

EFFECT OF EGG YOLK EXTRACT ON PROLIFERATION AND MYOGENIC  
DIFFERENTIATION OF C2C12 MYOBLASTS

by

DEVIKA UMESH JOGLEKAR

A thesis submitted to the

Graduate School-New Brunswick

and

The Graduate School of Biomedical Sciences

Rutgers, The State University of New Jersey

In partial fulfillment of the requirements

For the degree of

Master of Science

Graduate Program in Biomedical Engineering

Written under the direction of

Joseph W. Freeman

And approved by

---

---

---

New Brunswick, New Jersey

October 2016

## ABSTRACT OF THE THESIS

### **Effect of Egg Yolk Extract on Proliferation and Myogenic Differentiation of C2C12**

#### **Myoblasts**

By DEVIKA UMESH JOGLEKAR

Thesis Advisor: Dr Joseph W. Freeman

Muscle wasting disorders like atrophy and sarcopenia are characterized by compromised skeletal muscle mass and function. One of the many causes of muscle wasting reported is the failure of satellite cells to fuse and form myofibres. Satellite cells are an integral part of skeletal muscle regeneration because of their self-renewal and differentiation abilities. Muscle regeneration research with respect to satellite cells has stimulated interest in discovering factors that enhance proliferation and differentiation as a first step in the regenerative process. The importance of nutrition in regulating muscle mass and muscle protein synthesis is well established. However, limited literature is available regarding the role of nutrition in muscle regeneration; specifically with respect to satellite cells. As for nutrition, chicken egg yolk appears to be a promising candidate. Egg yolks are rich in nutrients such as vitamins, minerals, amino acids and proteins which can provide sustained supply of nutrition to the cells in-vitro. In the present study, we tested the effect of different fractions and concentrations of egg yolk extract on C2C12 myoblasts. For this purpose, egg yolk extract was supplemented in growth media and cells were grown in this nutrient rich media for one week. Cell viability, fluorescence imaging and mRNA expression of muscle specific genes were analyzed at specific time points of proliferation

and differentiation stages of myoblast. The study results showed that supplementation of the egg yolk extract in media enhanced proliferation and differentiation of myoblasts in a dose dependent manner which was analyzed by increase in cell viability, number of nuclei, number of myofibres and mRNA expression of muscle specific genes such as MyoD and myogenin.

## ACKNOWLEDGEMENTS

I thank my advisor Dr Joseph Freeman for his constant and endless support throughout this project. I would also like to thank my committee members, Dr Ronke Olabisi and Dr Francois Berthiaume for their guidance and appreciation.

In addition, I thank all my fellow lab members of MoTR for their encouragement. I am also grateful to Dr David Shreiber, the director of Biomedical Engineering program at Rutgers University.

I would like to dedicate my thesis to my parents for their unwavering support and love. Finally, I am thankful to Almighty God for giving me strength and hope.

## Table of Contents

ABSTRACT OF THE THESIS.....	ii
ACKNOWLEDGEMENTS.....	iv
Table of Contents.....	v
List of Figures.....	ix
List of Tables.....	xii
CHAPTER 1- Introduction.....	1
1.1 Overview.....	1
1.2 Objectives.....	3
CHAPTER 2- Literature Review.....	5
2.1 Overview of Muscle Regeneration.....	5
2.2 Satellite Cells in Skeletal Muscle Regeneration: Activation, Differentiation and Self- Renewal.....	6
2.3 C2C12 Myoblasts: Model of Skeletal Muscle Regeneration.....	8
2.4 Potential Therapeutic Strategies for Muscle Regeneration.....	8
CHAPTER 3- Cell Viability, Fluorescence Imaging and mRNA Expression of Muscle Specific Genes of C2C12 Myoblasts.....	12
3.1 Materials and Methods.....	12
3.1.1 Preparation of Egg Yolk Solution.....	12
3.1.2 Cell Culture.....	12
3.1.2.1 Proliferation of C2C12 Myoblasts in Growth Media (10% FBS) Supplemented with Egg Yolk Extract.....	13

3.1.2.2 Proliferation of C2C12 Myoblasts in Growth Media (10% FBS) Supplemented with Higher Concentration of Egg Yolk Extract.....	13
3.1.2.3 Myogenic Differentiation of C2C12 Myoblasts in Differentiation Media (2% FBS) Supplemented with Egg Yolk Extract.....	14
3.1.2.4 Myogenic Differentiation of C2C12 Myoblasts in Differentiation Media (2% FBS) Supplemented with Higher Concentration of Egg Yolk Extract.....	14
3.1.3 Cell Viability.....	15
3.1.4 Cytochemical Technique and Florescence Imaging.....	15
3.1.5 Real Time Quantitative Polymerase Chain Reaction (qPCR).....	16
3.1.6 Statistical Analysis.....	17
3.2 Results.....	18
3.2.1 Cell Viability Studies.....	18
3.2.1.1 Proliferation of C2C12 Myoblasts in Growth Media Supplemented with Egg Yolk Extract.....	18
3.2.1.2 Proliferation of C2C12 Myoblasts in Growth Media Supplemented with Higher Concentration of Egg Yolk Extract –Filtered Media.....	19
3.2.1.3 Proliferation of C2C12 Myoblasts in Growth Media Supplemented with Higher Concentration of Egg Yolk Extract –Unfiltered Media.....	20
3.2.1.4 Myogenic Differentiation of C2C12 Myoblasts in Differentiation Media Supplemented with Egg Yolk Extract.....	21
3.2.1.5 Myogenic Differentiation of C2C12 Myoblasts in Differentiation Media Supplemented with Higher Concentration Egg Yolk Extract-Unfiltered Media.....	22

3.2.1.6 Myogenic Differentiation of C2C12 Myoblasts in Differentiation Media Supplemented with Higher Concentration Egg Yolk Extract-Filtered Media.....	23
3.2.2 mRNA Expression Of Muscle Specific Genes.....	23
3.2.2.1 Filtered Media.....	24
3.2.2.2 Unfiltered Media.....	27
3.2.3 Fluorescence Image Analysis.....	30
3.2.3.1 Proliferation of C2C12 Myoblasts in Growth Media Supplemented with Egg Yolk Extract.....	30
3.2.3.2 Proliferation of C2C12 Myoblasts in Growth Media Supplemented with Higher Concentration of Egg Yolk Extract –Filtered Media.....	31
3.2.3.3 Proliferation of C2C12 Myoblasts in Growth Media Supplemented with Higher Concentration of Egg Yolk Extract –Unfiltered Media.....	33
3.2.3.4 Myogenic Differentiation of C2C12 Myoblasts in Differentiation Media Supplemented with Egg Yolk Extract.....	34
3.2.3.5 Myogenic Differentiation of C2C12 Myoblasts in Differentiation Media Supplemented with Higher Concentration Egg Yolk Extract-Unfiltered Media.....	36
3.2.3.6 Myogenic Differentiation of C2C12 Myoblasts in Differentiation Media Supplemented with Higher Concentration Egg Yolk Extract-Filtered Media.....	37
3.3 Discussion.....	39
3.3.1 Effect of Egg Yolk Extract on Myoblast Proliferation and Growth.....	39
3.3.2 Effect of Egg Yolk Extract on Myoblast Differentiation and Fusion.....	43
CHAPTER 4- Conclusion and Future Perspectives.....	47
REFERENCES.....	49

## List of Figures

Figure 1: Cell Viability Using Presto Blue Assay (n=4). Media with 10% Fetal Bovine Serum (FBS) Supplemented with 500µg/ml Egg Yolk Extract.....	18
Figure 2: Cell Viability Using Presto Blue Assay (n=4). Filtered Media with 10% Fetal Bovine Serum (FBS) Supplemented with Higher Concentration of Egg Yolk Extract.....	19
Figure 3: Cell Viability Using Presto Blue Assay (n=4). Unfiltered Media with 10% Fetal Bovine Serum (FBS) Supplemented With Higher Concentration of Egg Yolk Extract.....	20
Figure 4: Cell Viability Using Presto Blue Assay (n=4). Media with 2% Fetal Bovine Serum (FBS) Supplemented with 500µg/ml Egg Yolk Extract.....	21
Figure 5: Cell Viability Using Presto Blue Assay (n=4). Unfiltered Media with 2% Fetal Bovine Serum (FBS) Supplemented with Higher Concentration of Egg Yolk Extract.....	22
Figure 6: Cell Viability Using Presto Blue Assay (n=4). Filtered Media with 2% Fetal Bovine Serum (FBS) Supplemented with Higher Concentration of Egg Yolk Extract.....	23
Figure 7: mRNA Expression of MyoD on Day 3. Filtered Media with 2% Fetal Bovine Serum (FBS) Supplemented with Different Concentrations of Egg Yolk Extract.....	24
Figure 8: mRNA Expression of Myogenin 1 on Day 3. Filtered Media with 2% Fetal Bovine Serum (FBS) Supplemented with Different Concentrations of Egg Yolk Extract.....	25



Figure 9: mRNA Expression of Myogenin 4 on Day 3. Filtered Media with 2% Fetal Bovine Serum (FBS) Supplemented with Different Concentrations of Egg Yolk Extract.....	26
Figure 10: mRNA Expression of MyoD on Day 3. Unfiltered Media with 2% Fetal Bovine Serum (FBS) Supplemented with Different Concentrations of Egg Yolk Extract.....	27
Figure 11: mRNA Expression of Myogenin 1 on Day 3. Unfiltered Media with 2% Fetal Bovine Serum (FBS) Supplemented with Different Concentrations of Egg Yolk Extract.....	28
Figure 12: mRNA Expression of Myogenin 4 on Day 3. Unfiltered Media with 2% Fetal Bovine Serum (FBS) Supplemented with Different Concentrations of Egg Yolk Extract.....	29
Figure 13: Fluorescence Images of C2C12 Myoblasts on Day 7. Proliferation of C2C12 Myoblasts in A: Control Media, B: Filtered 500µg/ml Egg Yolk Supplemented Media and C: Unfiltered 500µg/ml Egg Yolk Supplemented Media.....	30
Figure 14: Fluorescence Images of C2C12 Myoblasts on Day 7. Proliferation of C2C12 Myoblasts in A: Control Media, B: Filtered 1mg/ml Egg Yolk Supplemented Media and C: Filtered 10mg/ml Egg Yolk Supplemented Media.....	32
Figure 15: Fluorescence Images of C2C12 Myoblasts on Day 7. Proliferation of C2C12 Myoblasts in A: Control Media 1, B: Unfiltered 1mg/ml Egg Yolk Supplemented Media, C: Control Media 2 and D: Unfiltered 10mg/ml Egg Yolk Supplemented Media.....	33
Figure 16: Fluorescence Images of C2C12 Myoblasts on Day 7. Myogenic Differentiation of C2C12 Myoblasts in A: Control Media, B: Filtered 500µg/ml Egg Yolk	

Supplemented Media and C: Unfiltered 500µg/ml Egg Yolk Supplemented Media.....35

Figure 17: Fluorescence Images of C2C12 Myoblasts on Day 7. Myogenic Differentiation of C2C12 Myoblasts in A: Control Media, B: Unfiltered 1mg/ml Egg Yolk Supplemented Media and C: Unfiltered 10mg/ml Egg Yolk Supplemented Media.....36

Figure 18: Fluorescence Images of C2C12 Myoblasts on Day 7. Myogenic Differentiation of C2C12 Myoblasts in A: Control Media, B: Filtered 1mg/ml Egg Yolk Supplemented Media and C: Filtered 10mg/ml Egg Yolk Supplemented .....38

## List of Tables

Table 1: qPCR Primer Sequences.....	17
Table 2: Image Analysis Using ImageJ for Filtered and Unfiltered 500µg/ml Egg Yolk Supplemented Media (10%FBS).....	31
Table 3: Image Analysis Using ImageJ for Filtered 1mg/ml and 10mg/ml Egg Yolk Supplemented Media (10%FBS).....	32
Table 4: Image Analysis Using ImageJ for Unfiltered 1mg/ml and 10mg/ml Egg Yolk Supplemented Media (10%FBS).....	34
Table 5: Image Analysis Using ImageJ for Filtered and Unfiltered 500µg/ml Egg Yolk Supplemented Media (2%FBS).....	35
Table 6: Image Analysis Using ImageJ for Unfiltered 1mg/ml and 10mg/ml Egg Yolk Supplemented Media (2%FBS).....	37
Table 7: Image Analysis Using ImageJ for Filtered 1mg/ml and 10mg/ml Egg Yolk Supplemented Media (2%FBS).....	38
Table 8: Summary of Proliferation Study.....	42
Table 9: Summary of Differentiation Study.....	46

## **CHAPTER 1 - Introduction**

### **1.1 Overview**

Skeletal muscle is a highly organised striated muscle tissue which comprises of long multinucleated cells called myofibres. It is the most abundantly found muscle type in the body. In developmental stages, mononucleated myogenic progenitors called myoblasts fuse together to form multinucleated myofibres through a multistep process known as myogenesis. This differentiation of myoblasts into myofibres is achieved in neonatal stages and the number of fibres remains constant thereafter [1, 2]. However, these myofibres grow in size in postnatal stages by fusing with muscle stem cells. In normal conditions, the skeletal muscle is stable and promotes sporadic satellite cell fusion if required for muscle turnover [2]. In mammals, skeletal muscle regeneration takes place at molecular, cellular or tissue level which is driven by various intrinsic and extrinsic factors.

