RESEARCH ARTICLE

MCBS
Mol Cell Biomed Sci. 2026; 10(3): 166-74
DOI: 10.21705/mcbs.v10i3.813

Medium Volume Modulates C2C12 Myoblasts Differentiation Efficiency and Expression of *Myod1* and *Myog*

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Background: Differentiation of C2C12 myoblasts into multinucleated myofibers is a well-established model for studying skeletal muscle biology. While numerous factors influencing myogenesis have been thoroughly investigated, the effects of medium volume during differentiation of myoblasts have not been extensively explored, and most studies do not report the specific medium volumes used during differentiation. In this study, we investigated how medium volume affects myogenic differentiation, metabolic activity, and gene expression in C2C12 cultures.

Materials and methods: The impact of different medium volumes (100, 250, and 400 µL/cm²) on C2C12 differentiation was evaluated over a five-day period, with medium replacement every two days. Morphological assessments and metabolic activity assays were performed at the end of differentiation. Gene expression analyses for *Myod1*, *Myog*, *Myf6*, *Myh1* and *Myh7* were conducted through RT-qPCR on initial cultures and on the final day of differentiation for comparison.

Results: The expression levels of the early myogenic regulatory factors *Myod1* and *Myog* were inversely correlated with medium volume, whereas the late differentiation markers *Myh1* (type II) and *Myh7* (type I) remained unaffected. Cultures differentiated with higher medium volume presented both increased metabolic activity and a higher differentiation index at the end of differentiation period.

Conclusion: Medium volume significantly affects the differentiation of C2C12 myoblasts. Higher medium volumes increased myofiber thickness, differentiation index, and metabolic activity, and reduced the expression of the early myogenic markers Myod1 and Myog. These findings highlight medium volume as an important experimental parameter that should be reported alongside medium composition to improve reproducibility and physiological relevance of in vitro skeletal muscle differentiation studies.

Keywords: *C2C12 differentiation, myogenesis, myogenic regulatory factors, skeletal muscle in vitro model*

Submission: May 12, 2026
Last Revision: June 22, 2026
Accepted for Publication: July 1, 2026

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Print ISSN: 2527-4384, Online ISSN: 2527-3442
DOI: 10.21705/mcbs.v10i3.813

Introduction

The culture and differentiation of myoblast cell lines serve as valuable tools for studying muscle physiology, pathology, and the development of therapeutic drugs. Although in vitro models do not fully replicate the complexity of whole-organ studies, they provide a controlled environment for the investigation of muscle-related activities.[1-6] To facilitate research, several myoblast cell lines derived from human and animal models have been developed and are commercially available, reducing the need for repeated myoblast isolation from fresh tissues.[3,7]

The C2C12 cell line, an immortalized murine myoblast lineage, is widely used for studies of muscle development and metabolism.[8-10] Serum growth factors and cell density are important for preventing C2C12 myoblasts from cell-cycle withdrawal and terminal differentiation into myofibers. Differentiation is typically induced by reducing the concentration of growth factors in the culture medium once the cells reach near confluence, replacing culture medium with differentiation medium.[11-13] The differentiation medium typically has a lower serum supplementation in its composition.

Significant protocol variations exist across the literature, potentially resulting in cultures with differing myofiber qualities and quantities.[14-16] Interestingly, our literature review revealed that most studies do not report the specific medium volumes used during C2C12 differentiation. Since metabolic rates change considerably as myoblasts differentiate into myofibers, variations in medium volume and replacement frequency may influence the adequate supply of nutrients for differentiation.[16,17] To address this gap, we investigated the influence of different medium volumes on C2C12 differentiation and myofiber formation.

