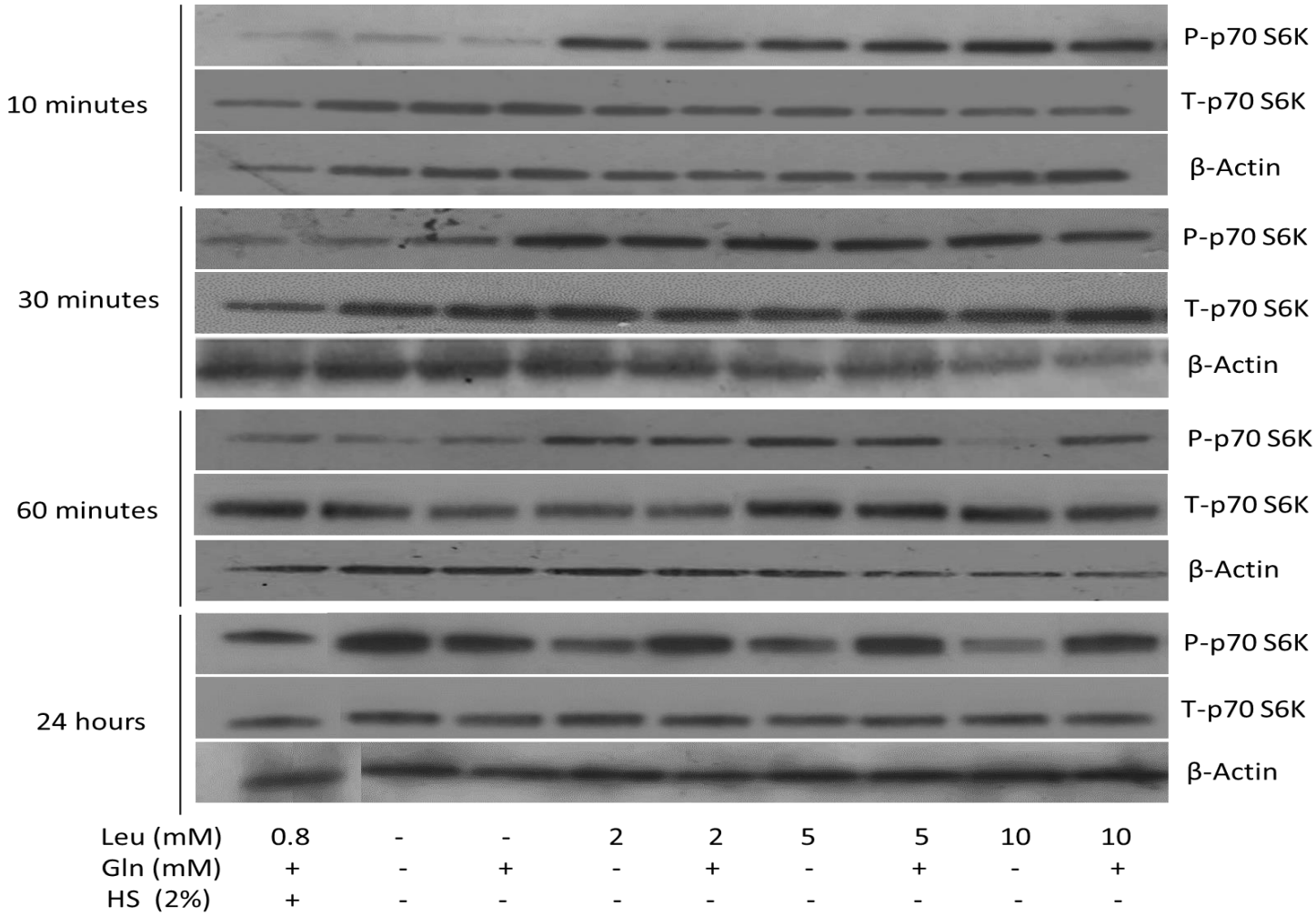


Figure 6.3. Representative western blots showing the phosphorylation of p70 S6K following exposure of C2C12 myotubes to leucine and/or glutamine. Representative blots from one experiment are shown ($n = 4$). C2C12 myotubes were maintained in DM or exposed to leucine, glutamine or leucine combined with glutamine, added to StM, for 10 min, 30 min, 60 min and 24 h. Abbreviations: P = phosphorylated; T = total; p70 S6K = ribosomal protein S6 kinase; mM = millimolar; Leu = L-leucine; Gln = L-glutamine; HS = horse serum.

6.4.3. Examining the phosphorylation of 4E-BP1 in C2C12 myotubes following exposure to leucine and/or glutamine

The phosphorylation of 4E-BP1 was investigated following exposure of differentiated C2C12 cells to leucine and/or glutamine. Results revealed that short-term exposure to different concentrations of leucine stimulated the phosphorylation of 4E-BP1 in a dose dependent-manner (Figures 6.4 A& B and 6.5). However, the phosphorylation of 4E-BP1 was inversely proportional to leucine concentrations upon medium- and long-term exposure (Figures 6.4 C&D and 6.5). Results also demonstrated that only exposure to a high concentration (10 mM) of leucine significantly increased the phosphorylation of 4E-BP1($P < 0.05$), from positive and negative controls after 10 min (Figures 6.4 A and 6.5). On the other hand, all the leucine concentrations increased the phosphorylation of 4E-BP1, when compared with both controls after 30 min (Figures 6.4 B and 6.5). Notably, medium- and long-term exposure to leucine alone, or leucine combined with glutamine, failed to significantly increase the phosphorylation of 4E-BP1 above the levels of controls (Figure 6.4 C&D and 6.5). No significant difference in the phosphorylation of 4E-BP1 was recorded following short-term exposure to leucine and glutamine combined (Figures 6.4 A&B and 6.5). Nevertheless, a significant difference among leucine and leucine + glutamine groups took place after medium-term exposure in which glutamine exerted antagonistic effects when combined with leucine. It decreased the phosphorylation of 4E-BP1, compared with leucine alone, when combined with low concentrations (2 mM and 5 mM) of leucine ($P < 0.001$) (Figures 6.4 C and 6.5), while increased the phosphorylation of 4E-BP1, compared with leucine alone, when combined with a high (10 mM) concentration of leucine ($P < 0.001$) (Figures 6.4 C&D and 6.5). The same results were also evident following long-term exposure to leucine and glutamine combined (Figures 6.4 D and 6.5).

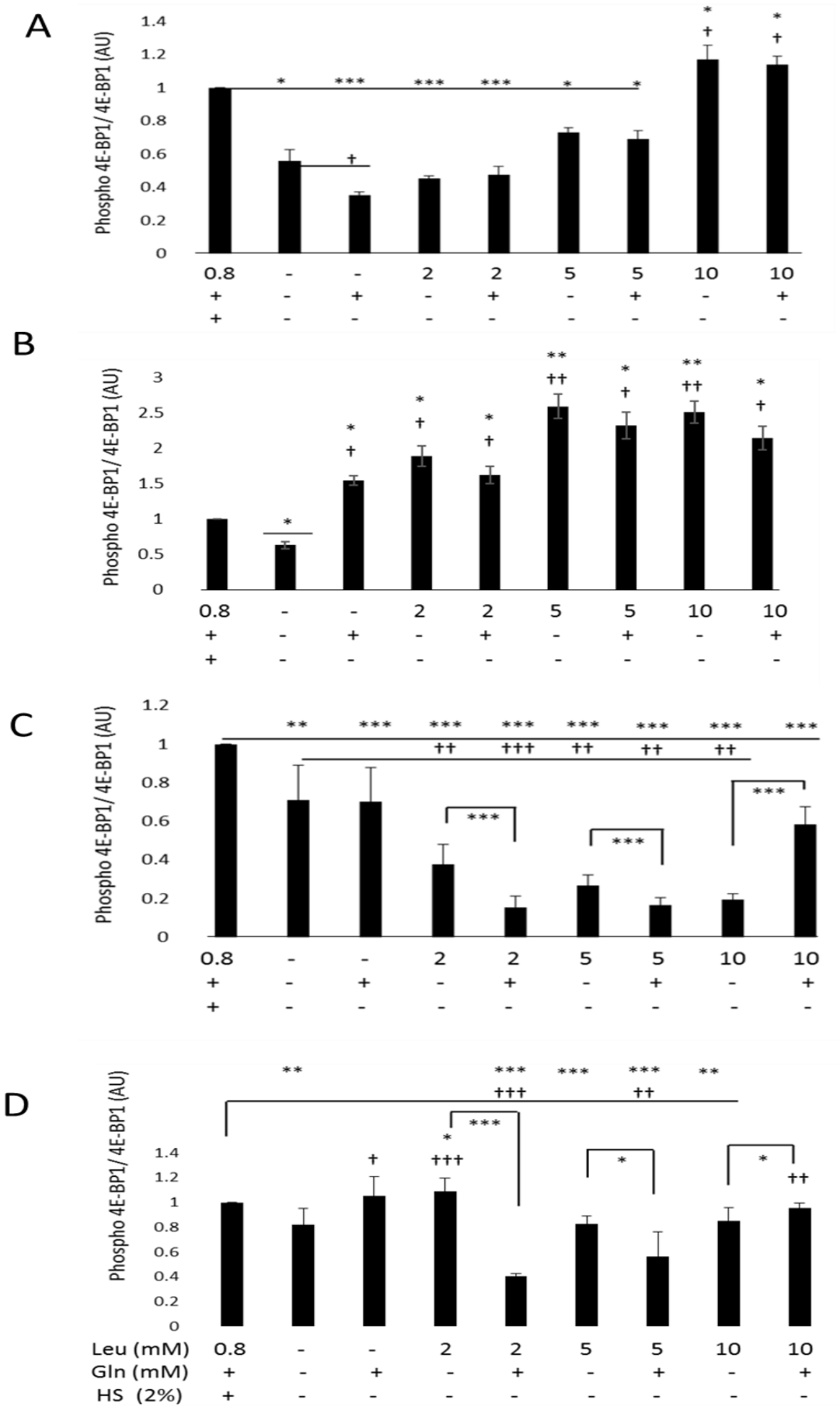

A
B
C
D
Phospho 4E-BP1/ 4E-BP1 (AU)
Leu (mM)
Gln (mM)
HS (2%)
0.8
2
5
10

Figure 6.4. The phosphorylation of 4E-BP1 following exposure of C2C12 myotubes to leucine and/or glutamine. C2C12 myotubes were maintained in DM or exposed to leucine, glutamine or leucine combined with glutamine, added to StM, for 10 min (A), 30 min (B), 60 min (C) and 24 h (D). Data are presented as means ± SD from three biological replica ($n = 3$). Asterisks denote significant increase compared with the positive control (first column to the left) and significant difference between leucine and leucine + glutamine groups $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$. Crosses denote significant increase compared with the negative control (second column to the left) $^{+}P < 0.05$, $^{++}P < 0.01$ and $^{+++}P < 0.001$. Asterisks with bars indicate significant decrease compared with the positive control $\bar{}^{*}P < 0.05$, $\bar{}^{**}P < 0.01$ and $\bar{}^{***}P < 0.001$. Crosses with bars indicate significant decrease compared with the negative control $^{\pm}P < 0.05$, $^{\pm\pm}P < 0.01$ and $^{\pm\pm\pm}P < 0.001$. Analysed by one-way ANOVA using Microsoft Excel followed by Tukey's HSD *post hoc* test using SPSS Statistics 20. Abbreviations: Leu = L-leucine; Gln = L-glutamine; 4E-BP1 = eukaryotic translation initiation factor 4E binding protein 1; Phospho = phosphorylated; HS = horse serum; mM = millimolar.

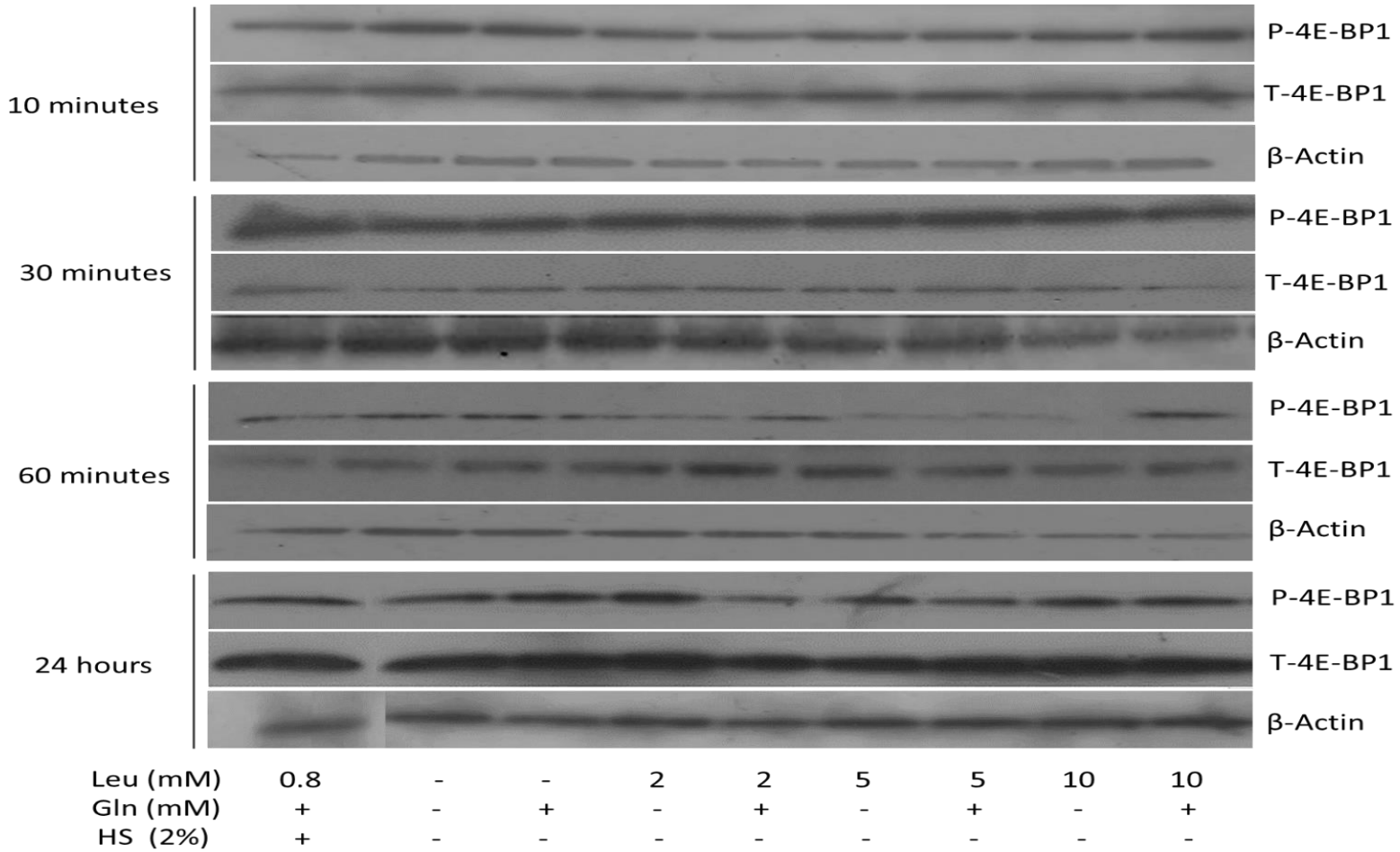


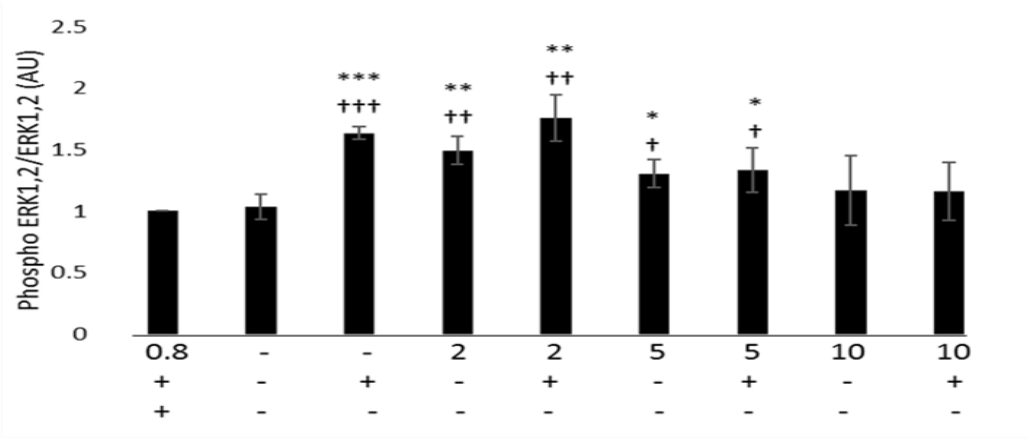
Figure 6.5. Representative western blots showing the phosphorylation of 4E-BP1 following exposure of C2C12 myotubes to leucine and/or glutamine. C2C12 myotubes were maintained in DM or exposed to leucine, glutamine or leucine combined with glutamine, added to StM, for 10 min, 30 min, 60 min and 24 h. Representative blots from one experiment are shown (n = 4). Abbreviations: P = phosphorylated; T = total 4E-BP1 = eukaryotic translation initiation factor 4E binding protein 1; Leu = L-leucine; Gln = L-glutamine HS = horse serum; mM = millimolar.

6.4.4. Phosphorylation of ERK1/2 following stimulation of C2C12 myotubes with leucine and/or glutamine

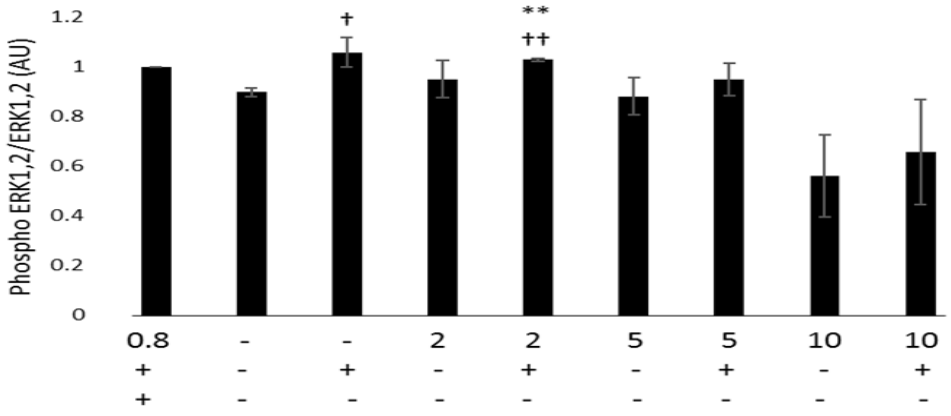
The phosphorylation of ERK1/2 was investigated following exposure of C2C12 myotubes to leucine and/or glutamine. Our analysis showed that the phosphorylation of ERK1/2 was inversely proportional to leucine concentrations at all tested time points (Figures 6.6 and 6.7). Interestingly, short-term exposure to low concentrations (2 mM and 5 mM) of leucine for 10 min transiently increased phosphorylation of ERK1/2, compared with controls ($P < 0.01$ and < 0.05, respectively) (Figures 6.6 A and 6.7). However, the same concentrations failed to maintain the phosphorylation of ERK1/2 above control levels at 30 min (Figures 6.6 B and 6.7). A significant decline in the phosphorylation of ERK1/2, compared with positive and negative controls ($P < 0.001$ and < 0.05, respectively), took place after 60 min exposure to 5 mM leucine (Figure 6.6 C). Finally, a significant decline ($P < 0.001$) in the phosphorylation of ERK1/2, compared with both controls, occurred following long-term exposure to all leucine concentrations (Figures 6.6 D and 6.7).

Our analysis also showed that short-term exposure to glutamine for 10 min significantly increased the phosphorylation of ERK1/2, when compared with positive and negative controls ($P < 0.001$) (Figures 6.6 A and 6.7). Similarly, short-term exposure to glutamine for 30 min significantly increased the phosphorylation of ERK1/2, when compared with the negative control ($P < 0.05$), (Figures 6.6 B and 6.7). However, medium-term exposure to glutamine did not alter phosphorylation of ERK1/2 (Figure 6.6 C), while long-term exposure to glutamine resulted in a significant decrease in ERK activity, when compared with the positive control ($P < 0.05$) (Figures 6.6 D and 6.7). Interestingly, combining glutamine and leucine increased the phosphorylation of ERK1/2 when compared with leucine groups (Figures 6.6 and 6.7).

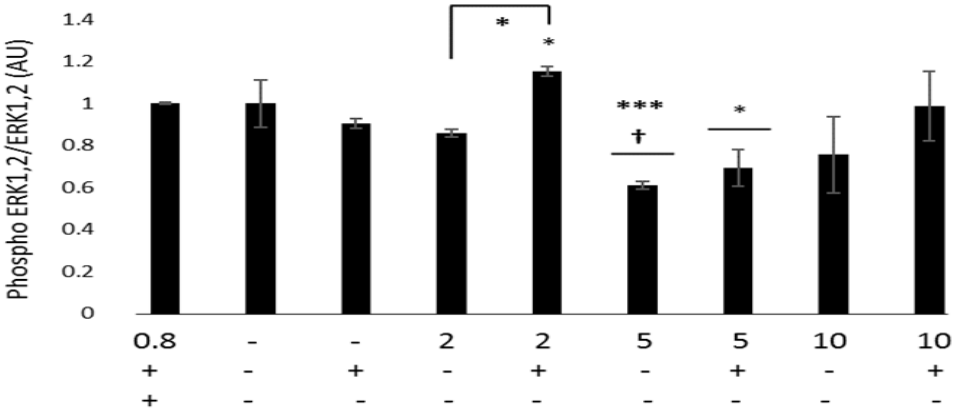
A


Phospho ERK1,2/ERK1,2 (AU)
2.5
2
1.5
1
0.5
0
*** †††
** ††
** ††
* †
* †
0.8 - - 2 2 5 5 10 10
+ - + - + - + - +
+ - - - - - - - -

B


Phospho ERK1,2/ERK1,2 (AU)
1.2
1
0.8
0.6
0.4
0.2
0
†
** ††
0.8 - - 2 2 5 5 10 10
+ - + - + - + - +
+ - - - - - - - -

C


Phospho ERK1,2/ERK1,2 (AU)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
*
*
*** †
*
0.8 - - 2 2 5 5 10 10
+ - + - + - + - +
+ - - - - - - - -

D

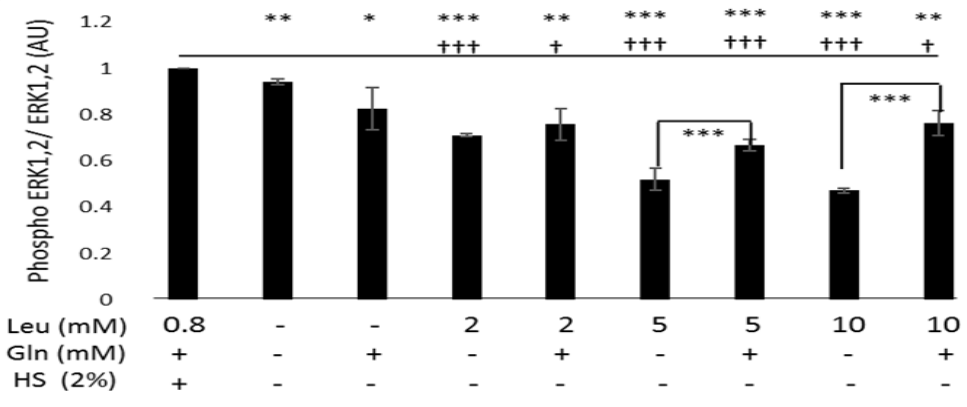

Phospho ERK1,2/ ERK1,2 (AU)
1.2
1
0.8
0.6
0.4
0.2
0
** * *** +++ ** † *** +++ *** +++ *** +++ ** †
*** ***
Leu (mM) 0.8 - - 2 2 5 5 10 10
Gln (mM) + - + - + - + - +
HS (2%) + - - - - - - - -

Figure 6.6. The phosphorylation of ERK1/2 following exposure of C2C12 myotubes to leucine and/or glutamine. C2C12 myotubes were maintained in DM or exposed to leucine, glutamine or leucine combined with glutamine, added to StM, for 10 min (A), 30 min (B), 60 min (C) and 24 h (D). Data are presented as means ± SD from three biological replica ($n = 3$). Asterisks indicate significant increase compared with the positive control (first column to the left) and significant difference between leucine and leucine + glutamine groups $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$. Crosses indicate significance increase compared with the negative control (second column to the left) $^{+}P < 0.05$, $^{++}P < 0.01$ and $^{+++}P < 0.001$. Asterisks with bars indicate significant decrease compared with the positive control $\overline{^{*}}P < 0.05$, $\overline{^{**}}P < 0.01$ and $\overline{^{***}}P < 0.001$. Crosses with bars indicate significant decrease compared with the negative control $\overline{^{+}}P < 0.05$ and $\overline{^{+++}}P < 0.001$. Analysed by one-way ANOVA using Microsoft Excel followed by Tukey's HSD *post hoc* test using SPSS Statistics 20. Abbreviations: Phospho = phosphorylated; ERK = extracellular signal regulated kinase; Leu = L-leucine; Gln = L-glutamine; mM = millimolar HS = horse serum.

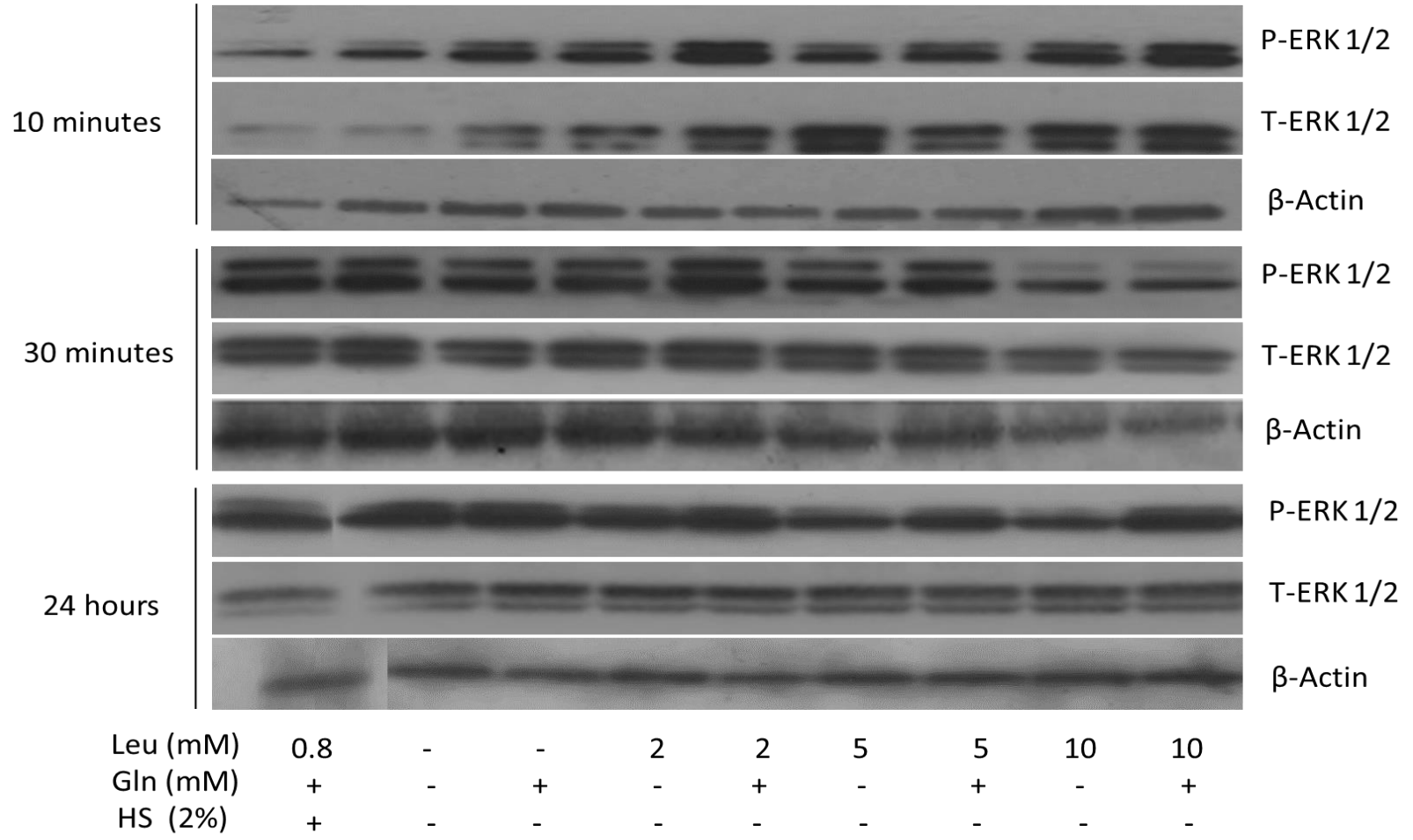


Figure 6.7. Representative western blots showing the phosphorylation of ERK1/2 following exposure of C2C12 myotubes to leucine and/or glutamine. C2C12 myotubes were maintained in DM or exposed to leucine, glutamine or leucine combined with glutamine added to StM. Representative blots from one experiment are shown (*n* =4). Abbreviations: P = phosphorylated; T = total; ERK = extracellular signal regulated kinase; Leu = L-leucine; Gln = L-glutamine; HS = horse serum; mM = millimolar.