Alleviating poor muscle regeneration or ameliorating dysfunctional muscle regeneration can be achieved by implementing these new therapeutic strategies that are targeted at cellular level. There are different aspects of skeletal muscle regeneration that individually and later collectively contribute to reformation of innervated and vascularised muscle tissue. These aspects include the proliferation or differentiation of myogenic stem cells, regulation of molecules, up regulation or down regulation of muscle specific genes or

ageing to name a few [3-8]. Efforts are being made to explore each of these aspects to better understand and improve research in muscle regeneration.

Muscle wasting disorders like atrophy and sarcopenia are characterized by compromised or loss of skeletal muscle mass and function [9-13]. The failure of satellite cells to fuse and form myofibres or decrease in their number and function are reported to cause such disorders [14, 15]. The regenerative ability is thought to be compromised due to the decreased response of these cells to various growth factors and agents or due to decrease in availability of sufficient growth factors that help in maintenance and sustenance of muscle mass and satellite cells [2].

Importance of satellite cells in skeletal muscle regeneration, be it in neonatal stage or postnatal stage can be attributed to their self-renewal and differentiating abilities [24]. Muscle regeneration research with respect to satellite cells has stimulated interest in discovering factors that enhance proliferation and differentiation as the first step of regenerative process. It is seen that these cells respond well in-vitro to different growth factors in a way that is dose or concentration dependent [8]. Numerous studies have also revealed that hormones, stimulation, innervations and vasculature can highly influence the proliferation and differentiation of satellite cells [2, 17]. Together it can be deduced that microenvironment of satellite cells do affect their behaviour and function.

Dysregulation of myogenic precursors that contribute to muscle loss is an emerging field of research. The importance of nutrition in regulating muscle mass and muscle protein

synthesis is well established [9]. However, limited literature is available regarding the role of nutrition on muscle regeneration; specifically with respect to satellite cells. As for nutrition, chicken egg yolk appears to be a promising candidate for providing sustained supply of nutrition to the cells in-vitro. Chicken egg yolk contains cholesterol, vitamins, minerals, amino acids and proteins. In the present study, we tested the effect of different concentrations of fertilized egg yolk extract on C2C12 myoblasts. For this purpose, egg yolk extract was supplemented in growth media and cells were grown in this nutrient rich media for one week. Cell viability, fluorescence imaging and mRNA expression of genes were analyzed at specific time points of proliferation and differentiation stages of myoblasts.

## **1.2 Objectives**

Specific aims of this study were:

1. To assess effect of egg yolk extract on myoblast proliferation and growth
2. To assess effect of egg yolk extract on myoblast fusion and differentiation

In order to achieve these objectives, following experiments were designed, conducted and analyzed:

C2C12 myoblasts were grown in Dulbecco modified eagle medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS) and 1% penicillin and streptomycin (P/S). The cell line was maintained in growth medium at 37°C in 5% CO<sub>2</sub> atmosphere. The cell studies were carried out in two sets of condition media: proliferation

media (10%FBS) supplemented with egg yolk extract and differentiation media (2%FBS) supplemented with egg yolk extract. To determine the effects of different concentration of egg yolk extract on cell proliferation and differentiation: 500 $\mu$ g/ml, 1mg/ml and 10mg/ml concentrations were used. All the cell studies were carried out for 7 days. Cell viability was measured on days 1, 3 and 7 of every cell study. Cell viability was determined using Presto Blue Assay with incubation period of 1 hour. The results were calculated in form of absorbance values using average values and standard deviations. The cells were fixed on day 7 and prepared for staining using cytochemical technique. The cells were stained with phalloidin and DAPI to detect nuclei and actin fibres. Fluorescence images were captured using a confocal microscope. Image analysis was carried out using ImageJ software to determine number of nuclei, number of myotubes and fusion index. For mRNA expression, PCR was carried out on day 3. The cells were lysed on day 3 to extract RNA and then reverse transcribed to form cDNA. qPCR was then carried out using cDNA and specific gene primers using SYBR green technique.

## CHAPTER 2- LITERATURE REVIEW

### 2.1 Overview of Muscle Regeneration

Muscle regeneration is a process that has three main sequential stages: 1) inflammation; 2) satellite cell activation, differentiation and self-renewal; and 3) maturation and remodelling of regenerated myofibres [1, 2]. These stages could also overlap when necessary. Muscle degeneration occurs when myofibre undergoes necrosis caused due to trauma, injury or mechanical damage to the muscle tissue [1, 2]. This event is marked by disruption of organised sarcolemma within myofibres which eventually breaks the myofibre integrity. Necrosis then activates cascade of inflammatory responses which attracts neutrophils and leucocytes to the injured area. Macrophages then invade the site of injury and remove the necrotic muscle tissue. During this stage, cytokines (e.g. TNF- $\alpha$ ) and interleukins (e.g. IL-1) are released [1, 2]. All these events are reported to facilitate proliferation and differentiation of satellite cells (myoblasts). Proliferation and differentiation of satellite cells is the hallmark of muscle regeneration process. Proliferation of satellite cells results in increased number of nuclei for differentiation and fusion which is required for muscle repair. Few studies have reported that muscle regenerative capacity is drastically challenged if the cell proliferation is blocked by treatments such as irradiation or colchicines [18, 19]. Following proliferation, satellite cells differentiate and fuse together to form myofibres. For regeneration of complete functional muscle, reinnervation and maturation of newly formed muscle fibres is required. Maturation of regenerated myofibres is associated with increase in size of



myofibres and migration of myonuclei to the peripheral region of the plasma membrane [1, 2, 20, 21].

Dysfunctional muscle regeneration is characterized by reduced differentiation and fusion of satellite cells into myofibres or incomplete fusion of regenerated myofibres which lead to muscle disorders as in case of sarcopenia or atrophy. Abnormal muscle regeneration is characterized by formation of branched myofibres as in case of hypertrophy. There are many causes of muscle wasting that are directly associated with the impairment of regenerative ability of the atrophying muscle. However, one of the prevailing hypothesis in the field of skeletal muscle regeneration associated with muscle wasting disorders such as cachexia, sarcopenia or atrophy is the impairment of satellite cell proliferation, differentiation or fusion of myoblasts [2, 9-13, 22] . It is reported that in atrophied muscle number of precursor cells or the satellite cells decrease over time and it could also result due to myoblast fusion defects [12]. In such cases, the satellite cells or the precursor cells are active which is marked by increased number of muscle precursor cells. However, due to fusion defect the myoblasts are prevented from differentiating and fusing into muscle fibres.

## **2.2 Satellite Cells in Skeletal Muscle Regeneration: Activation, Differentiation and Self-Renewal**

During embryonic stage, proliferated embryonic myogenic cells (myoblasts) differentiate and finally fuse to form multinucleated myofibres. This process is termed as myogenesis. Mammalian skeletal muscle which is otherwise stable recovers immediately from normal

wear and tear of muscle [2]. Postnatal myogenesis occurs by fusion of myoblasts with pre-existing myofibres. Satellite cells are required for muscle regeneration under pathological and physiological conditions. This is supported by studies where the satellite cells that were ablated in adulthood resulted in decreased muscle regeneration [2].

Myogenesis comprises of activation, differentiation and fusion of myoblasts. This process is controlled and regulated by appropriate and specific transcription factors. Satellite cells are located in sublamina of the intact muscle and are quiescent. These quiescent cells express paired-box protein-7 (Pax-7) which maintains their population [5-7, 23-24]. Satellite cells exit their quiescent stage when exposed to trauma or any kind of muscle damage. At this stage, myogenic factor 5 (Myf-5) along with Pax-7 activate and expand satellite cells (myoblasts) [5, 7]. Once activation, these satellite cells which are now termed as adult myoblasts proliferate. Satellite niche and its microenvironment are reported to influence the proliferation of myoblasts. Proliferating myoblasts are characterized by rapid expression of myoblast determination protein (MyoD) which predicts the differentiation potential of the proliferating myoblasts. After certain amount of time, the satellite cells are programmed to enter into myogenic differentiation phase where each cell fuses into a small myofibre. Differentiating myoblasts are characterized by expression of myogenin and myocyte enhancer factor 2 (MEF 2) [23, 25]. Finally the cells fuse together to form nascent multinucleated myotubes which then mature and are characterized by expression of myosin heavy chain (MHC) [23]. Together, expression of all these muscle specific genes is necessary for proper formation of skeletal muscle and down-regulation or up-regulation of any of the genes can result in changes in the

morphology and function of the skeletal muscle. These transcription factors also dictate a proper balance between proliferation and differentiation [5-7, 24, 26]. Imbalance of these events can result in muscle disorders as seen in muscle wasting.

### **2.3 C2C12 Myoblasts: Model of Skeletal Muscle Regeneration**

C2C12 murine cell line is an excellent and extensively used in vitro model to study myoblast proliferation and differentiation [27]. They are immortalized cells capable of differentiating into myofibres within three days of culture. At the undifferentiated stage, these myoblasts are flat and fusiform mononucleated structures. They are fast replicating cells and become confluent within 24-48 hours in high serum concentrations. Differentiation can be initiated by serum removal or providing low serum after reaching confluence. Differentiation is prominent after 72 hours in low serum concentration. At this stage, mononucleated myoblasts fuse to form multinucleated myotubes (intermediate differentiation) and the transcription factors such as MyoD and myogenin are expressed [23, 27]. These then increase in size and length and appear as large myotubes with numerous nuclei which are characterized by muscle specific protein, MHC (late differentiation).

### **2.4 Potential Therapeutic Strategies for Muscle Regeneration**

Therapeutic options for ameliorating muscle wasting are constantly evolving. Many pharmacological and non-pharmacological options including the effects of exercise, electrical stimulation, laser therapy, follistatin, growth factors [28-31] etc. have been

explored which have shown positive effects in-vitro and in-vivo and can contribute to the emerging therapies for sarcopenia and atrophy.

Nutrition is an emerging field in muscle regeneration which seems to have positive effects in improving muscle wasting. Nutritional compounds such as resveratrol (found in grapes) and epigallocatechin gallate (found in green tea) [32,33] have been studied and reported to enhance satellite cell proliferation in in-vitro and in-vivo models. Effects of resveratrol (RSV) on proliferation, differentiation and hypertrophy were studied in C2C12 cells [33]. The study showed that RSV has the potential to regulate the cell cycle thereby inducing and promoting hypertrophy in vitro. Its clinical application in conditions of muscle impairment would further confirm its efficacy. Green tea extract (GTE) is another nutritional compound that is reported to stimulate regenerative capacity of the atrophied muscle. Its effect was studied in dystrophic mice where their diet was supplemented with GTE. The study showed that in dystrophic mice, GTE was able to protect hindlimb muscle from necrosis and improving the function of the muscle. It is suggested that RSV and epigallocatechin gallate act as anti-oxidant thereby reducing reactive oxygen that favours proliferation and differentiation of satellite cells [32, 33]. Coffee is another compound that improves and enhances satellite cell function for muscle regeneration. The study showed that treatment of coffee was able to increase the regenerative capacity of the injured muscles in vivo and in vitro increased proliferation of satellite cells isolated from aged mice and attenuated cell cycle [34]. Vitamin D is also reported to have positive effects on muscle cells in skeletal muscle recovery.

Supplementation of vitamin D3 in-vitro was reported to enhance and improve cell migration, myoblast fusion and differentiation [35].

Advances in stem cell therapy have presented the scope of using muscle satellite cells for muscle development and repair. Furthermore, importance of stem cell and stem cell niche has been established which can directly or indirectly affect the homeostasis and regeneration processes. To support this, few studies have shown impairment of muscle satellite cell niche can negatively influence skeletal muscle development and result in sarcopenia [2, 3, 14]. Thus, delivery of stem cells seems to be an effective therapeutic strategy. However, direct delivery of stem cells at the damaged muscle site has challenges such as limited migration and exposure to inflammatory response. To overcome this, bioengineered cell engraftment matrices have been explored recently [36]. The basic idea behind developing these matrices is that the matrices can act as delivery systems for cells along with the key elements of stem cell niche. Incorporating the key elements in these engineered matrices would enhance the function of the satellite cells in muscle regeneration. Many natural and synthetic hydrogel-based biomaterials can be used to deliver the satellite muscle cells in vitro. Collagen gels have been used to encapsulate the C2C12 myoblasts which promoted myogenesis (early muscle development) in vitro [37]. Alginate which is derived from brown algae is also used to encapsulate myoblasts in vitro. Studies have shown that RGD-conjugated alginate can successfully encapsulate myoblasts which proliferate, differentiate and fuse into multinucleated myofibres [38].

Finally, there is no specific therapeutic option for muscle wasting. Therapy would vary from person to person. All the therapeutic strategies explored so far need to be tested on humans and transition of these therapies at clinical level is needed for their application. Combination of therapies can also be explored for better efficacy.