Materials and methods

C2C12 Cell Culture and Differentiation

The C2C12 cell line of mouse myoblasts (RRID:CVCL_0188) was obtained from the Rio de Janeiro Cell Bank (Duque de Caxias, RJ, Brazil; https://bcrj.org.br/) in September 2023, with confirmed identity and mycoplasma-free status. The cells were cultured at a density of 8×10^3 cells/cm² in T-25 flasks (Cat No. 83.3910.002, Sarstedt, Nümbrecht, Germany) containing 5 mL of Dulbecco's Modified Eagle Medium, High Glucose (DMEM High Glucose; Cat No. 12100046; Gibco, NY, USA), supplemented with 44 mM sodium bicarbonate (Cat No. S5761, Sigma–Aldrich, St. Louis, USA), 1 mM sodium pyruvate (Cat No.11360070, Gibco, NY, USA), and 25 mM HEPES (Cat No. SRE0065, Gibco, NY, USA), adjusted to pH 7.4 with 1 M HCl and 0.1 M HCl solutions. Measurements were made with a K39-0014PA pH meter (Kasvi, Pinhais, PR, Brazil). The medium was further supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS; Cat No. A525670; Gibco, NY, USA), 1% (v/v) penicillin–streptomycin (10,000 U/mL; Cat No. 15140122, Gibco, NY, USA), and 1% (v/v) GlutaMAX (200 mM) (Cat No. 35050061, Gibco, NY, USA). The culture medium was sterile-filtered using 0.22 µm PES membrane filters (Cat No. 83.3941.501, Sarstedt, Nümbrecht, Germany). Cultures were maintained at 37 °C in a humidified atmosphere of 5% CO_2. The culture medium was replaced every 48 hours during cell subculturing. The C2C12 cell line was cultured for 8 days before seeding for differentiation.

For differentiation assays, C2C12 cells were seeded in 24-well plates (Cat No. 3524, Corning, NY, USA) at a density of 2.5×10^4 cells/cm², and 250 µL/cm² of culture medium was used. After 48 h, when the cultures reached >80% confluency, the culture medium was replaced with differentiation medium supplemented with 2% (v/v) heat-inactivated horse serum (Cat No. 16050122, Gibco, NY, USA). Differentiation was induced using 100, 250, or 400 µL/cm² of differentiation medium, which was renewed every 48 h. Volumes used were the equivalent to 200, 500, and 800 µL per well on 24-well plates respectively. The day on which the culture medium was replaced with differentiation medium was designated Day 0 of differentiation. Two control groups were included: a confluent control group, in which C2C12 cells were seeded under the same conditions as those used in the differentiation assays and cultured for 48 h until they reached confluency (>80%), and a subconfluent control group, in which C2C12 cells were seeded at 1.25×10^4 cells/cm² and cultured for 48 h until they reached ~50% confluence.

Microscopy-Based Analysis of Cell Morphology and Differentiation Indices

Live differentiations were photographed on Days 1, 3, and 5 using a Leica DMI6000B inverted microscope equipped with a DFC365 FX camera (Leica Microsystems, Wetzlar,

Germany). On Day 5, the cells were washed twice with phosphate-buffered saline (PBS; Cat No. 70011044, Gibco, NY, USA) and fixed with 4% (w/v) paraformaldehyde (Cat No. P6148; Sigma–Aldrich, MO, USA) in PBS for 30 min. Permeabilization was then performed with 0.3% (v/v) Triton X-100 (Cat No. 282103; Sigma–Aldrich, MO, USA) in PBS for 15 min at room temperature, followed by blocking in 1% (w/v) bovine serum albumin (BSA;Cat No. A4737; Sigma–Aldrich, MO, USA) in PBS for one hour at room temperature. The cells were subsequently incubated overnight at 4°C with an anti-MyHC antibody (clone MF-20; Developmental Studies Hybridoma Bank, Iowa City, USA) diluted 1:100 in 1% BSA. The following day, the wells were washed with PBS and incubated with AlexaFluor488-conjugated donkey anti-mouse IgG (Cat No. A21202, Invitrogen, Waltham, USA) diluted 1:100 in 1% BSA for an hour at room temperature in the dark. Next, the cells were washed with PBS and stained for nuclei with Hoechst 33342 (1 µg/mL in PBS, Cat No. 62249, Thermo Scientific, MA, USA) for 10 min at room temperature in the dark. Finally, after an additional PBS wash, images were acquired via a Leica DMI6000B microscope and an IN Cell Analyzer 2200 (GE Healthcare, Chicago, IL, USA). For quantification of the differentiation index, eight fixed fields per well were captured using the IN Cell Analyzer. Each field was divided into three sections of approximately 48,000 µm² for analysis. The images were processed for contrast, brightness, and size adjustments using ImageJ software, and the nuclei were manually counted. The differentiation index was defined as the percentage of nuclei located within myofibers, defined as MyHC-positive with 3 or more nuclei and was calculated.[18,19]