6.4.5. The impact of blocking ERK1/2 on protein accumulation within C2C12 myotubes following exposure to leucine and/or glutamine

The total protein content of U0126-treated C2C12 myotubes was determined following exposure to glutamine, different concentrations of leucine or different concentrations of leucine combined with glutamine. We found that exposure to all leucine concentrations failed to increase the total protein content of U0126-treated C2C12 myotubes compared with untreated (vehicle control), positive and negative controls (Figure 6.8). The same finding was recorded when different concentrations of leucine were combined with glutamine (Figure 6.8).

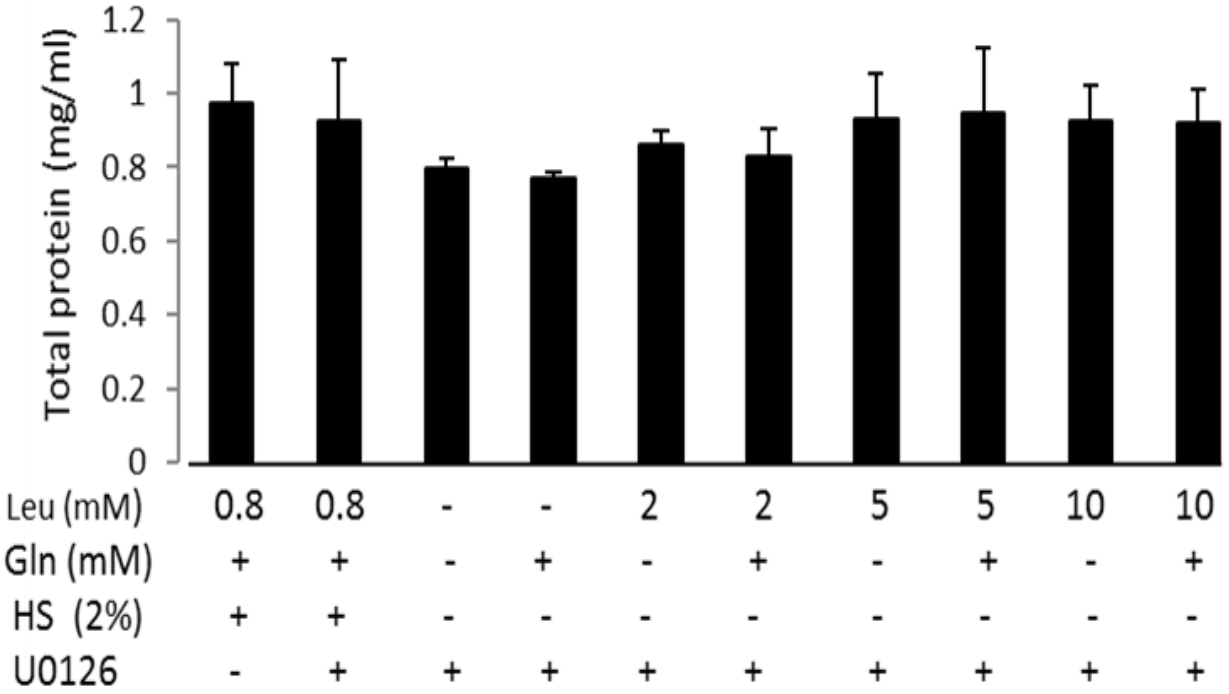


Figure 6.8. Total protein content of U0126-treated C2C12 myotubes following exposure to leucine and/or glutamine; assessed using a BCA protein assay. U0126-treated C2C12 myotubes were maintained in DM or exposed to leucine, glutamine or leucine combined with glutamine, added to StM, for 30 min. Data are presented as means ± SD from three biological replica (n = 3). Analysed by one-way ANOVA using Microsoft Excel. First column on the left represents vehicle control, second column represents positive control and third column represents negative control. Abbreviations: mg = milligrams; ml = millilitre; Leu = L-leucine; Gln = L-glutamine; mM = millimolar; HS = horse serum.

6.4.6. The impact of blocking ERK1/2 signalling on the phosphorylation of p70 S6K in C2C12 myotubes following exposure to leucine and/or glutamine

The phosphorylation of p70 S6K was determined in U0126-treated C2C12 myotubes following exposure to glutamine, different concentrations of leucine or different concentrations of leucine combined with glutamine. Results indicated that treatment with U0126 successfully inhibited the phosphorylation of ERK1/2 in C2C12 myotubes (Figure 6.9). A significant decrease ($P < 0.001$) in the phosphorylation of p70 S6K was recorded between U0126-treated and untreated (vehicle control) cells (Figure 6.10). Notably, exposing U0126-treated C2C12 myotubes to different concentrations of leucine stimulated the phosphorylation of p70 S6K in a dose-dependent manner (Figure 6.10). All leucine concentrations induced a significant increase in the phosphorylation of p70 S6K when compared with the negative control (Figure 6.10). However, 10 mM was the only leucine concentration to induce a significant increase in the phosphorylation of p70 S6K above the positive control ($P < 0.05$) (Figure 6.10). Finally, no differences were observed among leucine and leucine + glutamine groups (Figure 6.10).

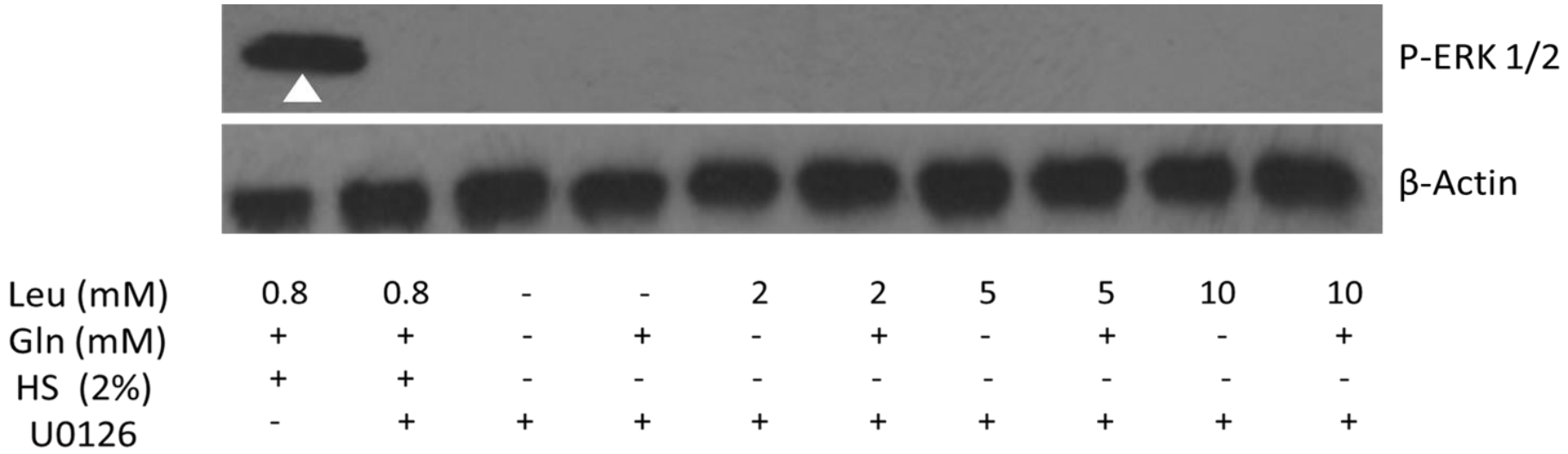


Figure 6.9. Representative western blots showing the phosphorylation of ERK1/2 following the exposure of U0126-treated C2C12 myotubes to leucine and/or glutamine. U0126-treated C2C12 myotubes were maintained in DM or exposed to leucine, glutamine or leucine combined with glutamine, added to StM, for 30 min. Representative blots from one experiment are shown (n = 4). U0126 treatment inhibited the phosphorylation of ERK1/2 in C2C12 myotubes. White arrowhead points to the band of vehicle control (untreated cells). Abbreviations: P = phosphorylated; ERK = extracellular signal regulated kinase; Leu = L-leucine; Gln = L-glutamine; mM = millimolar; HS = horse serum.

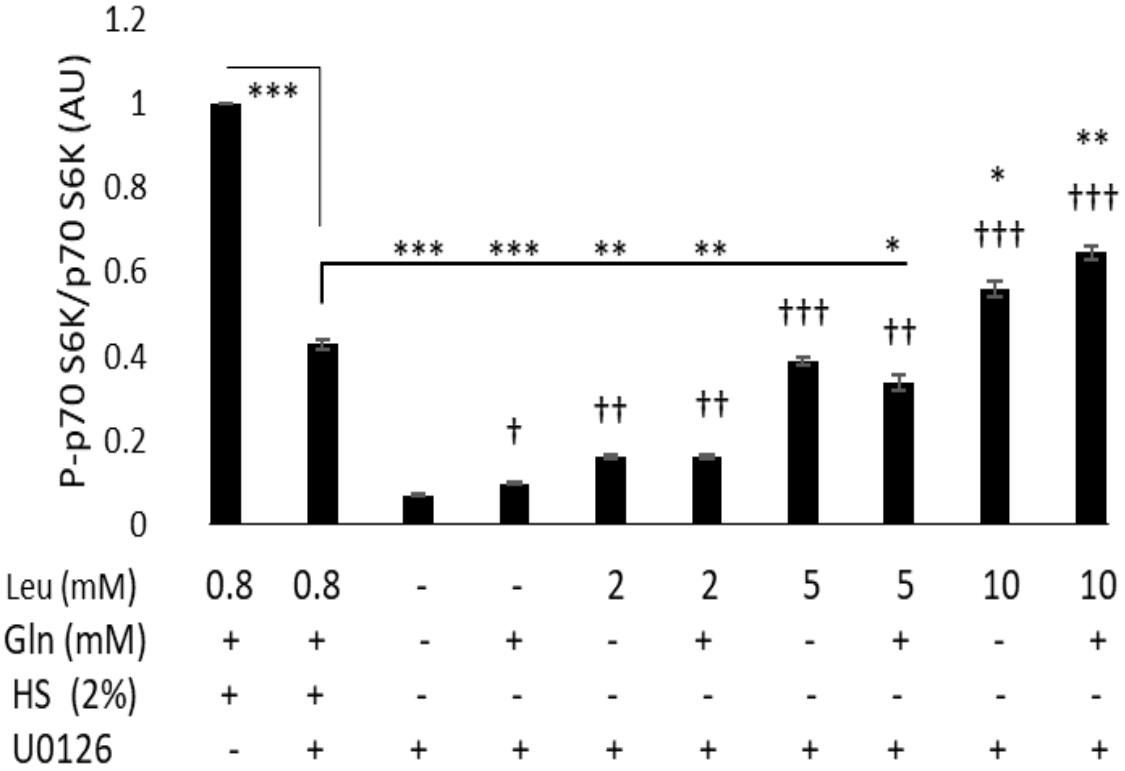


Figure 6.10. The phosphorylation of p70 S6K following the exposure of U0126-treated C2C12 myotubes to leucine and/or glutamine. U0126-treated C2C12 myotubes were maintained in DM or exposed to leucine, glutamine or leucine combined with glutamine, added to StM, for 30 min. Data are presented as means ± SD from three biological replica (n = 3). Asterisks indicate significant difference between untreated (vehicle control, first column to the left) and U0126-treated (positive control, second column to the left) groups, and indicate significant increase above the positive control $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$. Crosses indicate significance increase compared with the negative control (third column to the left) $^{+}P < 0.05$, $^{++}P < 0.01$ and $^{+++}P < 0.001$. Asterisks with bars indicate significant decrease compared with the positive control $\overset{*}{-}P < 0.05$, $\overset{**}{—}P < 0.01$ and $\overset{***}{—}P < 0.001$. Analysed by one-way ANOVA using Microsoft Excel followed by Tukey's HSD *post hoc* test using SPSS Statistics 20. Abbreviations P = phosphorylated; p70 S6K = ribosomal protein S6 kinase; Leu = L-leucine; Gln = L-glutamine; mM = millimolar; HS = horse serum.

6.4.7. The impact of blocking ERK1/2 signalling on the phosphorylation of 4E-BP1 following exposure of C2C12 myotubes to leucine and/or glutamine

The phosphorylation of 4E-BP1 was determined following exposure of U0126-treated C2C12 myotubes to leucine and/or glutamine. Results indicated that treatment with U0126 inhibited the phosphorylation of ERK1/2 in C2C12 myotubes (Figure 6.9). As seen with p70 S6K, a significant decrease ($P < 0.05$) in the phosphorylation of 4E-BP1 took place between U0126-treated and untreated (vehicle control) cells (Figure 6.11). however, no difference in the phosphorylation of 4E-BP1, compared to controls, was evident after exposure of U0126-treated C2C12 myotubes to leucine, glutamine or leucine and glutamine combined (Figure 6.11).

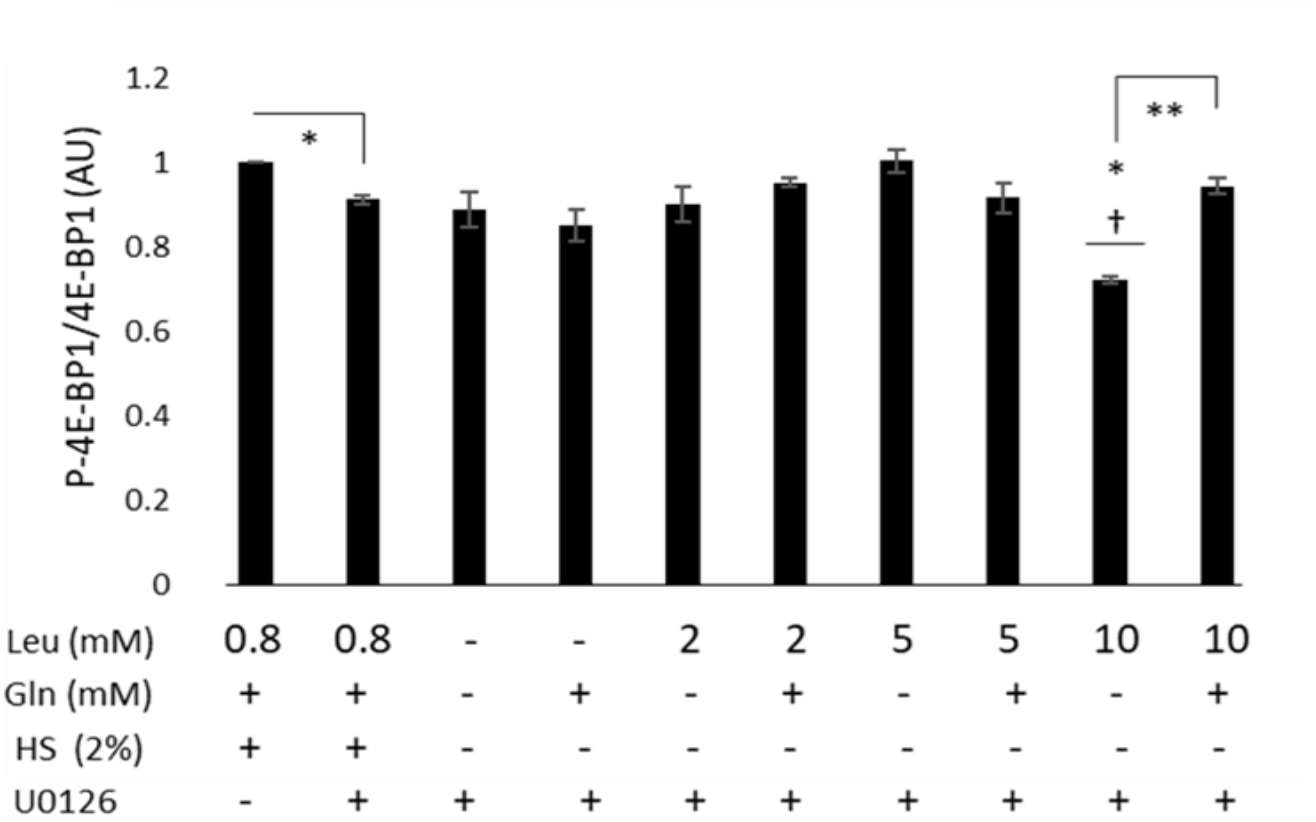


Figure 6.11. The phosphorylation of 4E-BP1 following the exposure of U0126-treated C2C12 myotubes to leucine and/or glutamine. U0126-treated C2C12 myotubes were maintained in DM or exposed to leucine, glutamine or leucine combined with glutamine, added to StM, for 30 min. Data are presented as means ± SD from three biological replica (n = 3). Asterisks indicate significant difference between untreated (vehicle control, first column to the left) and U0126-treated (positive control, second column to the left) groups and indicate significant difference between leucine and leucine + glutamine groups $^{*}P < 0.05$, $^{**}P < 0.01$. Asterisks with bars indicate significant decrease compared with the positive control $^{*}P < 0.05$. Crosses with bars indicate significant decrease compared with the negative control (third column to the left) $^{\pm}P < 0.05$. Analysed by one-way ANOVA using Microsoft Excel followed by Tukey's HSD *post hoc* test using SPSS Statistics 20. Abbreviations: P = phosphorylated; 4E-BP1 = eukaryotic translation initiation factor 4E binding protein 1; Leu = L-leucine; Gln = L-glutamine; mM = millimolar; HS = horse Serum.

6.4.8. The impact of blocking the PI3K/Akt pathway on the total protein content of C2C12 myotubes following exposure to leucine and/or glutamine

The PI3K/Akt signalling pathway was blocked by LY294002 in C2C12 myotubes. Results indicated that exposure to leucine, in the presence and absence of glutamine, did not induce protein accumulation in LY294002-treated C2C12 myotubes (Figure 6.12). Interestingly, exposure to glutamine significantly decreased ($P < 0.05$) the total protein content of cells when compared with the negative control (Figure 6.12). finally, exposure to leucine and glutamine, combined decreased the total protein when compared with the leucine groups (Figure 6.12), but the only significant decrease was noticed in 5 mM groups ($P < 0.01$).

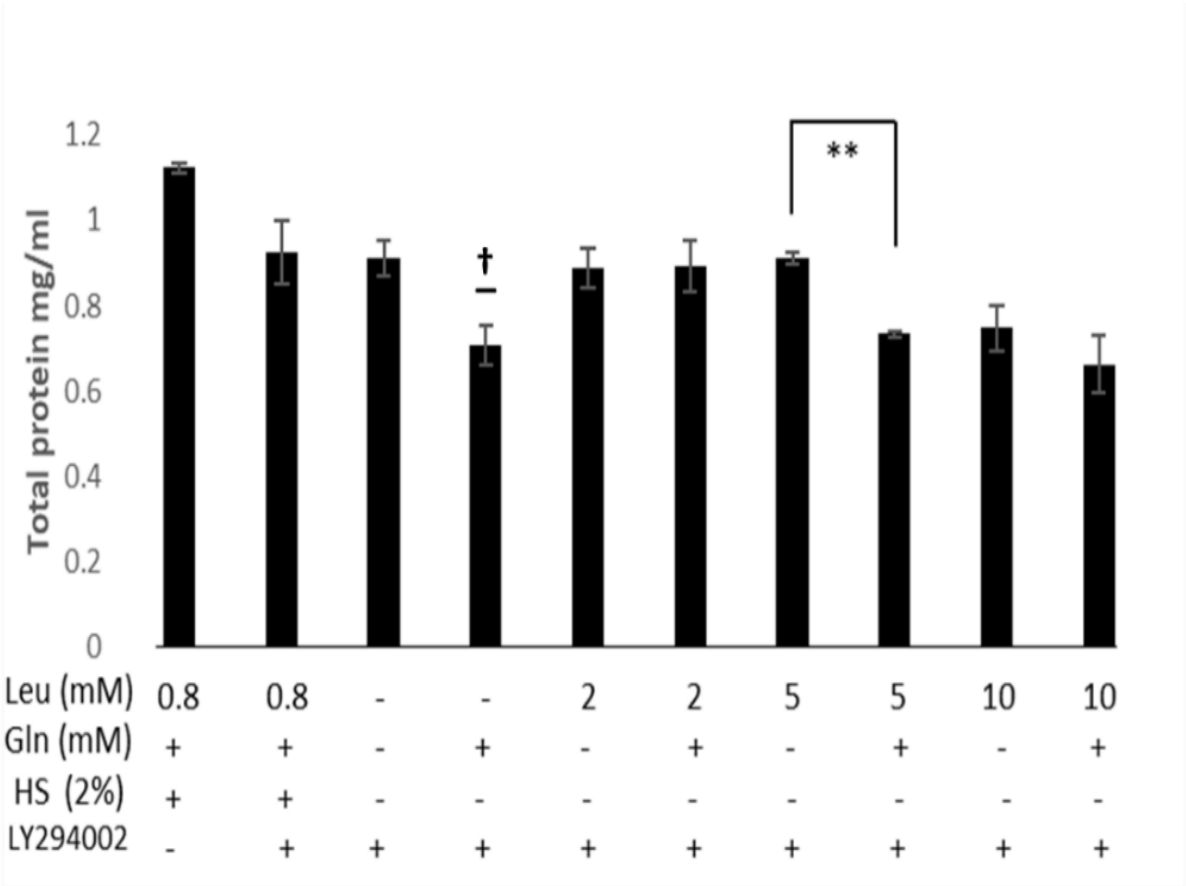


Figure 6.12. Total protein content of LY294002-treated C2C12 myotubes following exposure to leucine and/or glutamine. LY294002-treated C2C12 myotubes were maintained in DM or exposed to leucine, glutamine or leucine combined with glutamine, added to StM, for 30 min. Data are presented as means ± SD from three biological replica (n = 3). The first column on the left represents untreated vehicle control, the second column represents LY294002-treated positive control and the third column on the left represents LY294002-treated negative control. Asterisks indicate significant difference between leucine and leucine + glutamine groups $^{**}P < 0.01$. Cross with bar indicates significant decrease compared with the negative control $^{\pm}P < 0.05$. Analysed by one-way ANOVA using Microsoft Excel followed by Tukey's HSD *post hoc* test using SPSS Statistics 20. Abbreviations: mg = milligrams; ml = millilitre; Leu = L-leucine; Gln = L-glutamine; mM = millimolar; HS = horse serum.

6.4.9. Assessing the impact of blocking the PI3K/Akt pathway on the phosphorylation of p70 S6K and 4E-BP1 in C2C12 myotubes following exposure to leucine and/or glutamine

The phosphorylation of p70 S6K and 4E-BP1 was determined in LY294002-treated C2C12 myotubes following exposure to glutamine, different concentrations of leucine or different concentrations of leucine combined with glutamine. Results indicated that treatment with LY294002 totally blocked the phosphorylation of Akt and p70 S6K in C2C12 myotubes (Figure 6.13). Additionally, the phosphorylation of 4E-BP1 was barely detected in LY294002-treated C2C12 myotubes (Figure 6.13).

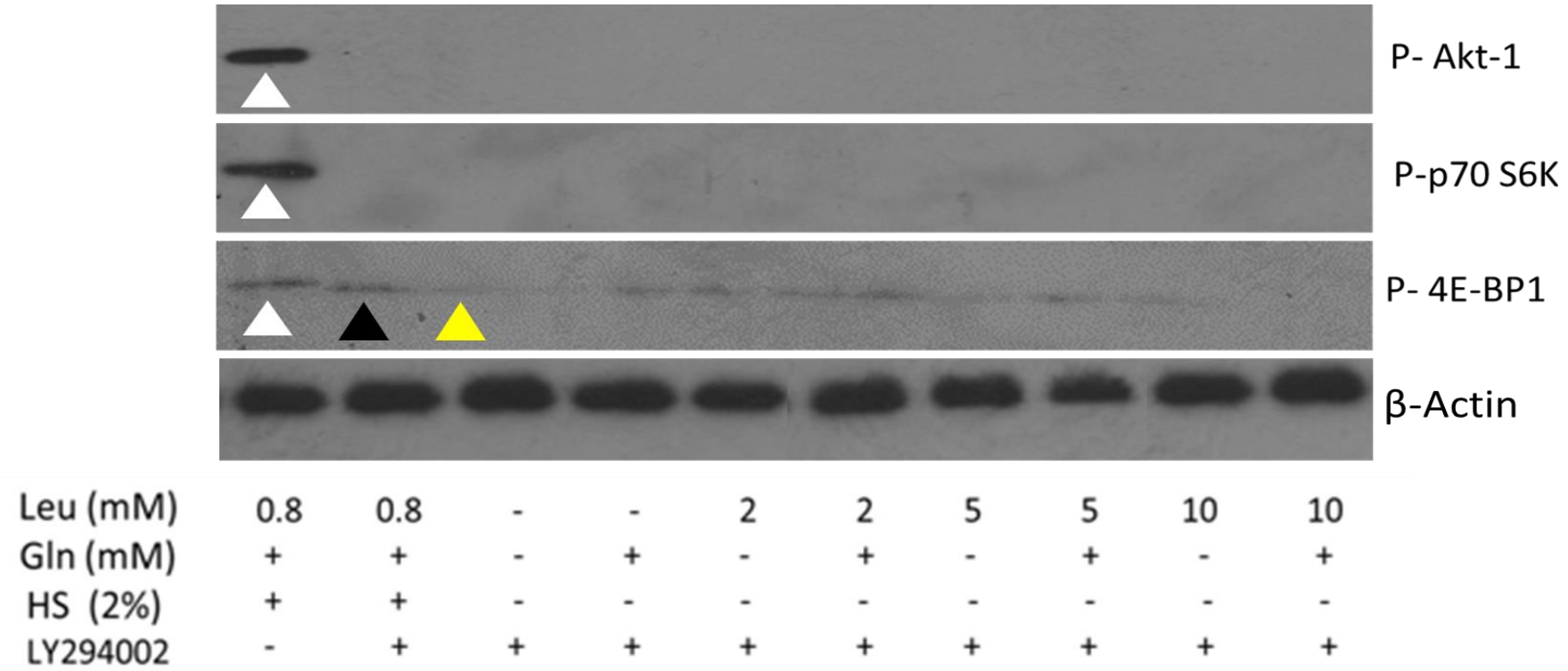


Figure 6.13. Western blots representing the phosphorylation of Akt-1, p70 S6K and 4E-BP1 following the exposure of LY294002-treated C2C12 myotubes to leucine and/or glutamine. LY294002-treated C2C12 myotubes were maintained in DM or exposed to leucine, glutamine or leucine combined with glutamine, added to StM, for 30 min. Representative blots from one experiment are shown ($n = 4$). LY294002 treatment inhibited the phosphorylation of Akt and p70 S6K in C2C12 myotubes. White arrowheads point to the bands of vehicle control. Black and yellow arrowheads point to the bands of positive and negative controls, respectively. Abbreviations: P = phosphorylated; Akt = protein kinase B; p70 S6K = ribosomal protein S6 kinase; 4E-BP1 = eukaryotic translation initiation factor 4E binding protein 1; Leu = L-leucine; Gln = L-glutamine; mM = millimolar; HS = horse serum.

6.4.10. PathScan® Intracellular Signaling Membrane Arrays

In this experiment the PathScan® Intracellular Signaling Membrane Array kit was used to gain insight into the phosphorylation events that took place after stimulation of differentiated C2C12 cells with leucine alone, and leucine combined with glutamine. The arrays did detect the phosphorylation of GSK-3β, proline rich Akt-substrate 40 (PRAS40), ERK1/2, mTOR, S6 ribosomal protein and Bad in control and treated groups (Figure 6.14). On the other hand, the phosphorylation of Akt, heat shock protein 27, p53 and stress-activated protein kinase/ c-Jun N-terminal kinase, and the cleavage of Poly (ADP-ribose) polymerase (PARP) and caspase-3 were barely detected in control or treated groups (Figure 6.14). The arrays did not indicate significant differences among control (Figure 6.14 A), leucine (Figure 6.14 B) and leucine + glutamine (not shown) groups.