## **CHAPTER 3- Cell Viability, Fluorescence Imaging and mRNA Expression of Muscle Specific Genes of C2C12 Myoblasts**

### **3.1 Materials and Methods**

#### **3.1.1 Preparation of egg yolk solution**

Fertilized egg yolk extract (stored at -20°C) obtained from Myos. Corp was first crushed into powder using mortar and pestle. The egg yolk extract powder was then dissolved in PBS to make egg yolk stock solution. Finally, the egg yolk solution was supplemented in media at respective concentrations.

#### **3.1.2 Cell culture**

C2C12 (ATCC) cells were expanded in Dulbecco modified eagle medium (DMEM) (Fischer Scientific) supplemented with 10% (v/v) fetal bovine serum (FBS) (Fischer Scientific) and 1% (v/v) penicillin and streptomycin (P/S) (Gibco® by Life Technologies). The cell line was maintained in the growth medium (DMEM+ 10%FBS+ 1%P/S) at 37°C in 5% CO<sub>2</sub> atmosphere. Cell culture studies were performed when about 80% cell confluence was attained. For cell studies, the cells were fed with 400µl of condition media every day. The cell proliferation, differentiation and myofibre formation were monitored daily until day 7 using a light microscope (Olympus CKX31).

### **3.1.2.1 Proliferation of C2C12 myoblasts in growth media (10% FBS) supplemented with egg yolk extract**

Cells were seeded at seeding density of 10,000 cells/cm<sup>2</sup>. The cells were grown in growth medium (DMEM+ 10%FBS+ 1%P/S) until 80% confluent. Media was then replaced with growth media supplemented with 500µg/ml egg yolk (Myos Corp) stock solution (day 0). Furthermore, the egg yolk supplemented media was filtered through 0.2µm sterile filter (Fischer Scientific). Together, the cell study had 3 groups of condition media (n=4): 1) Control media (no egg yolk extract), 2) Unfiltered growth media (500µg/ml egg yolk extract) and 3) Filtered growth media (500µg/ml egg yolk extract).

### **3.1.2.2 Proliferation of C2C12 myoblasts in growth media (10% FBS) supplemented with higher concentration of egg yolk extract**

Cells were seeded at seeding density of 10,000 cells/cm<sup>2</sup>. The cells were grown in growth medium (DMEM+ 10%FBS+ 1%P/S) until 80% confluent. Media were then replaced with growth media supplemented with 1mg/ml and 10mg/ml egg yolk stock solution respectively (day 0). The study had filtered and unfiltered media. Together, the cell study had 3 groups of condition media (n=4) for filtered and unfiltered respectively: 1) Control media (no egg yolk extract), 2) Growth media (1mg/ml egg yolk extract) and 3) Growth media (10mg/ml egg yolk extract).



### **3.1.2.3 Myogenic differentiation of C2C12 myoblasts in differentiation media (2% FBS) supplemented with egg yolk extract**

Cells were seeded at seeding density of 10,000 cells/cm<sup>2</sup>. The cells were grown in growth medium (DMEM+ 10%FBS+ 1%P/S) until 80% confluent. Media were then replaced with differentiation media (DMEM+ 2%FBS+ 1%P/S) supplemented with 500µg/ml egg yolk stock solution (day 0). Furthermore, egg yolk supplemented media was filtered through 0.2µm sterile filter. Together, the cell study had 3 groups of condition media (n=4): 1) Control media (no egg yolk extract), 2) Unfiltered differentiation media (500µg/ml egg yolk extract) and 3) Filtered differentiation media (500µg/ml egg yolk extract).

### **3.1.2.4 Myogenic differentiation of C2C12 myoblasts in differentiation media (2% FBS) supplemented with higher concentration of egg yolk extract**

Cells were seeded at seeding density of 10,000 cells/cm<sup>2</sup>. The cells were grown in growth medium (DMEM+10%FBS+1%P/S) until 80% confluent. Media were then replaced with differentiation media (DMEM+ 2%FBS+ 1%P/S) supplemented with 1mg/ml and 10mg/ml egg yolk stock solution respectively (day 0). The study had filtered and unfiltered media. Together, the cell study had 3 groups of condition media (n=4) for filtered and unfiltered respectively: 1) Control media (no egg yolk extract), 2) Differentiation media (1mg/ml egg yolk extract) and 3) Differentiation media (10mg/ml egg yolk extract).

### **3.1.3 Cell viability**

Cell viability assay was performed on days 1, 3 and 7 during cell studies. Cell viability was analyzed using Presto Blue® (Invitrogen) assay. The assay measured mitochondrial activity which indirectly detected cell viability. The assay was characterized by change in color (blue to dark violet) which was measured in form of fluorescence units. Presto Blue® solution was made by diluting the reagent (1:10) with media in dark condition. Media was removed from all wells and washed with PBS. The Presto Blue solution was then added to each well and incubated for 1 hour in dark. After incubation period, Presto Blue® solution from each well was collected in a new well plate. The readings were measured using a plate reader (Infinite M200 Pro, Tecan) with help of i-control 1.9 software. Relative absorbance for every sample was calculated using the average values and standard deviation.

### **3.1.4 Cytochemical Technique and Fluorescence Imaging**

Cytochemical technique was carried out directly on cells in well-plates on day 7. Cells were washed with PBS and fixed with 4% paraformaldehyde (PFA) at R.T. for 20 min. The cells were washed again with PBS and stored at 4°C overnight. Cells were then treated with triton-X (0.5%) solution for 15 min, washed with PBS and incubated in 1% bovine serum albumin (BSA) (Fischer Scientific) for 30 min. The cells were then stained with fluorescein phalloidin (1:20) (Life Technologies) for 20 min at R.T., washed with PBS and counter stained with DAPI (Life Technologies) for 5 min.

All the wells were imaged with spinning disc confocal microscope (Olympus IX81) to view actin fibres and nuclei of cells at 20X objective. The slidebook 5 software by 3i intelligent imaging innovations was used to capture images of the samples. ImageJ software was used to analyze number of nuclei, number of myotubes and fusion index. Fusion index was calculated using the formula: number of nuclei on myotubes / total number of nuclei [42]. All the values were calculated using average and standard deviation.

### **3.1.5 Real Time Quantitative PCR analysis**

qPCR was performed on day 3 to analyze mRNA expression of muscle specific genes such as MyoD and myogenin in cells. The cells were lysed using RLT Plus Buffer with  $\beta$ -mercaptoethanol (10  $\mu$ l of  $\beta$ -mercaptoethanol to every 1ml of RLT Plus Buffer) and RNA was extracted using RNeasy Plus mini kit (Qiagen). Total RNA was quantified using Nanodrop 2000c spectrophotometer (Thermo Scientific) and reverse transcribed to create cDNA library using high-capacity cDNA reverse transcription kit (Life Technologies) using Peltier Thermal Cycler (PTC-200, MJ Research). The resultant cDNA was amplified using the primers (Harvard Medical School Primer Bank) listed in Table 1 [43, 44, 45]. PikoReal 96™ Real-Time PCR System (Thermo Fisher Scientific) was used to analyze the PCR products. qPCR measurement was performed in triplicates. SYBR green technique was used to detect the PCR products. The relative quantification of real time PCR was carried out according to the method mentioned in [38, 39]. The values were normalized to a reference gene, GAPDH.

**Table 1:** qPCR Primer Sequences

Gene	Primer Sequence (5'-3')
<b>MyoD</b> - Forward [43]	CGG GAC ATA GAC TTG ACA GGC
<b>MyoD</b> - Reverse [43] PrimerBank ID: 170172578c1	TCG AAA CAC GGG TCA TCA TAG A
<b>Myogenin 1</b> - Forward [44]	GAG ACA TCC CCC TAT TTC TAC CA
<b>Myogenin 1</b> - Reverse [44] PrimerBank ID: 13654247a1	GCT CAG TCC GCT CAT AGC C
<b>Myogenin 4</b> - Forward [45]	CGA TCT CCG CTA CAG AGG C
<b>Myogenin 4</b> - Reverse [45] PrimerBank ID: 162287254c3	GTT GGG ACC GAA CTC CAG T
<b>GAPDH</b> - Forward [46]	AGG TCG GTG TGA ACG GAT TTG
<b>GAPDH</b> - Reverse [46] PrimerBank ID: 126012538c1	GGG GTC GTT GAT GGC AAC A

### 3.1.6 Statistical Analysis

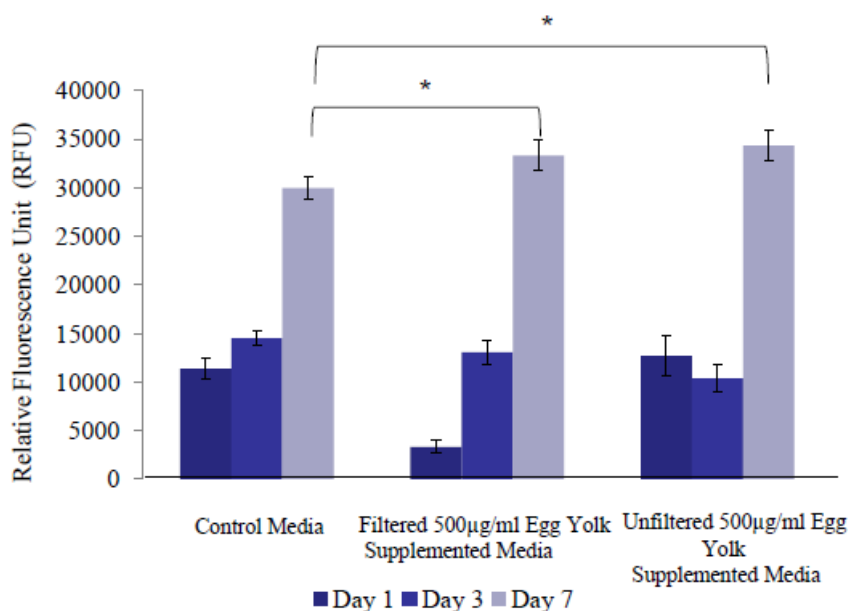
One way analysis of variance (ANOVA) was carried out using Synergy software by Kleidagraph® to determine statistical significance. A value of  $p \leq 0.05$  was considered significant using Fischer's least significant difference comparison. All the data are represented as  $\pm$  mean standard deviation ( $\pm$ S.D).

## 3.2 Results

### 3.2.1 Cell viability studies

#### 3.2.1.1 Proliferation of C2C12 myoblasts in growth media supplemented with egg yolk extract

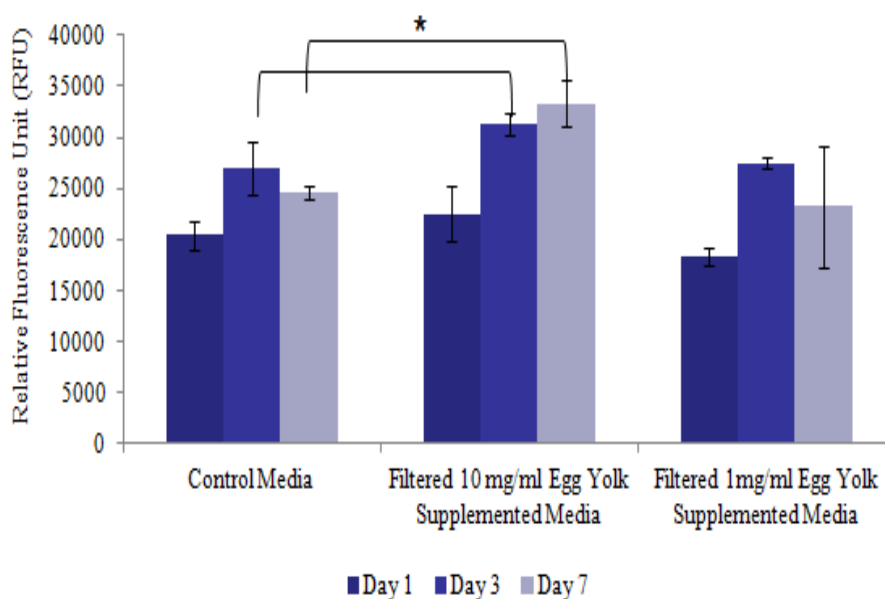
Cell viability increased from day 1 to day 7 for control media and filtered media supplemented with 500µg/ml egg yolk. However, the cell viability decreased on day 2 and then increased on day 7 for unfiltered media supplemented with 500µg/ml egg yolk. Together, the cell viability was highest on day 7 as expected. Also, the cell viability on day 7 was significantly higher ( $p \leq 0.05$ ) in egg yolk supplemented groups (filtered and unfiltered) as compared to the control group.



**Figure 1:** Cell viability using Presto Blue Assay (n=4). Media with 10% fetal bovine serum (FBS) supplemented with 500µg/ml egg yolk extract.

