Morphometric Characteristics of Human Skeletal Muscle Cells in Primary Culture

Características Morfométricas de Células Musculares Esqueléticas Humanas en Cultivo Primario

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SUMMARY: The study of cell morphology has contributed to the innovation of clinical techniques and biomedical research. Primary cell culture techniques are well standardized; however, knowledge about morphometric parameters under cell culture conditions is scarce. Variations in morphology can affect cell physiology and responses. The aim of this study was to use morphometric tools to describe the growth and development of skeletal muscle cells under standard cell culture conditions. A photographic database was generated, and morphometric data was obtained for nine cell characteristics (n = 559 cells). Four muscular cell shapes (spherical, irregular outline, triangular and spindle/fusiform) were characterized with wide ranges in variation. The maximum cell length (110-262 µm), width (35-66 µm), area (2,642 - 9,480 µm²), projection lengths (45 - 127 µm), and nucleus diameter (28 ± 11 µm) were obtained by day 23 of culture. A single centrally positioned nucleus was observed in each cell; nucleoli diameter (5 ± 2 µm) and number (1 - 5) varied. In general, cyclic changes in cell sizes were identified during culture, whereas cell length, width, and area increased in spurts. These results suggest that morphometric parameters can be used to monitor skeletal muscle cell development under standard culture conditions.

KEY WORDS: Cell biology; Histology; Morphology; Muscle.

INTRODUCTION

Satellite cells, a precursor of skeletal muscle cells, remain in an undifferentiated state and display little activity in mature tissues, although myoblast proliferation can occur following satellite cell activation (Yablonka-Reuveni, 2011). Myoblasts are mononuclear cells that divide and merge to generate multinucleated myocytes, a precursor of muscle fibers (Almeida *et al*., 2016). Cell culture techniques allow the detailed study of the development and metabolism of cells at different states and under diverse conditions (Gil-Loyzaga, 2011). Bioassays performed under controlled conditions facilitate the study of issues ranging from the adaptation of cells exposed to extreme environmental conditions (Slentz *et al*., 2001), to the metabolic effects of toxic substances such as caffeine and nicotine (Kordosky-Herrera & Grow, 2009). Morphometric comparison of muscle cells under culture conditions has contributed to the understanding of biochemical processes that lead to apoptosis (Sandri & Carraro, 1999) and

the progression of diseases such as dystrophinopathies (Delaporte *et al*., 1984), where morphometric information sets the basis for the development of promising therapies (Dumont & Rudnicki, 2016; Piga *et al*., 2019).

As with other cell types, morphological analyses of muscle cells developing under basal, normal, or standard conditions are of biological interest as information regarding the cells themselves, the individual, or species they were derived from can be obtained from the cell's appearance. This morphological approximation is also useful when diagnosing lesions or pathologic processes, and as a prognostic tool as changes in the characteristics of the cytoplasm and organelles can be important for the interpretation of cell state and metabolism (True, 1996; Kumar *et al*., 2017). Image analyses, including cytometry and the quantification of cellular components and morphometry, are generally used for these

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purposes (Meijer *et al*., 1997). Morphometry quantitatively describes the geometric characteristics of tissues, cells, nuclei, and nucleoli in either bidimensional or tridimensional contexts (Raskin & Meyer, 2010). Knowing the possible range of variation for a particular characteristic allows the limits of a phenotype to be identified and contributes to the understanding and interpretation of metabolism under typical and anomalous conditions (Ressel, 2018). For example, in dystrophinopathies, variations in the size of muscle fibers and atypical positioning of the nuclei have been described with respect to normal muscle cells (Kumar *et al*.). Under cell culture conditions, the cellular length, width, and number of nuclei are of interest in the description of Duchenne's muscle dystrophy, a progressive disease that produces severe muscle weakness and atrophy (Delaporte *et al*.).

Even with the advances in muscle cell knowledge, research regarding morphological development under cell culture conditions is still pertinent. The objective of this study was to use morphometric tools to describe the development of human muscle cells in standard cell culture conditions, from the initial steps of primary culture to the propagation phase.