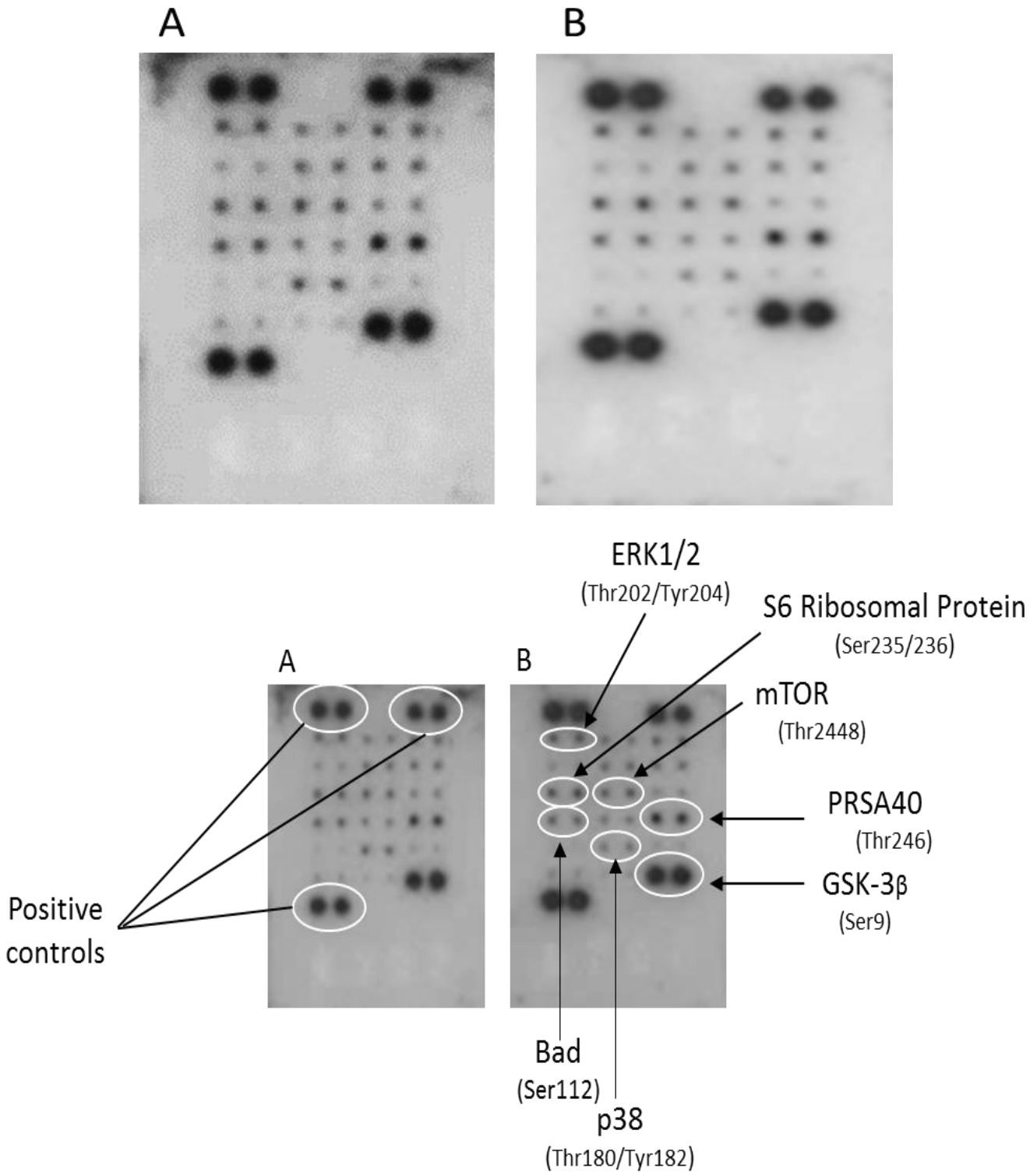


Figure 6.14. PathScan® Intracellular Signaling Membrane Arrays of C2C12 myotubes before (A) and after (B) exposure to leucine. C2C12 myotubes were exposed to 0 mM or 5mM L-leucine for 30 min. (*n* = 1). Abbreviations: ERK = extracellular signal regulated kinase; mTOR = mammalian target of rapamycin; GSK-3β = glycogen synthase kinase 3β; PRSA40 = proline-rich Akt-substrate 40; Ser = serine; Thr = threonine; Tyr = tyrosine.

6.4.11. The phosphorylation of GSK-3β following exposure of C2C12 myotubes to leucine and/or glutamine

The phosphorylation of GSK-3β was determined in C2C12 myotubes, following exposure to glutamine, different concentrations of leucine or different concentrations of leucine combined with glutamine. Results indicated that the phosphorylation of GSK-3β was directly proportional to leucine concentrations at 10 min, although only the exposure to 10 mM leucine induced a significant increase in the phosphorylation of GSK-3β ($P < 0.05$), compared with positive and negative controls (Figures 6.15 A and 6.16). At 30 min, exposure of C2C12 myotubes to all leucine concentrations induced a significant increase in the phosphorylation of GSK-3β above the controls (Figures 6.15 B and 6.16). However, the phosphorylation of GSK-3β returned to below control levels at 60 min (Figure 6.15 C and 6.16). Results also indicated that combining leucine with glutamine did not result in significant differences between leucine and leucine + glutamine groups at 10 or 30 min (Figures 6.15 A&B and 6.16). Nonetheless, a significant increase in the phosphorylation of GSK-3β, compared to leucine, took place in leucine + glutamine groups at 60 min (Figures 6.15 C and 6.16).

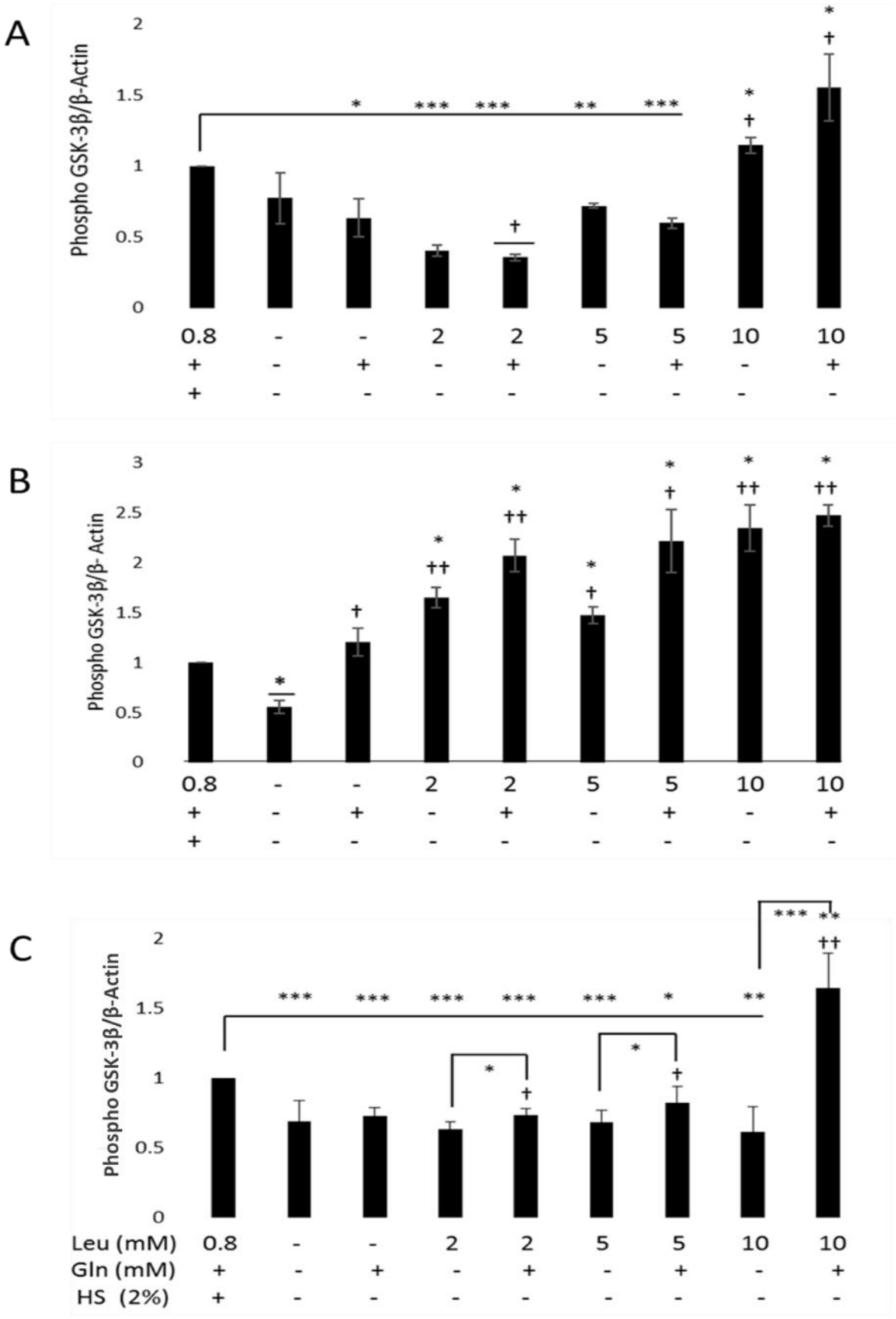

A
Phospho GSK-3β/β-Actin
0
0.5
1
1.5
2
0.8 - - 2 2 5 5 10 10
+ - + - + - + - +
+ - - - - - - - -
B
Phospho GSK-3β/β- Actin
0
0.5
1
1.5
2
2.5
3
0.8 - - 2 2 5 5 10 10
+ - + - + - + - +
+ - - - - - - - -
C
Phospho GSK-3β/β-Actin
0
0.5
1
1.5
2
Leu (mM) 0.8 - - 2 2 5 5 10 10
Gln (mM) + - + - + - + - +
HS (2%) + - - - - - - - -

Figure 6.15. The phosphorylation of GSK-3β following exposure of C2C12 myotubes to leucine and/or glutamine. C2C12 myotubes were maintained in DM or exposed to leucine, glutamine or leucine combined with glutamine, added to StM, for 10- (A), 30- (B) and 60- (C) min. Data are presented as means ± SD of three biological replica (n = 3). Asterisks indicate significant increase above the positive control (first column to the left) and indicate significant differences between leucine and leucine + glutamine groups $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$. Crosses indicate significant increase above the negative control (second column to the left) $^{+}P < 0.05$ and $^{++}P < 0.01$. Asterisks with bars indicate significant decrease compared with the positive control $\overline{^{*}}P < 0.05$, $\overline{^{**}}P < 0.01$ and $\overline{^{***}}P < 0.001$. Crosses with bars indicate significant decrease compared with the negative control $^{\pm}P < 0.05$. Analysed by one-way ANOVA using Microsoft Excel followed by Tukey's HSD *post hoc* test using SPSS Statistics 20. Abbreviations: Phospho = phosphorylated; GSK-3β = glycogen synthase kinase 3β; Leu = L-leucine; Gln = L-glutamine; mM = millimolar; HS = horse serum.

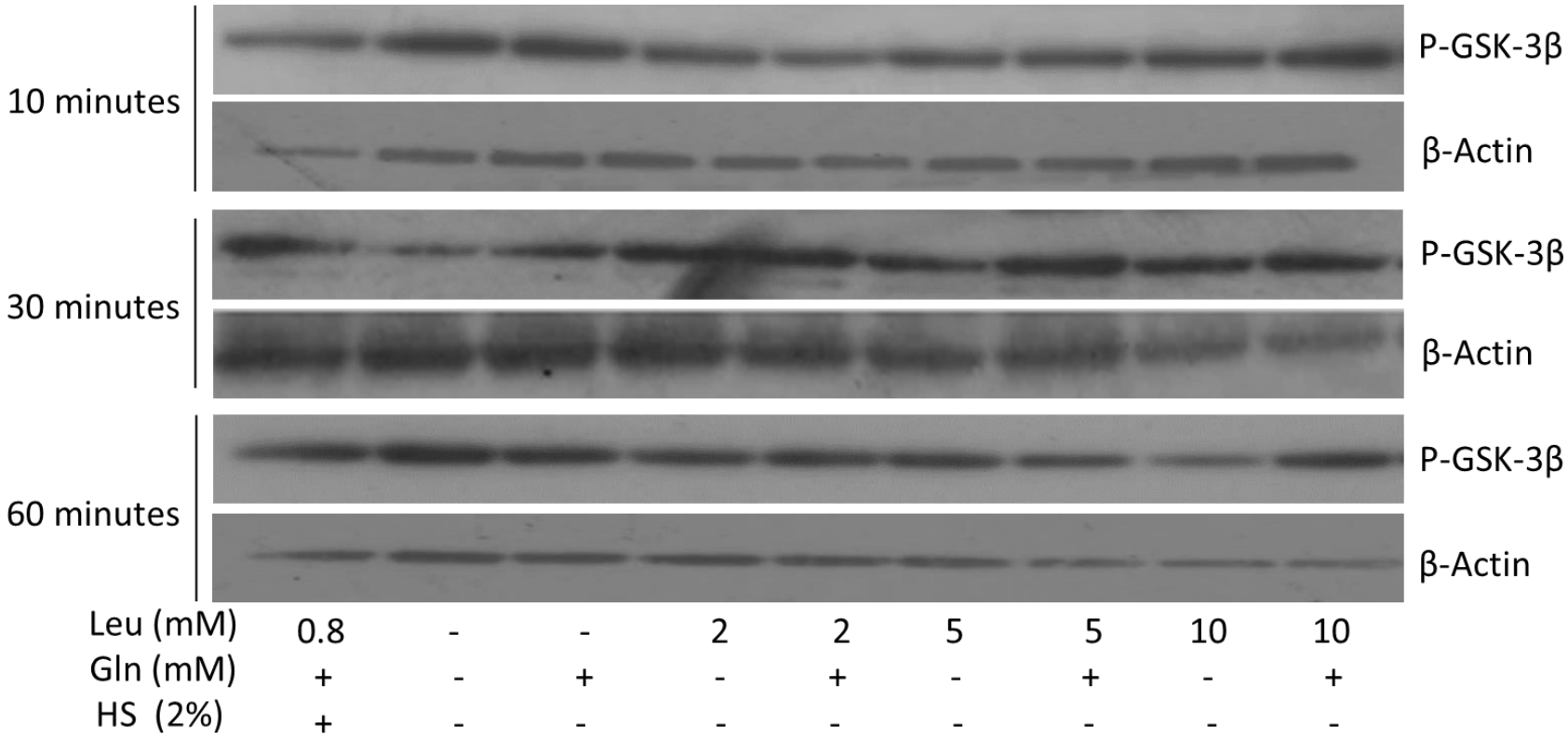


Figure 6.16. Representative western blots showing the phosphorylation of GSK-3β following exposure of C2C12 myotubes to leucine and/or glutamine. C2C12 myotubes were maintained in DM or exposed to leucine, glutamine or leucine combined with glutamine, added to StM, for 10-, 30- and 60 min. Representative blots from one experiment are shown (*n* = 4). Abbreviations: P = phosphorylated; GSK-3β = glycogen synthase kinase 3β; Leu = L-leucine; Gln = L-glutamine; mM = millimolar; HS = horse serum.

6.4.12. Protein accumulation in C2C12 myoblasts following exposure to leucine and/or glutamine

The total protein content of C2C12 myoblasts was determined following exposure to glutamine, different concentrations of leucine or different concentrations of leucine combined with glutamine. Results revealed that exposing C2C12 myoblasts to all concentrations of leucine resulted in a significant decrease in their total protein contents, compared with the positive control, at all tested time points (Figure 6.17). No significant differences in protein accumulation were noticed among different leucine concentrations at 10 min (Figure 6.17 A). However, a significant decrease in total protein was recorded after exposure to a high concentration (10 mM) of leucine, compared to lower concentrations (2 mM and 5 mM) of leucine for 30- ($P < 0.001$) and 60 min ($P < 0.05$ and < 0.01, respectively) (Figure 6.17 B and C). Finally, combining leucine with glutamine resulted in a decrease in total protein, compared with leucine alone, at 10 min (Figure 6.17A). Interestingly, when glutamine was combined with physiological concentrations of leucine (2 mM and 5 mM) no difference in total protein, compared to leucine groups, was recorded at 30 or 60 min (Figure 6.17 B and C, respectively). However, a significant increase in total protein, above leucine alone, was recorded when glutamine was combined with a high concentration (10 mM) of leucine for 30 and 60 min ($P < 0.05$) (Figure 6.17 B and C, respectively).

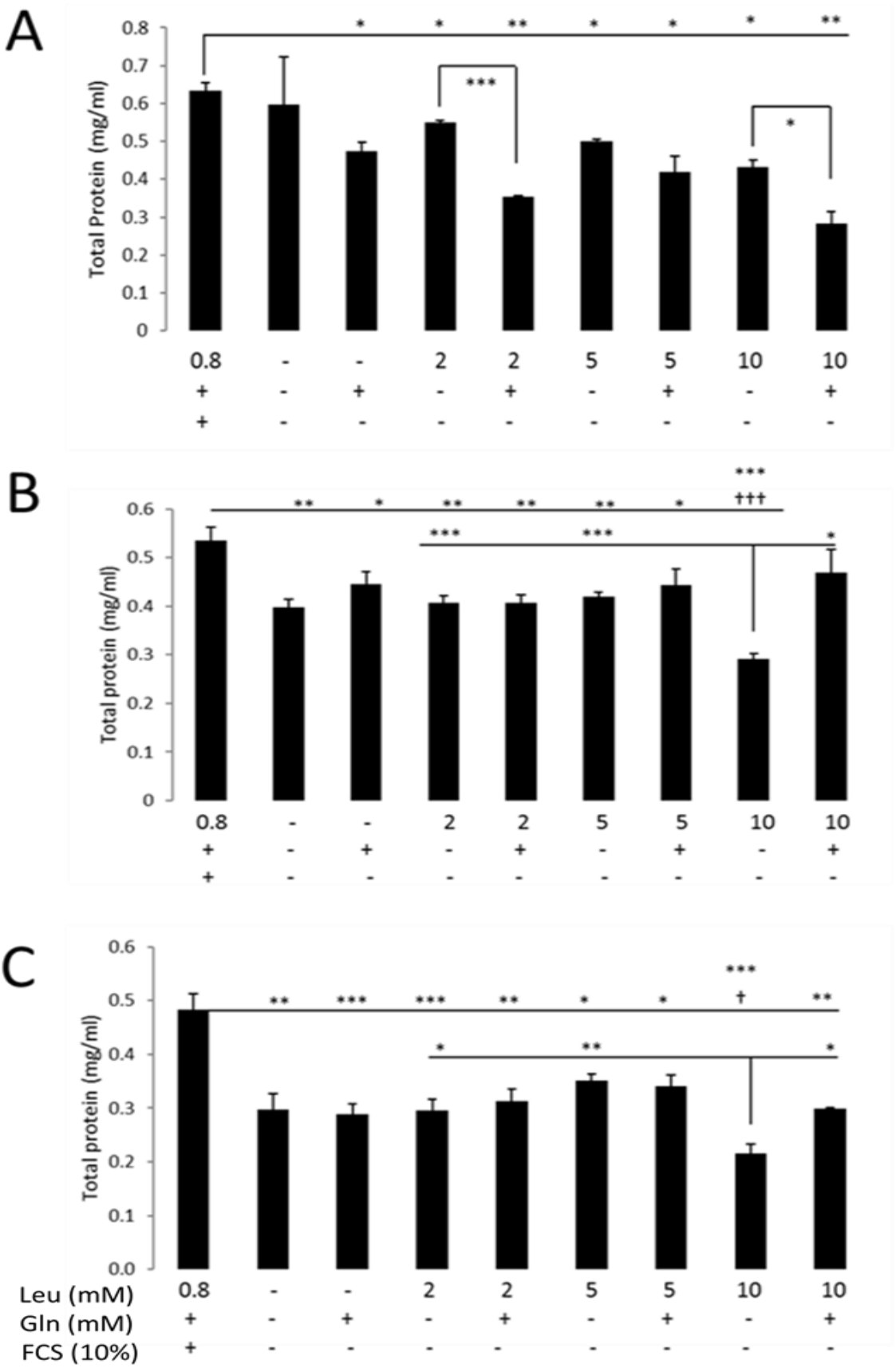

A
Total Protein (mg/ml)
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0.8 - - 2 2 5 5 10 10
+ - + - + - + - +
+ - - - - - - - -
B
Total protein (mg/ml)
0.6
0.5
0.4
0.3
0.2
0.1
0
0.8 - - 2 2 5 5 10 10
+ - + - + - + - +
+ - - - - - - - -
C
Total protein (mg/ml)
0.6
0.5
0.4
0.3
0.2
0.1
0.0
Leu (mM) 0.8 - - 2 2 5 5 10 10
Gln (mM) + - + - + - + - +
FCS (10%) + - - - - - - - -

Figure 6.17. Total protein content of C2C12 myoblasts following exposure to leucine and/or glutamine. C2C12 myoblasts were maintained in GM or exposed to leucine, glutamine or leucine combined with glutamine, added to StM, for 10- (A), 30- (B) and 60- (C) min. Data are presented as means ± SD of three biological replica ($n = 3$). Asterisks indicate significant increase above the positive control (first column on the left) and indicate significant difference between leucine and leucine + glutamine groups $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$. Asterisks with bars indicate significant decrease compared with the positive control $\overline{^{*}}P < 0.05$, $\overline{^{**}}P < 0.01$ and $\overline{^{***}}P < 0.001$. Crosses with bars indicate significant decrease compared with the negative control (second column on the left) $\overline{^{+}}P < 0.05$ and $\overline{^{+++}}P < 0.001$. Analysed by one-way ANOVA using Microsoft Excel followed by Tukey's HSD *post hoc* test using SPSS Statistics 20. Abbreviations: Leu = L-leucine; Gln = L-glutamine; FCS = foetal calf serum; mg = milligrams; ml = millilitre; mM = millimolar.

6.4.13. The phosphorylation of ERK1/2 following exposure of C2C12 myoblasts to leucine and/or glutamine

The phosphorylation of ERK1/2 was determined after exposure of C2C12 myoblasts to leucine and/or glutamine. Results indicated a significant decrease in the phosphorylation of ERK1/2, when compared with the controls, following 10 min exposure to leucine alone ($P < 0.01$ and < 0.001) (Figure 6.18 A). At 30 min, the phosphorylation of ERK1/2 in response to leucine stimulation returned to basal levels (Figure 6.18 B) and maintained this state at 60 min (Figure 6.18 C). Short-term stimulation with glutamine alone failed to increase the phosphorylation of ERK1/2 at 10 min. However, exposure to glutamine for 30 and 60 min significantly increased the phosphorylation of ERK 1/2 above the negative control ($P < 0.05$ and < 0.001, respectively). Notably, combining physiological concentrations of leucine with glutamine significantly increased the phosphorylation of ERK1/2, when compared with leucine alone, at 30 min ($P < 0.01$) (Figure 6.18 B). However, a significant increase in the phosphorylation of ERK1/2 took place after combining a supraphysiological concentration of leucine (10 mM) with glutamine for 60 min ($P < 0.05$) (Figure 6.18 C).

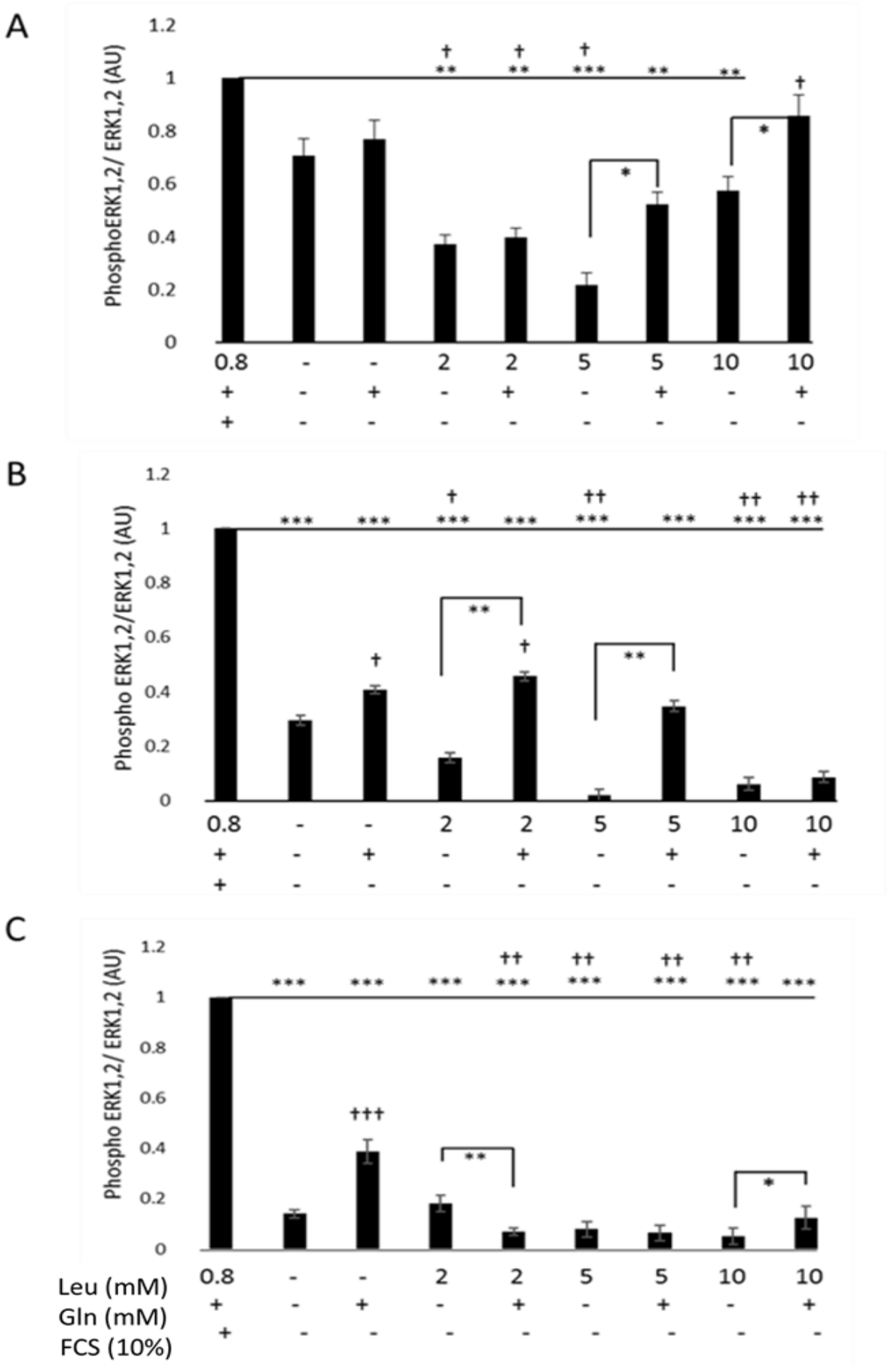

A
PhosphoERK1,2/ ERK1,2 (AU)
1.2
1
0.8
0.6
0.4
0.2
0
0.8 - - 2 2 5 5 10 10
+ - + - + - + - +
+ - - - - - - - -
B
Phospho ERK1,2/ERK1,2 (AU)
1.2
1
0.8
0.6
0.4
0.2
0
0.8 - - 2 2 5 5 10 10
+ - + - + - + - +
+ - - - - - - - -
C
Phospho ERK1,2/ ERK1,2 (AU)
1.2
1
0.8
0.6
0.4
0.2
0
0.8 - - 2 2 5 5 10 10
Leu (mM) + - + - + - + - +
Gln (mM) + - - - - - - - -
FCS (10%)

Figure 6.18. Phosphorylation of ERK1/2 following exposure of C2C12 myoblasts to leucine and/or glutamine. C2C12 myoblasts were maintained in GM or exposed to leucine, glutamine or leucine combined with glutamine, added to StM for 10- (A), 30- (B) and 60- (C) min. Data are presented as means ± SD of three biological replica (n = 3). Asterisks indicate significant increase above the positive control (first column to the left) and indicate significant difference between leucine and leucine + glutamine groups $^{*}P < 0.05$ and $^{**}P < 0.01$. Crosses indicate significant increase above the negative control (second column to the left) $^{+}P < 0.05$ and $^{+++}P < 0.001$. Asterisks with bars indicate significant decrease compared with the positive control $\overline{}^{**}P < 0.01$ and $\overline{}^{***}P < 0.001$. Crosses with bars indicate significant decrease compared with the negative control $^{\pm}P < 0.05$ and $^{\pm\pm}P < 0.01$. Analysed by one-way ANOVA using Microsoft Excel followed by Tukey's HSD *post hoc* test using SPSS Statistics 20. Abbreviations: Leu = L-leucine; Gln = L-glutamine; ERK = extracellular signal regulated kinase; FCS = foetal calf serum; mM = millimolar.