### 3.2.1.2 Proliferation of C2C12 myoblasts in growth media supplemented with higher concentration of egg yolk extract –Filtered Media

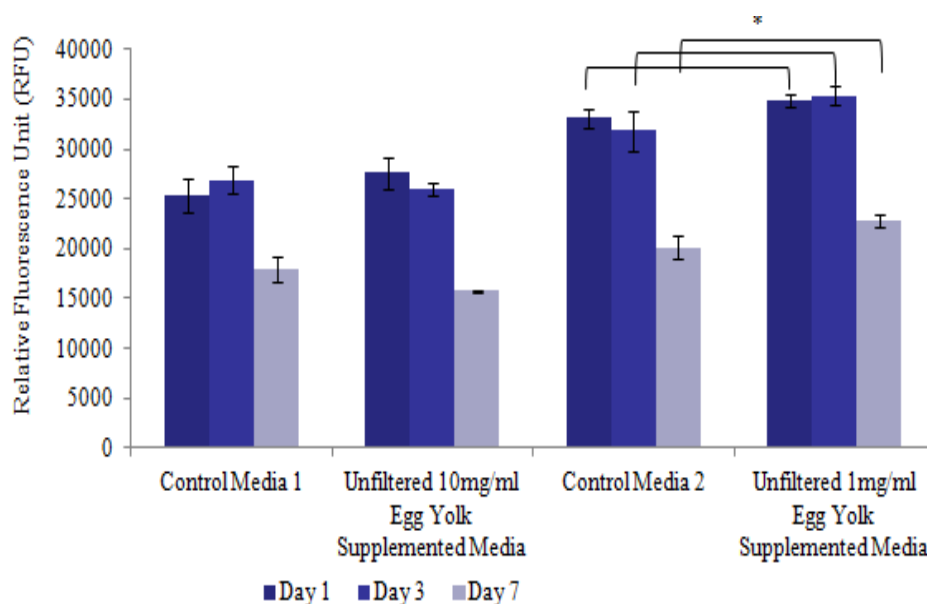
For the filtered media, cell viability increased from day 1 to day 3 and then decreased on day 7 for control media and 1mg/ml egg yolk supplemented media. However, the cell viability increased from day 1 to day 7 for 10mg/ml egg yolk supplemented media. Also, the cell viability on day 7 was highest for the 10mg/ml egg yolk supplemented media group. Statistically significant differences ( $p \leq 0.05$ ) were seen on day 3 and day 7 between control media and filtered 10mg/ml fertilized egg yolk supplemented media.



**Figure 2:** Cell viability using Presto Blue Assay (n=4). Filtered media with 10% fetal bovine serum (FBS) supplemented with higher concentration of egg yolk extract

### 3.2.1.3 Proliferation of C2C12 myoblasts in growth media supplemented with higher concentration of egg yolk extract –Unfiltered Media

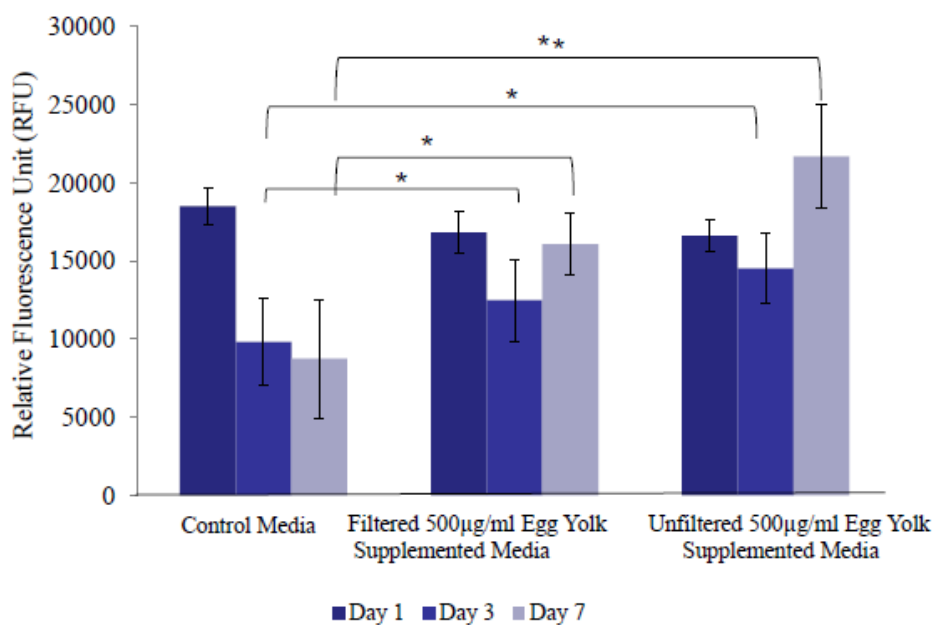
For unfiltered media, cell viability decreased from day 1 to day 7 for all the groups. There were two separate controls for 1mg/ml and 10mg/ml egg yolk supplemented media groups. The cell viability was higher for unfiltered 1mg/ml egg yolk supplemented media as compared to control media2 on day 1, 3 and 7. Cell viability was almost same for unfiltered 10mg/ml egg yolk supplemented media and control media2. Statistically significant differences ( $p \leq 0.05$ ) were seen on day 1, day 3 and day 7 between control2 and unfiltered 1mg/ml egg yolk supplemented media. No statistical significance was seen between control media1 and unfiltered 10mg/ml egg yolk supplemented media.



**Figure 3:** Cell viability using Presto Blue Assay (n=4). Unfiltered media with 10% fetal bovine serum (FBS) supplemented with higher concentration of egg yolk extract

### 3.2.1.4 Myogenic differentiation of C2C12 myoblasts in differentiation media supplemented with egg yolk extract

Cell viability decreased from day 1 to day 7 for the control media. However, for the egg yolk supplemented media the cell viability decreased on day 2 and then eventually increased on day 7. The cell viability on day 3 and day 7 was significantly higher ( $p \leq 0.05$ ) for the 500 $\mu$ g/ml egg yolk supplemented media (filtered and unfiltered) as compared to the control media.

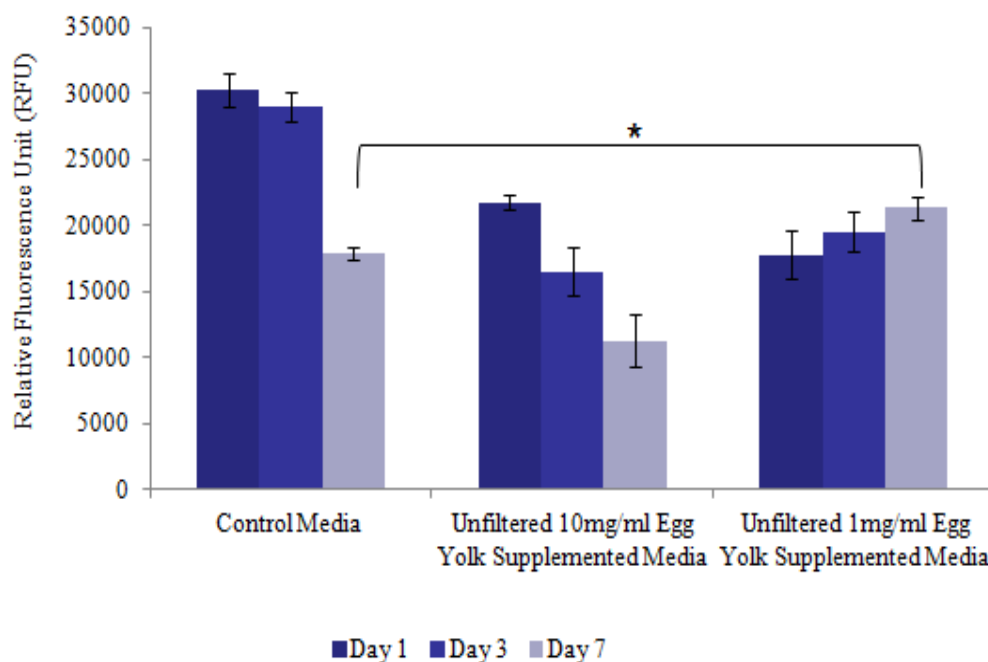


**Figure 4:** Cell viability using Presto Blue Assay (n=4). Media with 2% fetal bovine serum (FBS) supplemented with 500 $\mu$ g/ml egg yolk extract



### 3.2.1.5 Myogenic differentiation of C2C12 myoblasts in differentiation media supplemented with higher concentration egg yolk extract-Unfiltered Media

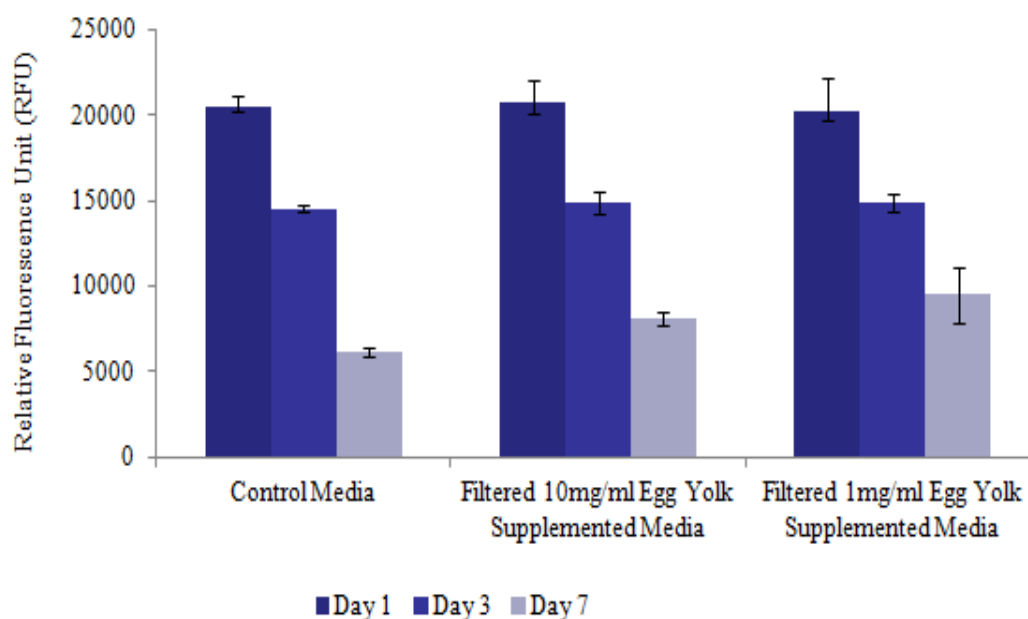
For unfiltered, the cell viability decreased from day1 to day 7 for control media and unfiltered 10mg/ml egg yolk supplemented media. The cell viability increased from day 1 to day 7 for unfiltered 1mg/ml egg yolk supplemented media. On day 7, the cell viability was significantly higher ( $p \leq 0.05$ ) for unfiltered 1mg/ml egg yolk supplemented media as compared to the control media.



**Figure 5:** Cell viability using Presto Blue Assay (n=4). Unfiltered media with 2% fetal bovine serum (FBS) supplemented with higher concentration of egg yolk extract

### 3.2.1.6 Myogenic differentiation of C2C12 myoblasts in differentiation media supplemented with higher concentration egg yolk extract-Filtered Media

For filtered media, the cell viability decreased from day 1 to day 7 for all the groups. The cell viability was slightly higher on day 7 for egg yolk supplemented media as compared to the control media. However, statistically significant differences were not seen between groups.