Metabolic Activity Assay

The metabolic activity of the cells after differentiation was assessed by measuring their ability to reduce 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Cat No. M6494, Invitrogen, MA, USA) into formazan. The differentiation medium was supplemented with 10% (v/v) 5 mg/mL MTT stock solution prepared in distilled water. The cell cultures were differentiated in 24-well plates, and on Day 5, they were washed twice with PBS at room temperature. This was followed by a 1-hour incubation at 37 °C in 500 µL of differentiation medium containing MTT in the dark. After incubation, the culture supernatant was aspirated, and 500 µL of dimethyl sulfoxide (DMSO; Cat No. 276855, Sigma–Aldrich, MO, USA) was added to each well. The plates were then shaken gently for 15 min in the dark. Optical density readings were taken at 570 nm using a SpectraMax M2 microplate reader (Molecular Devices, CA, USA).

Gene Expression Analysis by Quantitative Real-Time PCR (qPCR)

Total RNA was extracted using TRIzol Reagent (Cat No. 15596026; Invitrogen, MA, USA) according to the manufacturer's instructions, and the resulting extracts were subsequently resuspended in UltraPure distilled water (Cat No. 10977035; Invitrogen, MA, USA). The RNA concentration and purity were assessed using a NanoDrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA, USA), and only samples with A260/A280 ratios between 1.9 and 2.1 were used for subsequent experiments. For DNA removal, 500 ng of total RNA was treated with DNase I (Cat No. AMPD1; Sigma–Aldrich, MO, USA) following the manufacturer's protocol. cDNA was synthesized using high-capacity cDNA reverse transcription kit (Cat No. 4368814; Applied Biosystems, MA, USA) in combination with an RNaseOUT ribonuclease inhibitor (Cat No. 10777019; Invitrogen, MA, USA), according to the appropriate protocols. The resulting cDNA samples were diluted 1:1 in UltraPure distilled water prior to PCR. qPCR was carried out in 20 µL reaction, containing 1 µL of diluted cDNA, 10 µL of RT² SYBR Green FAST Mastermix (Cat No. 330600; Qiagen, Hilden, Germany), 0.5 µL of 10 µM forward primer, and 0.5 µL of 10 µM reverse primer. Primers for Myod1, Myog, Myf6, Myh1, and Myh7 were designed considering RefSeq RNA Database, using Primer-BLAST tool to span exon–exon junctions of the target transcripts (Table 1). The primers used were synthesized by Exxtend Biotecnologia Ltda. (Paulínia, SP, Brazil).

Reactions were performed in duplicates using a Rotor-Gene Q Cycler (Qiagen, Hilden, Germany), and the results were normalized to Gapdh expression, based on its validated stability during C2C12 differentiation.20 The specificity of target amplification was assessed through melting curve analysis. Cycle threshold (Ct) values and corresponding amplification efficiencies, provided by the Q-Rex software (Qiagen, Hilden, Germany), were averaged for each sample and used to determine relative expression levels

Print ISSN: 2527-4384, Online ISSN: 2527-3442
DOI: 10.21705/mcbs.v10i3.813

Table 1. List of primers used for qPCR.