MATERIAL AND METHOD

Ethical aspects. This study was conducted in strict accordance to the principles expressed in the Declaration of Helsinki, in conformity with the Ley General de Salud (2020), and the official laws in Mexico (NOM-007-SSA2-2016). Sampling and research protocols, as well as the informed consent were approved by and registered with Coordinación Nacional de Investigación en Salud del Instituto Mexicano del Seguro Social (IMSS, Ref. F-CNIC 2019-174; R 2000-785-008) and Coordinación Nacional de Bioética (CONBIOÉTICA, Ref. B5-61-2800/202000). All donors were previously informed of the procedure, the research purpose, and destination of the biological samples; informed consent was obtained prior to sampling.

Criteria for donor selection. Human skeletal muscle samples were obtained from healthy female donors subjected to scheduled caesarean section at the Hospital General de Zona 1, IMSS, La Paz, Baja California Sur, Mexico. Inclusion criteria included adult women whose health status had been monitored in the previous months, were diagnosed as clinically healthy by a certified MD, and were scheduled for routine (non-emergency) cesarean section. Exclusion criteria included the presence of chronic diseases, the necessity for emergency cesarean intervention, or the unwillingness to sign the informed consent.

Sample collection. Muscle biopsies were collected between January 2019 and February 2020. During the programmed surgery, approximately 3 g of rectus abdominis muscle was collected from each donor. Samples were immediately rinsed using Dulbecco's phosphate-buffered saline (DPBS 1X, Gibco™) containing 2 % penicillin/streptomycin (10,000 U mL^{-1}, Gibco™). Each sample was placed in a Falcon tube (50 mL, Corning™) containing DMEM/F12 medium (1X, Gibco™) and 1 % penicillin/streptomycin mix (10,000 U mL^{-1}, Gibco™), placed on ice and transported to the Oxidative Stress Laboratory at the Centro de Investigaciones Biológicas del Noroeste (CIBNOR), S.C., La Paz, Baja California Sur, Mexico, following standard biological security criteria (NOM-051-SCT2/2011, 2011; World Health Organization, 2015). Samples were immediately processed to initiate primary cell cultures.

Primary cell cultures. Using a Class II Type A2 laminar flow hood (Esco Labculture™, E-Series), samples were transferred to sterile Petri plates and rinsed with DPBS containing 1 % penicillin/streptomycin mix. Unwanted tissues (e.g., fat, connective tissue) were removed and samples were mechanically disaggregated. Small (<1 mm) muscle pieces were plated in cell culture flasks (25 mL, Falcon™) and 2 mL of growth medium (1X DMEM/F12, 12.4 % v/v fetal calf serum, 1 % 10,000 U mL^{-1} penicillin/ streptomycin, 100X GlutaMAX, 100 mM sodium pyruvate, and 1M HEPES) was added. Flasks were placed in an incubator (Thermo Scientific™, Series 8000) at 37°C (± 0.5°C) in 5 % CO_2 and 95 % (± 5 %) humidity. Following 24 hours of incubation, samples were supplied with 1 mL of growth medium and returned to the incubator to allow adhesion of the tissue fragments to the flask surface. On day three of incubation, detritus and tissue fragments that had not adhered to the flasks were removed; growth medium was replaced every three days (Neville *et al*., 1997). Cell cultures were continuously monitored using an inverted microscope (Zeiss, Axio Vert. A1). Once the skeletal muscle cells had spread to cover most of the growth surface (90 - 100 % confluency), a standardized trypsinization protocol was used to remove adherent cells (Bischoff, 1974) which were moved to new flasks (70 mL, Falcon™). Flasks were placed in an incubator using the aforementioned conditions and cell growth was monitored with routine maintenance every third day. Transfer was performed when cells reached > 90 % confluency.

