Ann Med Res

Current issue list available at AnnMedRes

Annals of Medical Research

journal page: www.annalsmedres.org

Morphological examination is more effective than cell viability assays in the characterization of myotube atrophy

Ugur Akpulat

Kastamonu University, Faculty of Medicine, Department of Medical Biology, Kastamonu, Türkiye

ARTICLE INFO

Keywords:
Skeletal muscle atrophy
Myotube atrophy
MTT assay
Myotube morphology

Received: Jul 31, 2023
Accepted: Nov 27, 2023
Available Online: 27.11.2023

DOI:
10.5455/annalsmedres.2023.07.170

Abstract

Aim: Skeletal muscle atrophy is a significant health problem associated particularly with aging, cancer, and metabolic diseases, leading to a decline in quality of life and responses to treatment. During skeletal muscle atrophy, the balance between protein synthesis and degradation in the tissue is disrupted in favor of protein degradation. This condition results in a reduction in muscle mass and loss of strength and function in the muscles. In order to elucidate the molecular mechanisms causing skeletal muscle atrophy, prevent the processes leading to atrophy, and test the effectiveness of emerging therapeutics, in vitro models that mimic atrophic conditions are commonly used. In these models, typically utilizing primary myoblasts obtained from tissues or myoblast cell lines, either cell viability assays or myotube morphology analyses are employed to determine the degree of atrophy occurring during differentiation. In this study, the two different approaches, which are generally used interchangeably, have been mutually evaluated for their ability to characterize the features of myotube atrophy.

Materials and Methods: The myotube atrophy model was established by subjecting differentiating C2C12 cells from myoblasts to myotubes to varying doses of a synthetic glucocorticoid, dexamethasone. Atrophic changes occurring dose-dependently in myotubes were evaluated using the commonly employed MTT cell viability assay and myotube diameter measurement analysis.

Results: While myotube diameter measurement can grade the extent of atrophy in myotubes, the MTT assay, although unable to directly assess the degree of myotube atrophy, demonstrated that the decrease in cell viability indicates the cause of myoblast atrophy.

Conclusion: While cell viability tests and myotube morphology analyses are commonly used interchangeably to assess myotube atrophy, these two approaches can characterize different aspects of myotube atrophy. The choice of approach should be determined based on the research nature, considering their abilities to characterize myotube atrophy.

Introduction

Skeletal muscle constitutes about 40% of the total body weight and is the main protein reservoir due to the high amount of contractile proteins. The protein mass increases to a greater extent than in any other life period during neonatal life to provide an organism's prerequisite functions including breathing, eating, and moving. The muscle mass is maintained by cell and protein turnover regulatory pathways responsively to environmental cues to sustain those functions throughout the life cycle [1]. The coordination in regulatory pathways and balance between protein synthesis and degradation are critical for long-term health and quality of life. Malnutrition, aging, cancer cachexia, muscle disuse, and various muscular dystrophy conditions lead skeletal muscle fibers to undergo atrophy [2]. During muscle atrophy, the protein degradation rate exceeds protein synthesis, leading to muscle mass loss and a decline in muscle function. Muscle atrophy can give rise to weakness and fatigability, a reduced ability to perform daily activities, mobility disorders, delayed recovery from acute diseases, impaired efficacy of treatments for chronic diseases, and an imbalance in metabolism and cognition. Thus, avoiding muscle atrophy or minimizing muscle loss is crucial for preventing functional limitations, mobility loss, metabolic diseases, and maintaining muscle strength [3, 4].

Understanding the molecular mechanisms that mediate the process of atrophy, developing novel therapeutic agents to prevent muscle loss, and discovering mediators that ex-

*Corresponding author:
Email address: uakpulat@kastamonu.edu.tr (Ugur Akpulat)

acerbate protein breakdown require responsive experimental systems and measurement methods to assess the magnitude of the response. Myoblast cultures provide valuable insights into many intracellular signaling pathways involved in skeletal muscle atrophy. Generally, primary skeletal myoblast cells obtained from human or model animal tissue biopsies and immortalized cell lines such as murine C2C12 cells or rat L6 cells are used to study skeletal muscle atrophy [5, 6].

The pathophysiology of muscle atrophy is commonly modeled in vitro by exposing differentiated myoblasts (myotubes) to various substances such as inflammatory cytokines (e.g., TNF-alpha), H_2O_2, and dexamethasone. These agents lead to protein breakdown and shrinkage of myotubes by inducing proteolysis mechanisms, oxidative stress, apoptosis, endoplasmic reticulum stress, and mitochondrial dysfunction [6]. During these experimental systems, either viability assays or interpreting morphological changes of myotubes are generally applied to measure the atrophy rate or the impact of anti-atrophic candidates [7-9]. Even though both approaches are widely performed, since both indirectly determine the atrophic development and assess different aspects of the process, one should consider which is more suitable for the experimental design. In this study, the two most preferred tests, the MTT cell viability method and myotube diameter measurement, were evaluated in terms of the extent to which atrophy conditions affected myotubes.