Gene	Primer	Sequence	NCBI reference sequence	Amplicon size (bp)	Annealing Temperature (°C)
Myod1	Forward	CTCACCAGACCTGCGCTTTT	NM_010866.2	134	58
	Reverse	CATGCTCATCCTCACGAGCG			
Myog	Forward	CCCTACAGACGCCCACAATC	NM_031189.2	102	58
	Reverse	CAGTTGGGCATGGTTTCGTC			
Myf6	Forward	CCACAGATCGTCGGAAAGCA	NM_008657.3	113	62
	Reverse	CAGTCTCTGGTTGGGGTTGG			
Myh1	Forward	CGGTCGAAGTTGCATCCCT	NM_030679.2	145	60
	Reverse	GAGCCTCGATTCGCTCCTTT			
Myh7	Forward	ACCTGTCCAAGTTCCGCAAGG	NM_080728.3	159	60
	Reverse	CCCTTGGAGCTGGGTAGCACA			
Gapdh	Forward	ACTGGCATGGCCTTCCGTGTTC	NM_008084.4	228	61
	Reverse	ATTGAGAGCAATGCCAGCCCCG			

using the Pfaffl method, which accounts for differences in amplification efficiency between target and reference genes.[21]

Statistical Analysis

The results are expressed as the mean ± standard deviation (SD). Analysis of variance (ANOVA) was performed via Tukey's post hoc test for multiple comparisons. Statistical analyses were performed using GraphPad Prism (La Jolla, CA, USA). Immunofluorescence images are representative of 2 independent experiments performed with 3 technical replicates each. Differentiation index was calculated from 4 independent experiments performed with 2 technical replicates each. For RT-qPCR experiments, differentiations were performed as 3 independent experiments, with 3 technical replicates. A single reverse transcription reaction was performed for each technical replicate, and 2 technical replicates of the qPCR were made for each reverse transcribed sample. The mean Ct value and amplification efficiency were used for gene expression analysis. MTT Assay experiments were performed as 2 independent experiments with 5 technical replicates. pH measurements were performed on 3 independent experiments with a single technical replicate each.

Results

Differentiation Medium Volume Affects Fiber Morphology, Differentiation Rate and Cell Metabolism

C2C12 myoblasts seeded at a density of 2.5×10^4 cells/cm² consistently reached over 80% confluency by Day 2 after plating, at which point differentiation was initiated. By Day 5 of differentiation, morphological changes became evident, with individual cells being fused and forming fibers (Figure 1A). Cultures began to show morphological differences on Day 3 of differentiation, and fiber thickness increased steadily until Day 5, after which no noticeable day-to-day differences were observed. Upon the initiation of differentiation, medium acidification was observed under all the tested conditions, with higher volumes more effectively mitigating acidification. The supernatant of day 5 cultures was collected for pH measurement with a K39-0014PA pH meter. Measured values were: 400 µL/cm²: 7.23 ± 0.11; 250 µL/cm²: 7.15 ± 0.10; 100 µL/cm²: 7.08 ± 0.01. Notably, the fiber morphology was strongly and consistently influenced by the medium volume. A lower medium volume resulted in thinner fibers and a greater number of fibers whereas a higher volume appeared to produce fewer but thicker fibers (Figure 1A), with these visual characteristics consistently perceived through microscopy.