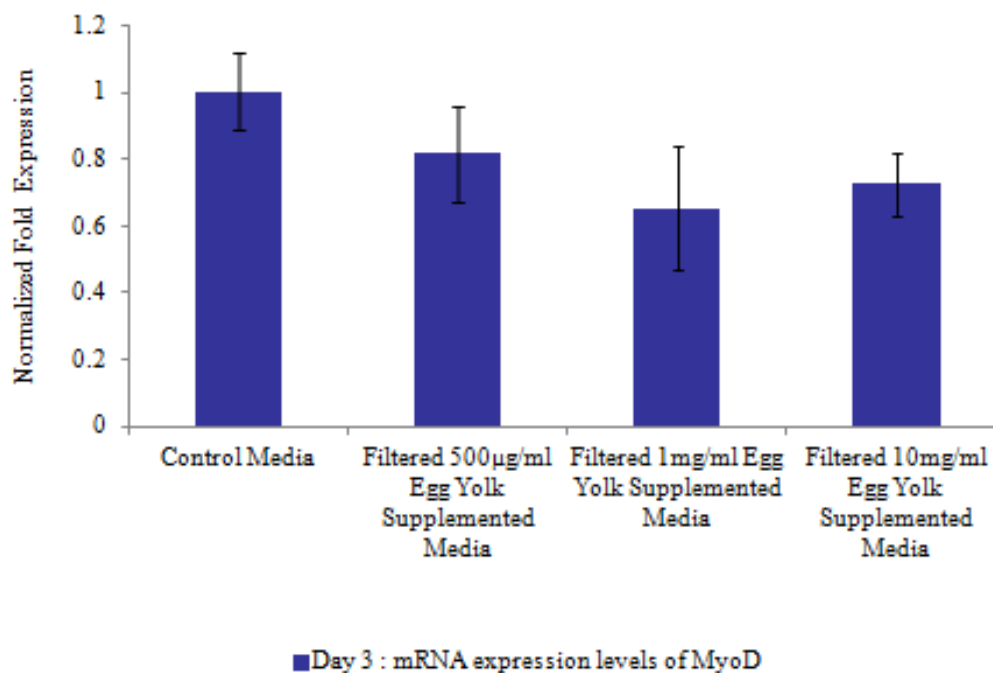
**Figure 6:** Cell viability using Presto Blue Assay (n=4). Filtered media with 2% fetal bovine serum (FBS) supplemented with higher concentration of egg yolk extract

### 3.2.2 mRNA expression of muscle specific genes

mRNA expressions of muscle specific genes: MyoD and myogenin were analyzed on day 3 for C2C12 myoblasts grown in control media and egg yolk supplemented media :

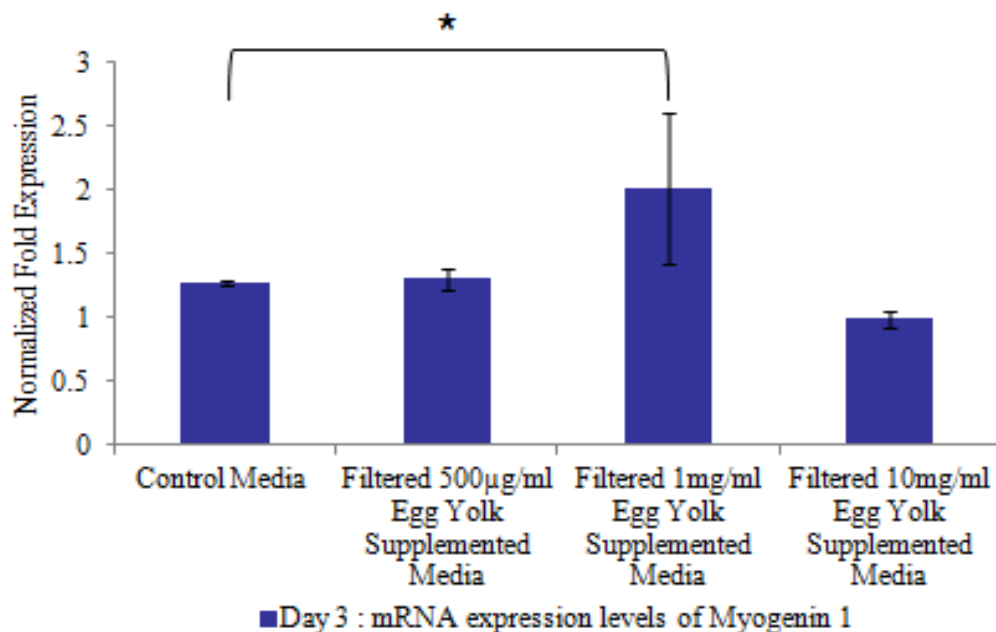
500 $\mu$ g/ml, 1mg/ml and 10mg/ml respectively. Filtered and unfiltered media was used for the respective concentrations.

### 3.2.2.1 Filtered Media



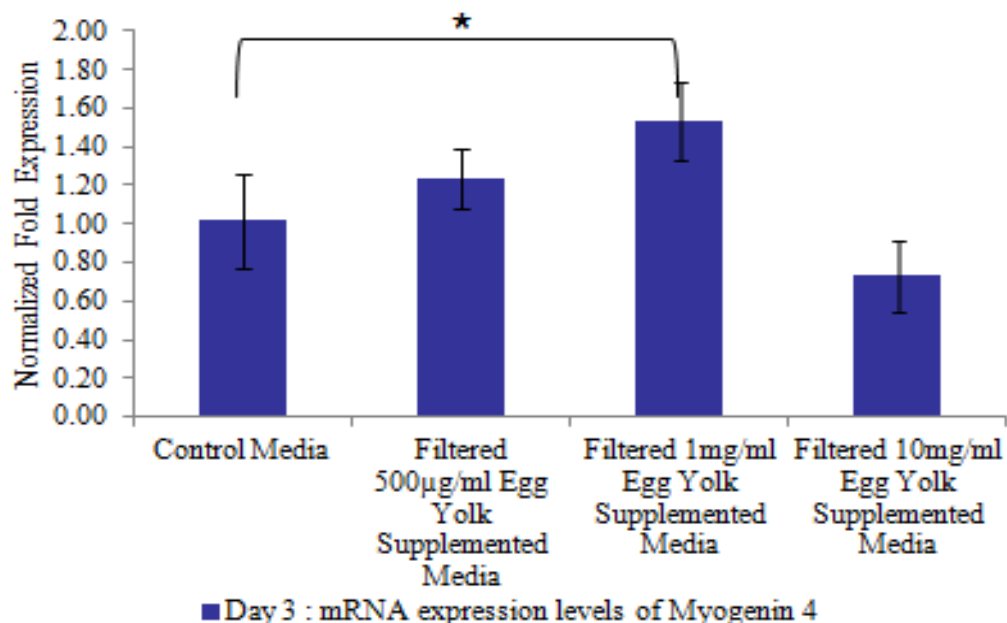
**Figure 7:** mRNA expression of MyoD on day 3. Filtered media with 2% fetal bovine serum (FBS) supplemented with different concentrations of egg yolk extract

Statistical differences in mRNA expression of MyoD were not seen in the filtered egg yolk supplemented groups as compared to the control on day 3 (Fig.7)



**Figure 8:** mRNA expression of Myogenin 1 on day 3. Filtered media with 2% fetal bovine serum (FBS) supplemented with different concentrations of egg yolk extract

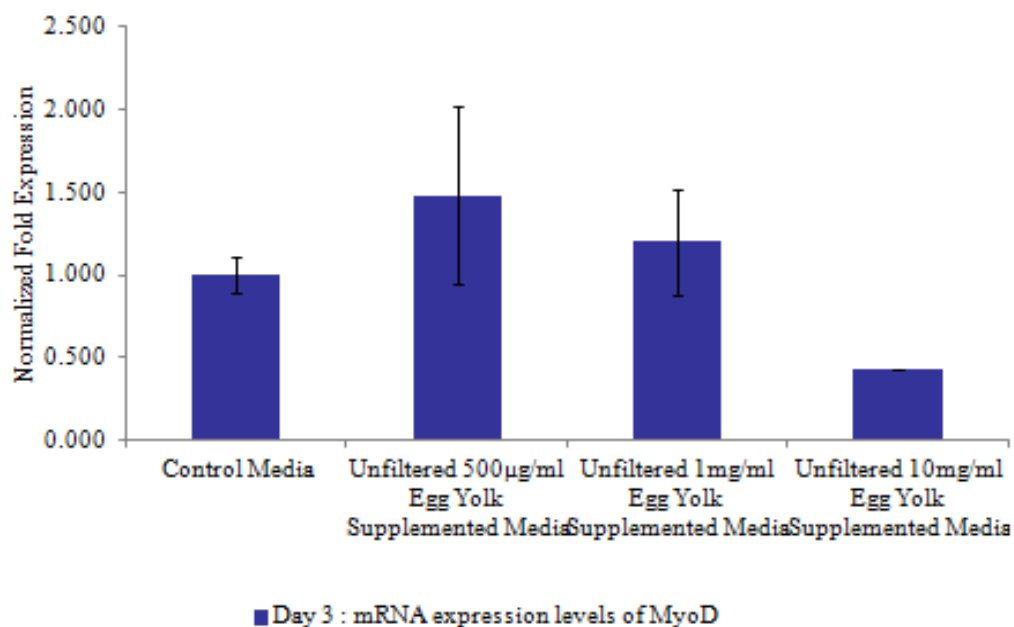
mRNA expression of Myogenin-1 was significantly higher ( $p \leq 0.05$ ) for filtered 1mg/ml egg yolk supplemented media. mRNA expression of Myogenin-1 was relatively same for control media and filtered 500µg/ml egg yolk and lowest for filtered 10mg/ml egg yolk supplemented media. However, there was no significant difference between control media, filtered 500µg/ml egg yolk supplemented media and filtered 10mg/ml egg yolk supplemented media (Fig. 8).



**Figure 9:** mRNA expression of Myogenin 4 on day 3. Filtered media with 2% fetal bovine serum (FBS) supplemented with different concentrations of egg yolk extract

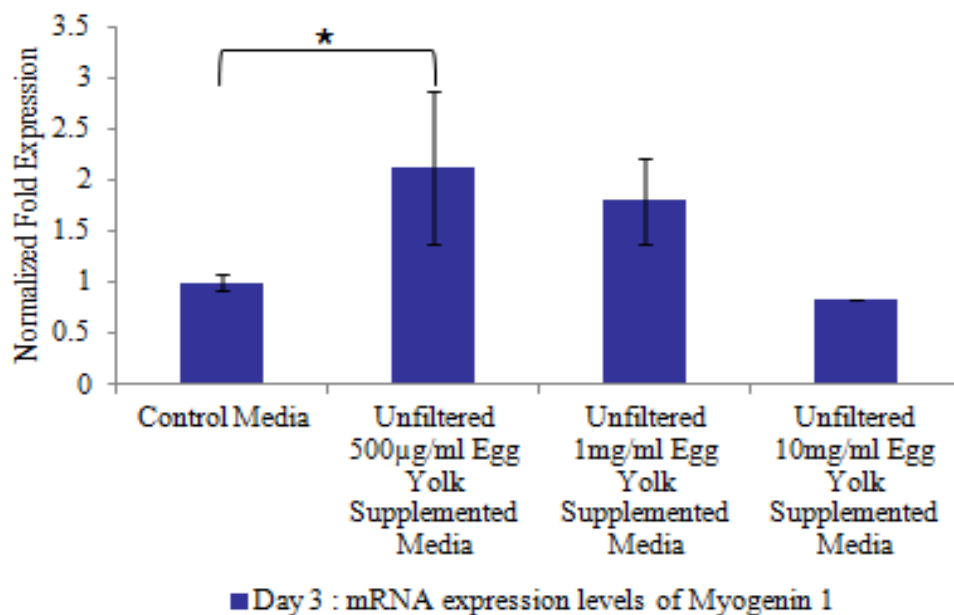
mRNA expression of Myogenin-4 was significantly higher ( $p \leq 0.05$ ) for filtered 1mg/ml egg yolk supplemented media. mRNA expression of Myogenin-4 is slightly higher for filtered 500µg/ml egg yolk and slightly lower for filtered 10mg/ml egg yolk supplemented media as compared to the control media. However, there was no significant difference between control media, filtered 500µg/ml egg yolk supplemented media and filtered 10mg/ml egg yolk supplemented media (Fig. 9).

### 3.2.2.2 Unfiltered Media



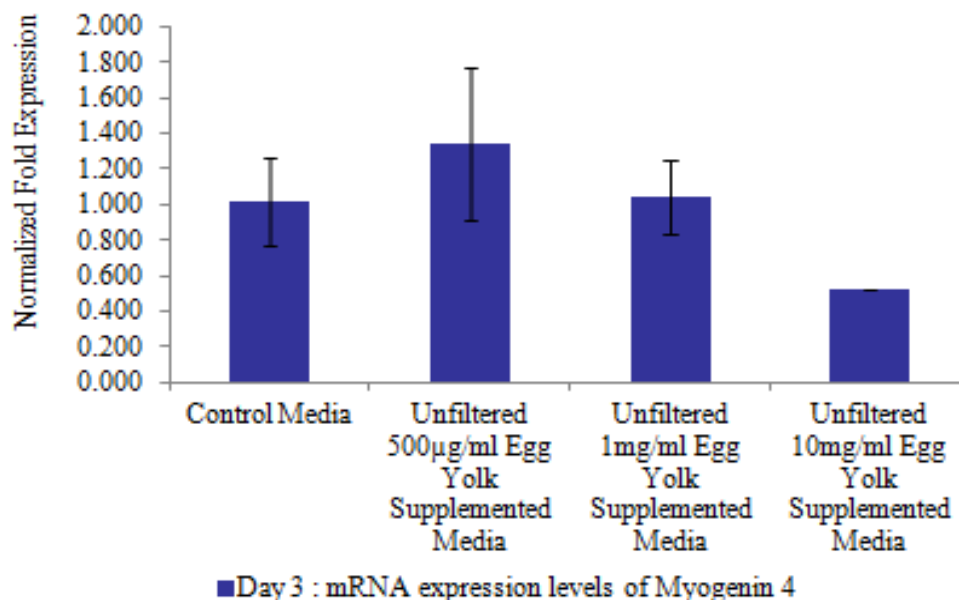
**Figure 10:** mRNA expression of MyoD on day 3. Unfiltered media with 2% fetal bovine serum (FBS) supplemented with different concentrations of egg yolk extract

mRNA expression of MyoD-1 was slightly higher for unfiltered 500µg/ml egg yolk supplemented group and unfiltered 1mg/ml egg yolk supplemented group. However, there was no statistical significance between the two groups. The mRNA expression was lowest for unfiltered 10mg/ml egg yolk supplemented media (Fig.10).