6.5. Discussion

Both leucine, a BCAA, and glutamine, a conditional EAA, have powerful protein anabolic properties. Leucine is well known for enhancing the rate of MPS (Anthony *et al*., 2001, Anthony *et al*., 2002); whereas glutamine is known to decrease the rate of MPB (Holecek *et al*., 2000). In this study, protein accumulation and the phosphorylation of selected proteins (p70 S6K, 4E-BP1, ERK1/2 and GSK-3β) were examined following stimulation of C2C12 cells with glutamine, increasing concentrations of leucine, and co-stimulation with leucine and glutamine, at different time points. Additionally, the impact of blocking the ERK1/2 and PI3K/Akt signalling pathways on protein accumulation and on the phosphorylation of mTOR effectors p70 S6K and 4E-BP1, in C2C12 cells following exposure to leucine and/or glutamine was also investigated.

Results from chapter 4 emphasised the need to examine the anabolic effects of leucine in the presence of serum, in order to preserve cell viability and prevent against protein loss. Therefore, the phosphorylation of the aforementioned proteins, after exposure of proliferating and differentiated C2C12 cells to leucine and/or glutamine, was first examined in GM and DM respectively. However, it was found that a spontaneous phosphorylation of ERK1/2 may occur secondary to introducing serum to starved cells, which may impede the effects of supplemented AAs (Marchetti *et al*., 2005). Consequently, a shift was made to test the phosphorylation of these proteins in StM. A second adjustment was to introduce a positive control (full DM and full GM for differentiated and proliferating C2C12 cells, respectively) in addition to the already existing negative control.

6.5.1. Total protein content of C2C12 cells following exposure to leucine

Results showed that both short- and long-term exposure to all leucine concentrations not only failed to increase, but significantly decreased the total protein content of C2C12 myotubes compared with the positive control. This inability of leucine to induce protein accumulation, above the levels observed in the positive control, may be related to protein degradation in response to starvation prior to and during leucine treatment. It could also be attributed to the scarcity of nutrients in StM, which did not support the protein anabolic effects of leucine. Nonetheless, long-term stimulation with leucine + glutamine resulted in a significant increase in total protein above the positive and negative controls. This finding suggests that glutamine

is essential for achieving the protein anabolic effects of leucine. However, since this effect was only noticed following long-term exposure, therefore glutamine may be essential for preventing protein loss.

Another notable finding was the positive correlation between total protein and leucine concentrations following short-term exposure, versus the inverse correlation between total protein and leucine concentrations that occurred after long-term exposure of C2C12 myotubes to leucine. An increase in total protein following short-term exposure to a high leucine concentration was anticipated and agrees with our first hypothesis. However, the inability of this leucine concentration to sustain this increase in total protein may have resulted from the depletion of the AA pool within the cells, a conclusion that agrees with the finding of a previous study (Wilson *et al*., 2010). On the other hand, exposure to lower leucine concentrations did not deplete the AA reserve during early stages; a putative reason why higher protein contents were recorded after long-term exposure of C2C12 myotubes to these concentrations.

6.5.2. Activation of mTOR signalling following exposure of C2C12 myotubes to leucine

The protein-enhancing properties of leucine have been attributed to the activation of the mTOR signalling pathway (Lynch *et al*., 2003; Tokunaga *et al*., 2004). Especially the phosphorylation and activation of its immediate target p70 S6 and the phosphorylation and deactivation of its second downstream target 4E-BP1. Together, p70 S6K and 4E-BP1 trigger translation initiation for protein synthesis (Lynch *et al*., 2003; Tokunaga *et al*., 2004; Hara *et al*., 1998). Therefore, it was essential to examine the phosphorylation of p70 S6K and 4E-BP1, following exposure of differentiated C2C12 myotubes to leucine and/or glutamine.

6.5.2.1. The phosphorylation of p70 S6K following exposure of C2C12 myotubes to leucine

AAs are thought to initiate protein synthesis via the phosphorylation of the p70 S6K (Brown *et al*., 1995). The phosphorylation of p70 S6K is an indication of mTOR phosphorylation (Farrari and Thomas, 1994) and is often used as a proxy measure for MPS (Drummond *et al*., 2009). In the present study, short-term exposure of C2C12 myotubes to leucine induced a significant increase in the phosphorylation of p70 S6K in a dose-dependent manner. However, further exposure to leucine resulted in an inverse correlation between leucine concentrations and the

phosphorylation of p70 S6K. Notably, all leucine concentrations failed to sustain the phosphorylation of p70 S6K in C2C12 myotubes, which fell below control levels following long-term exposure. These results suggest a concentration- and time-dependent effect of leucine on p70 S6K phosphorylation. A direct correlation between leucine concentrations and the phosphorylation of p70 S6K following short-term stimulation was previously reported in C2C12 myotubes (Areta *et al*., 2014) and agrees with our first hypothesis. However, to the best of our knowledge, an inverse correlation between leucine concentrations and p70 S6K phosphorylation following long-term stimulation has not previously been recorded. As with the results of protein accumulation, it is possible that stimulating C2C12 myotubes with a high concentration of leucine over-stimulated the phosphorylation of p70 S6K, which returned to basal levels following long-term exposure. On the other hand, stimulation with lower leucine concentrations induced, and sustained, a relatively modest phosphorylation of p70 S6K for a longer period.

6.5.2.2. The phosphorylation of p70 S6K following exposure of C2C12 myotubes to leucine plus glutamine

Many studies have suggested that glutamine exerts its protein anabolic effect via the phosphorylation of mTORC1 signalling – although the activation of mTOR in response to glutamine supplementation has been shown to be cell-specific. Glutamine was required for the activation of mTORC1 in hepatocytes, HEK293T and HeLa cells, but less by mouse embryonic fibroblasts, and inhibited the phosphorylation of mTOR in muscle cells (Krause *et al*., 2002; Deldicque *et al*., 2008; Carroll *et al*., 2016). In the present study, results following short-term stimulation indicated that glutamine did not exert any added effect on the phosphorylation of p70 S6K when it was combined with leucine. Interestingly, following medium-term stimulation, glutamine exerted two different effects. First, it caused a significant decrease in the phosphorylation of p70 S6K when combined with physiological concentrations of leucine. Second, it caused a significant increase in the phosphorylation of p70 S6K when combined with a supraphysiological concentration of leucine. Results following long-term exposure indicated that, when glutamine was combined with leucine, it sustained the phosphorylation of p70 S6K and resulted in a significant increase above the leucine-treated groups.

The published literature suggests that glutamine plays a role in initiating protein synthesis in many cell types, but not in C2C12 myotubes (Deldicque *et al*., 2008). Therefore, the significant

increase and decrease in the phosphorylation of p70 S6K, compared with leucine alone, following stimulation of C2C12 myotubes with leucine and glutamine combined, is most likely due to the ability of glutamine to sustain the leucine-induced phosphorylation of p70 S6K, rather than its ability to increase, or decrease it. In other words, a significant increase in the phosphorylation of p70 S6K in leucine plus glutamine groups may be the result of a significant decrease in the phosphorylation of p70 S6K in the corresponding leucine groups, and vice versa. Another plausible explanation is that long-term stimulation of differentiated C2C12 cells with leucine and glutamine provided an adequate signal to stimulate mTOR signalling (Gleason *et al*., 2007; Xu *et al*., 2001), as opposed to leucine alone. Finally, the fact that glutamine is essential for delivering leucine into cells (Nicklin *et al*., 2009) suggests that shortage of glutamine, in leucine groups, might have hampered the transport of leucine into cells. This may have resulted in weak phosphorylation of p70 S6K following stimulation of C2C12 myotubes with leucine alone.

6.5.2.3. The phosphorylation of 4E-BP1 following exposure of C2C12 myotubes to leucine

The 4E-BP1 is another downstream effector of mTOR. 4E-BP1 plays an important role in protein synthesis, the phosphorylation and deactivation of 4E-BP1 allow the binding of eIF4E to eIF4G and the formation of active eIF4F complex. The latter is essential for the initiation of protein synthesis (Coldwell *et al*., 2013). On the other hand, unphosphorylated 4E-BP1 confines the availability of free eIF4E and prevents a cap-dependent initiation of translation for protein synthesis (Beretta *et al*., 1996). Previous studies suggest that 4E-BP1 becomes phosphorylated in response to leucine administration (Hara *et al*., 1998; Norton and Layman., 2006; Deldicque *et al*., 2008; Areta *et al*., 2014). It has also been shown that leucine-induced protein synthesis is achieved, in part, by the phosphorylation of 4E-BP1 (Fox *et al*., 1998).

In the present study, as with p70 S6K, short-term stimulation of C2C12 myotubes with leucine induced a dose-dependent increase in the phosphorylation of 4E-BP1. However, medium- and long-term exposure to leucine resulted in an inverse correlation between the phosphorylation of 4E-BP1 and leucine concentrations. Although, these findings agree with our first hypothesis, there is no evidence in the literature to support the finding that short-term exposure to leucine induces a concentration-dependent phosphorylation of 4E-BP1. Instead, a plateau in the phosphorylation of 4E-BP1 has been reported following acute stimulation of C2C12 myotubes with different concentrations of leucine (Areta *et al*., 2014). The conflict between the present

results and those reported by Areta and colleagues (2014) can presumably be attributed to different experimental conditions. In that study, differentiated C2C12 cells were incubated with leucine in saline, which lacks glucose and EAAs. These nutrient-limited conditions may have affected the response of C2C12 cells to increasing concentrations of leucine. Our results following medium- and long-term exposure suggest AA depletion occurs with higher concentrations of leucine.

In general, the phosphorylation of 4E-BP1 after exposure to leucine followed the same pattern as p70 S6K. However, in contrast to p70 S6K, the ability of physiological leucine concentrations to induce a significant increase in 4E-BP1 phosphorylation was evident following 30 min. In p70 S6K, a significant increase in phosphorylation was recorded following 10 min exposure to the same leucine concentrations. A further difference was the transient nature of 4E-BP1 phosphorylation, which fell to a low level following medium-term exposure – in contrast to the relatively sustainable phosphorylation of p70 S6K.

6.5.2.4. The phosphorylation of 4E-BP1 following exposure of C2C12 myotubes to leucine plus glutamine

As with p70 S6K, short-term stimulation with leucine and glutamine combined, failed to increase the phosphorylation of 4E-BP1, when compared to the leucine groups. However, following medium-term stimulation, glutamine exerted two contrasting effects when combined with leucine; it significantly decreased phosphorylation of 4E-BP1, when combined with low concentrations, while it increased phosphorylation of 4E-BP1 when combined with a high concentration of leucine. The reason behind these differential effects may be due to the availability of protein substrates after medium- and long-term stimulation of C2C12 myotubes with low concentrations of leucine. This contrasts with an early depletion following stimulation with a high concentration of leucine. On the contrary to p70 S6K, glutamine was not essential to sustain the phosphorylation of 4E-BP1 in C2C12 myotubes following long-term exposure to leucine.

6.5.3. Activation of MAPK signalling following exposure of C2C12 myotubes to leucine

There is growing evidence that leucine's protein anabolic properties are not merely dependent on mTOR activation. For example, blocking mTOR signalling did not completely block leucine-mediated protein synthesis in the skeletal muscles of rats (Anthony *et al*., 2000). Also, a notable increase in MPS was evident in diabetic rats following leucine administration, without an increase in the phosphorylation of p70 S6K and 4E-BP1 (Anthony *et al*., 2002). Additionally, an increase in the phosphorylation of 4E-BP1 and p70 S6K that occurred following leucine treatment was not associated with increased rates of MPS (Deldicque *et al*., 2008; Haegens *et al*., 2012; Areta *et al*., 2014). Taken together, these studies suggest that leucine might exert its protein anabolic properties, in part, via signalling pathway (s) other than mTOR.

MAPK is an anabolic signalling pathway, which controls many aspects of cell function (Crew *et al*., 1992; Cargnello and Roux., 2011). A crosstalk between mTOR and MAPK signalling has been suggested in the literature, and it has recently been reported that ERK1/2 contributes to the activation of mTOR (Carriere *et al*., 2011). Therefore, the phosphorylation of ERK1/2, the most recognised member of MAPK (Cargnello and Roux., 2011), was determined in C2C12 myotubes after short-, medium- and long-term exposure to leucine and/or glutamine. Results from the present study indicated that exposure to leucine modulated the phosphorylation of ERK1/2 in C2C12 myotubes. The published literature does not seem to point to a direct effect of leucine on the phosphorylation of ERK in muscle cells. Instead, leucine caused an increase in the magnitude of ERK phosphorylation in response to insulin treatment (Camillo *et al*., 2014).

Our results also showed that ERK1/2 phosphorylation was inversely proportional to leucine concentrations at all time points. This result indicates that ERK1/2 was less sensitive to the depletion of the AA pool after long-term exposure to a high leucine concentration. This finding contradicts the observations of p70 S6K and 4E-BP1 phosphorylation and indicates a different effect of leucine on mTOR and MAPK signalling pathways.

To further investigate the role played by ERK1/2 in mediating leucine's activation of mTOR and protein accumulation, a specific MEK inhibitor (U0126) was used to block the activity of ERK1/2 in C2C12 myotubes. Results revealed that blocking ERK1/2 reduced differences in total protein content between control, leucine and leucine + glutamine groups. Blocking ERK

signalling also attenuated the magnitude of p70 S6K phosphorylation following short-term exposure to leucine and/or glutamine. These results agreed with our second hypothesis and agrees with the findings of a previous study, which reported that ERK1/2 directly phosphorylates Raptor, which in turn activates mTORC1 for protein synthesis (Carriere *et al*., 2011). Our results suggest an essential role for ERK1/2 in mediating the activation of mTOR signalling in response to leucine. Our results also suggest that the protein anabolic effects of leucine are regulated by both the mTOR and MAPK signalling pathways.

6.5.4. The PI3K/Akt signalling pathway

It has previously been shown that leucine treatment increased the phosphorylation of PI3K/Akt in the skeletal muscles of rats and mice (Saha *et al*., 2010; O'Neill *et al*., 2010). In the present study, a putative role for PI3K in mediating the phosphorylation of mTOR substrates p70 S6K and 4E-BP1 in C2C12 myotubes, following short-term exposure to leucine and/or glutamine, was investigated by blocking PI3K/Akt signalling. Results showed that blocking the PI3K signalling pathway inhibited the phosphorylation of p70 S6K in C2C12 myotubes after exposure to leucine alone, or leucine in combination with glutamine. This finding suggests that PI3K is located upstream of p70 S6K in the signalling pathway, which agrees with our third hypothesis and concurs with numerous reports of the ability of PI3K inhibitors to suppress the phosphorylation of p70 S6K in pancreatic beta-cells, rat skeletal muscles, 293 cells and hepatoma cells following exposure to AAs (Xu *et al*., 1998; Patti *et al*., 1998; Dardevet *et al*., 2000, Weng *et al*., 1995). Notably, blocking PI3K signalling attenuated, but did not inhibit, the phosphorylation of 4E-BP1 in C2C12 myotubes following exposure to leucine and/or glutamine. This result suggests that p70 S6K and 4E-BP1 are regulated differently.

6.5.5. The PathScan® Intracellular Signaling Arrays

We examined the phosphorylation and cleavage of 18 different intracellular signalling factors in myotubes after short-term exposure to leucine and leucine + glutamine in addition Remarkably, results showed no difference in phosphorylation intensity between control, leucine and leucine + glutamine arrays. The lack of differences between control and treated groups could be due to the fact that these arrays are semi-quantitative. Nevertheless, the most significant finding from using these arrays was the prominent phosphorylation of GSK-3β in

all groups. For this reason, the phosphorylation of GSK-3β was investigated in C2C12 myotubes following exposure to leucine in the presence and absence of glutamine.

6.5.6. The phosphorylation of GSK-3β following exposure to leucine and/or glutamine

GSK-3β is a serine-threonine kinase, which controls many cell functions. Normally, GSK3 is in a constant unphosphorylated/active state. Upon stimulation with insulin or leucine, the phosphorylation of PI3K and the subsequent phosphorylation of Akt occur which, in turn, lead to the phosphorylation and deactivation of GSK3 (Saltiel and Kahn, 2001; Lizcano and Alessi, 2002). The deactivation of GSK prevents its inhibitory effect on downstream substrates and permits the activation of glycogen synthas and eIF2B, to promote glycogen deposition and protein synthesis, respectively (Parker *et al*., 1983; Welsh and Proud, 1993; Doble and Woodgett, 2003). Interestingly, the pattern of GSK-3β phosphorylation matched the total protein content of C2C12 myotubes after exposure to leucine and/or glutamine. This suggests an important role for GSK-3β in mediating protein accumulation in C2C12 cells following exposure to leucine.

Our results also showed that leucine mediated a dose-dependent phosphorylation of GSK-3β. An increase in the phosphorylation of GSK-3β following leucine treatment is consistent with a previous study, which reported enhanced phosphorylation of GSK-3β following incubation of L6 muscle cells with leucine (Peyrollier *et al*., 2000). Notably, in the present study, glutamine when combined with leucine, increased GSK-3β phosphorylation above the leucine groups. Moreover, glutamine sustained the phosphorylation of GSK-3β in C2C12 myotubes, which reached low levels after medium-term exposure to leucine. This finding supports an important role for glutamine in sustaining the phosphorylation of GSK-3β following exposure to leucine. This result has not been previously reported and merits further investigation.

6.5.7. Total protein contents of C2C12 myoblasts following exposure to leucine

As previously mentioned, the presence of serum in culture medium may change the phosphorylation of ERK signalling. Therefore, the phosphorylation of ERK1/2 in C2C12

myoblasts were examined following exposure to leucine and/or glutamine in the absence of serum. Given the adverse impact of lack of serum on cell viability, it was unfeasible to examine the long-term effect of leucine and/or glutamine on the phosphorylation events in C2C12 myoblasts. Instead, the impact of short- and medium-term exposure to leucine and/or glutamine, was examined.

Our results indicated that exposure to all tested concentrations of leucine decreased the total protein content of C2C12 myoblasts, when compared with the positive control. In addition, all leucine concentrations failed to increase the total protein content of C2C12 myoblasts, compared to the negative control. These results are consistent with the findings obtained in chapter 4, wherein leucine was only able to increase protein content in the presence of serum. Interestingly, the greatest reduction in total protein was observed in cells incubated with a high concentration of leucine. The significant decrease in total protein following prolonged exposure to a high concentration of leucine was also recorded in chapter 5 and is likely to be a consequence of the depletion of the AA pool. Notably, combining glutamine with a high concentration of leucine increased the total protein content of C2C12 cells above leucine alone. The significant differences in protein content between leucine and leucine + glutamine groups suggest an anti-proteolytic effect of glutamine. This finding supports a previous study demonstrating glutamine's ability to decrease the rate of MBP (Holecek *et al*., 2000).

6.5.8. The phosphorylation of MAPK signalling following exposure of C2C12 myoblasts to leucine

Short-term exposure of C2C12 myoblasts to leucine induced a transient and weak phosphorylation of ERK1/2. However, the phosphorylation of ERK1/2 in response to leucine reached very low levels following further exposure. Interestingly, this decrease in ERK1/2 phosphorylation was prevented when leucine was combined with glutamine. The early decrease in ERK1/2 phosphorylation could be due to the activation of the "son the sevenless" gene (*sos*) in C2C12 myoblasts (Orton *et al*., 2009). The activation of the *sos* gene prevents uncontrolled cell proliferation. The ability of glutamine to rescue leucine-mediated phosphorylation of ERK1/2, is consistent with its ability to induce the phosphorylation of ERK1/2 (Zhu *et al*., 2015; Yuan *et al*., 2015).

6.6. Conclusion

Results in the present chapter demonstrated that exposure to leucine modulated the phosphorylation of p70 S6K, 4E-BP1, GSK-3β and ERK1/2 in differentiated C2C12 cells. Results also revealed that glutamine combined with leucine mitigated the reduction of p70 S6K and ERK1/2 phosphorylation, which occurred following long-term stimulation of C2C12 myotubes with leucine. Additionally, blocking the ERK1/2 signalling pathway reduced the phosphorylation of p70 S6K and 4E-BP1 in C212 myotubes, following stimulation with leucine and/or glutamine. However, blocking PI3K/Akt signalling prevented the phosphorylation of p70 S6K in C2C12 myotubes after stimulation with leucine and/or glutamine. Finally, leucine induced a weak and transient activation of ERK1/2 in C2C12 myoblasts, which reached low levels after medium-term exposure. However, when glutamine was combined with leucine, the former rescued the phosphorylation of ERK1/2 in C2C12 myoblasts.

7. Assessing the temporal expression of muscle-specific microRNAs in C2C12 myotubes after exposure to leucine alone or combined with glutamine

7.1. Introduction

miRNAs are short non-coding RNAs that play an important role in regulating gene expression (He and Hannon, 2004). They are estimated to control ~ 60% of the mammalian genome through miRNA-mRNA interactions, which silence gene expression at the post-transcriptional level (Friedman *et al.*, 2009). Muscle specific miRNAs (myomiRs) are a group of miRNAs enriched in the skeletal and cardiac muscles and include miR-1, miR-133a, miR-133b, miR-206, miR-208a, miR-208b and miR-499 (McCarthy *et al*., 2008). Increasing evidence indicates that myomiRs play a central role in muscle development by influencing the expression of muscle specific genes (Chen *et al*., 2006). MyomiRs also mediate the response of muscle cells to various atrophic and hypertrophic stimuli (Eisenberg *et al*., 2009; Horak *et al*., 2016).

Leucine has a well-recognised protein enhancing properties (Kimball and Jefferson, 2001; Dardevet *et al*., 2000; Anthony *et al.*, 2001; Lynch *et al.*, 2003). However, knowledge regarding the role of miRNAs in mediating the protein-enhancing effects of leucine on muscle cells is limited. One study reported alterations in the expression of a few miRNAs (miR-1, miR-499, miR-23a and miR-208b) within the skeletal muscles of humans after ingesting a mixture of EAAs (Drummond *et al*., 2009). However, the role of leucine in that study could not be specifically identified because it could have been confounded by the effects of other co-administered AAs. Another study reported an upregulation of miR-27a concomitant with a downregulation of *myostatin* following a short-term incubation of C2C12 myoblasts with leucine (Chen *et al*., 2013). These two studies suggest a possible role of miRNAs in regulating the response of muscle cells to AAs supplementations, including leucine. Therefore, the present study examined the temporal expression of miR-1 a, miR-133a, miR-133b, miR-206 and miR-499, following the deprivation and the exposure of C2C12 myotubes to leucine and/or glutamine.

7.2. Research hypotheses

We hypothesised that exposing differentiated C2C12 myotubes to leucine alone, or combined with glutamine, alters the expression of myomiRs. This hypothesis is based on the fact that myomiRs play an important role in regulating different aspects of muscle biology (Chen *et al*., 2006), and that ingestion of EAAs alters the expression levels of myomiRs in humans (Drummond *et al*., 2009).

7.3. The aims of this chapter

1. To assess the expression of mature miR-1, miR-133a, miR-133b, miR-206 and miR-499 in C2C12 myotubes following short-, medium- and long-term leucine deprivation.
2. To assess the expression of mature miR-1, miR-133a, miR-133b, miR-206 and miR-499 in C2C12 myotubes following short-, medium- and long-term incubation with leucine.
3. To assess the expression of mature miR-1, miR-133a, miR-133b, miR-206 and miR-499 in C2C12 myotubes following short-, medium- and long-term incubation with leucine and glutamine, combined.

7.4. Results

7.4.1. Assessing the quality of isolated RNA

The quality of total RNA, including miRNAs, extracted from C2C12 myotubes was assessed using the Agilent 2100 Bioanalyzer system. Results of the gel indicated high integrity of isolated miRNAs as shown by the fully intact two bands, representing the 28S and 18S subunits of ribosomal RNA (rRNA), without any sign of degradation (Figure 7.1). Electrograms also showed two clearly defined peaks for 28S and 18S rRNA in which the 28S peaks were higher than the 18S (Figure 7.2). The RIN of all samples was ≥ 9.9 (Figure 7.2).

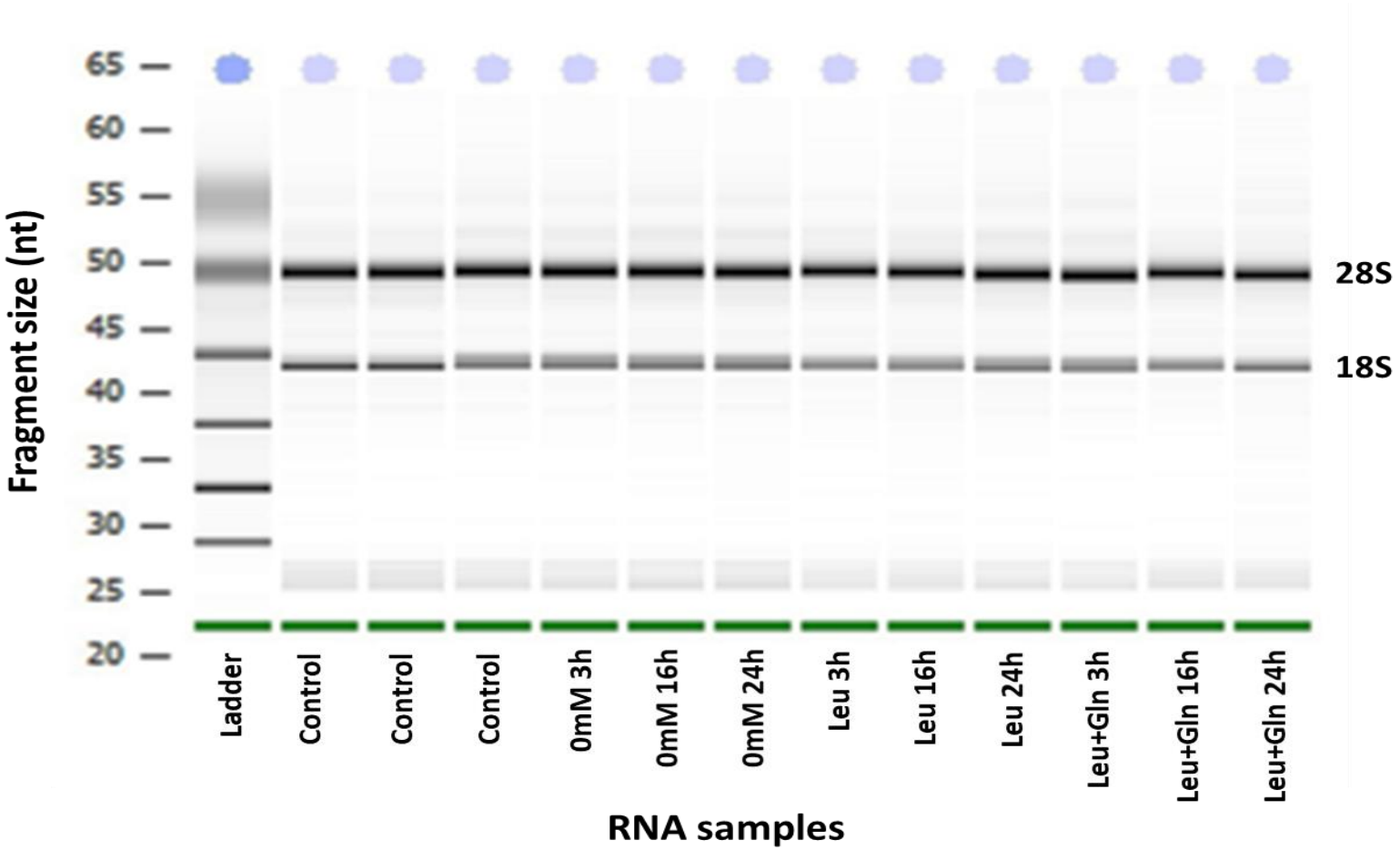


Figure 7.1. Gel image showing the quality of isolated RNA; obtained using an Agilent RNA 6000 Nano Kit and Agilent 2100 Bioanalyzer system. RNA was extracted from C2C12 myotubes prior to (control) and following 3-, 16- and 24 h of exposure to leucine alone (Leu) and leucine combined with glutamine (Leu + Gln)**.** An Agilent RNA 6000 Nano Ladder is used. The 28S rRNA band is visible at 50 nt and the 18S rRNA band is visible at 44 nt. Abbreviations: nt = nucleotides; mM = millimolar; Leu = L-leucine; Gln = L-glutamine; h = hour.