Materials and Methods

Cell culture and induction of atrophy in myotubes

C2C12 murine skeletal muscle cells (ATCC; American Type Culture Collection) were cultured in a humidified incubator (37 °C, 5% CO_2) at low density with high glucose DMEM (Dulbecco's modified Eagle's medium; Invitrogen, Gibco) containing 10% FBS (Fetal Bovine Serum; GE Healthcare) and 1% P/S (Penicillin-Streptomycin, 10,000 U/mL; Invitrogen, Gibco). To induce spontaneous differentiation of myoblast to myotubes, the medium was replaced with high glucose DMEM containing 2% HS (Horse Serum; Invitrogen, Gibco) and 1% P/S when the cells reached about 80% confluence. Dexamethasone (DEX; Sigma-Aldrich) was used to stimulate muscle atrophy in myotubes. Following 2 days of differentiation, the differentiating cells were treated with 25 µM, 50 µM, and 100 µM DEX for 2 more days. In the control group, the myoblasts were allowed to undergo 4 days of differentiation without DEX treatment. All experimental conditions were performed in triplicate wells.

Staining of myotubes and myotube diameter measurement

LADD staining protocol was used to readily distinguish myotubes from unfused myoblasts and thus measure the myotube diameter. LADD stain was prepared freshly by dissolving 0.27 g fuchsin (Sigma-Aldrich) and 0.73 g toluidine blue (Sigma-Aldrich) in a final volume of 100 mL of 30% ethanol solution. Briefly, the cells were washed with PBS (phosphate-buffered saline) and fixed in 70% ethanol at room temperature (RT) for 10 minutes. The ethanol was aspirated, and the cells were covered with LADD stain solution at RT for 1 minute. Afterward, cells were washed with distilled water several times to remove unbound staining until no further water discoloration was evident. The cells were allowed to air dry before visualization by phase contrast brightfield microscopy. Images were captured at 100X magnification. The diameters of at least 100 myotubes from 12 random fields of the triplicate wells of each condition were measured. The mean diameter of each myotube was calculated from three approximately equidistant points along their length using the Image J software (NIH).

MTT cell viability assay

The viability of the myotubes exposed to varying concentrations of dexamethasone was determined by MTT assay, as described before with minor modifications [10]. The cells differentiated for a total of four days, with the last two days in the presence or absence of dexamethasone, were incubated in MTT medium supplemented with 5mg/mL MTT reagent and 0.5% FBS in DMEM for 4 h at 37°C. After removing the medium, the formed formazan crystals were dissolved in DMSO, and the absorbance of the lysate was measured at a wavelength of 570 nm using a microplate reader. The viability of dexamethasone-treated myotubes was expressed as a percentage relative to the untreated control ones.

Results

Influence of atrophy induction on morphological appearance of myotubes

After a two-day differentiation period, cells that continued to differentiate in a culture medium containing dexamethasone for an additional 48 hours exhibited all the characteristic morphological features of myotube atrophy. Myotubes showed significant morphological differences in length, width, and surface area, varying degrees in accordance with the dosage of dexamethasone. The myotubes exposed to dexamethasone displayed cell membrane shrinkage, a significant reduction in their diameters, and exhibited misalignment with each other. Moreover, the placement and distribution of myonuclei along the myotube revealed substantial differences compared to control cells (Figure 1A).

Cell viability under atrophic conditions

In myotubes stimulated with dexamethasone to induce atrophy, the commonly used MTT assay was applied to determine the extent to which tests measuring cell viability could represent myotube atrophy. During the differentiation process from myoblasts to myotubes, cells exposed to dexamethasone for two days exhibited a similar loss of viability compared to the control cells, regardless of the difference in dosage (Figure 1B). Despite exposure to different doses of dexamethasone, approximately a 20% decrease in cell viability was observed in all cells within the applied dosage range compared to the control group. Dexamethasone has caused a significant reduction in cell viability; however, the MTT assay results did not reflect the dose-dependent morphological differences in myotubes that could be clearly distinguished by microscopic examination.