**Figure 11:** mRNA expression of Myogenin 1 on day 3. Unfiltered media with 2% fetal bovine serum (FBS) supplemented with different concentrations of egg yolk extract

mRNA expression of Myogenin-1 was significantly higher ( $p \leq 0.05$ ) for unfiltered 500µg/ml egg yolk supplemented media. The mRNA expression was almost same for control and unfiltered 10mg/ml egg yolk supplemented media. There was no significant difference between control and the unfiltered 1mg/ml egg yolk supplemented group (Fig. 11).



**Figure 12:** mRNA expression of Myogenin 4 on day 3. Unfiltered media with 2% fetal bovine serum (FBS) supplemented with different concentrations of egg yolk extract

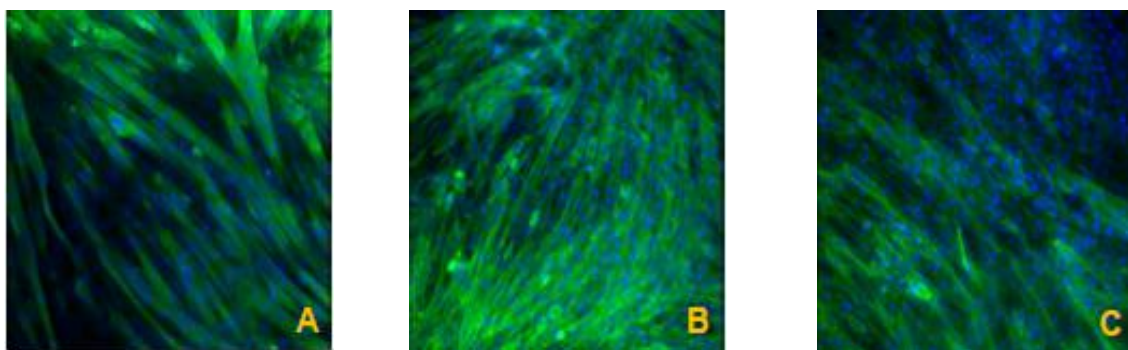
mRNA expression of Myogenin-4 was lowest for unfiltered 10mg/ml egg yolk supplemented media. The mRNA expression was almost same for control media and unfiltered 1mg/ml egg yolk supplemented media. The mRNA expression was slightly higher for unfiltered 500µg/ml egg yolk supplemented media. However, there were no significant differences between control and unfiltered egg yolk supplemented groups (Fig. 12).



### 3.2.3 Fluorescence Image Analysis

#### 3.2.3.1: Proliferation of C2C12 myoblasts in growth media (10% FBS) supplemented with egg yolk extract

There was no significant difference in number of nuclei and number of myotubes between the control and 500 $\mu$ g/ml egg yolk supplemented groups. However, there was a significant difference ( $p \leq 0.05$ ) in fusion index between control media and 500 $\mu$ g/ml egg yolk supplemented media (unfiltered and filtered).



**Figure 13:** Fluorescence images of C2C12 myoblasts on day 7. Proliferation of C2C12 myoblasts in A: Control Media, B: Filtered 500 $\mu$ g/ml Egg Yolk Supplemented Media and C: Unfiltered 500 $\mu$ g/ml Egg Yolk Supplemented Media.

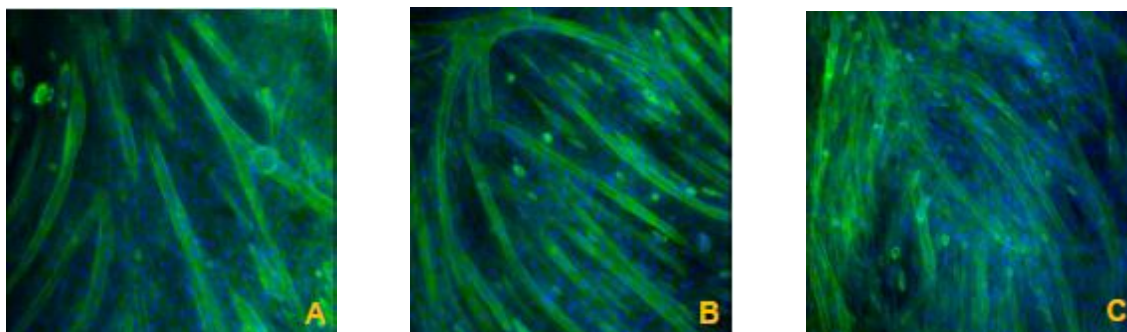
**Table 2:** Image Analysis using ImageJ for filtered and unfiltered 500µg/ml egg yolk supplemented media (10%FBS)

Groups	# Nuclei	# Myotubes	Fusion Index(%)
Control Media	908±160	45±7	40.3±0.7
Filtered 500µg/ml Egg Yolk Supplemented Media	751±54	33±2	59±0.7
Unfiltered 500µg/ml Egg Yolk Supplemented Media	926±179	48±4	77±7.86

\*


### 3.2.3.2: Proliferation of C2C12 myoblasts in growth media (10% FBS) supplemented with higher concentration of egg yolk extract –Filtered Media

Significant difference ( $p \leq 0.05$ ) was seen in number of nuclei and number of myotubes between control and filtered 10mg/ml egg yolk supplemented media. However, there was no significant difference in number of nuclei and number of myotubes between control and filtered 1mg/ml egg yolk supplemented media. There was also no significant difference in fusion index between control and filtered egg yolk supplemented media.



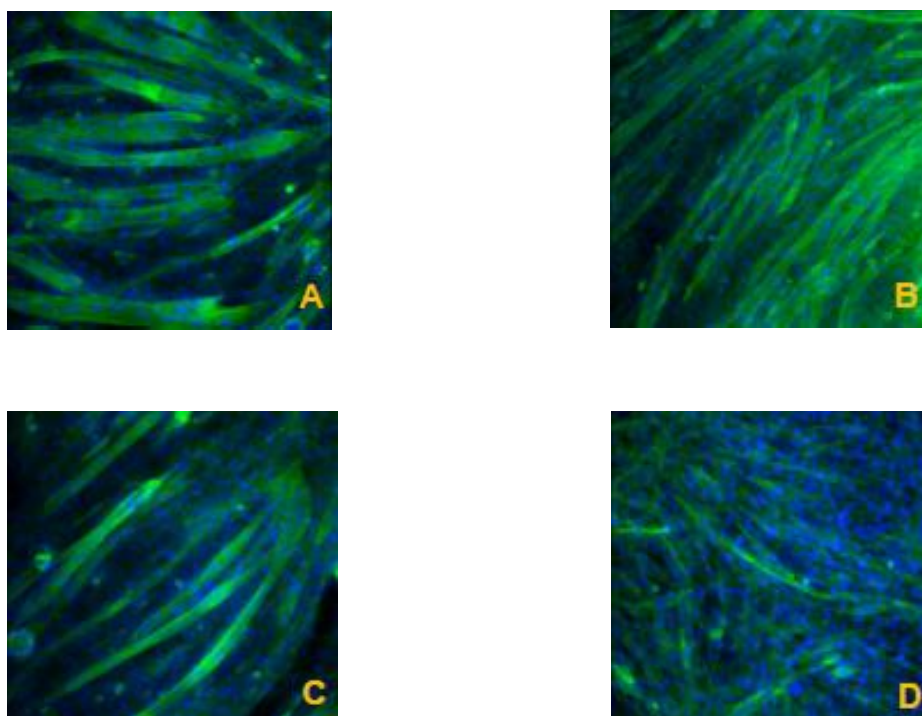
**Figure 14:** Fluorescence images of C2C12 myoblasts on day 7. Proliferation of C2C12 myoblasts in A: Control Media, B: Filtered 1mg/ml Egg Yolk Supplemented Media and C: Filtered 10mg/ml Egg Yolk Supplemented Media

**Table 3:** Image Analysis using ImageJ for filtered 1mg/ml and 10mg/ml egg yolk supplemented media (10%FBS)

Groups	# Nuclei	# Myotubes	Fusion index (%)
Control	971±145	38±10	58.2±20.10
Filtered 1mg/ml Egg Yolk Supplemented Media	1226±180	45±10	53±10.10
Filtered 10mg/ml Egg Yolk Supplemented Media	1226±103	48±9	60.4±9.31

### 3.2.3.3: Proliferation of C2C12 myoblasts in growth media (10% FBS) supplemented with higher concentration of egg yolk extract –Unfiltered Media

Significant difference was not seen in number of nuclei between the control and unfiltered egg yolk supplemented groups. However, there was significant difference ( $p \leq 0.05$ ) in number of myotubes for unfiltered 1mg/ml and unfiltered 10mg/ml egg yolk supplemented media when compared to the control media. Significant difference ( $p \leq 0.05$ ) was also seen in fusion index for unfiltered 1mg/ml egg yolk supplemented media when compared to the control.



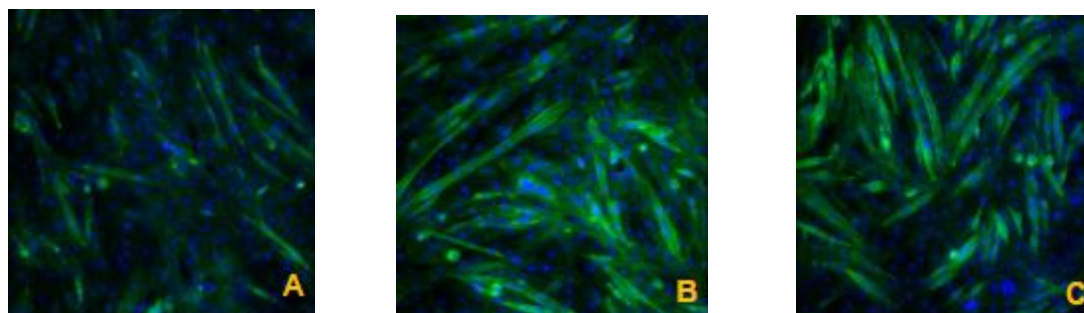
**Figure 15:** Fluorescence images of C2C12 myoblasts on day 7. Proliferation of C2C12 myoblasts in A: Control Media 1, B: Unfiltered 1mg/ml Egg Yolk Supplemented Media, C: Control Media 2 and D: Unfiltered 10mg/ml Egg Yolk Supplemented Media

**Table 4:** Image Analysis using ImageJ for unfiltered 1mg/ml and 10mg/ml egg yolk supplemented media (10%FBS)

Groups	#Nuclei	#Myotubes	Fusion Index (%)
Control Media 1	1001±61	136±26	47.43±12.61
Unfiltered 1mg/ml Egg Yolk Supplemented Media	1028±53	182±18	66.29±8.93
Control Media 2	985±97	156±20	70.50±6.71
Unfiltered 10mg/ml Egg Yolk Supplemented Media	903±26	223±42	78.35±8.45

**3.2.3.4: Myogenic differentiation of C2C12 myoblasts in differentiation media (2% FBS) supplemented with egg yolk extract**

There was no significant difference in number of nuclei, number of myotubes and fusion index between control and egg yolk supplemented groups.



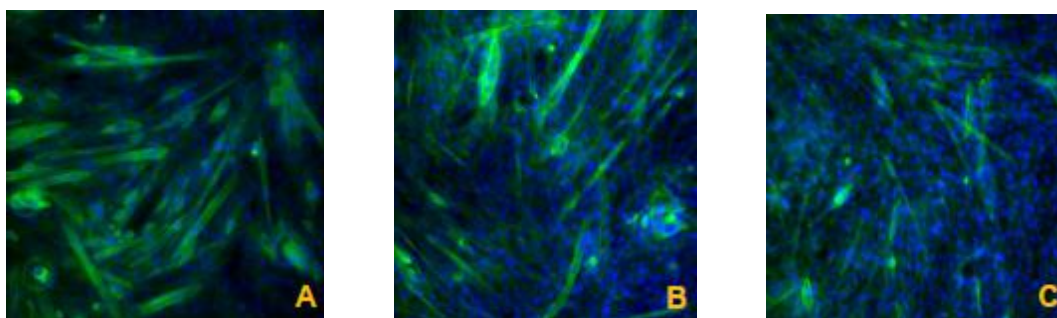
**Figure 16:** Myogenic differentiation of C2C12 myoblasts in A: Control Media, B: Filtered 500µg/ml Egg Yolk Supplemented Media and C: Unfiltered 500µg/ml Egg Yolk Supplemented Media

**Table 5:** Image Analysis using ImageJ for filtered and unfiltered 500µg/ml egg yolk supplemented media (2%FBS)

Groups	#Nuclei	#Myotubes	Fusion Index (%)
Control	438±45	33±4	46.80±4.5
Filtered 500µg/ml Egg Yolk Supplemented Media	459±63	50±17	51.00±7.36
Unfiltered 500µg/ml Egg Yolk Supplemented Media	456±117	53±12	57.8±2

### 3.2.3.5: Myogenic differentiation of C2C12 myoblasts in differentiation media (2% FBS) supplemented with higher concentration egg yolk extract-Unfiltered

Significant difference ( $p \leq 0.05$ ) was seen in number of nuclei between control and both unfiltered egg yolk supplemented media. Significant difference ( $p \leq 0.05$ ) was also seen in number of myotubes for unfiltered 10mg/ml egg yolk supplemented media when compared to the control media. For fusion index, there was significant difference ( $p \leq 0.05$ ) between control and unfiltered 10mg/ml egg yolk supplemented media.