Cell density and viability. At each transfer, cell density and viability were estimated following methods modified from Doyle & Griffiths (1998). Cell suspensions were diluted with DPBS, stained with Trypan Blue (0.4 %, Gibco™), and incubated for 5 minutes at room temperature (25 °C). Cell dilutions were placed in a Neubauer chamber (0.0025 mm²,

bright-line, Marienfeld™) and the number of the viable and non-viable cells was evaluated, in triplicate, using an optical microscope (10X objective, ZEISS™) (Louis & Siegel, 2011). The percentage of viable cells was estimated (Doyle & Griffiths; Louis & Siegel) using the equation:

$$\% \text{ Viable Cells} = (\text{Number of viable cells} / \text{Total number of cells}) * 100$$

Skeletal muscle cell morphometric characterization. Color (32 bpp, RGB) photomicrographs were obtained from each flask at each transfer using an inverted microscope coupled with a digital camera (AxioCam, ICc5™) under phase contrast illumination. Eighty-four photomicrographs in JPEG high-resolution format (5 mp; 2,452 x 2,056 pixels) were selected for analysis.

Nine morphologic characteristics were considered for cell description (Table I) (Ressel). Using ImageJ 1.53c (Rasband, 2018) software, the photomicrographs were transformed to gray color scale (8 bpp) without modifying resolution or relative size. Prior to recording cell measures, each photomicrograph was calibrated with a standard length (digital grid, ZEISS™), to set 1.4 pixels:µm, 2.9 pixels:µm and 5.8 pixels:µm ratio scales for 10X, 20X, and 40X objectives, respectively. Finally, scaling bars were added as a reference to cell size.

Statistical analysis. Atypical values were identified and re-moved using Grubbs' test for two opposite outliers. Morphometric variables were $\log_{10}$ transformed (Sokal & Rohlf, 2009), and normality and homoscedasticity were analyzed using the Lilliefors-Kolmogorov-Smirnov and Levene's tests, respectively. A Kruskal-Wallis rank sum test was applied to determine equality between data from different cell cultures and dates. A Tukey's post hoc test was performed to identify differences between samples (Daniel & Cross, 2018). Significance in all cases was assumed when $p < 0.05$ ($\alpha = 5$ %). For all statistical analyses and graphs, R 3.6.3 software (R Core Team, 2020) with graphics interface Rstudio 1.2.5042 (RStudio Team, 2020) was used. Data

tendency lines were plotted using the geom_smooth function (Wickham, 2016) for smoothed conditional means, based on generalized additive models, from ggplot2 R library (Wood, 2020).

RESULTS

Skeletal muscle cell morphometric characterization. Morphometric measures were obtained from 558 skeletal muscle cells. Four general forms were identified (Table I). No significant differences ($p > 0.05$) were observed in the cellular characteristics between independent cultures (between different individuals). In general, the morphological characteristics showed the same tendencies up to 60 days in culture.

Human skeletal muscle cell development under cell culture. Unless otherwise specified, all intervals are presented as the quartiles Q25 % and Q75 %. Appearance and morphometric data recorded for human skeletal muscle cells during 38 days of primary culture are shown in Figures 1, 2, 3, and 4. From day 2 of cell culture, spindle/fusiform-shaped mononucleated cells (Fig. 2B), measuring 68±17 µm in cell length and 810 ± 219 µm² in area, were observed. In these cells, the nucleus was centrally positioned, with a diameter of 12±3 µm, and variable nucleoli numbers (1 - 5) were observed. At this time, cell projections ranged from 10 to 23 µm in length.

By day 8, an increase in cell number and cell size was observed ($p < 0.05$). Cell length ranged from 89 to 207 µm, cell area ranged from 2,401 to 4,637 µm², and cell projection lengths ranged from 24 to 75 µm. At this time, the diameter of the nucleus was 20 ± 7 µm, centrally positioned, and with nucleoli numbers ranging from 1 to 5 (Fig. 2C). Between day 9 and 13, few changes in cell size were observed, although cell divisions continued (Fig. 2D). On day 9, cell area decreased ($p < 0.05$), coinciding with the removal of debris in the culture media.