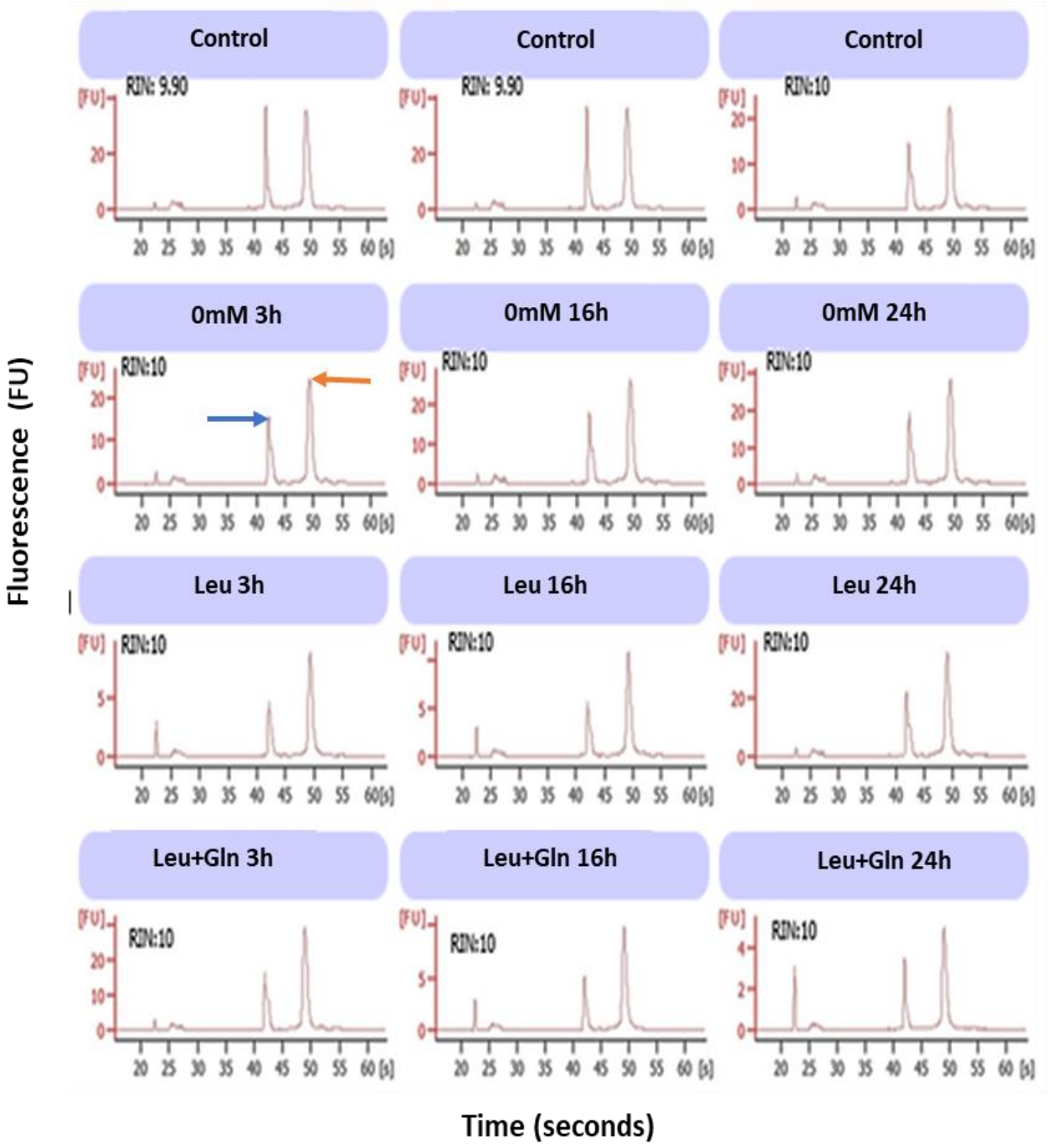


Figure 7.2. Electropherograms showing the quality of isolated RNA; obtained using an Agilent RNA 6000 Nano Kit and Agilent 2100 Bioanalyzer system. RNA was extracted from C2C12 myotubes prior to (control) and following 3-, 16- and 24 h of exposure to leucine alone (Leu) and leucine combined with glutamine (Leu + Gln). Electropherograms result from the application of fluorescence (fluorescent units) over time (in seconds). The blue arrow points to the peak of the 18S, while the orange arrow points to the 28S peak. Abbreviations: FU = fluorescent units; S = seconds; RIN = RNA integrity number; mM = millimolar; Leu = L-leucine; Gln = L-glutamine; h = hour.

7.4.2. Temporal expression of miR-1

The expression of miR-1 was examined in C2C12 myotubes. Results indicated that leucine deprivation, exposure to leucine or exposure to leucine combined with glutamine did not induce a significant upregulation in the expression of miR-1 at any of the tested time points (Figure 7.3). However, the expression of miR-1 increased, compared to other groups, following short- and long-term stimulation with leucine (Figure 7.3 A and C, respectively). Also, the expression of miR-1, compared to other groups, in response to short- and long-term leucine deprivation (Figure 7.3 A and C, respectively). On the other hand, a significant decrease in miR-1 expression, from the control, was observed after short-term exposure to leucine and glutamine combined ($P < 0.05$) (Figure 7.3 A). In general, there was a time-dependent increase in the expression of miR-1. Therefore, the plotting of miRNA expression data distribution using Q-Q plot was implemented. A good agreement in the Q-Q plot was observed for miR-1 expression as indicated by a straight line of slope less than 1 (Figure 7.4).

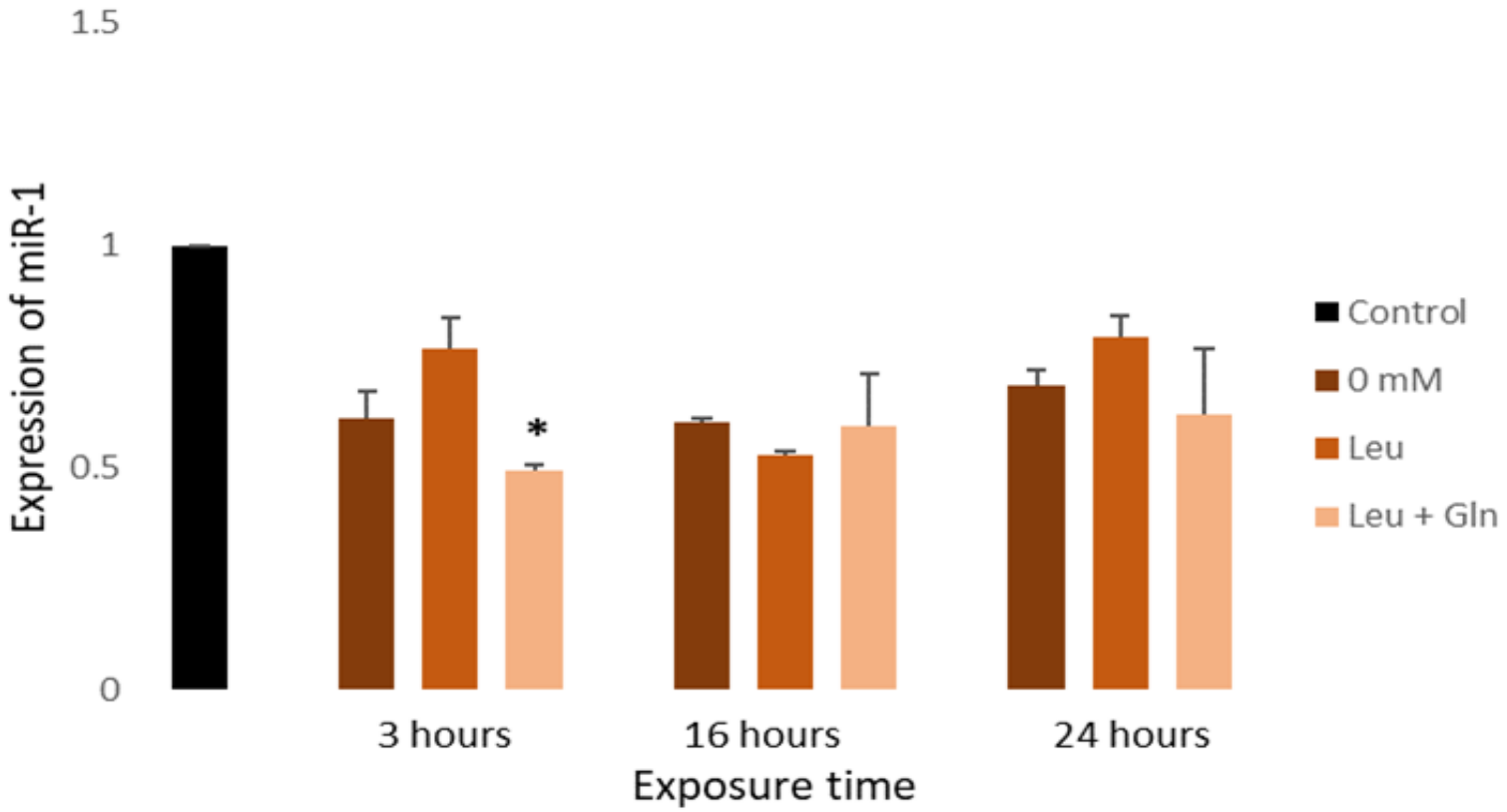


Figure 7.3. Expression of miR-1 in C2C12 myotubes; examined using a a quantitative real-time polymerase chain reaction. C2C12 myotubes were deprived of leucine (0 mM), supplemented with 5 mM leucine (Leu) or supplemented with 5 mM leucine plus 2 mM glutamine (Leu + Gln) for 3-, 16- and 24 h. Data are presented as means ± SD of normalised fold changes (n = 3-4). Asterisk indicates significant decrease compared to the baseline/control $^{*}P < 0.05$. Analysed by one-way ANOVA using Microsoft Excel followed by Tukey's HSD *post hoc* test using SPSS Statistics 20. Abbreviations: Leu = L-leucine; Gln = L-glutamine; mM = millimolar; miR = microRNA gene.

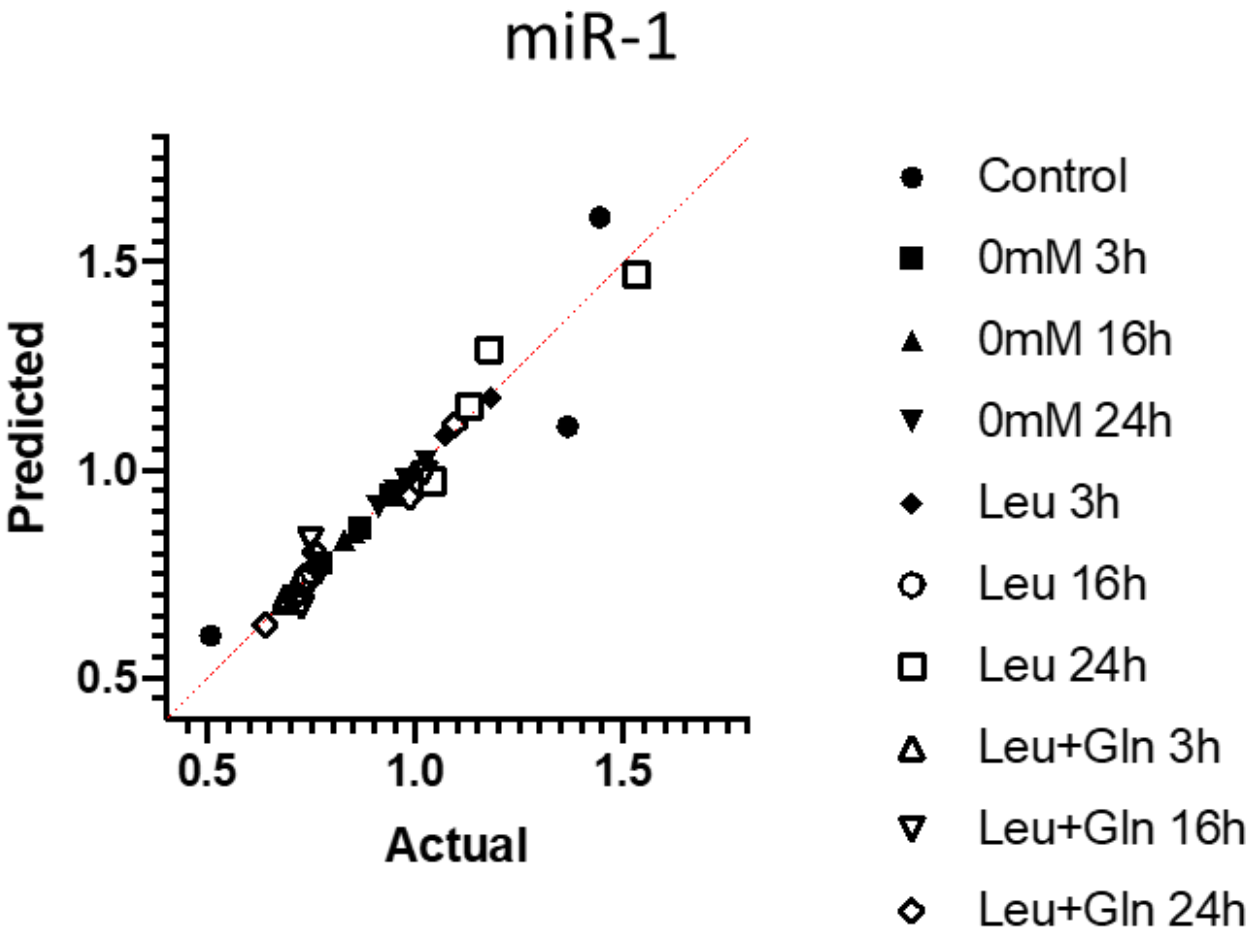


Figure 7.4. Quantile-quantile plot of predicted versus actual expression values of miR-1 in C2C12 myotubes. Analysed by normality and lognormality tests using GraphPad Prism 7.0 software. (n = 3-4). C2C12 myotubes were starved of leucine (0 mM), exposed to 5 mM leucine alone (Leu) or 5 mM leucine combined with 2 mM glutamine (Leu+Gln) for 3-, 16- and 24 h. The plot compared the distribution of the actual P values of miR-1 expression for the different treatment conditions against the predicted normal distribution. Abbreviations: Leu = L-leucine; Gln = L-glutamine; mM = millimolar; h = hour; miR = microRNA gene.

7.4.3. Temporal expression of miR-499

The expression of miR-499 was assessed in C2C12 myotubes. As with miR-1, all treatments did not induce a significant increase in the expression of miR-499 from the control (Figure 7.5). Nonetheless, an increase in the expression of miR-499 was noticed following medium- and long-term leucine deprivation (Figure 7.5 B and C, respectively). An increase in the expression of miR-499 was also evident after short- and long-term exposure to leucine, when compared to other groups (Figure 7.5 A and C, respectively). However, medium-term exposure to leucine resulted in a significant decrease in the expression of miR-499 ($P < 0.05$) compared with the control (Figure 7.5 B).

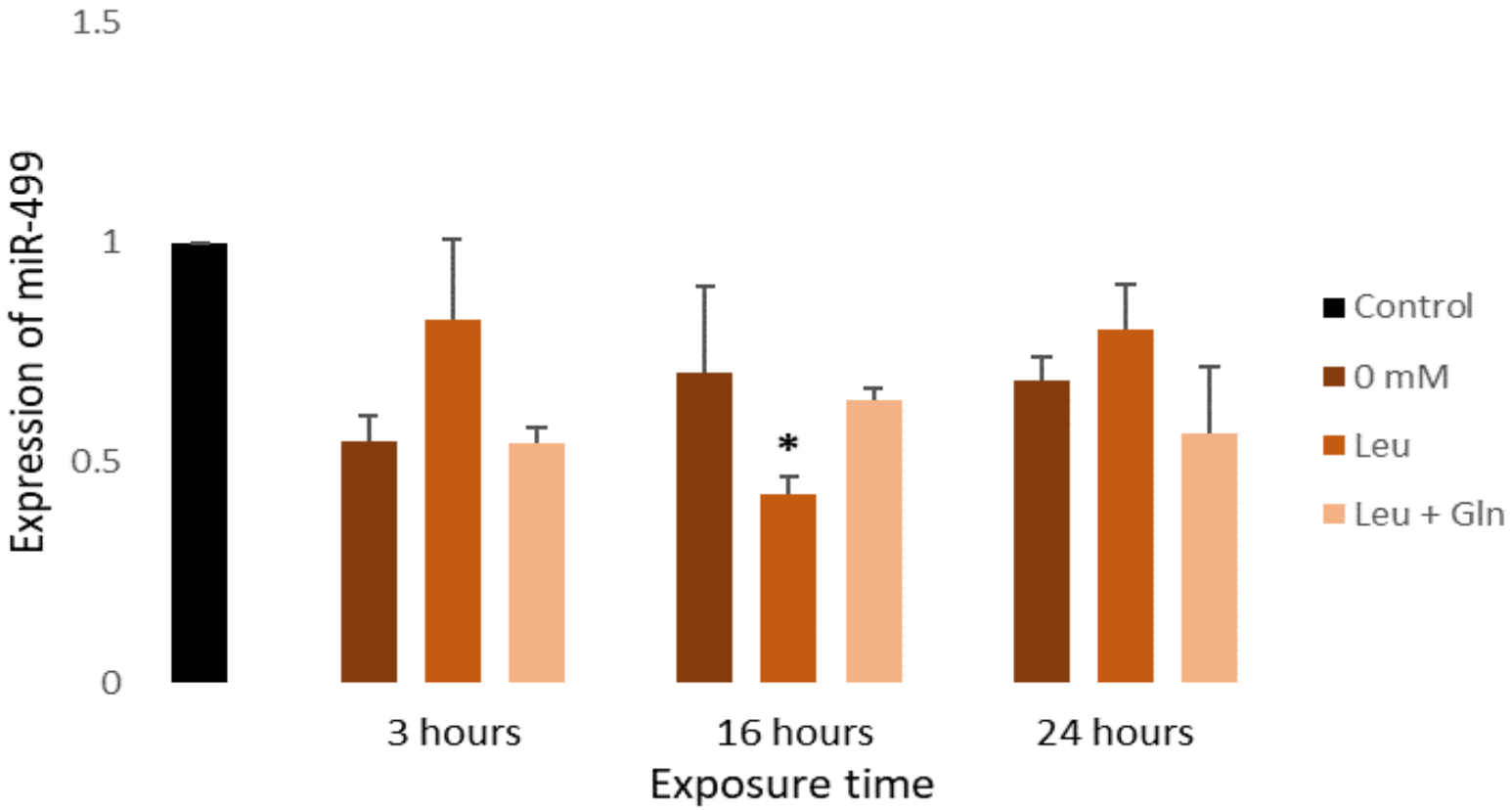


Figure 7.5. Expression of miR-499 in C2C12 myotubes; examined using a quantitative real-time polymerase chain reaction. C2C12 myotubes were deprived of leucine (0 mM), supplemented with 5 mM leucine (Leu) or supplemented with 5 mM leucine plus 2 mM glutamine (Leu + Gln) for 3-, 16- and 24 h. Data are presented as means ± SD of normalised fold changes (n = 3-4). Asterisk indicates significant decrease compared to the control $^{*}P < 0.05$. Analysed by one-way ANOVA using Microsoft Excel followed by Tukey's HSD *post hoc* test using SPSS Statistics 20. Abbreviations: Leu = L-leucine; Gln = L-glutamine; mM = millimolar; miR = microRNA gene.

7.4.4. Temporal expression of miR-206

No changes in the expression levels of miR-206 were noticed following short-term leucine deprivation, exposure to leucine alone or exposure to leucine plus glutamine (Figure 7.6 A). However, medium- and long-term leucine deprivation significantly decreased the expression of miR-206 compared with the control ($P < 0.05$) (Figure 7.6 B and C, respectively). Similarly, medium- and long-term exposure to leucine plus glutamine resulted in a significant decrease in the expression of miR-206 ($P < 0.05$) compared with the control (Figure 7.6 B and C, respectively). A significant decrease ($P < 0.05$) in the expression of miR-206 compared to the control, was recorded following medium-term exposure to leucine alone (Figure 7.6 B). However, an increase in the expression of miR-206, from other groups, was noticed following long-term exposure to leucine (Figure 7.6 C).

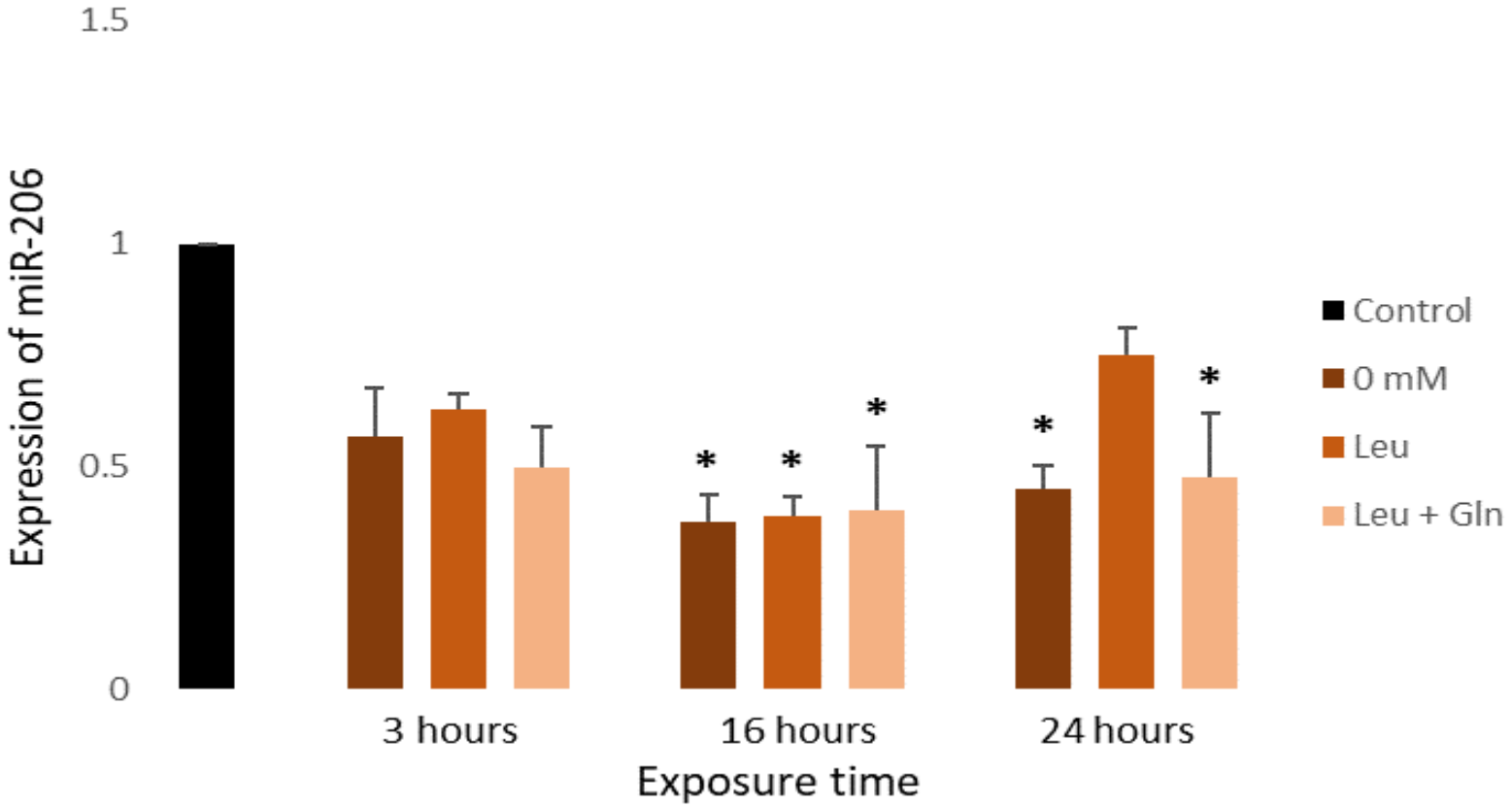


Figure 7.6. Expression of miR-206 in C2C12 myotubes; examined using a quantitative real-time polymerase chain reaction. C2C12 myotubes were deprived of leucine (0 mM), supplemented with 5 mM L-leucine (Leu) or supplemented with 5 mM leucine plus 2 mM L-glutamine (Leu + Gln) for 3-, 16- and 24 h. Data are presented as means ± SD of normalised fold change (n = 3-4). Asterisks indicate significant decrease compared to the control/baseline $^{*}P < 0.05$. Analysed by one-way ANOVA using Microsoft Excel followed by Tukey's HSD *post hoc* test using SPSS Statistics 20. Abbreviations: Leu = L-leucine; Gln = L-glutamine; mM = millimolar; miR = microRNA gene.

7.4.5. Temporal expression of miR-133a

Results indicated that short- and medium-term leucine deprivation did not alter the expression of miR-133a in C2C12 myotubes (Figure 7.7 A and B, respectively). However, an increase in the expression of miR-133a, when compared with the other time points, took place following long-term leucine deprivation (Figure 7.7 C). No alteration in the expression of miR-133a was noticed following short-term exposure to leucine alone (Figure 7.7 A). However, medium-term exposure to leucine resulted in a significant decrease in the expression of miR-133a, when compared to the control ($P < 0.05$) (Figure 7.7 B), while long-term exposure to leucine increased the expression of miR-133a, when compared with the other treatment groups (Figure 7.7 C). Results also showed that both short- and medium-term exposure to leucine plus glutamine did not alter the expression of miR-133a (Figure 7.7 A). However, long-term exposure to both AAs induced an increase in the expression of miR-133a, when compared to other time points (Figure 7.7 C).

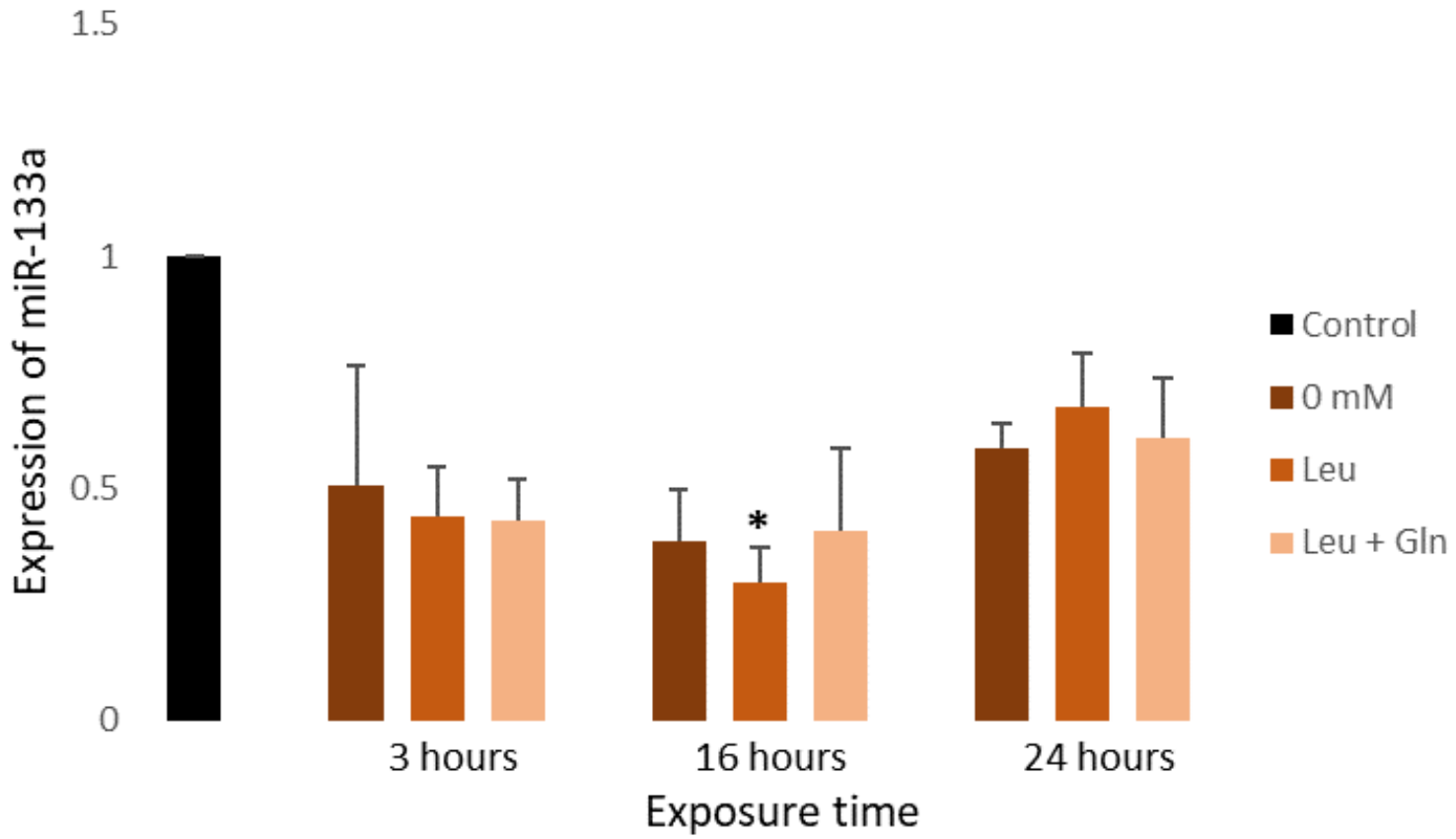


Figure 7.7. Expression of miR-133a in C2C12 myotubes; examined using a quantitative real-time polymerase chain reaction. C2C12 myotubes were deprived of leucine (0 mM), supplemented with 5 mM leucine (Leu) or supplemented with 5 mM leucine plus 2 mM glutamine (Leu + Gln) for 3-, 16- and 24 h. Data are presented as means ± SD of normalised fold change (n = 3-4). Asterisk indicates significant decrease compared to the control $^{*}P < 0.05$. Analysed by one-way ANOVA using Microsoft Excel followed by Tukey's HSD *post hoc* test using SPSS Statistics 20. Abbreviations: Leu = L-leucine; Gln = L-glutamine; mM = millimolar; miR = microRNA gene.