**Figure 17:** Fluorescence images of C2C12 myoblasts on day 7. Myogenic differentiation of C2C12 myoblasts in A: Control Media, B: Unfiltered 1mg/ml Egg Yolk Supplemented Media and C: Unfiltered 10mg/ml Egg Yolk Supplemented Media

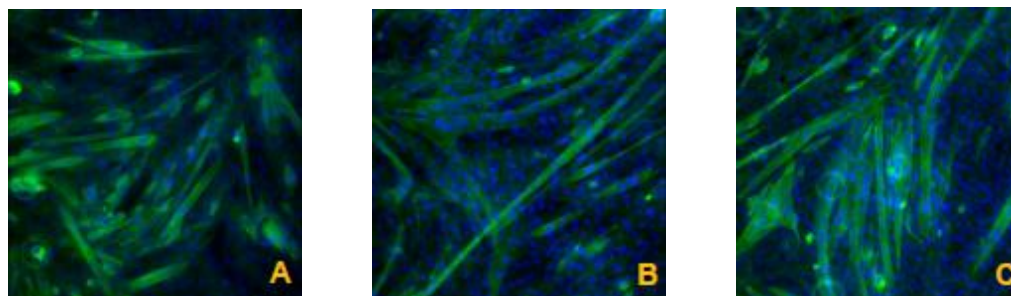
**Table 6:** Image Analysis using ImageJ for unfiltered 1mg/ml and 10mg/ml egg yolk supplemented media (2%FBS)

Groups	#Nuclei	#Myotubes	Fusion Index (%)
Control	694±70	119±22	40.77±11.33
Unfiltered 1mg/ml Egg Yolk Supplemented Media	1002±7 *	174±81 *	46.00±25 *
Unfiltered 10mg/ml Egg Yolk Supplemented Media	1011±18	269±23	63.71±7

### 3.2.3.6: Myogenic differentiation of C2C12 myoblasts in differentiation media (2% FBS) supplemented with higher concentration egg yolk extract-Filtered

Significant difference ( $p \leq 0.05$ ) was seen in number of nuclei for both filtered 1mg/ml and filtered 10mg/ml egg yolk supplemented media when compared to the control media. Significant difference ( $p \leq 0.05$ ) was also seen in number of myotubes and fusion index for filtered 10mg/ml egg yolk supplemented media when compared to the control media. However, there was no significant difference in number of myotubes or fusion index between control and filtered 1mg/ml egg yolk supplemented media.





**Figure 18:** Fluorescence images of C2C12 myoblasts on day 7. Myogenic differentiation of C2C12 myoblasts in A: Control Media, B: Filtered 1mg/ml Egg Yolk Supplemented Media and C: Filtered 10mg/ml Egg Yolk Supplemented Media

**Table 7:** Image Analysis using ImageJ for filtered 1mg/ml and 10mg/ml egg yolk supplemented media (2%FBS)

Groups	#Nuclei	#Myotubes	Fusion Index (%)
Control	694±70	119±22	40.77±11.3
Filtered 1mg/ml Egg Yolk Supplemented Media	826±51	153±37	53.00±11.08 *
Filtered 10mg/ml Egg Yolk Supplemented Media	906±106	212±24	56.74±8.33

### **3.3 Discussion**

Compromised muscle regenerative capacity as seen in atrophy and sarcopenia can be caused due to the reduced number or function of myoblasts [37]. To address this issue, in-vitro studies are being developed which typically involve studying the process of myogenesis in muscle cells at conditions that promote cell growth. As mentioned earlier, nutritional intervention to ameliorate skeletal muscle function at cellular or molecular level can be a promising therapeutic strategy. In the present study, we have studied the effect of egg yolk extract on proliferation and differentiation of C2C12 myoblasts. This was achieved by supplementing different concentrations of egg yolk in growth media. Three end points: cell viability, mRNA expression and fluorescence imaging were used to analyze the effects of egg yolk on myogenesis of C2C12 myoblasts. C2C12 cell line is an ideal model to study myogenesis in vitro. Since the process of myogenesis can be divided into two stages: proliferation and differentiation, the study was also divided into these respective stages. The end points were analyzed in both the phases to see whether supplementation of egg yolk in growth media directly or indirectly affects the process of myogenesis.

#### **3.3.1 Effect of egg yolk extract on myoblast proliferation and growth**

To study the effect of egg yolk extract on proliferation of C2C12 myoblasts, the egg yolk extract was supplemented in 10% FBS media. This maintained the growth of myoblasts until day 7. In proliferative phase, the formation of myotubes took place from day 5. On day 7, well defined and mature myotubes were seen.

Cell viability increased from day 1 to day 7 for the filtered and unfiltered 500µg/ml egg yolk supplemented groups as expected and was significantly higher than the control on day 7. This was the lowest concentration of egg yolk that was used which had a positive effect on cell viability. Thus, it appeared that supplementation of egg yolk enhanced and maintained myoblast proliferation at low concentration. To confirm whether an increase in the concentration of egg yolk supplemented in media would change cell viability data, higher concentrations of egg yolk: 1mg/ml and 10mg/ml were used. Here again filtered and unfiltered media was used. This was done to see if the filtration of egg yolk had any effects on the myoblast growth and proliferation. Filtration can be considered as a way of fractionating the egg yolk extract. The cell viability was significantly higher for the 10mg/ml egg yolk supplemented media after filtration when compared to control media. However, for the unfiltered media, cell viability was higher for 1mg/ml egg yolk supplemented media. This clearly showed that filtration of the egg yolk supplemented media affected the myoblast growth and proliferation. This could be because of the concentration range of egg yolk needed to be supplemented in the media. It appeared that high concentration of egg yolk had a positive effect after filtration and low concentration of egg yolk had a positive effect without filtration. One of the reasons could be that the active agent/factor that promoted myoblast proliferation present in low and high egg yolk concentration got filtered out during the process which directly affected the myoblast growth. Another reason could be that the cells required the growth promoting agent/factor at a particular concentration range. However, we do not exactly know if lipids, proteins or lipoproteins present in egg yolk could be the active agents/factors that promoted myoblast growth. Together, the cell viability results showed that the egg yolk

extract supplementation in media enhanced and promoted myoblast growth and proliferation which was dose dependent.

To see if the cell viability results correlate with the morphology of the myoblasts, fluorescence images of the myoblasts were captured on day 7. It can be seen clearly in the images that there are dense myotubes in egg yolk supplemented media as compared to the control media. Image analysis for the myoblasts grown in 500 $\mu$ g/ml egg yolk supplemented media showed that there was no significant difference in number of nuclei between groups. However, fusion index (number of nuclei on myotubes/ total number of nuclei) which determined myoblast fusion into myotubes was significantly higher for filtered and unfiltered 500 $\mu$ g/ml egg yolk supplemented media than the control media. This showed that the egg yolk supplementation enhanced fusion of myoblasts into myotubes. For filtered 10mg/ml egg yolk supplemented in the media, the number of nuclei was significantly higher than control which showed that the egg yolk affected the myoblast proliferation by increasing the number of nuclei in filtered media. Also, the number of myotubes in filtered 10mg/ml egg yolk supplemented media was significantly higher than the control which showed that at higher, filtered concentrations egg yolk enhanced myotube formation. However, fusion index for all groups are not significantly different. For higher concentrations of egg yolk supplemented in the media, the number of nuclei is not significantly different for unfiltered media when compared with the control. However, the number of myotubes in unfiltered 1mg/ml and 10mg/ml egg yolk supplemented media was significantly different than the control which showed that egg yolk promoted myotube formation. Also, the fusion index was higher and significantly

different for unfiltered 1mg/ml egg yolk supplemented media than the control. Together, the image analysis showed that the higher filtered egg yolk concentration and the lower unfiltered egg yolk concentration supplemented in the growth media promoted and enhanced myoblast growth and fusion.

Looking at the trend of the results summarised in Table 8, it appeared that lower concentration of egg yolk extract enhanced cell viability and promoted fusion of myoblasts. However, higher concentration of egg yolk extract enhanced cell viability and proliferation which was characterized by increase in number of nuclei and myotubes. Together, it appeared that higher concentration of egg yolk along with high serum concentration drove the cells to the proliferation phase and low concentration of egg yolk helped in differentiation and fusion of myoblasts.

**Table 8:** Summary of proliferation study.

Groups	Cell Viability	Image Analysis		
		#Nuclei	#Myotubes	FI
Filtered 500µg/ml EY supplemented media	✱	✱	✱	✱
Unfiltered 500µg/ml EY supplemented media	✱	✱	✱	✱
Filtered 1mg/ml EY supplemented media	✱	✱	✱	✱
Unfiltered 1mg/ml EY supplemented media	✱	✱	✱	✱
Filtered 10mg/ml EY supplemented media	✱	✱	✱	✱
Unfiltered 10mg/ml EY supplemented media	✱	✱	✱	✱

✱ Higher/more than control (significantly different)  
 ✱ Same as control (not significantly different)

### **3.3.2 Effect of egg yolk extract on myoblast differentiation and fusion**

To study the effect of egg yolk on differentiation and fusion of C2C12 myoblasts, the egg yolk was supplemented in 2% FBS media. In differentiation phase, the formation of myotubes took place from day 3. By day 7, well defined and mature myotubes were seen.

Cell viability for the control group went on decreasing from day 1 to day 7. This was probably because of the low serum available for the cells. However, the low serum did promote differentiation from day 3. The cell viability for 500µg/ml egg yolk supplemented groups and control was almost same on day 1. The cell viability dropped on day 3 for all groups but the cell viability was slightly higher for 500µg/ml egg yolk supplemented groups when compared to the control. On day 7, the cell viability increased for 500µg/ml egg yolk supplemented groups and it decreased even further for the control. Looking at this pattern it can be seen that the egg yolk extract in the media to some extent maintained myoblast growth and differentiation. Also, the unfiltered egg yolk groups had highest cell viability which again shows that the filtration may have removed the active agent that enhanced cell proliferation. For higher concentration of egg yolk extract in the unfiltered media, the cell viability was highest on day 7 for 1mg/ml egg yolk supplemented media and was significantly higher than the control. Looking again at the increasing cell viability pattern for egg yolk supplemented media groups; it appeared that the egg yolk maintained cell viability while promoting differentiation of myoblasts. For higher concentration of egg yolk extract in the filtered media, the cell viability was almost same for all groups and not significantly different than control on day 7. This shows that the filtration of low serum media with the egg yolk did not improve cell

viability but the unfiltered low serum concentration media improved cell viability. This indirectly tells us that the agent or factor present in egg yolk which enhanced myogenesis needs to be present at a specific concentration and may get filtered out during filtration process thereby affecting the cell viability. Together, the egg yolk extract in presence of low serum concentration promoted differentiation and maintained cell viability.

To see how the egg yolk affects the muscle specific genes during myogenesis, mRNA expressions of MyoD and myogenin were analyzed on day 3 since the fusion of myoblasts into myotubes were apparent on day 3 and all the muscle specific markers for early differentiation are usually expressed on day 3. To initiate differentiation low serum concentration was used in the media. MyoD and myogenin are myogenic transcription factors for skeletal muscle which determine the fate of the myoblasts in their early differentiation stage. These are not the only factors that are expressed during the process of myogenesis but their expression would suggest that the myoblasts do fuse into myotubes and could be considered as a positive effect. The mRNA expression levels of MyoD in filtered egg yolk supplemented media showed that the MyoD expression was not significantly different between the groups which suggested that the egg yolk did not affect the MyoD expression but also did not interfere with the myogenesis process at gene level. The mRNA expression levels for MyoD in unfiltered egg yolk supplemented media showed that the MyoD expression was not significantly different between the egg yolk groups and the control. The myogenin 1 and myogenin 4 expressions was significantly higher in filtered 1mg/ml egg yolk supplemented media than the control. The expression was almost same for the filtered 500µg/ml and filtered 10mg/ml egg yolk

supplemented groups when compared with the control. mRNA expression for myogenin 1 was significantly higher in unfiltered 500 $\mu$ g/ml egg yolk supplemented media when compared to the control. The myogenin 1 expression was not significantly different for the unfiltered 1mg/ml and 10mg/ml egg yolk supplemented groups. The myogenin 4 expression was almost same for all the groups and there were no significant differences between the groups. Myogenin 1 and myogenin 4 were two different primers for the same marker with different sensitivity. This showed that myogenin 1 was more sensitive than myogenin 4. Together, it appeared that there was no enhanced MyoD expression in myoblasts on day 3 as compared to the control but there was increased myogenin expression in myoblasts grown in filtered 1mg/ml egg yolk supplemented media and increased myogenin expression in myoblasts grown in unfiltered 500 $\mu$ g/ml egg yolk supplemented media.