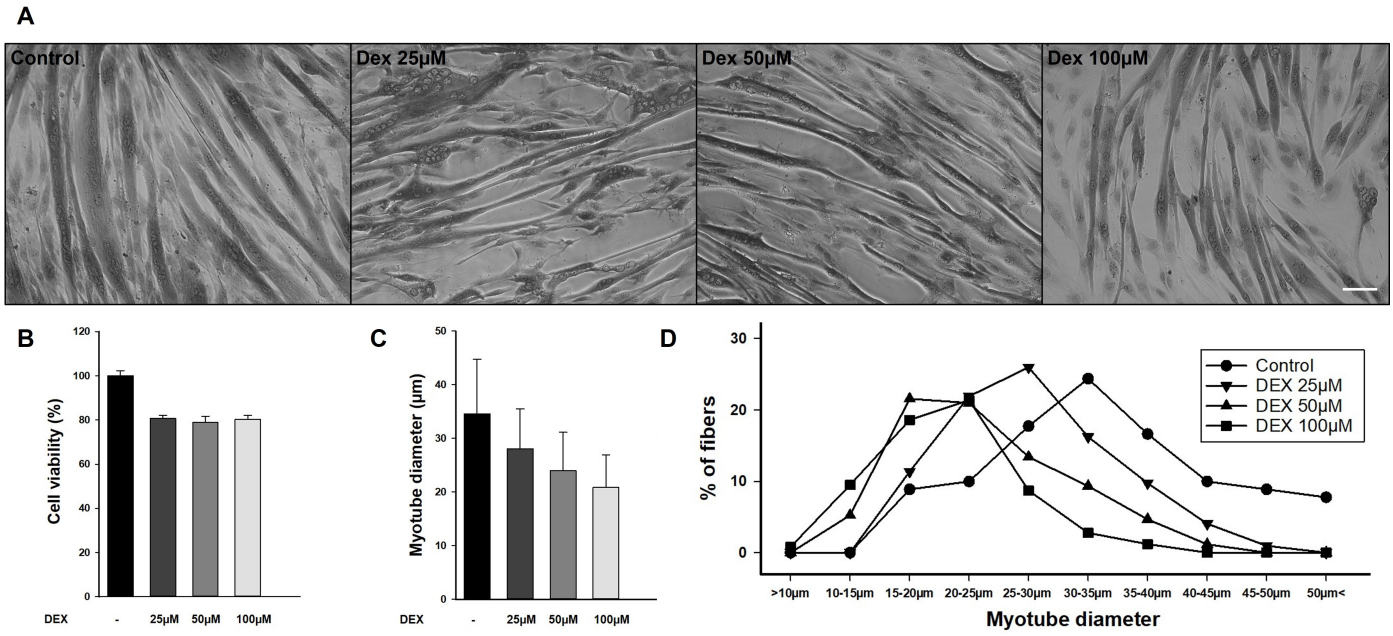

Figure 1. Characterization of dexamethasone-induced myotube atrophy. (A) Representative images showing the morphological appearance of myotubes exposed to different doses of dexamethasone during the differentiation process. (B) Demonstration of the effect of different doses of dexamethasone on the total cell viability in culture compared to the control using the MTT assay. (C) Mean myotube diameters. (D) Distribution of myotube diameters within each group. The scale bar is 100 µm.

Determination of atrophy by myotube diameter measurement

Although morphological observations demonstrated that dexamethasone caused atrophy in myotubes, the diameter of myotubes was measured to assess the extent to which they were affected by different doses of dexamethasone. For this purpose, analyses were conducted to evaluate the mean myotube diameter and the distribution of myotube diameters in response to dexamethasone. The results revealed that the mean diameter of myotubes decreased dose-dependently in dexamethasone-treated groups compared to the control (Figure 1C). Furthermore, as the dose of dexamethasone increased, the number of larger myotubes decreased while the number of smaller myotubes increased (Figure 1D).

Discussion

Skeletal muscle atrophy is a condition where the muscle fibers experience a size reduction. Glucocorticoids (GC) significantly impact skeletal muscle, reducing protein synthesis and increasing protein breakdown, ultimately contributing to muscle atrophy. When animals are administered high doses of GC, it decreases muscle mass and dysfunction, characterized by weakened force and diminished strength [11, 12]. Dexamethasone is a synthetic corticosteroid that mimics the actions of natural glucocorticoids produced by the adrenal glands. In in vitro experiments, when C2C12 myotubes are exposed to dexamethasone, it inhibits the formation of myotubes and reduces the diameter of existing myotubes. This effect is achieved through the process of ubiquitin-proteasome-mediated protein degradation [13-15].

The MTT calorimetric assay is used to evaluate the capability of viable cells to convert soluble tetrazolium salt into an insoluble formazan precipitate. Due to the direct correlation between metabolic activity and cell count in dividing cells, MTT assay is commonly employed to determine the cytotoxic effect of a compound on dividing cells in a culture medium [16]. During differentiation, myoblasts undergo fusion with each other or with existing myotubes [17]. Throughout the differentiation process, the cellular composition of the culture medium consists of both myoblasts and myotubes. Therefore, the metabolic activity measured by the MTT assay represents the total metabolic activity, including the myotubes and the differentiating myoblasts. Although the MTT assay partially demonstrates that dexamethasone leads to myotube atrophy by reducing metabolic activity during differentiation, the extent of atrophy in myotubes cannot be entirely determined. However, myotube diameter measurement analysis can effectively scale the atrophy developed in myotubes depending on the dose of dexamethasone, morphologically. The findings demonstrate that myotube diameter measurement is more sensitive than cell viability assays in defining and quantifying myotube atrophy.