7.4.6. Temporal expression of miR-133b

The expression levels of miR-133b were assessed in C2C12 myotubes following leucine deprivation, exposure to leucine and exposure to leucine and glutamine combined. Results indicated that short-term exposure to leucine and leucine plus glutamine, resulted in a significant decrease in the expression of miR-133b ($P < 0.05$) compared to the control (Figure 7.8 A). However, no significant changes in miR-133b expression were noticed following medium- and long-term exposure (Figure 7.8 B and C, respectively). Results indicated a gradual time-dependent increase in the expression of miR-133b in all groups (Figure 7.8). Therefore, the quantiles of the observed distribution of miR-133b expression were plotted against the quantiles of the fitted normal distribution using Q-Q plot. Results indicated a good agreement in the Q-Q plot for miR-133b as indicated by a straight line of slope less than 1 (Figure 7.9).

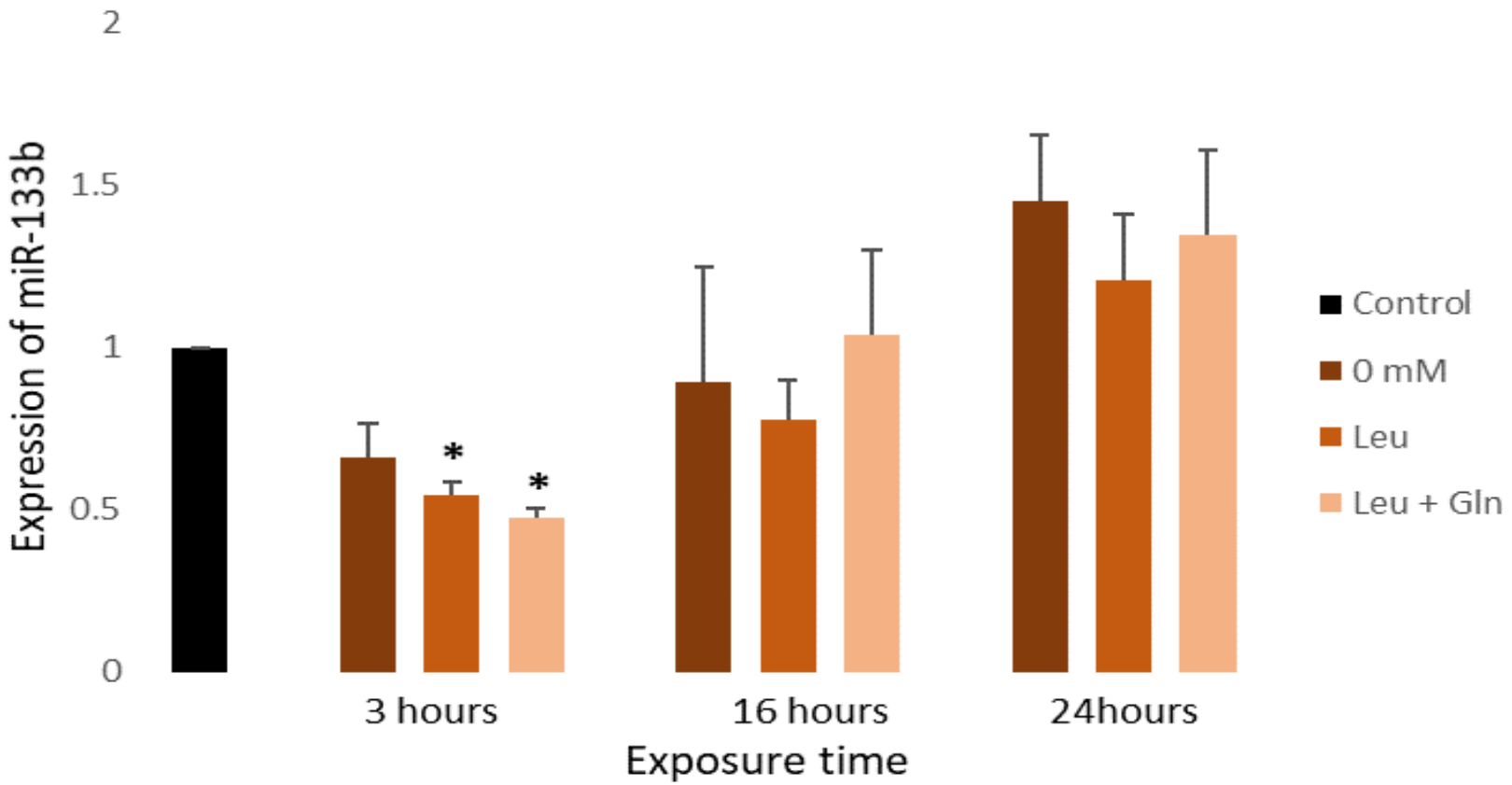


Figure 7.8. Expression of miR-133b in C2C12 myotubes; examined using a a quantitative real-time polymerase chain reaction. C2C12 myotubes were deprived of leucine (0 mM), supplemented with 5 mM leucine (Leu) or supplemented with 5 mM leucine plus 2 mM glutamine (Leu + Gln) for 3-, 16- and 24 h. Data are presented as means ± SD of normalised fold change (n = 3-4). Asterisks indicate significant decrease compared to the control/baseline $^{*}P < 0.05$. Analysed by one-way ANOVA using Microsoft Excel followed by Tukey's HSD *post hoc* test using SPSS Statistics 20. Abbreviations: Leu = L-leucine; Gln = L-glutamine; mM = millimolar; miR = microRNA gene.

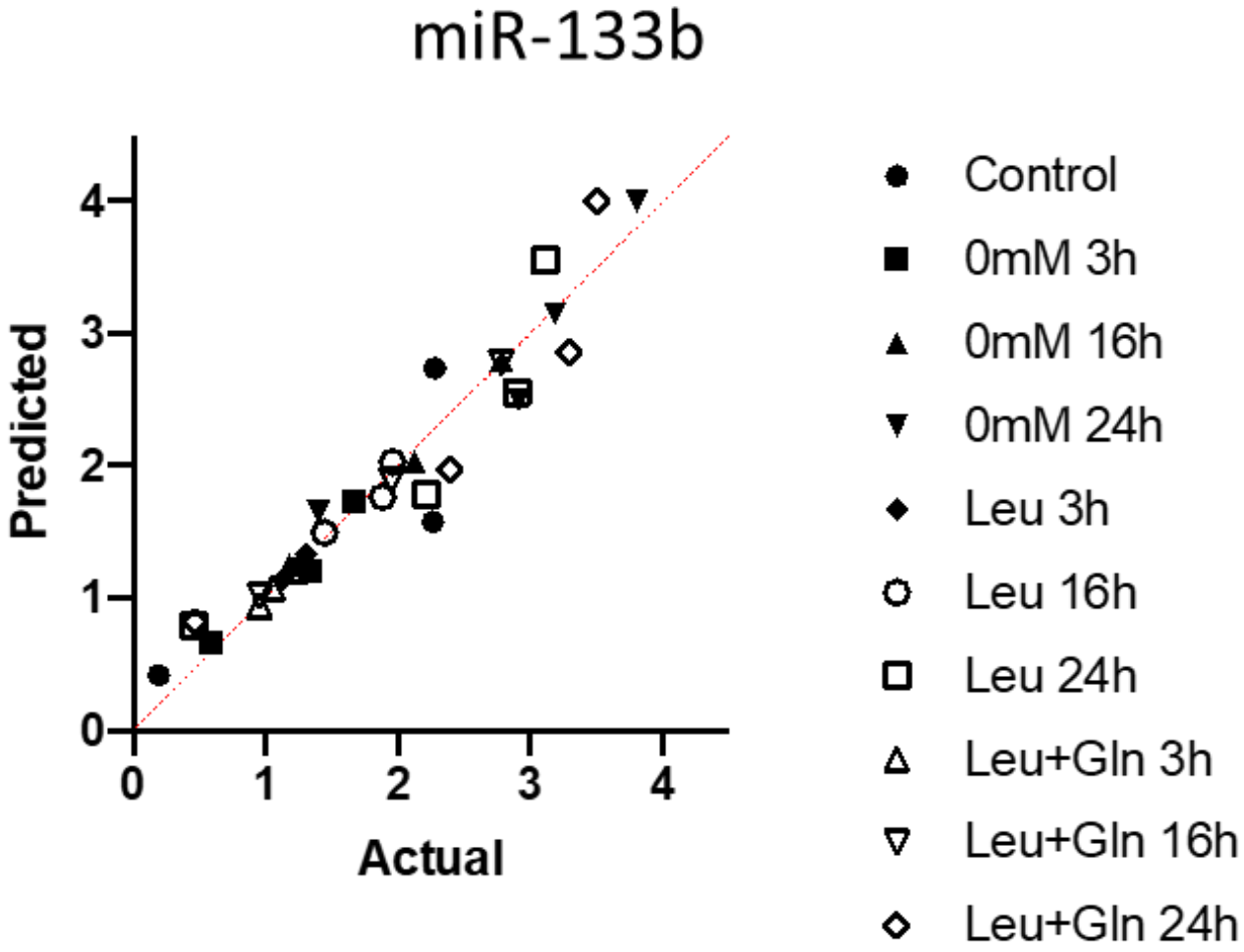


Figure 7.9. Quantile-quantile plot of predicted versus actual expression values of miR-133b in C2C12 myotubes. Analysed by normality and lognormality tests using GraphPad Prism 7.0 software. (*n* = 3-4). C2C12 myotubes were starved of leucine (0 mM) or exposed to 5 mM leucine alone (Leu) or 5 mM leucine combined with 2 mM glutamine (Leu+Gln) for 3-, 16- and 24 h. The plot compared the distribution of the actual *P* values of miR-1 expression for the different treatment conditions against the predicted normal distribution. Abbreviations: Leu = L-leucine; Gln = L-glutamine; mM = millimolar; h = hour; miR = microRNA gene.

7.4.7. Quality control

A stable internal control is necessary for an accurate and reliable testing the expression of miRNAs. Herein, the Hs_RNU6B was used as an internal control. Results indicated that Hs_RNU6B was stable across treatments with Cp differences of approximately 1 (Appendix 13). Also, analysis of serial dilutions of exogenous internal positive control (IPC) was applied. Results revealed that the IPC amplified normally, indicating the absence of amplification inhibitors. Additionally, analysis of melting and standard dilution curves revealed that all primers produced single amplicons (Appendix 14) and all reactions were of comparable high efficiencies (Appendix 15). Moreover, no template control (NTC) was applied in every reaction, by replacing DNA template with nuclease-free water, to monitor for contamination. Results showed no amplification in any of the NTC reactions, indicating the absence of extraneous nucleic acid contaminants and the absence of primer-dimer formation.

7.5. Discussion

MiRNAs represent a class of small (21-23 nt) non-protein coding RNAs. They control more than two thirds of the mammalian genome (Friedman *et al*., 2009) via interfering with the post-transcriptional events (Hamilton and Baulcombe, 1999; Reinhart *et al*., 2000). Generally, miRNAs are ubiquitously expressed in tissues. However, a number of miRNAs (miR-1, miR-133, miR-206, miR-208, miR-486 and miR-499) are enriched in skeletal and cardiac muscles (McCarthy and Esser, 2007; Callis *et al*., 2008; van Rooij *et al*., 2008; van Rooij *et al*., 2009). These miRNAs are known as 'myomiRs'. In general, myomiRs are abundant in both skeletal and cardiac muscles, however miR-206 has been shown to be specific for skeletal muscles (Kim *et al*., 2006), while miR-208a is specific for cardiac muscles (van Rooij *et al*., 2008). MyomiRs have a well-recognised role in regulating the proliferation, differentiation and fibre type specification of muscle cells (reviewed by Horaka *et al*., 2016). They also regulate the response of muscle cells to various stimuli via mediating muscle hypertrophy, atrophy and regeneration (reviewed by Horaka *et al*., 2016).

Unfortunately, studies investigating the role of myomiRs in mediating the response of muscles to nutritional stimuli are very limited. In addition, studies investigating the role of myomiRs in mediating the response of muscles to leucine are nearly non-existent (Drummond *et al*., 2010). One study examined the expression of miRNAs in the skeletal muscles of aged and young humans following exercise and ingestion of EAA (Drummond *et al*., 2008). However, the differential expression of miRNAs in that study was in response to two anabolic stimuli (exercise and EAA), accordingly the contribution of EAA was not clear. In a subsequent study, Drummond *et al*., (2009) demonstrated an upregulation of three myomiRs (miR-1, miR-208b and miR-499) in the vastus lateralis muscle of humans following the ingestion of an EAA mixture rich in leucine. Despite that this study clearly demonstrated that AAs alter the expression of myomiRs within skeletal muscles, the role of myomiRs in mediating the effect of leucine on differentiated muscle cells has not been clarified.

Therefore, the present study aimed at investigating the expression of five myomiRs following short-, medium- and long-term exposure of differentiated C2C12 myotubes to leucine in the presence or absence of glutamine. A leucine concentration of 5 mM was chosen for this study, this was based on the findings of chapters 4 and 5 in which long-term exposure to 5 mM leucine significantly increased the total protein content of C2C12 myotubes. Herein, the expression of

myomiRs was also examined following deprivation of leucine. Of note, only the expression of mature forms of myomiRs were examined in this study, due to the disassociation that often occurs between the expression of primary miRNAs and their mature transcripts in muscles (McCarthy and Esser, 2007; Drummond *et al*., 2009).

Results indicated that short-term exposure to leucine induced an increase in the expression of miR-1 and miR-499 when compared to other treatment groups. Interestingly, medium-term exposure to leucine significantly decreased the expression of all tested miRNAs. However, long-term exposure to leucine increased the expression of all examined miRNAs. Despite that the exact roles of tested miRNAs in mediating the response of muscle cells to leucine treatment remains undefined, literature indicated that miR-1and miR-206 regulate the differentiation of muscle cells. For example, miR-1 enhances the differentiation of muscle cells by targeting histone deacetylase 4 (HDAC4), a known repressor of myocyte enhancer factor-2 (Chen *et al*., 2006; Potthoff *et al*., 2007). Whereas miR-206 promotes muscle differentiation by supressing the helix–loop–helix protein Id, a negative regulator of MyoD (Kim *et al*., 2006, McCarthy, 2008). It has been reported that the upregulation of miR-206 in proliferating C2C12 cultures resulted in the same effect of serum withdrawal and initiated cell differentiation (Zhang *et al*., 2011). Literature also indicated that miR-133 controls both the proliferation and differentiation of muscle cells by regulating the expression of HDAC4 and serum response factor, respectively (Chen *et al*., 2006). Finally, miR-499 has been shown to alter muscle fibre specification by affecting repressors of the β MyHC gene, thus favouring a slow muscle fibre phenotype (van Rooij *et al*., 2008; McCarthy *et al*. 2009).

It is important to clarify that it is hard to link the expression of one miRNA to a single target nor a single function (Drummond, 2010). The published literature showed that increased expression of certain miRNAs in muscles was not associated with a decrease in the expression of their targets (Drummond *et al*., 2009; Nielsen *et al*., 2010). Also, a single miRNA may exert opposing effects on the same gene (Chuang *et al*., 2015). For example, miR-206 was downregulated during muscle atrophy (Allen *et al*., 2009). However, inhibition of miR-206 increased the muscle mass in tilapia fish (Yan *et al*., 2013) and the girth of C2C12 myotubes (Winbanks *et al*., 2013). Finally, miRNAs do not act solely, they rather act in a concerted manner to alter the expression of their target genes, in what is referred to as "hunt in packs" (Lanceta *et al*., 2010).

In the present study, it was particularly difficult to associate the expression of tested myomiRs to alterations in the proliferation or differentiation of C2C12 myotubes, because these cells are post-mitotic fully differentiated syncytia. Nevertheless, miR-1, miR-206 and miR-499 have been shown to target *myostatin*, a well-known negative regulator of muscle mass (Clop *et al*., 2006; Liu *et al*., 2011; Drummond *et al*., 2009). Herein, a downregulation of *myostatin* by leucine via the upregulation of miR-1, miR-206 and miR-499 could be a plausible explanation to the anabolic protein effects of leucine on C2C12 cells. Results also indicated a significant increase in the expression of miR-1, miR-133a and miR-499 following long-term leucine deprivation. Apoptosis of various cells following leucine withdrawal has been suggested in published literature (Sheen *et al*., 2011; Xiao *et al.*, 2016). Therefore, the increased expression of these myomiRs following long-term leucine deprivation is probably attributed to their roles in regulating cell survival and apoptosis (Xu *et al*., 2007; Shieh *et al*., 2011). A previous study showed that miR-1 was upregulated in cardiac cells during oxidative stress, suggesting a pre-apoptotic role of miR-1 (Xu *et al*., 2007). On the other hand, miR-133a repressed the caspase-9 gene in cardiomyocytes, which suggests an anti-apoptotic effect of this miRNA (Shieh *et al*., 2011). Finally, the upregulation of miR-499 inhibited the mitochondrial apoptosis pathway following hydrogen peroxide-induced injury of cultured cardiomyocytes, implying that miR-499 might be an antiapoptotic miRNA (Wang *et al*., 2014).

7.6. Conclusion

Results obtained in the present study showed a differential expression of myomiRs (miR-1, miR-133a, miR-133b, miR-206 and miR-499) in response to leucine withdrawal, exposure to leucine and exposure to leucine plus glutamine. An increase in the expression of all examined myomiRs was observed following long-term exposure of C2C12 myotubes to leucine. This elevated expression could be related to a downregulation of *myostatin*. Also, an increase in the expression of miR-1, miR-133a and miR-499 was detected after long-term leucine deprivation. This finding may be related to the role of these three miRNAs in regulating cell apoptosis. The results of this study are preliminary and too limited to speculate about their possible use in clinical practice. Therefore, more research is clearly needed to further improve the current knowledge of the roles of myomiRs and identifying their regulatory targets in differentiated C2C12 myotubes in response to leucine treatment and leucine deprivation.

8. General discussion

The exceptional high growth of broiler chickens poses a number of challenges, particularly those related to animal welfare. Genetic selection of broilers has been aimed at producing larger breast muscles, as a favourable marketing trait, leaving leg muscles unaffected by this selection (Arthur and Albers, 2003). This has resulted in conformational changes associated with many issues, such as impaired locomotion, leg deformities and mortality among these birds (Julian, 1998; Julian, 2005, Jones *et al*., 2005). These issues are some of the most pressing welfare and economic concerns associated with the broiler industry, and much work is needed to identify the mechanism(s) that underlie this phenomenon. In humans, muscle wasting, also known as muscle atrophy, is a debilitating condition characterised by progressive loss of muscle mass and function. Poor quality of life, increased morbidity and mortality are some of the adverse consequences associated with muscle wasting (Powers *et al*., 2016). Muscle wasting also places a significant burden on healthcare systems (Steffl *et al*., 2017). It is widely accepted that promoting muscle gain or hypertrophy is a primary goal to combat muscle wasting (Bamman *et al*., 2018).

Any muscle hypertrophy in postnatal life occurs via two mechanisms, proliferation of muscles cellular components and acceleration of the rate of protein synthesis within muscle cells. Accordingly, any successful strategy to induce muscle hypertrophy and combat muscle wasting should aim to increase the proliferative capacities of SCs and/or increasing the rate of MPS. Proper understanding of the different cues that underlie the proliferation and differentiation of SCs will contribute to a strategy for mitigating or even reversing muscle wasting. A number of studies have investigated the characteristics of SCs *in vitro* using cell lines and primary cultures and *in vivo* using different animal models. In the past few years, there have been enormous efforts to translate results gained from these studies to the human benefits (Snijders *et al*., 2015). Typically, SCs are dormant in postnatal life, however knowing that SCs isolated from animals that have been genetically selected for high growth rate are highly responsive to anabolic stimuli (Duclos *et al*., 1996; Merly *et al*., 1998; Mathison *et al*., 1989). This gives hope for modulating the activation of these cells to induce muscle hypertrophy. The existence of two genetically similar chicken breeds, one is selected for high growth (broilers) and the other exhibits normal growth (layers), provides a good opportunity to understand the role of SCs in muscle growth and hypertrophy.

Enhancing the rate of MPS is considered the most effective approach to achieve muscle hypertrophy and consequently mitigate muscle wasting (Bamman *et al*., 2018). There is a notion that nutritional intervention, in particular protein supplementation, is the most efficient approach to combat muscle loss in various muscle wasting conditions (Little and Phillips, 2009; Mourtzakis and Bedbrook, 2009; Goisser *et al*., 2015). Indeed, protein intake reversed muscle atrophy by enhancing the rate of MPS in many studies (Little and Phillips, 2009; Mourtzakis and Bedbrook, 2009; Glover and Phillips, 2010). Notably, all the anabolic powers of protein have been attributed to a single BCAA, leucine (Buse and Reid, 1975; Li and Jefferson, 1978; Escobar *et al*., 2006; Rieu *et al*., 2006; Katsanos *et al*., 2006). Many studies have reported the ability of leucine to increase the rate of protein synthesis in the skeletal muscles of animals and humans (Hong and Layman, 1984; Greiwe *et al*., 2001; Lynch *et al*., 2002; Escobar *et al*., 2005; Crozier *et al*., 2005; Escobar *et al*., 2006; Drummond *et al*., 2008; Norton *et al*., 2009; Wilson *et al*., 2010). Also, many studies have reported the ability of leucine to reduce and reverse muscle wasting caused by different medical conditions (i.e., cachexia) (Rieu *et al*., 2006; Maki *et al*., 2012; Ribeiro *et al*., 2015; Matsui *et al*., 2019). Additionally, a recent evidence-based study showed that leucine supplementation was the best approach to treat muscle loss associated with ageing (i.e., sarcopenia) (Gielen *et al*., 2020).

Therefore, this thesis was conducted to investigate the growth and differentiation capacities of SCs isolated from the muscles of young broiler and layer chickens. The differential response of these primary SCs to leucine supplementation was also investigated. This thesis also tested the effects of leucine on C2C12 cells, an *in vitro* model of muscle cells. Here, the effects of increasing concentrations of leucine and/or glutamine over different exposure times and under different culture conditions, on various parameters in proliferating and fully differentiated C2C12 cells were investigated. This thesis indicated that the different proliferation and differentiation capacities of SCs derived from the breast and leg muscles of broiler and layer chickens, may be underlying causes for lameness in modern broilers. This thesis also clarified the relationship between leucine concentration and exposure time, and their role in shaping the outcome of leucine treatment on cultured muscle cells. In addition, this thesis revealed the crucial role of optimal experimental conditions in promoting the anabolic effects of leucine on muscle cells *in vitro*. The data obtained from this thesis provide new insights that may ultimately improve the health and welfare of broiler chickens and mitigate muscle wasting in humans.

8.1. Comparing the growth and differentiation of muscle stem cells isolated from two chicken breeds with different growth rates

We examined any potential differences between SCs isolated from selected (breast) and unselected (leg) muscles of young broiler and layer chickens. However, studying the functions of these cells requires knowledge of the different culture conditions of each cell type. Several protocols have been described for the isolation and cultivation of SCs from the skeletal muscle of many animals, including chicken. However, the available protocols for isolating CSCs were validated using the breast muscles of broiler chickens at their marketing age. The isolation and cultivation conditions of these cells are not applicable to SCs from the breast muscles of layer chickens or SCs from the leg muscles of both chicken breeds. Information in the published literature on the cultivation requirements for CSCs in general, and SCs derived from the leg muscles of chickens in particular, is scarce. Therefore, it was essential to tackle this challenge and optimise the culture conditions for these cell types. The findings of this thesis will enable further study of the properties of different populations of primary chicken muscle cells *in vitro*, to assist in explaining the mechanisms that led to the divergent growth rates between broiler and layer chickens.

Our results indicated that the breast muscles of broiler and layer chickens yielded more myogenic cells, when compared with the leg muscles, which yielded heterogeneous cell preparations heavily contaminated with connective tissue cells. The heterogeneous nature of primary cultures derived from the leg muscles made examining the properties of SCs incredibly challenging and hampered the use of sensitive quantitative assays. More importantly, due to the contamination of SCCs with non-myogenic cells, results gained from these primary cultures may not be reliable. The inaccuracy of results generated from heterogeneous SC populations has been a cause of concern for many years (Matsuda *et al*., 1983; Baquero-Perez *et al*., 2012). Our results point to the need for enriching SCs isolated from the leg muscles of chickens, in order to allow for direct comparison with breast-derived cells. Unfortunately, we were unable to enrich SCs using FACS because Pax7, a hallmark for CSCs, is intracellular. Also, enriching CSCs by differential platting did not lead to any efficient result. Thus, an effective method of enriching CSCs would be of great value.

It has been proposed that selection for high growth has increased the proliferative capacities of SCs (Knizetova *et al*., 1972; Penney *et al*., 1983; Brown and Stickland, 1994; Rehfeldt *et al*.,

2002). In support of this, our results demonstrated that SCs isolated from the breast muscles of broiler chickens, were more proliferative than those isolated from the leg muscles of the same breed and the breast and leg muscles of layers. Accordingly, the nutritional requirements of SCs isolated from the breast muscles of broiler chickens, were higher than leg-derived ones. In the same line, SCs isolated from breast muscles, in contrast to cells isolated from leg muscles, were less tolerant to serum restriction. These findings highlight the different nutritional requirements for SCs isolated from selected (breast) and unselected (leg) muscles of broiler chickens and should be taken into consideration when culturing these cells *in vitro*.

Our results suggest that genetic selection has also affected the differentiation capacities of CSCs. Herein, proteins associated with myogenic differentiation were highly expressed in cultures derived from the leg muscles of layer chickens, when compared with cultures derived from the same muscles of broilers. This low differentiation capacity of broilers' leg muscle cells may be the reason that underpins lameness – a common observation in modern broiler farms, which has serious welfare and economic implications (Julian, 1998; Julian, 2005, Jones *et al*., 2005; Sinclair *et al*., 2019). Manipulating the expression of these proteins in broilers' leg muscles may promote the differentiation of these muscles, and consequently improve the welfare of broiler chickens.

A large number of studies have attributed leg deformities that occur in modern broiler chickens to leg infections and/or poor housing conditions (reviewed by Granquist *et al*., 2019). However, this thesis is the first to propose that low proliferation and differentiation capacities of broiler's leg-derived SCs; as important underlying causes for lameness in modern broilers. Finally, our results indicated that leucine had different effects on SCs derived from selected (breast) and unselected (leg) muscles of the same bird. Exposure to leucine enhanced the proliferation, and consequently the differentiation, of SCs isolated from the leg muscles of broiler chickens. On the other hand, SCs isolated from the breast muscles of broiler chickens could not tolerate high concentrations of leucine. This finding suggests that leucine administration may represent an effective nutritional strategy to improve the mass and function of broilers leg muscles and consequently the welfare of these birds.