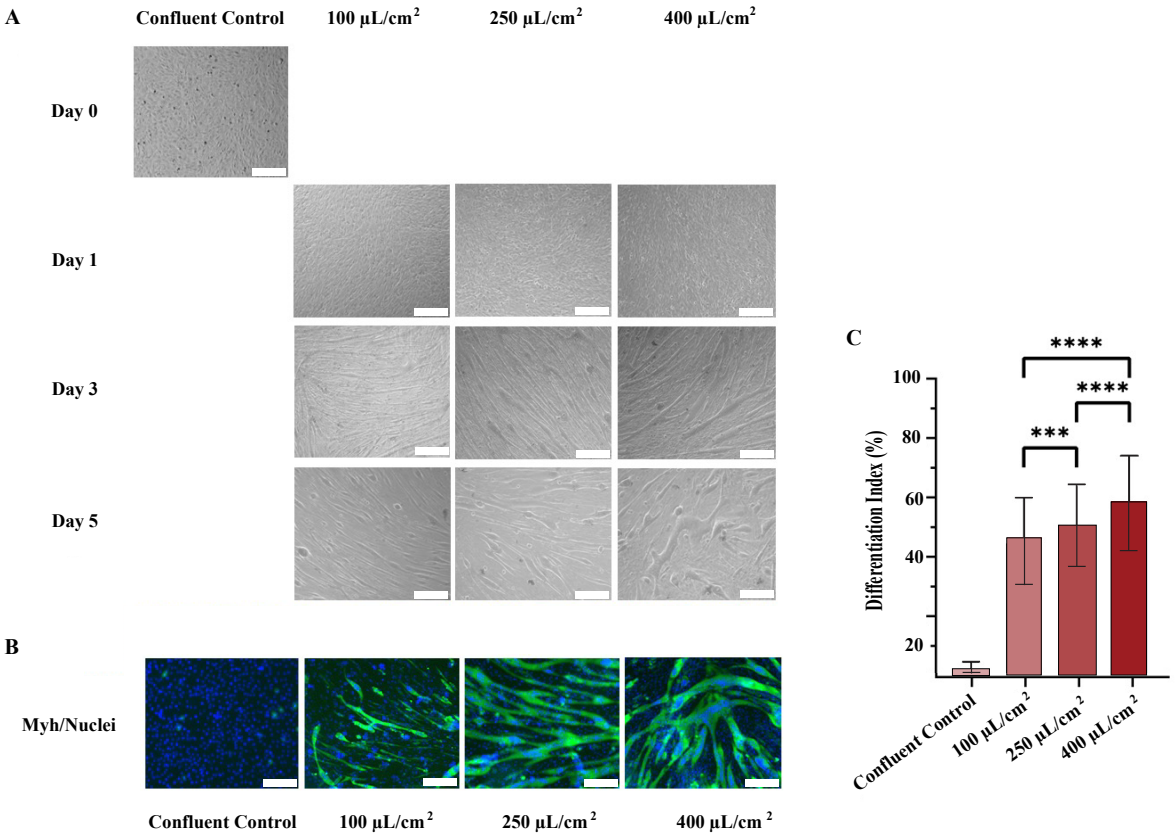


Figure 1. Effect of medium volume on C2C12 cell differentiation and morphology. Highly confluent C2C12 cells were subjected to differentiation via various volumes of differentiation medium. (A) Representative brightfield images showing cell morphology over 5 days of cultivation in the indicated medium volumes. (B) On Day 5, the cells were stained for total myosin heavy chain and nuclei. Images are representative of three independent experiments. (C) Differentiation index based on four independent experiments. ***p<0.001; ****p<0.0001. Comparisons between the control group and all volume groups revealed significant differences. White bar = 100 µm.

To confirm C2C12 differentiation, immunofluorescence staining for sarcomeric myosin heavy chain (MYH) was performed. Confluent cultures on Day 0 presented little to no fluorescence, confirming their undifferentiated state. In contrast, by Day 5, MYH-positive cells were present throughout the entire culture area, indicating successful differentiation. Notably, medium volume qualitatively influenced fiber morphology, with higher volumes leading to fibers with greater cytoplasmic content (Figure 1B).

To make our observations more quantitative, the differentiation index was determined using the acquired microscopy images. The differentiation index, also known as the fusion index or myogenic index, is a commonly used parameter to assess myoblast differentiation in vitro.[11,19] Compared with the other groups, the cultures differentiated with 400 µL/cm² medium presented the highest differentiation index (Figure 1C). A positive correlation was observed between the medium volume and the differentiation index, indicating a clear trend of increasing differentiation with increasing volume. Specifically, the differentiation index increased by more than 5% when 100 µL/cm² was compared with 250 µL/cm² and when 250 µL/cm² was compared with 400 µL/cm². Despite the relatively large standard deviation, the statistical analysis confirmed the significance of this trend. The upper limit of the 95% confidence interval (95% CI) for 100 µL/cm² did not overlap

Print ISSN: 2527-4384, Online ISSN: 2527-3442
DOI: 10.21705/mcbs.v10i3.813

with the lower limit of the 95% CI for 250 µL/cm², and the same was observed between 250 µL/cm² and 400 µL/cm². This lack of overlap, combined with the ANOVA results (p<0.05), indicates that the differentiation index increases significantly with medium volume. Importantly, the random distribution of fibers, together with fiber size and overlap, can bias the analysis. Due to these factors, more than 190 images per condition were analyzed to ensure more reliable counting and statistical analysis.