Image analysis of 500 $\mu$ g/ml egg yolk supplemented groups for day 7 showed no significant differences in number of nuclei, number of myotubes and fusion index between the groups. For higher concentration of egg yolk in unfiltered media, the number of nuclei was significantly higher for unfiltered 1mg/ml egg yolk, unfiltered 10mg/ml egg yolk and filtered 10mg/ml egg yolk supplemented groups when compared to the control. Number of myotubes was significantly higher in filtered and unfiltered 10mg/ml egg yolk supplemented groups. Fusion index was significantly higher only in filtered and unfiltered 10mg/ml egg yolk supplemented groups. Together, the image analysis showed that the higher filtered or unfiltered egg yolk concentration supplemented in the low



serum concentration media contributed to the myoblast differentiation and fusion which was characterized by increase in number of nuclei, myotubes and fusion index.

Looking at the trend of the results summarized in Table 9, it appeared that low egg yolk concentration in differentiation media only maintained and enhanced cell viability. However, higher egg yolk concentration in differentiation media drove the cells to differentiation phase even though the viability was comparatively same with the control. Also, the expression of myogenin on day 3 for low egg yolk extract supplemented media suggested that low egg yolk concentration has the ability to enhance myogenin expression; however the concentration is not enough to promote fusion. Increased myogenin expression was not seen in filtered and unfiltered 10mg/ml egg yolk supplemented groups on day 3 which suggests that it was too early for the myogenin to get expressed in cells grown in 10mg/ml egg yolk supplemented media thus presenting the need for more timepoints.

**Table 9:** Summary of differentiation study.

Groups	Cell Viability	Image Analysis			qPCR	
		#Nuclei	#Myotubes	F.I	<u>MyoD</u>	<u>Myogenin</u>
Filtered 500µg/ml EY supplemented media	✱✱	✱✱	✱✱	✱✱	✱✱	✱✱
Unfiltered 500µg/ml EY supplemented media	✱✱	✱✱	✱✱	✱✱	✱✱	✱✱
Filtered 1mg/ml EY supplemented media	✱✱	✱✱	✱✱	✱✱	✱✱	✱✱
Unfiltered 1mg/ml EY supplemented media	✱✱	✱✱	✱✱	✱✱	✱✱	✱✱
Filtered 10mg/ml EY supplemented media	✱✱	✱✱	✱✱	✱✱	✱✱	✱✱
Unfiltered 10mg/ml EY supplemented media	✱✱	✱✱	✱✱	✱✱	✱✱	✱✱

✱✱ Higher/more than control (significantly different)

✱✱ Same as control (not significantly different)

## **CHAPTER 4 - Conclusion and Future Perspectives**

Overall, supplementation of egg yolk extract enhanced proliferation and differentiation of myoblasts which was dose dependent. This was characterized by increased cell viability, number of nuclei, number of myotubes, fusion index and mRNA expression of myogenin. Also, filtration can be used as a method of fractionating the egg yolk. However, depending on the concentration of the egg yolk supplemented, filtration can result in loss of active components (e.g. lipids, lipoproteins) of egg yolk that promotes and enhances proliferation or differentiation. More endpoints such as immunostaining (e.g. MHC, myogenin), increasing the number of samples and time points are needed to determine ideal concentration of egg yolk extract that can be supplemented. Thus, this study highlights the potential use of egg yolk as a therapeutic agent to improve myogenesis.

Stem cells are ideal cues for skeletal muscle regeneration. Currently, use of stem cells in regenerative medicine is not easily applicable because of the difficulties in handling and isolating these cells. However, stem cell based therapies would greatly benefit from the future studies that are targeted towards ameliorating the function of stem cells during muscle regeneration using different natural compounds. Microenvironment and stem cell niche should be taken into account to influence the efficacy of stem cell therapies. Thus, developing cell engraftment matrices with growth factors and natural compounds that promote or enhance cell function would greatly benefit the stem cell therapy for muscle regeneration. Egg yolk extract can also be considered as a natural compound that could

be incorporated in the biomaterial based matrices for developing cell delivery systems. PEGDA seems to be a potential hydrogel for incorporating egg yolk extract. However, various factors have to be considered while using this technique such as denaturation of egg yolk while crosslinking PEGDA, concentration of egg yolk and stability of the hydrogels. Besides PEGDA, collagen and hyaluronic acid can also be explored. Nutrition in muscle regeneration is a new field and has promising effects in regenerative medicine. Nutritional intervention can improve the microenvironment and function of cells in aging muscles. Thus, future research should focus on developing such nutrition based therapies for clinical applications.

## REFERENCES

1. Takeshi Endo. Molecular mechanisms of skeletal muscle development, regeneration and osteogenic conversion. *Bone*. 2015; 80: 2–13.
2. Hang Yin, Feodor Price and Michael A. Rudnicki. Satellite cells and the muscle stem cell niche. *Physiological Reviews*. 2013; 93: 23–67.
3. Erin E. Talbert and Denis C. Guttridge. Impaired regeneration: A role for the muscle microenvironment in cancer cachexia. *Seminars in Cell & Developmental Biology*. 2016; 54: 82–91.
4. Jasdeep Saini, et al. Regenerative function of immune system: Modulation of muscle stem cells. *Ageing Research Reviews*. 2016; 27: 67–76.
5. Hugo C. Olguin, et al. Reciprocal inhibition between Pax7 and muscle regulatory factors modulates myogenic cell fate determination. *The Journal of Cell Biology*. 2007; 177(5): 769-779.
6. Charlotte A. Berkes and Stephen J. Tapscott. MyoD and the transcriptional control of myogenesis. *Seminars in Cell & Developmental Biology*. 2005; 16: 585–595.
7. R. N. Cooper, et al. In vivo satellite cell activation via Myf5 and MyoD in regenerating mouse skeletal muscle. *Journal of Cell Science*. 1999; 112: 2895-2901.
8. J. Menetrey, et al. Growth factors improve muscle healing in vivo. *Journal of Bone and Joint Surgery [Br]*. 2000; 82-B: 131-7.
9. Carla Domingues-Faria, et al. Skeletal muscle regeneration and impact of aging and nutrition. *Ageing Research Reviews*. 2016; 26: 22–36.
10. Vanessa C. Vaughan, Peter Martin and Paul A. Lewandowski. Cancer cachexia: impact, mechanisms and emerging treatments. *Journal of Cachexia, Sarcopenia and Muscle*. 2013; 4:95–109.
11. Kamran A. Mirza, et al. Attenuation of muscle wasting in murine C2C12 myotubes by epigallocatechin-3-gallate. *Journal of Cachexia, Sarcopenia and Muscle*. 2014; 5:339–345.
12. Cathleen Drescher, et al. Loss of muscle mass: current developments in cachexia and sarcopenia focused on biomarkers and treatment. *Journal of Cachexia, Sarcopenia and Muscle*. 2015; 6: 303–311.

13. Maurizio Bossola, et al. Skeletal muscle regeneration in cancer cachexia. *Clinical and Experimental Pharmacology and Physiology*. 2016; 43: 522-527.
14. S. S. Jejurikar and W. M. Kuzon, Jr. Satellite cell depletion in degenerative skeletal muscle. *Apoptosis*. 2003; 8: 573–578.
15. Naomi E. Brooks and Kathryn H. Myburgh. Skeletal muscle wasting with disuse atrophy is multi-dimensional: the response and interaction of myonuclei, satellite cells and signaling pathways. *Frontiers in Physiology*. 2014; 5(99).
16. Peter S. Zammit, et al. Muscle satellite cells adopt divergent fates: a mechanism for self-renewal? *The Journal of Cell Biology*. 2004. 166: 347–357.
17. Indong Jun, Sungin Jeong and Heungsoo Shin. The stimulation of myoblast differentiation by electrically conductive sub-micron fibers. *Biomaterials*. 2009; 30: 2038-2047.
18. Pietsch J. The effects of colchicine on regeneration of mouse skeletal muscle. *The Anatomical Record*. 1961; 167-172.
19. Quinlan J. G., Lyden S. P., Cambler D. M., Johnson S. R., Michaels S. E., Denman D. L., Radiation inhibition of mdx mouse muscle regeneration: dose and age factors. *Muscle Nerve*. 1995; 28: 201-206.
20. Francesco Saverio Tedesco, et al. Repairing skeletal muscle: regenerative potential of skeletal muscle stem cells. *Journal of Clinical Investigation*. 2010; 120: 11–19.
21. Michael J.O. Wakelam, et al. The fusion of myoblasts. *Biochemical Journal*. 1985; 228: 1-12.
22. Barbara Cisterna, et al. Adapted physical exercise enhances activation and differentiation potential of satellite cells in the skeletal muscle of old mice. *Journal of Anatomy*. 2016; 228: 771–783.
23. Stephane Dedieu, et al. Involvement of myogenic regulator factors during fusion in cell line C2C12. *The International Journal of Developmental Biology*. 2002; 46.
24. C.K. Smith II, M.J. Janney and R.E. Allen. Temporal expression of myogenic regulatory genes during activation, proliferation, and differentiation of rat skeletal muscle satellite cells. *Journal of Cellular Physiology*. 1994; 159: 379-385.
25. Sabourin L. A and Rudnicki M. A. The molecular regulation of myogenesis. *Clinical Genetics*. 2000; 57: 16–25.

26. Zipora Yablonka-Reuveni, et al. The Transition from proliferation to differentiation is delayed in satellite cells from mice lacking MyoD. *Developmental Biology*. 1999; 210: 440–455.
27. S. Burattini, et al. C2C12 murine myoblasts as a model of skeletal muscle development: morpho-functional characterization. *European Journal of Histochemistry*. 2004; 48(3): 223-234.
28. Alves A. N, et al. Comparative effects of low-level laser therapy pre- and post-injury on mRNA expression of MyoD, myogenin, and IL-6 during the skeletal muscle repair. *Lasers in Medical Science*. 2016; 31(4): 679-85.
29. Christoph Handschin, et al. External physical and biochemical stimulation to enhance skeletal muscle bioengineering. *Advanced Drug Delivery Reviews*. 2015; 82–83: 168–175.
30. Yaden B. C, et al. Follistatin: a novel therapeutic for the improvement of muscle regeneration. *Journal of Pharmacology and Experimental Therapeutics*. 2014; 349(2): 355-71.
31. Laumonier T, Menetrey J. Muscle injuries and strategies for improving their repair. *Journal of Experimental Orthopaedics*. 2016; 3:15.
32. Olivier M. Dorchies, et al. Green tea extract and its major polyphenol (-)-epigallocatechin gallate improve muscle function in a mouse model for Duchenne muscular dystrophy. *American Journal of Physiology: Cell Physiology*. 2006; 290: C616-C625.
33. Montesano A. et al. Resveratrol promotes myogenesis and hypertrophy in murine myoblasts. *Journal of Translational Medicine*. 2013; 11: 310.
34. Yinting Guo, et al. Coffee treatment prevents the progression of sarcopenia in aged mice in vivo and in vitro. *Experimental Gerontology*. 2014; 50: 1–8.
35. Owens D. J, et al. A system based investigation into vitamin D and skeletal muscle repair, regeneration, and hypertrophy. *American Journal of Physiology: Endocrinology Metabolism*. 2015; 309: E1019–E1031.
36. W. M. Han, et al. Engineered matrices for skeletal muscle satellite cell engraftment and function. *Matrix Biology*. 2016.
37. C. Rhim D. A. Lowell M. C. Reedy, D.H. Slentz, S.J. Zhang, W.E. Kraus, G. A. Truskey, Morphology and ultrastructure of differentiating three-dimensional mammalian skeletal muscle in a collagen gel. *Muscle Nerve*. 2007; 37: 71-80.

38. Lee K.Y, Mooney D. J. Alginate: properties and biomedical applications. *Progress in Polymer Science*. 2012; 37: 106-126.
39. Michael W. Pfaffl. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Research*. 2001; 29(9): 2002-2007.
40. Michael W. Pfaffl, Graham W. Horgan and Leo Dempfle. Relative expression software tool (REST©) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. *Nucleic Acids Research*. 2001; 30 (9): 1-10.
41. James G. Ryall, Jonathan D. Schertzer and Gordon S. Lynch. Cellular and molecular mechanisms underlying age-related skeletal muscle wasting and weakness. *Biogerontology*. 2008; 9: 213–228.
42. Der Vartanian A, et al. Protein *O*-fucosyltransferase 1 expression impacts myogenic C2C12 cell commitment via the Notch signaling pathway. *Molecular and Cellular Biology*. 2015; 35: 391–405.
43. Wolf J. R, Hirschhorn R. R and Steiner S. M. Growth factor responsiveness: role of MyoD and myogenin. *Experimental Cell Research*. 1992; 202 (1): 105-112.
44. Laurent P. Bogdanik, et al. A MusD retrotransposon insertion in the mouse *Slc6a5* gene causes alterations in neuromuscular junction maturation and behavioral phenotypes. *PLoS ONE*. 2012; 7(1): e30217.
45. Chaturvedi V, et al. Interactions between skeletal Mmuscle myoblasts and their extracellular matrix revealed by a serum free culture system. *PLoS ONE*. 2015; 10(6): e0127675.
46. Karavia E. A, et al. Apolipoprotein A-I modulates processes associated with diet-induced nonalcoholic fatty liver disease in mice. *Molecular Medicine*. 2012; 18: 901-912.