8.2. The murine (C2C12) muscle cells as model for myogenic studies

Cell lines are valuable tools for biological studies (Allen *et al.*, 2005). Over the past half-century, the mouse C2C12 myoblast cells have been extensively used as a model for myogenic

studies (Burattini *et al*., 2009). The extensive use of C2C12 cells as an *in vitro* model for skeletal muscles is mainly due to the difficulty of conducting *in vivo* research, the unfeasibility of identifying a specific biological role for a particular compound in the complex interactions, among a myriad of factors, that take place *in vivo* and the controversial results that are often generated from *in vivo* studies (Vignais and Vignais, 2010). It is worth mentioning that current knowledge of the molecular mechanisms underlying leucine-mediated MPS is mostly derived from *in vitro* studies. Data on the protein effects of leucine that were obtained from *in vivo* studies were often inconsistent, possibly due to difficulties in isolating the independent effect of leucine from the various events that occur secondary to leucine administration, such as increased plasma insulin levels (Wilkinson *et al*., 2013). Additionally, temporal monitoring of the dynamic phosphorylation events following leucine treatment, which requires rapid collection of many samples, is only possible using *in vitro* cultures.

8.3. Measuring the viability of C2C12 cells

Assessing the viability of *in vitro* cultured cells provides an accurate prediction of the outcomes of various treatmentsand compounds on living systems. However, assessing cell viability has been neglected in almost all leucine studies to date. There are numerous commercial assays for the quantitative analysis of cell proliferation and viability. However, due to the shortcomings of some viability assays, relying on a single assay may be unreliable and lead to misinterpretation of results. Therefore, it is preferable to utilise multiple methods simultaneously for viability assessment (Uzunoglu *et al*., 2010; Posimo *et al*., 2014; Śliwka *et al*., 2016). Herein, we utilised a number of viability assays including the AB, VB-48^{TM}, VCC and Mitochondrial Membrane Potential assays, to measure the viability of C2C12 cells.

Interestingly, our findings suggested that the choice of assay can influence the measurements as results varied significantly depending on the type of assay used. Results from the VB-48^{TM} and the VCC assays indicated that short-term serum starvation undermined the viability and survival of C2C12 myoblasts. In marked contrast, results generated by the AB assay suggested a boost in cell viability following short-term serum starvation. The reason for such conflicting results is, perhaps, related to differences in the sensitivity or performance of the different assays. The AB assay measures the reducing environment of cells through the reduction of a cell permeable dye (resazurin). Thus, it reflects the effect of serum shortage on cellular metabolism rather than cell integrity. Whereas the VB-48^{TM} assay is based on quantification of the

intracellular levels of free thiol, while the VCC assay measures the ability of cells to preferentially uptake or exclude certain dyes. Therefore, the VB-48^{TM} and the VCC assays are less affected by the metabolic state of C2C12 cells. This makes these assays VB-48^{TM} metabolically independent and, consequently, more accurate methods for measuring the viability of metabolically active muscle cells. Nevertheless, the results of the AB assay, despite differing from results obtained using other viability assays, point to an adaptive mechanism employed by C2C12 myoblasts to mitigate the impact of short-term serum deprivation and this warrants further investigation.

Interestingly, all viability assays produced comparable results, with respect to the effect of long-term serum restriction. They all indicated that long-term serum restriction caused a significant reduction in the viability of C2C12 myoblasts, which was concomitant with low counts of total and live cells. This finding shows the detrimental effect of long-term serum restriction on proliferating C2C12 cells and concurs with many reports of apoptosis in various cells subjected to prolonged serum deprivation (Smith *et al*., 1999; Khalyfa *et al*., 2005; Goyeneche *et al*., 2006; Maldonado and Muñoz-Pinedo; 2011). Although the nature of serum and its full effects on biological systems are not fully known, serum starvation has been categorised as "environmental stress" and an "apoptotic trigger" (Bousette *et al*., 2010; Liu *et al*., 2010). Therefore, based on the results of current study, we strongly recommend that the duration of serum starvation should be decreased to the minimum possible, in order to maintain the viability, proliferation and survival of the cultured cells.

Our results showed that 5 mM leucine had the most desired effect on the viability of C2C12 myoblasts. However, a prominent finding was that long-term leucine deprivation enhanced the viability of C2C12 myoblasts. This is rather unusual because it has been shown elsewhere that leucine deprivation halts proliferation, undermines viability and initiates apoptosis in numerous tumour cell lines (Sheen *et al*., 2011; Xiao *et al.*, 2016). Interestingly, herein, long-term leucine withdrawal did not result in significant cell death. In contrast, leucine deprivation was associated with a relative increased number of live cells. Both are indicative of cell cycle arrest, a conclusion that is in line with the findings of a previous study (Everhart and Prescott, 1972). However, further work is required to validate this conclusion and to delineate the mechanisms by which C2C12 cells tolerate leucine deprivation. Importantly, this observation questions the practicality of using leucine deprivation as a control for studies investigating the effect of leucine on muscle cells; and argues for the need for an alternative control to avoid false results.

Another notable finding of this study was the severe decline in cell viability following long-term incubation of C2C12 myoblasts with a supraphysiological concentration of leucine. High leucine intake is recommended to treat muscle wasting in elderly and critically ill humans (Dardevet *et al*., 2000; Katsanos *et al*., 2006). Results of the present work, however, showed a negative impact of high leucine concentrations on the viability of muscle cells, and should be taken into consideration when using leucine to combat muscle wasting. Finally, none of the aforementioned viability assays could be used for measuring the viability of differentiated C2C12 cells. C2C12 myotubes are multinucleated, non-proliferative and metabolically active cells, which are very difficult to assess for viability. Nonetheless, it is essential to assess the viability of C2C12 myotubes because the outcomes of different treatments may be confounded by variations in cell viability. This highlights the need for a viability assay that is not affected by the metabolic status of the cells and does not require cells to be in single cell suspensions, in order to reliably assess the viability of differentiated muscle cells.

8.4. Measuring the total protein content of C2C12 cells

Determining the total protein content of cells has the advantage of being indicative of the overall effect of different treatments on protein balance. Measuring the total protein content was particularly useful in this study for evaluating the effects of different treatments on differentiated C2C12 myotubes, since the viability of these cells could not be assessed as mentioned earlier. Our results indicated that both short- and long-term serum starvation decreased the total protein content of proliferating and differentiated C2C12 cells, which is consistent with a previous report (Pirkmajer and Chibalin, 2011). Although the rates of MPS and MPB were not measured in the current work, other studies have linked serum deprivation to increased rates of MPB and/or decreased rates of MPS (Hershko and Tomkins, 1971; Epstein *et al*., 1975). The current finding highlights the detrimental effect of serum deprivation on the protein content of muscle cells and indicates a need for reducing the duration of serum withdrawal to avoid unnecessary protein degradation.

Leucine is well-recognised for increasing the rate MPS (Kimball and Jefferson, 2001; Dardevet *et al*., 2000; Anthony *et al.*, 2001; Lynch *et al.*, 2003). However, its effect on total protein remains largely unknown. Previous studies have shown that enhanced MPS does not always correlate with muscle hypertrophy (Mitchell *et al*., 2012; Mitchell *et al*., 2013), pointing to a requirement to measure the net protein gain after leucine treatment. Results of the present work

indicated that exposure to physiologically relevant concentrations of leucine, in the presence of serum, induced a significant increase in the total protein content of proliferating and differentiated C2C12 cells. In contrast, exposure to the same leucine concentrations under reduced-serum, and serum free conditions failed to increase the total protein content of cells. These findings reinforce the key role of serum in mediating the protein anabolic effects of leucine on muscle cells. Previous leucine research has largely evaluated the protein effects of leucine under serum-restricted conditions (Kim *et al*., 2009; Huang *et al*., 2013; Zhang *et al*., 2014; Mao *et al*., 2015). Based on our findings, serum restriction may be an underlying cause of leucine's inability to increase the rate of MPS in some studies (Glynn *et al*., 2010; Wilson *et al*., 2011; Areta *et al*., 2014). Also, results gained from these studies cannot predict the outcomes of leucine administration in animals or humans, given that leucine is usually supplemented in combination with other nutrients. In support of this assumption ingestion of leucine, protein and carbohydrates significantly increased the rate of MPS and the net protein balance in the skeletal muscles of humans, while ingestion of leucine alone did not have any anabolic effect (Koopman *et al*., 2005).

Altogether our results strongly suggest that the protein anabolic effects of leucine on proliferating and differentiated C2C12 cells can only be attained under optimal experimental conditions. Therefore, it is highly recommended that an evaluation of the effects of leucine on cultured muscle cells is performed in the presence of serum and following short-term serum starvation of cultured cells. Importantly, based on our results, examining the effects of leucine under serum-restricted conditions may lead to false results, as muscle cells will develop adaptive mechanisms to overcome short-term serum restriction, or initiate apoptosis following long-term serum shortage. In support of this assumption, serum starvation totally changed the response of cultured muscle cells to various treatments (Whorwood *et al*., 2001; Al-Khalili *et al*., 2004). Our results also provide valuable information regarding the optimal leucine concentration that can positively alter protein levels in muscle cells. The published literature indicated that only high doses of leucine can induce MPS (McNurlan *et al*., 1982; Anthony *et al*., 1999). Accordingly, high protein intake is recommended to combat the muscle wasting in humans. However, our findings have shown that exposure to a high concentration of leucine compromised the viability of muscle cells. The decrease in the total protein content of C2C12 cells following long-term stimulation with a supraphysiological concentration of leucine is a further evidence of the adverse effects associated with high leucine concentrations. On the other hand, exposure to low concentrations of leucine enhanced the viability and increased the total

protein content of cells. Therefore, based on the findings of this thesis, it is highly recommended that leucine is given at physiologically relevant levels in order to achieve muscle hypertrophy in animals and alleviate muscle wasting in humans.

8.5. Signalling pathways that mediate the protein anabolic effects of leucine on C2C12 cells

We investigated the signalling events that may have mediated the significant alterations in the total protein content of C2C12 cells after leucine treatment.

8.5.1. The mTOR signalling

In the 1970's, leucine was shown to alter protein turnover by increasing the rate of protein synthesis in the skeletal muscles of rats (Buse and Reid, 1975; Li and Jefferson, 1978). Since then, research undertaken to elucidate the mechanism (s) underlying this phenomenon have revealed that leucine stimulates protein synthesis via activation of the mTOR signalling pathway (Lynch *et al*., 2003; Tokunaga *et al*., 2004; Stipanuk, 2007; Hara *et al*., 1998). However, the concentration- and time-dependent effect of leucine on the activation of mTOR signalling is incompletely understood. Herein, the phosphorylation of mTOR's substrates, p70 S6K and 4E-BP1, was examined because their phosphorylation is an indicator of mTORC1 activation (Nojima *et al*., 2003; Schalm *et al*., 2003; Proud, 2004).

Our results indicated that short-term stimulation of C2C12 myotubes with leucine induced the phosphorylation of p70 S6K and 4E-BP1 in a concentration-dependent manner, a result that is consistent with previous studies (Fox *et al*., 1998; Nakajo *et al*., 2005; Areta *et al*., 2014). Because the phosphorylation of p70 S6K is a proxy measure of MPS (Drummond *et al*., 2009). Therefore, our results are indicative of active concentration-dependent protein synthesis, following short-term stimulation of C2C12 myotubes with leucine. On the other hand, the phosphorylation of mTOR's substrates was inversely correlated with leucine concentrations following medium- and long-term stimulation. Although the reason for this finding is unclear, it indicates an early saturation of p70 S6K and 4E-BP1 by higher concentrations of leucine. It has been shown that overprovision of leucine disrupts the levels of EAAs – in particular levels of other BCAAs, such as valine and isoleucine (Tom and Nair, 2006) – due to "branched-chain amino acid antagonism" (Harper *et al*., 1984). It is also broadly accepted that leucine *per se*

cannot elicit MPS. Therefore, muscle cells must have continuous supply of AAs in order to respond to the protein anabolic stimuli of leucine. If this is the case, then stimulation of C2C12 cells with high concentrations of leucine must have decreased the intracellular levels of other AAs. This limited supply of AAs further reduced the phosphorylation of p70 S6K and 4E-BP1 in response to medium- and long-term stimulation with leucine. However, stimulation with lower concentrations of leucine did not deplete the AA pool in the early stages, causing a prolonged phosphorylation of p70 S6K and 4E-BP1 in response to leucine. This may explain why high doses of leucine failed to sustain MPS in another study (Atherton *et al*., 2010), however, this assumption requires further validation. Furthermore, our results demonstrated a decline in the phosphorylation of p70 S6K and 4E-BP1 following long-term exposure of C2C12 cells to all leucine concentrations. This explains the transient nature of leucine-induced MPS, which has been reported in other studies (Escobar *et al*., 2005; Wilson *et al*., 2010).

The published literature indicated that only high doses of leucine are capable of eliciting MPS (McNurlan *et al*., 1982; Anthony *et al*., 1999). Based on our findings, we concluded that exposure to high leucine concentrations would eventually decrease the total protein content of muscle cells, regardless of an early increase in MPS, inferred from the increased phosphorylation of p70 S6K. In support of this conclusion, a previous study showed decreased MPS in response to the overprovision of leucine (Atherton *et al*., 2010). Intriguingly, high protein intake is recommended to combat muscle wasting in the elderly and patients with chronic inflammatory illnesses. Ageing is associated with anabolic resistance in which physiological levels of AAs fail to alter the rate of MPS (Dardevet *et al*., 2000; Katsanos *et al*., 2006). Enhanced protein synthesis in elderly humans was only achieved by the consumption of high levels of AAs to induce a state of hyperaminoacidemia (Volpi *et al*. 1999; Arnal *et al*., 1999; Paddon-Jones *et al*., 2004; Ni Lochlainn *et al*., 2018). Similarly, inflammation reduces the ability of muscle cells to respond to AAs provision (McGaha *et al*., 2012). Inflammation also increases the catabolism of AAs, to synthesise much-needed glutamine (McGaha *et al*., 2012). However, high protein diets can cause irreversible damage to the kidneys, particularly in the elderly (Fliser *et al*., 1993). Additionally, consumption of high levels of EAAs, especially leucine, has been linked to insulin resistance and diabetes mellitus (Moghei, 2015). Therefore, based on the findings of the present work and evidence from the published literature (Fliser *et al*.,1993; Tom and Nair, 2006; Moghei, 2015), it is recommended that leucine is provided at physiological levels in order to induce a sustained MPS, maintain the viability of muscle cells and avoid possible systemic side effects.

Overall, our results indicated that an increase in MPS does not mean an increase in the total protein content of cells. Herein, the increased phosphorylation of mTOR substrates, indicative of protein synthesis, following exposure to a high concentration of leucine, was transient and was not associated with an increase in the total protein content of C2C12 cells. Therefore, the exclusive reliance on measuring the rate of MPS, following leucine treatment, does not justify the use of a particular leucine concentration to induce muscle hypertrophy. Instead, it is highly recommended that assessing the rate of MPS should be backed-up by assessing the total protein gain following leucine treatment.

8.5.2. The ERK1/2 signalling

The MAPK is a protein anabolic signalling pathway. It has been shown that ERK1/2, the prototypical pathway of MAPK signalling (Meloche and Pouysségur, 2007), acts as an upstream activator of mTOR (Carriere *et al*., 2011). However, the role of ERK1/2 in mediating the protein effects of leucine is controversial. Results of the current study showed that leucine modulated the phosphorylation of ERK1/2 in C2C12 cells, which contradicts previous findings (Peyrollier *et al*., 2000; Camillo *et al*., 2014). Interestingly, blocking ERK1/2 attenuated, but did not inhibit, the phosphorylation of mTOR substrates p70 S6K and 4E-BP1 in C2C12 myotubes stimulated with leucine. This finding indicates that ERK1/2 is required for the full phosphorylation of p70 S6K and 4E-BP1 following leucine provision, however the phosphorylation of p70 S6K and 4E-BP1 is independent of ERK1/2. It also verifies the link between the leucine-induced activation of mTOR and the MAPK signalling pathway.

8.5.3. The GSK-3β signalling

It has been proposed that leucine interacts with the insulin signalling pathway at multiple sites (Garlick and Grant, 1988; Balage *et al*., 2011; Macotela *et al*., 2011). GSK-3β is also a key controller of global protein synthesis (Proud, 2006). Our results showed that the phosphorylation pattern of GSK-3β in C2C12 myotubes, following exposure to leucine, mirrored the phosphorylation of mTOR substrates. Importantly, the maximum phosphorylation of GSK-3β in these cells was concomitant with the maximum phosphorylation of mTOR substrates. These findings suggest a role for GSK-3β deactivation in mTOR activation and, consequently, protein accretion following leucine treatment. This finding concurs with a

previous study showing the ability of GSK3 to activate p70 S6K in various cell lines (Shin *et al*., 2011). Inhibition of GSK3 also suppressed the phosphorylation of mTORC1 substrates in cultured muscle cells stimulated with AAs (Stretton *et al*., 2015). Hence, based on our results and evidence from other studies (Proud, 2006; Shin *et al*., 2011), it is clear that the GSK-3β signalling – at least in part – is relevant to the modulation of C2C12 cells total protein content by leucine.

8.5.4. The PI3K/Akt signalling

The published literature suggests a role for PI3K/Akt signalling in mediating the effects of leucine on muscles. For example, increased phosphorylation of PI3K/Akt occurred in the skeletal muscles of rats (Saha *et al*., 2010) and mice (O'Neill *et al*., 2010) following leucine treatment. It has also been proposed that Akt directly phosphorylates mTOR, with this effect being dependent on the availability of AAs (Nave *et al*., 1999). Additionally, present work indicated an involvement of GSK-3β, a downstream substrate of PI3K/Akt signalling, in mediating leucine's effects on C2C12 cells. Our results showed that blocking PI3K completely inhibited the phosphorylation of p70 S6K in C2C12 myotubes treated with leucine. This result suggests an essential role for PI3K/Akt signalling in mediating the protein effects of leucine in muscle cells. However, because the activation of p70 S6K by PI3K has been questioned in some studies (Ming *et al*., 1994; Hara *et al*., 1995), further work is required to identify the exact role PI3K/Akt signalling plays in mediating the protein effects of leucine on muscle cells.

8.6. Characterising the combined effect of leucine and glutamine on C2C12 cells

Glutamine is a non EAA, which shares a close metabolic relationship with BCAAs (Holeck, 2002). Glutamine also facilitates the delivery of leucine into cells (Nicklin *et al*., 2009). Nevertheless, data on the combined effect of leucine and glutamine is of limited availability and is inconsistent (Xu *et al*., 2001; Krause *et al*., 2002; Nakajo *et al*., 2005; Deldicque *et al*., 2008). Additionally, both leucine and glutamine are powerful activators of mTORC1 (Lynch *et al*., 2003; Tokunaga *et al*., 2004; Stipanuk, 2007; Yuan *et al*., 2015). However, the independent effect of glutamine on mTOR signalling remains undefined (Deldicque *et al*., 2008; Yuan *et*

al., 2015) and the combined effect of leucine and glutamine on mTOR remains contentious (Xu *et al.*, 2001; Krause *et al.*, 2002; Nakajo *et al.*, 2005).

Our results showed that combining glutamine and leucine promoted the viability, proliferation and survival of C2C12 cells. Glutamine and leucine combined, also promoted protein accumulation in C2C12 cells. These findings are in line with published studies, which showed that glutamine increased protein synthesis, proliferation and viability of different cells (Tannock *et al*., 1986; Ko *et al*., 1993; Turowski *et al*., 1994; Lingqin *et al*., 2015). Our results also showed that glutamine independently increased the phosphorylation of the mTOR downstream substrates p70 S6K and 4E-BP1. However, this result contradicts the findings of Nakajo *et al*., (2005) who reported an inhibition of mTOR phosphorylation following exposure of intestinal cells to glutamine. Our data also contradicts those of Deldicque *et al*., (2008) who reported that glutamine did not induce the phosphorylation of mTOR, nor its downstream substrates, in the same cells used in our study (C2C12 myotubes). The contradiction between our data and those of Nakajo could be attributed to the use of different cell lines. However, the difference between our results and those of Deldicque and colleagues is more likely due to different experimental designs. In the aforementioned study, C2C12 myotubes were starved of serum for 1 h prior to glutamine exposure, whereas, in our study, C2C12 myotubes were starved of serum for 4 h. If this is the case, then serum appears to be essential for shaping the outcome of glutamine treatment on muscle cells, but this result requires further validation.

Our results also showed that short-term exposure to glutamine failed to elicit an additional effect on leucine-induced phosphorylation of p70 S6K or 4E-BP1. However, following medium-term exposure, glutamine exerted two contrasting effects. On the one hand, glutamine increased the phosphorylation of mTOR substrates induced by a high concentration of leucine. On the other hand, glutamine decreased the phosphorylation of mTOR substrates induced by lower concentrations of leucine. Interestingly, following long-term stimulation, glutamine exerted an additional effect on the leucine-induced phosphorylation of mTOR and MAPK signalling pathways, and sustained the phosphorylation of p70 S6K and ERK1/2 in C2C12 myotubes, which reached low levels after stimulation with leucine alone.

These contrasting effects strongly suggest that the relationship between leucine and glutamine, at least in the context of the phosphorylation of mTOR, is both concentration- and time-dependent. However, the question remains, why was this additive effect of glutamine only evident following long-term stimulation? Although we do not have a definitive answer, this

result together with the results of chapter 5, suggest that glutamine is not essential for leucine-induced phosphorylation of mTOR, nor for leucine-mediated protein accumulation in C2C12 cells. However, the increase in the protein content and the phosphorylation of mTOR substrates in C2C12 cells treated with leucine plus glutamine, compared with those treated with leucine alone following the long-term treatment only; suggests that this apparent increase may be attributed to the anti-proteolytic effect of glutamine, which, in turn, limits protein degradation. Therefore, the increased protein content in C2C12 cells treated with a combination of leucine and glutamine is due to protein degradation in C2C12 cells treated with leucine alone.

To the best of our knowledge, the present work evaluated, for the first time, the signalling events that take place following long-term stimulation of C2C12 myotubes with leucine and glutamine combined. The present work showed that glutamine is essential for sustaining the leucine-induced phosphorylation of ERK1/2 and p70 S6K in differentiated C2C12 cells, after long-term stimulation. Also, the present work is the first to reveal that the combined effect of leucine and glutamine is both time- and concentration-dependent.

8.7. The temporal expression of myomiRs in C2C12 cells

For the past 20 years, miRNAs have been recognised as key regulators of gene expression. It is estimated that more than 60% of the mammalian genome and 30% of protein coding genes in humans are controlled by miRNAs (Berezikov *et al*., 2005; Friedman *et al*., 2009). MyomiRs (miR-1, miR-133a, miR-133b, miR-206 and miR-499) are a class of miRNAs enriched in cardiac and skeletal muscles. They regulate several aspects of muscle biology such as proliferation, differentiation, metabolism, hypertrophy and regeneration (reviewed by Horaka *et al*., 2016). It is well-established that nutritional interventions, especially AAs, control the expression of protein-coding genes in muscles (Clarke and Abraham, 1992). It has also been proposed that nutrients modulate the expression of miRNAs in muscle cells (Chen *et al*., 2013; Drummond *et al*., 2009). However, studies investigating the effect of AAs on the expression of myomiRs are extremely limited. It has been reported that EAAs, in general, upregulate the expression of miR-1, miR- 208b and miR-499 *in vivo* (Drummond *et al*., 2009).

Our results show that long-term stimulation of C2C12 myotubes with leucine induced the upregulation of miR-1, miR-133a, miR-206 and miR-499.Computational analysis of the putative targets for miRNAs indicated that one miRNA can target several genes; and that a single gene could be targeted by more than one miRNA (Lim *et al*., 2005). This difficulty in

determining the regulatory targets of miRNAs hinders the identification of their exact functions. Additionally, despite myomiRs having been proposed as powerful regulators of muscle mass, a recent study questioned this assumption by showing that life-long reduction in miRNA levels did not affect the skeletal muscle mass of mice (Vechetti *et al*., 2019). Furthermore, the upregulation of miR-1, miR-133a, miR-133b and miR-206 in muscle following endurance exercise, was not associated with the downregulation of their targets (Nielsen *et al*., 2010). All this hinders the identification of the exact role of myomiRs in mediating the protein effect of leucine on C2C12 cells. It is noteworthy that miRNAs only fine-tune the expression of target genes; rather than exerting an all-or-nothing control (Lee *et al*., 2007; Lanceta *et al*., 2010). It has also been proposed that miRNAs act in a co-ordinated manner, as a "pack", to exert a particular effect (Lanceta *et al*., 2010). This broad range of miRNA regulatory targets is likely required for altering certain signalling pathways (Drummond, 2010).

Herein, miR-1, miR- 133a, miR-133b, miR-206 and miR-499 appeared to mediate the response of C2C12 myotubes to leucine. Although somewhat speculative, our results suggest that the upregulation of these myomiRs, following leucine treatment, could have downregulated the activity of *myostatin*, a well-recognised inhibitor of muscle growth. It is generally accepted that *myostatin* is the *bona fide* target of miR-1, miR-206 and miR-499 (Clop *et al*., 2006; Liu *et al*., 2011; Drummond *et al*., 2009). Also, infusion of EAAs caused a 75% reduction in *myostatin* levels in humans' skeletal muscles (Drummond *et al*., 2009). In addition, leucine treatment downregulated the expression of *myostatin* in cultured C2C12 cells (Chen *et al*., 2013). However, this speculative conclusion requires further validation.

Taken together, the results of the current study provided new insights into a possible involvement of myomiRs in shaping the response of cultured muscle cells to leucine. Controlling the expression of these myomiRs will help in inducing muscle hypertrophy and/or reversing muscle atrophy. This would be of great benefit to the health and welfare of farm animals and humans, and further work is warranted to define the regulatory targets of these miRNAs.

8.8. Limitations and suggestions for future work

The main aim of this thesis was to evaluate the protein anabolic effects of the BCAA leucine on primary and established muscle cells. The results generated in this thesis suggest promising

directions for future work using leucine to induce muscle hypertrophy in animals and to alleviate muscle wasting in humans. Nonetheless, some limitations of this thesis should be taken into consideration.

8.8.1. Statistical significance versus biological relevance

It is noteworthy to highlight that the term 'significant' has been used in this thesis to show that the results were statistically significant (i.e., the differences between groups exceeded the significance level; alpha). In recent years, an increasing attention has been paid to the difference between 'statistical significance' and 'biological relevance', and what does this difference mean to the interpretation of results obtained from biological experiments. Statistical significance denotes whether an effect, or a difference exists, whereas biological significance indicates the magnitude of this effect. This means that the outcome of a statistical analysis although could be statistically significant, it does not necessarily mean the same *in vivo* and vice versa (Alderson, 2004; Altman and Bland, 1995). Unfortunately, no statistical test is capable of predicting whether a statistically significant effect equates to a significant difference *in vivo*. This leaves it to the researcher to hypothesise which appropriate effect size can achieve the desired *in vivo* outcome. Most likely, this assumption will be based on the knowledge and prior experience of the researcher, which can be subjective. To overcome this limitation, Lovell (2013) argued for more rigorous study design with an appropriate sample size and statistical power.

For *in vivo* studies, there are always ethical implications associated with the use of unnecessary large number of animals in research. Hence, there is always a drive to reduce the use of live animals in studies especially those studies that may inflict injury or suffering on the animals. In these *in vivo* experiments the number of animals needed to achieve the desired biological effect should be justified and pre-determined before the conduction of the experiment by calculating the power of study (Luus *et al.*, 1989). On the other hand, *in vitro* experiments do not have such ethical requirements of the sample size, but the challenge will be often related to the turnaround time, financial implication and other logistical resources. Another challenge associated with *in vitro* experiments is the requirement of a prior knowledge of the effect size of interest that is expected to be biologically significant. This specific information can be difficult to have for *in vitro* studies that have complex experimental designs involving multiple and dependent variables, such as those conducted in the present thesis.

In the present thesis, the anabolic effects of leucine were tested on cultured muscle cells. As mentioned earlier, *in vitro* systems offer a lot of advantages over *in vivo* studies especially when the aim is to test the effects of pharmaconutrients and drugs. Nevertheless, *in vitro* systems have limitations. The first and foremost limitation is the lack of *in vivo* complexity; cell models lack the extensive network of interactions that occur among different cell types in any given tissue. Other limitations of the *in vitro* systems include the difficulties associated with translating *in vitro* concentrations to *in vivo* doses, monitoring the impacts of long-term exposures and simulating *in vivo* responses to stress situations (Ghallab, 2013; Hartung, 2013). All these challenges make direct application of results gained from *in vitro* studies to predict biological responses in living systems (i.e., *in vivo* to *in vitro* extrapolation) non-feasible (Ghallab, 2013; Hartung, 2018). Taken together, while *in vitro* studies lay the foundation for *in vivo* investigation, clinically relevant data can be only obtained from *in vivo* studies conducted on the target species. Therefore, in future studies, the nature and size of anticipated biological effects should be considered at an early stage of the experimental design.