Conclusion

In experimental systems where skeletal muscle atrophy is modeled under in vitro conditions, assessing the extent to which a compound exacerbates or alleviates atrophy is commonly performed either through tests measuring cell

viability or by analyzing myotube morphology. In this study, where both approaches were evaluated for myotube atrophy, it was demonstrated that they are not mutually exclusive but rather complementary in providing a comprehensive assessment of atrophy.

Ethical approval

It is a cell culture study that does not require ethical approval.

References

1. Davis TA, Fiorotto ML. Regulation of muscle growth in neonates. Curr Opin Clin Nutr Metab Care. 2009;12(1):78-85.
2. Ebert SM, Al-Zougbi A, Bodine SC, Adams CM. Skeletal Muscle Atrophy: Discovery of Mechanisms and Potential Therapies. Physiology (Bethesda). 2019;34(4):232-9.
3. Yin L, Li N, Jia W, Wang N, Liang M, Yang X, et al. Skeletal muscle atrophy: From mechanisms to treatments. Pharmacol Res. 2021;172:105807.
4. Schiaffino S, Dyar KA, Ciciliot S, Blaauw B, Sandri M. Mechanisms regulating skeletal muscle growth and atrophy. FEBS J. 2013;280(17):4294-314.
5. Sharples AP, Stewart CE. Myoblast models of skeletal muscle hypertrophy and atrophy. Curr Opin Clin Nutr Metab Care. 2011;14(3):230-6.
6. Mankhong S, Kim S, Moon S, Kwak HB, Park DH, Kang JH. Experimental Models of Sarcopenia: Bridging Molecular Mechanism and Therapeutic Strategy. Cells. 2020;9(6).
7. Gao H, Ao M, Wang H, Yu L. Rapamycin represses myotube hypertrophy and preserves viability of C2C12 cells during myogenesis in vitro. Transplantation. 2014;98(2):139-47.
8. Roberts BM, Kolb AL, Geddis AV, Naimo MA, Matheny RW. The dose-response effects of arachidonic acid on primary human skeletal myoblasts and myotubes. J Int Soc Sports Nutr. 2023;20(1):2164209.
9. Tseng YT, Chang WH, Lin CC, Chang FR, Wu PC, Lo YC. Protective effects of Liuwei dihuang water extracts on diabetic muscle atrophy. Phytomedicine. 2019;53:96-106.
10. Sinan KI, Akpulat U, Aldahish AA, Celik Altunoglu Y, Baloglu MC, Zheleva-Dimitrova D, et al. LC-MS/HRMS Analysis, Anti-Cancer, Anti-Enzymatic and Anti-Oxidant Effects of Boerhavia diffusa Extracts: A Potential Raw Material for Functional Applications. Antioxidants (Basel). 2021;10(12).
11. Shah OJ, Kimball SR, Jefferson LS. Acute attenuation of translation initiation and protein synthesis by glucocorticoids in skeletal muscle. Am J Physiol Endocrinol Metab. 2000;278(1):E76-82.
12. Schakman O, Kalista S, Barbe C, Loumaye A, Thissen JP. Glucocorticoid-induced skeletal muscle atrophy. Int J Biochem Cell Biol. 2013;45(10):2163-72.
13. Massaccesi L, Goi G, Tringali C, Barassi A, Venerando B, Papini N. Dexamethasone-Induced Skeletal Muscle Atrophy Increases O-GlcNAcylation in C2C12 Cells. J Cell Biochem. 2016;117(8):1833-42.
14. Shen S, Liao Q, Liu J, Pan R, Lee SM, Lin L. Myricanol rescues dexamethasone-induced muscle dysfunction via a sirtuin 1-dependent mechanism. J Cachexia Sarcopenia Muscle. 2019;10(2):429-44.
15. Desler MM, Jones SJ, Smith CW, Woods TL. Effects of dexamethasone and anabolic agents on proliferation and protein synthesis and degradation in C2C12 myogenic cells. J Anim Sci. 1996;74(6):1265-73.
16. Kumar P, Nagarajan A, Uchil PD. Analysis of Cell Viability by the MTT Assay. Cold Spring Harb Protoc. 2018;2018(6).
17. Wong CY, Al-Salami H, Dass CR. C2C12 cell model: its role in understanding of insulin resistance at the molecular level and pharmaceutical development at the preclinical stage. J Pharm Pharmacol. 2020;72(12):1667-93.