Since mitochondria play a crucial role in the energy supply of skeletal muscle and undergo biogenesis during myogenesis, we evaluated C2C12 metabolic activity using MTT assays. Cultures with larger fibers presented a consistent increase in metabolic activity (Figure 2F). Myofibers differentiated with 400 µL/cm² presented over a 50% increase in their metabolic activity as measured by MTT when compared to those produced with 100 µL/cm² (400 µL/cm²: Mean = 1.58, SD = 0.06; 250 µL/cm²: Mean = 1.39, SD = 0.05; and 100 µL/cm²: Mean = 0.99, SD = 0.06).

Differentiation Medium Volume Influences Myod1 and Myog Gene Expression on Myofibers

The acquisition of a myofiber phenotype is largely driven by myogenic regulatory factors (MRFs), including myogenic regulatory genes, including *Myod1*, *Myog*, and *Myf6*.[22-25] On the basis of these findings, we examined the expression of these genes alongside type I (*Myh7*) and type II (*Myh1*) muscle markers to assess their relationship with myofiber formation under varying culture medium volumes.

Myod1 expression peaked at >80% confluency but declined to less than a 0.5-fold change relative to the confluent control group by Day 5 of differentiation (Figure 2A). C2C12 cells differentiated under lower medium volumes presented greater Myod1 expression, with Myod1 levels showing a negative correlation with volume/cm².

Myog expression was nearly undetectable in the subconfluent state (Figure 2B). Its expression increased significantly upon reaching confluence and increased several-fold during the differentiation period. Statistically significant differences in *Myog* expression were observed among the volume groups, with higher expression in the lower volume groups, following a pattern similar to *Myod1* expression. However, these differences were more pronounced than those in Myod1.

Myf6 expression remained very low and unchanged across the tested differentiation conditions, with significant expression detected only after cell confluence was reached (Figure 2C). Myf6 is involved primarily in the later stages of myogenesis, contributing to the maturation and maintenance of skeletal muscle fibers.[26]

To evaluate the effect of culture volume on fiber-type formation, we assessed the expression of *Myh1* and *Myh7*, which are predominant in fast-twitch (Type II) and slow-twitch (Type I) fibers, respectively.[27] The expression of these genes was very low or undetectable before differentiation (Figure 2D and 2E). However, after differentiation, the expression of both genes significantly increased across all the volume groups, with no significant differences among them. Owing to the very low expression levels, we used the 100 µL/cm2 group as the reference to calculate the fold change. This expression pattern aligns with the literature, which reports low *Myh1* and *Myh7* levels in myoblasts, followed by increased expression postdifferentiation.[19,28,29] No significant differences in Myh1 and Myh7 expression were observed between the tested conditions.

Discussion

The C2C12 myoblast cell line is widely used to study muscle development and regeneration. Its ability to differentiate into myotubes is highly sensitive to the culture environment, including nutrient uptake dynamics, growth factor signaling, gas exchange, and waste accumulation in the medium. However, there is limited information in the literature regarding the optimal medium volume for C2C12 differentiation. This aspect is particularly important, as the metabolic rate changes significantly from myoblast culture to myofiber culture.[16] Consequently, an insufficient supply of nutrients in the differentiation medium over time could impact the differentiation process, ultimately affecting the number and size of myofibers.