8.8.2. Comparing satellite cells isolated from broiler and layer chickens

This thesis compared the proliferation and differentiation capacities of SCs isolated from the breast and leg muscles of young broiler and layer chickens. Considerable precautions were taken to reduce variations due to diet, housing conditions, sex …etc. Nevertheless, variations due to the divergent growth rate between broilers and layers, and between breast and leg muscles remain a possibility. For decades, genetic selection of chickens aimed at improving the production and reproduction traits of broilers and layers, respectively (Emmerson, 1997). This resulted in significant variability in muscle development and growth between the two chicken breeds (Buzala and Janicki, 2016). It has been reported that the rate of muscle growth is two- to threefold greater in broilers than layers (Zheng *et al*., 2009). Additionally, the breast muscles of broiler chickens grow eight times as fast as in layers (Zheng *et al*., 2009). Moreover, during the first 2 weeks of life, the breast muscles of broilers and layers grow at faster rates than the leg muscles (Saunderson and Leslie, 1988). Herein, SCs were isolated from the breast and leg muscles of two-week-old broiler and layer chickens, because this is the age of maximum weight difference between the two breeds (Jones *et al*., 1986; Zheng *et al*., 2009). Monitoring the growth of the breast and leg muscles of broiler and layer chickens and examining the

characteristics of SCs isolated from these muscles at comparable developmental stage, is another possibility for future work.

8.8.3. Evaluating the effect of leucine on primary chicken satellite cells

Obtaining a pure population of SCs from the skeletal muscles of chickens was unfeasible in the present work. The contamination of isolated SCs with non-myogenic cells made cell counting very difficult, hampered the application of quantitative assays and interfered with accurate interpretations of the results. Accordingly, the proliferation and differentiation of CSCs was evaluated morphologically, which is a limitation in this study. Over the last 20 years, the main goal of satellite cell research has shifted from isolating SCs from the skeletal muscles of human and different animal species (Blau and Webster, 1981; Dodson *et al*., 1986; Dodson *et al*., 1987; Yablonka-Reuveni *et al*., 1987; McFarland et l., 1988; Powell *et al*., 1989) towards the isolation of pure SCs, or SCs with the least number of non-myogenic cell contaminants (Rhoads *et al*., 2008). In recent years, a significant progress has taken place in this field such as the development of genetically modified reporter mice and FACS. However, FACS is inapplicable to CSCs. Therefore, it is important that future work is aimed at purifying SCs isolated from the skeletal muscles of chickens.

8.8.4. Evaluating the viability of C2C12 cells

In the present work, it was essential to assess the impact of different nutrient starvation protocols, in order to choose a feasible starvation regimen that will not compromise the viability of C2C12 cells. Our results indicated that C2C12 cells tolerated the impact of short-term serum starvation, possibly by enhancing their metabolic activities. However, we are unsure of the events that mediated the adaptation of C2C12 cells to short-term serum withdrawal. Therefore, elucidating the mechanisms that led to such results could be another area for future work. Our results also indicated that long-term serum withdrawal decreased the viability of C2C12 cells, without causing massive cell loss. Therefore, assessing the apoptosis of serum starved C2C12 cells should be an aim of future work. As mentioned earlier, the viability of differentiated C2C12 myotubes could not be measured by any of the assays we applied to measure the viability of proliferating C2C12 myoblasts. Instead, the protein content of C2C12 myotubes was measured to evaluate the effects of different treatments. However, identification of assays

that reliably assess the viability of multinucleated syncytia, without being affected by the metabolic state of cells, would be particularly useful.

Herein, the viability of C2C12 cells following treatment with different leucine concentrations was compared to a negative control (i.e., leucine deprivation). Intriguingly, the viability of cells in the control groups were not severely affected by leucine deprivation. Results of differential cell counting, together with data from the published literature (Everhart and Prescott, 1972), point to the possibility that leucine deprivation may have induced cell cycle arrest. However, this conclusion requires further confirmation. Therefore, defining the mechanisms that mediated the response of C2C12 myoblasts to leucine deprivation is another possibility for future work. Importantly, this result suggests the need for an alternative control, other than leucine withdrawal, to evaluate the effects of leucine on muscle cells. The use of negative controls, especially in biology, is quite useful for detecting and reducing sources of suspected and unsuspected bias (Lipsitch *et al*., 2010). Nevertheless, our results suggest that leucine deprivation alters the physiology of C2C12 cells; therefore, its routine use as a control for leucine research is questionable.

Finally, exposure to a supraphysiological concentration of leucine compromised the viability of proliferating C2C12 cells. Yet again, because this was not associated with massive cell death, it is unclear if this decline in cell viability was due to a cytotoxic or a cytostatic effect of this high leucine concentration. In order to reach a conclusive answer, future studies should investigate the events that mediate the response of C2C12 myoblasts to long-term exposure to high leucine concentrations.

8.8.5. Evaluating the effect of leucine on the total protein content of C2C12 cells

Leucine is well-known for increasing the rate of MPS and decreasing the rate of MPB (Anthony *et al*., 2001; Lynch *et al*., 2003; Lang and Frost, 2005). However, the rates of MPS and MPB, following stimulation of C2C12 cells with leucine, were not assessed in this thesis. Alternatively, the total protein content of C2C12 cells was measured following leucine and/or glutamine treatment. Although measuring the total protein of cells is a direct reflection of the overall effect of leucine treatment, it does not discriminate whether a protein gain is due to enhanced MPS or decreased MPB. Therefore, assessing the rates of MPS and MPB could be a

target for future work. Importantly, as detailed earlier, relying on the mere assessment of MPS should be avoided. Instead, both the rate of MPS and the total protein content of muscle cells, should be measured to provide an accurate evaluation of the protein effects of leucine.

8.8.6. Investigating the signalling pathways that mediate the protein anabolic effects of leucine on C2C12 cells

Theoretically, leucine exerts its protein anabolic effects through the activation of mTOR signalling and the phosphorylation of its downstream substrates p70 S6K and 4E-BP1 (Von Manteuffel *et al*., 1996). However, results given in chapter 6 indicate a possible role for the GSK-3β and PI3/Akt signalling pathways in mediating the anabolic protein effects of leucine and the leucine-induced phosphorylation of mTOR substrates. Therefore, additional studies are required to determine the exact roles of PI3/Akt and MAPK in mediating the effects of leucine on C2C12 cells. Our results also indicate a possibility that other signalling pathways mediate the effects of leucine on muscle cells. We screened the phosphorylation and cleavage of 18 intracellular proteins, using the PathScan® Intracellular Signaling Membrane Arrays, to identify candidates for future work. However, as these arrays are semi-quantitative, there was no clear difference between the treated and control groups. Consequently, future studies need to aim at screening the phosphorylation of various signalling pathways in muscle cells treated with leucine and/or glutamine, using quantitative assays.

8.8.7. Examining the temporal expression of myomiRs in C2C12 cells

miRNAs are a relatively newly recognised class of non-coding RNAs. The identification of miRNAs as powerful regulators of gene expression unleashed new opportunities to control the expression of genes associated with many ailments. However, a common characteristic of all miRNAs is their ability to target tens, or even hundreds, of genes (Friedman *et al*., 2009). This pleiotropic property of miRNAs has hindered the identification of their targets and, consequently, the identification of their exact functions in biological systems. This thesis indicated that leucine alters the expression of myomiRs in C2C12 myotubes. However, due to the scarce information regarding the functions of myomiRs in differentiated muscle cells, we could not identify their precise role in mediating leucine action on C2C12 myotubes. Thus, identifying the possible targets of these miRNAs in differentiated muscle cells is warranted.

Also, we attempted to screen the expression of more miRNAs using high throughput (HTP) arrays. However, due to unforeseen events, we could not pursue this further. Therefore, HTP profiling of the expression of miRNAs in C2C12 cells following exposure to leucine poses an area for future work.

Finally, despite that qRT-PCR remains the gold standard for testing the expression levels of miRNAs, there is no consensus about the ideal normalization strategy for miRNAs in qRT-PCR studies (Schwarzenbach *et al*., 2015). Nonetheless, the non-coding small nuclear RNAs (snRNAs); RNU6A and RNU6B has been the most used reference genes in miRNA qRT-PCR assays (Schwarzenbach *et al*., 2015). In the present study the Hs_RNU6B was used as an internal control. The decision to use a single internal control was supported by many studies in the published literature. Our results showed that the differences in the Cp values of the internal control were around 1, which indicates the stability of our internal control across treatments. However, future studies should aim at implementing more than one internal control.

8.8.8. Final remarks

The consistent theme throughout this thesis was the assessment of the effects of leucine on primary and continuous muscle cells grown *in vitro*. Testing the effects of leucine on cultured cells has many advantages. Nonetheless, testing the effects of leucine *in vivo*, using animal models, will provide valuable insights into the actions of leucine in biological systems. Also, determining the optimal leucine dose and duration of treatment in these models, as pointed out in chapters 5 and 6, would provide criteria for a successful therapeutic strategy to induce muscle hypertrophy in animals and humans. Also, the effects of a few different concentrations of leucine, on C2C12 cells, were evaluated in this thesis. Future studies should aim at examining a wider range of leucine concentrations on different muscle cell lines. This would be important for identifying the optimal concentration of leucine required to achieve efficacy. It would also help identify if the protein anabolic effects of leucine are cell specific.

9. References

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Appendices

Appendix. 1

This shows the sources and dilutions of primary antibodies used in this thesis.

Antibodies	Source	Dilution
p/ERK1/2$^{thr202/tyr204}$	Cell Signaling Technology	1:5000
ERK1/2	Cell Signaling Technology	1:5000
p/p70 S6K^{thr389}	Cell Signaling Technology	1:100
p70 S6K	Cell Signaling Technology	1:2000
p/4E-BP1	Cell Signaling Technology	1:5000
4E-BP1	Cell Signaling Technology	1:5000
GSK-3β	Cell Signaling Technology	1:500
p/Akt^{ser473}	Cell Signaling Technology	1:1000
β-actin (13e5)	Cell Signaling Technology	1:15000
Desmin clone d33	Thermo Fisher Scientific	1:200
MyHC (*fast*)	Sigma-Aldrich	1:400
Pax7	R&D Systems	1:50
Mcad	Santa Cruz	1:100
MyoD	Santa Cruz	1:50

Abbreviations: p = phosphorylated; ERK = extracellular signal regulated kinase; p70 S6K = ribosomal protein S6 kinase; 4E-BP1 = eukaryotic translation initiation factor 4E binding protein; GSK = glycogen synthase kinase; Akt = protein kinase B; MyHC = myosin heavy chain; Mcad = m-cadherin; Pax7 = paired box 7; MyoD = myoblast determination protein 1.

Appendix. 2

This shows the compositions of media used in this thesis.

1. Full GM

DMEM + 4.5mg/L D-glucose + 0.8 mM L-leucine (Gibco, Thermo Fisher Scientific), supplemented with 10% *v/v* heat inactivated (HI) foetal calf serum (FCS) (Sigma-Aldrich), 2 mM L-glutamine (Sigma-Aldrich) and 1% *v/v* antibiotic/antimycotic mixture (Thermo Fisher Scientific).

2. GM

DMEM + 4.5mg/L D-glucose (MP biomedicals, USA) supplemented with 10% *v/v* HI FCS (Sigma-Aldrich) and 1% *v/v* antibiotic/antimycotic mixture (Thermo Fisher Scientific).

3. Full DM

DMEM + 4.5 mg/L D-glucose + 0.8 mM L-leucine (Gibco, Thermo Fisher Scientific), supplemented with 2% *v/v* HS (Sigma-Aldrich), 2 mM L-glutamine (Sigma-Aldrich) and 1% *v/v* antibiotic/antimycotic mixture (Thermo Fisher Scientific).

4. DM

DMEM + 4.5 mg/L D-glucose (MP Biomedicals) supplemented with 2% *v/v* HS (Sigma-Aldrich) + 1% *v/v* antibiotic/antimycotic mixture (Thermo Fisher Scientific).

5. SM

DMEM + 4.5 mg/L D-glucose + 0.8 mM L-leucine (Gibco, Thermo Fisher Scientific) supplemented with 2 mM L-glutamine (Sigma-Aldrich) and 1% *v/v* antibiotic/antimycotic mixture (Thermo Fisher Scientific).

6. StM

The StM was composed of DMEM + 4.5 mg/L D-glucose (MP Biomedicals) supplemented with 1% *v/v* antibiotic/antimycotic mixture (Thermo Fisher Scientific).

7. GM-Gln

DMEM + 4.5 mg/L D-glucose + 0.8 mM L-leucine (Gibco, Thermo Fisher Scientific) supplemented with 0.8 mM L-leucine, 10% *v/v* HI FCS (Sigma-Aldrich) and 1% *v/v* antibiotic/antimycotic mixture (Thermo Fisher Scientific).

8. dGM

DMEM + 4.5mg/L D-glucose + 0.8 mM L-leucine (Gibco, Thermo Fisher Scientific) supplemented with 10% *v/v* dialysed FCS (Sigma-Aldrich), 2 mM L-glutamine (Sigma-Aldrich) and 1% *v/v* antibiotic/antimycotic mixture (Thermo Fisher Scientific).

Appendix. 3

This shows the compositions of the Click-iT® Reaction Cocktail Mix for the Click-iT EdU proliferation assay.

Reagent	**Volume (µl)**
• 1X Click-It® Reaction Buffer	430
• $CUSO_4$	20
• Alexa Fluor® Azide	1.2
• Reaction Buffer Additive	50
• Total volume	500

Abbreviations: 1X = one time; µl = microlitre.

Appendix. 4

This shows the preparation of BSA standards for the BCA protein assay. BSA standards were prepared as serial dilutions from 2mg/ml BSA stock solution in RIPA lysis buffer.

Volume of BSA Stock solution (µl)	Vial	Volume of diluent (µl)	Final BSA concentration (µg/ml)
300 of stock solution	A	0	2000
375 of stock solution	B	125	1500
325 of stock solution	C	325	1000
175 of vial B	D	175	750
325 of vial C	E	325	500
325 of vial E	F	325	250
325 of vial F	G	325	125
100 of vial G	H	400	25
0	Blank	400	0

Abbreviations: BSA = bovine serum albumin; µl = microlitre; µg = microgram; ml = millilitre.

Appendix. 5

This shows protein sample preparation for gel electrophoresis. Protein samples were combined with 2X Tris-Glycine SDS Sample Buffer, double distilled water and 10X NuPAGE Reducing Agent and heated at 85°C for 2 min.

Reagents	**Volume (µl)**
• Protein sample	variable
• 2X Tris-Glycine SDS Sample Buffer	10
• Double distilled water	variable
• 10X NuPAGE Reducing Agent	2
• Total volume	20

Abbreviations: µl = microlitre; SDS = sodium dodecyl sulphate; 2X = two times; µl = microlitre.

Appendix. 6

This shows the compositions of 0.1 M glycine stripping buffer. The pH of stripping buffer was adjusted to 2.2 using HCL.

Reagents	**Weight and volume**
Glycine	15 g
SDS	1 g
Tween-20	20 ml
Double distilled water	till 1000 ml

Abbreviations: M = molar; SDS = sodium dodecyl sulphate; g = gram; ml = millilitre; HCL = hydrochloric acid.

Appendix. 7

This shows the compositions of Reverse Transcription Mix for the reverse transcription reaction.

Reagents	Volume (µl)
• 5X miScript HiSpec Buffer	2
• 10X miScript Nucleics Mix	4
• RNase-free water	variable
• miScript Reverse Transcriptase Mix	2
• Template RNA	variable
• Total volume	20

Abbreviations: 5X = five times; 10X = ten times; µl = microlitre; RNA = ribonucleic acid.

Appendix. 8

This shows the miRNA forward primers.

Primers	miRbase ID	Sanger accession No.
miR-1	MIMAT0000123	MI0000139
miR-133a	MIMAT0000145	MI0000159
miR-133b	MIMAT0000769	MI0000821
miR-199a	MIMAT0000229	MI0000241
miR-206	MIMAT0000239	MI0000249
miR-499	MIMAT0003482	MI0004676
Hs_RNU6	_____	MS00033740

Abbreviations: miR= microRNA gene; ID = identification number; No. = number.

Appendix. 9

This shows the Reaction Mix for qRT-PCR.

Reagents	Volume (µl)
• 2X QuantiTect SYBR Green PCR Master Mix	12.5
• 10X miScript Universal Primer	2.5
• 10X miScript Primer Assay	2.5
• Template cDNA	2
• RNase free water	5.5
• Total volume	25

Abbreviations: 2X = two times; 10X = ten times; µl = microlitre; PCR = polymerase chain reaction; cDNA = complimentary DNA.

Appendix. 10

This shows the cycling conditions for qRT-PCR.

Steps	Temperature	Duration
Initial activation step	95 ºC	15 minutes
Denaturation	94 ºC	15 seconds
		(45 cycles)
Annealing	55 ºC	30 seconds
Extension	70 ºC	30 seconds

Abbreviation: ºC = degree centigrade.

Appendix. 11

This shows the weights of broiler and layer chickens in grams at the hatching, 7th and 14th day of life.

	Broilers	Layers
Body weight at hatching (g)	46	36
	44	44
	43	31
	52	29
	48	35
	54	33
	47	31
	48	34
	48	35
Body weight at 7 days (g)	128	84
	115	85
	121	69
	132	73
	112	81
	129	71
	114	66
	103	56
	118	75
Body weight at 14 days (g)	279	137
	266	153
	318	159
	281	171
	259	155
	223	147
	318	161

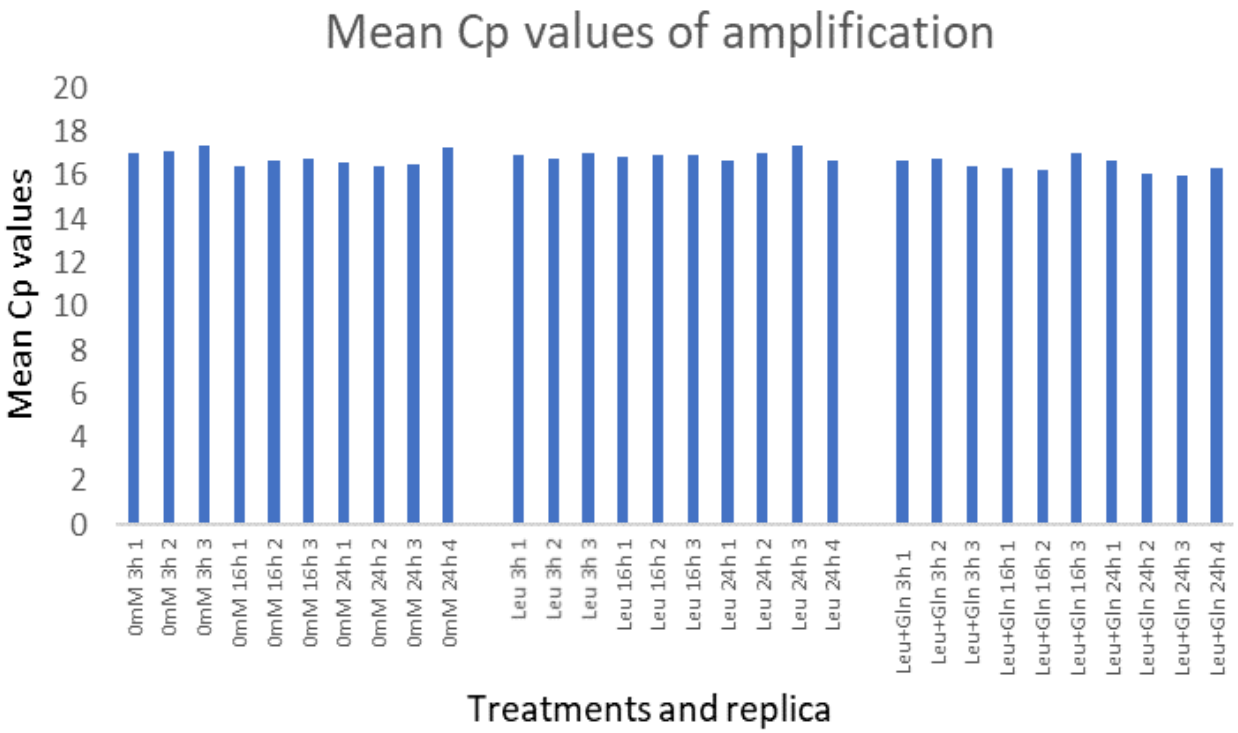
Appendix. 12

This shows the dietary requirements for broiler and layer chickens during the first 6 weeks of life.

Dietary requirements	Broilers	Layers
Energy (Kcal)	3200	2900
Leucine (%)	20-23	18
Total Protein (%)	1.18-1.35	1

(Nutrient Requirements of Chickens and Turkeys, 1993, Joffre D. Firman, Department of Animal Sciences, University of Missouri). Abbreviation: Kcal = kilocalorie.

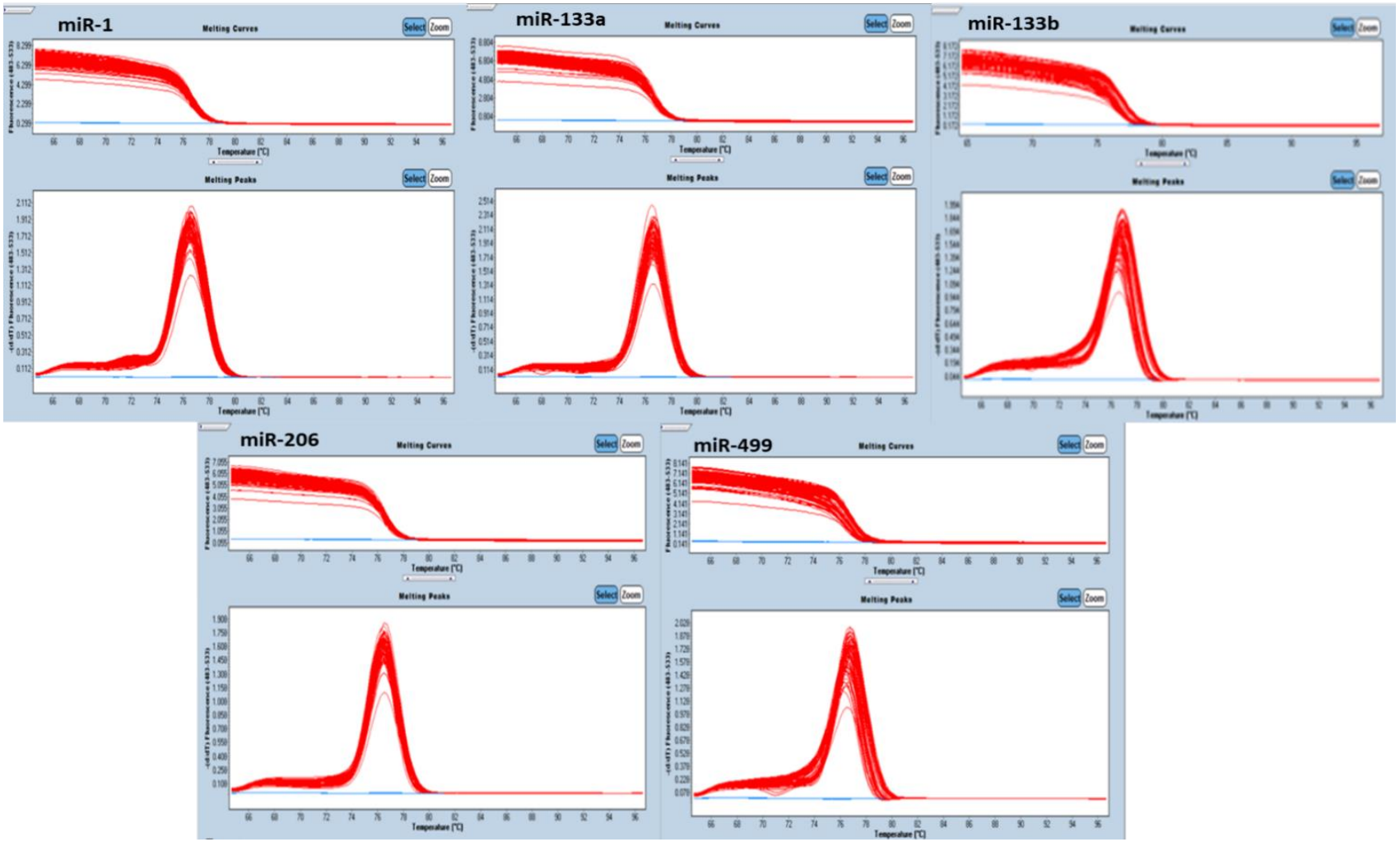
Appendix. 13



A bar chart showing the mean Cp values of Hs_RNUB amplification across treatments. Abbreviations: Leu =leucine, Gln = glutamine; mM = millimolar, h = hour; Cp = crossing points.

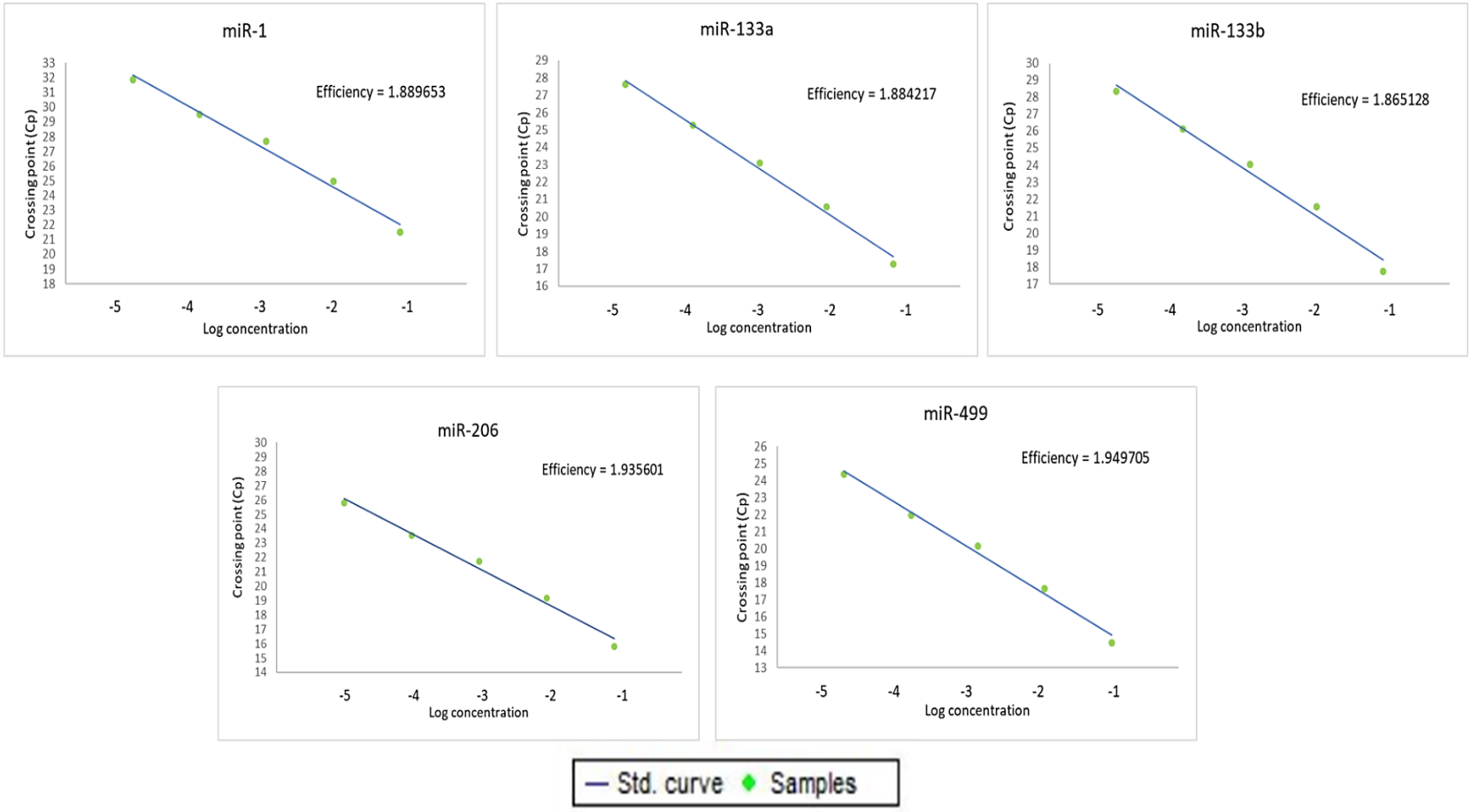
Appendix. 14

The shows the melting curves and melting peaks of miR-1, miR-133a, miR-133b, miR-206 and miR-499 amplification.



Appendix. 15

This shows the standard curves and efficiencies of miR-1, miR-133a, miR-133b, miR-206 and miR-499.



Abbreviations: Std. = standard; miR = microRNA gene; Cp = crossing points.