The presence of undifferentiated myoblasts was significant and consistent across all the differentiation experiments, even after five days of differentiation. It has been suggested previously that the differentiation index may reach an upper threshold because secreted factors inhibit differentiation and promote the formation of reserve cells.[19] The impact of medium volume on cellular metabolism has been reported by others using different cell models.[30-32] Previous studies on myoblasts have also demonstrated that the composition of the differentiation medium used results in myofibers with distinct phenotypes and metabolic profiles, while serum-free differentiations also lead to earlier expression of sarcomeric actin.[15,16] As mentioned previously, in all the experiments and across all the tested

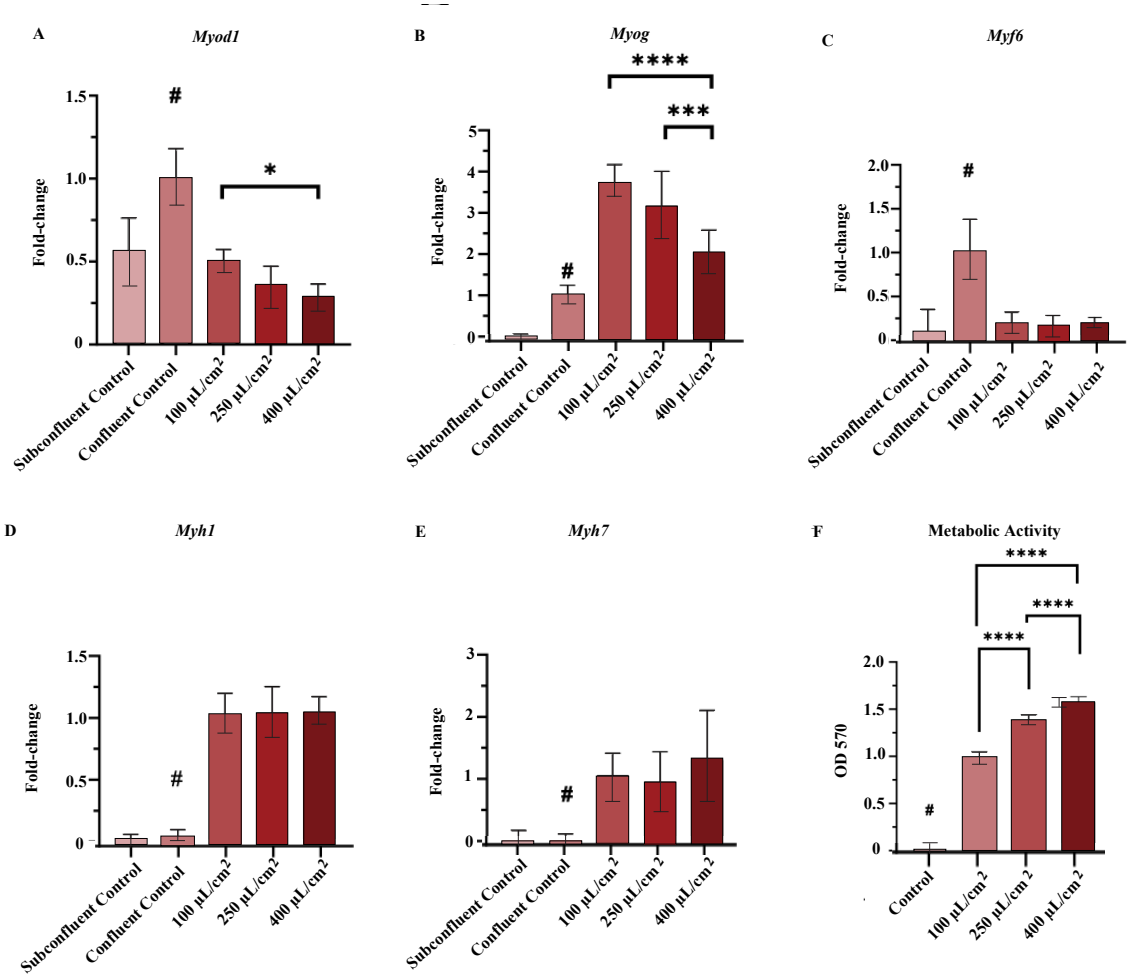


Figure 2. Effect of medium volume on gene expression and metabolism. Subconfluent, confluent, and 5-day differentiated C2C12 cells were analyzed via RT–qPCR for the expression of key myogenesis regulators and sarcomere components: *Myod1* (A), *Myog* (B), *Myf6* (C), *Myh1* (D), and *Myh7* (E). Fold changes were calculated relative to the Confluent Control. For *Myh1* and *Myh7*, the 100 µL/cm² group was used as the reference for the fold-change calculations because the Confluent Control group presented very low expression levels of these genes. The data represent the means of two independent experiments performed in triplicate. (F) Differentiation metabolic activity was assessed using the MTT assay, with data compiled from two independent experiments, each performed in quintuplicate. Statistical significance: **p*<0.05; ***p*<0.01; ****p*<0.001; *****p*<0.0001; #: comparisons between the control group (confluent control (A-E) and Control (F)) and all volume groups revealed significant differences (**p*< 0.05). OD570 nm: optical density at 570 nm.

volumes, the rate of medium acidification increased noticeably after the initiation of differentiation, indicating an increase in metabolic activity. Larger myofibers are expected to require more energy due to the increased demand for protein synthesis, and cellular maintenance. MTT assays measure the conversion of MTT into formazan by cellular oxidoreductases, primarily mitochondrial dehydrogenases.[33] Therefore, the higher metabolic rate observed in cells cultured with larger medium volumes likely reflects the increase in mitochondrial activity and the total differentiated biomass.

Because *Myod1* is a master regulator gene that promotes myogenic lineage commitment, observing its peak expression at confluence followed by a decrease during differentiation is a well-documented pattern.[22,24] In this context, the lower *Myod1* expression observed with larger culture volumes is also a consistent finding. *Myog* gene plays a key role in muscle cell differentiation and

Print ISSN: 2527-4384, Online ISSN: 2527-3442
DOI: 10.21705/mcbs.v10i3.813

maturation, functioning to drive the transition from early to late differentiation phases.[11,34,35] Thus, its higher expression by Day 5 of differentiation, compared with that on Day 0, follows an expected pattern. Once myoblasts transition into fully differentiated myotubes, the role of *Myog* diminishes, leading to a natural decline in its expression.[35,36] Therefore, the increased expression of *Myog* at lower volumes is consistent with that of *Myod1*. While *Myog* is more critical for myoblast differentiation, *Myf6* plays a greater role in muscle fiber maintenance and the final stages of differentiation. The consistently low *Myf6* expression observed on Day 5 may reflect the temporal delay in its activation or suggest that further progression of myotube maturation is still required under the tested conditions.

This study has some limitations that should be acknowledged. Our findings may not fully extrapolate to other myoblastic cell lines or primary myoblast cultures. Besides, the molecular analysis was limited to a small panel of myogenic markers, making further investigation with a more comprehensive panel necessary to better understand the effects observed.

Conclusion

This study demonstrated that the volume of differentiation medium significantly influences C2C12 myoblast differentiation, affecting myofiber morphology, differentiation efficiency, and metabolic activity. Higher medium volumes were associated with visually thicker myofibers, higher differentiation indices, and increased mitochondrial activity, likely due to nutrient availability and/or reduced accumulation of metabolic byproducts. Conversely, lower medium volumes led to a seeming greater number of thinner fibers and higher expression of the early myogenic markers (*Myod1* and *Myog*), indicating a delayed or less efficient differentiation process. Despite these differences, no significant alterations were observed on the expression of *Myh1* and *Myh7*, implying that medium volume influences efficiency without affecting fiber-type-associated markers.

Acknowledgment

This study was funded by the São Paulo Research Foundation [FAPESP; grant #2023/03903-3], the National Council for Scientific and Technological Development [CNPQ; grant #406018/2022-7], and the Brazilian Federal Agency for Support and Evaluation of Graduate Education [CAPES; grants #88887.903165/2023-00 and #88887.615277/2021-00].

Authors' Contributions

BS, AVHL and SWH were involved in concepting and planning the research, BS and AVHL performed the data acquisition/collection and also calculated the experimental data and performed the analysis, BS and SWH drafted the manuscript and designed the figures, SWH aided in interpreting the results. All authors took parts in giving critical revision of the manuscript.

Ethical Statement

No human participants, human biological materials, or animals were involved in this study. Therefore, ethical approval from an institutional review board or ethics committee was not required.

Conflict of Interest

The authors declare that they have no conflicts of interest or competing interests related to the content of this manuscript.

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