Table I. Morphological characteristics analyzed in skeletal muscle cells grown under culture conditions.

| Character	Description
Length	Maximum distance between the most extreme points of the cell
Width	Perpendicular distance to cell length, measured at nucleus height
Area	Surface covered by the whole cell
Projections length	Length of the greatest cytoplasmic prolongation
Nucleus diameter	Larger nucleus diameter, if more than one
Nucleolus diameter	Largest diameter of the largest nucleolus
Number of nuclei	Number of clearly defined nuclei in a cell
Number of nucleoli	Number of clearly defined nucleoli in a cell
Shape	General appearance considering the cell contour. Irregular, Sphere, Spindle, Triangular
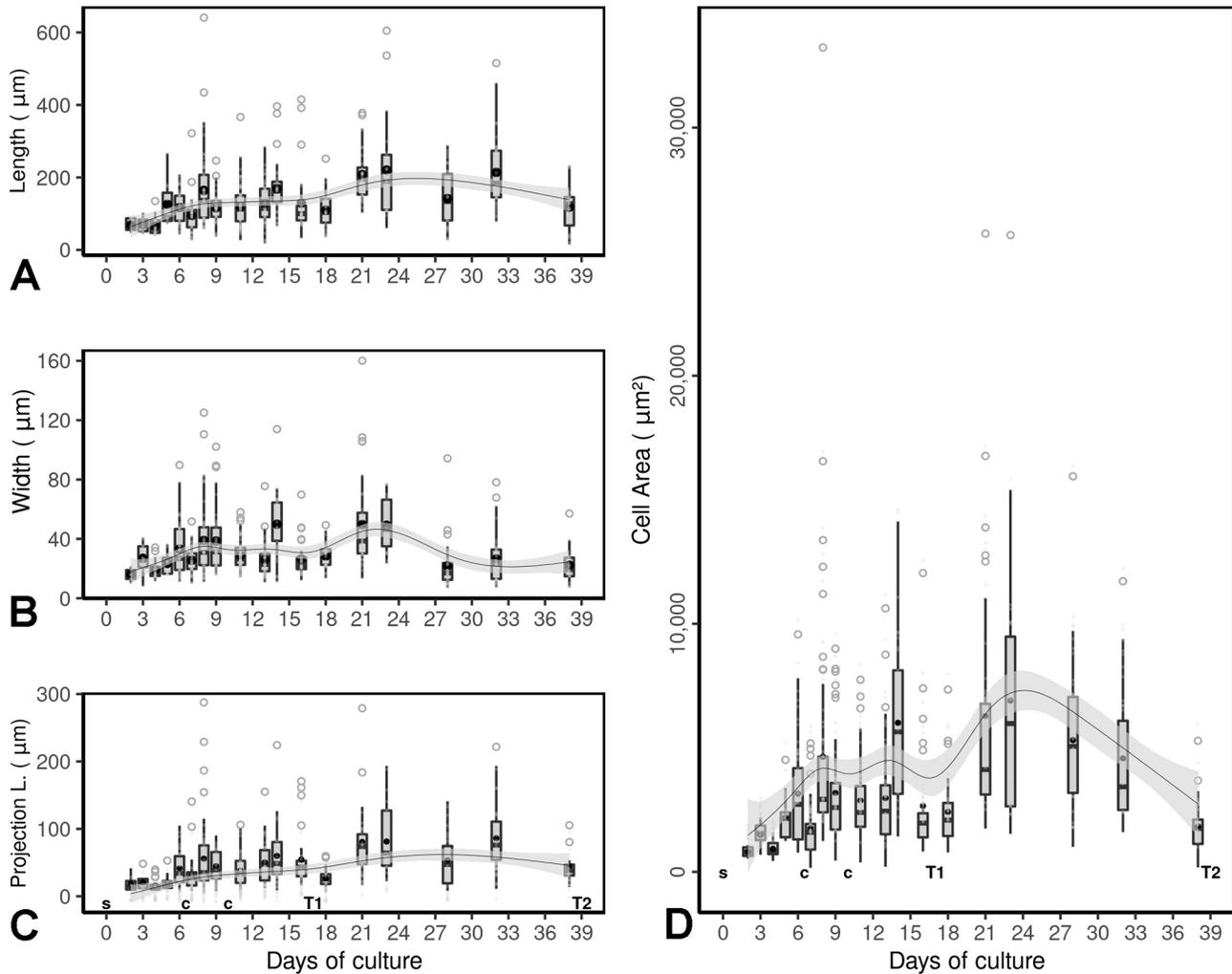
Fig. 1. (A) Variations in cellular length, (B) width, (C) projection lengths, and (D) area of human skeletal muscle cells during 38 days in culture. After seeding in culture flasks with a volume of 25 mL (s), detritus was removed from the cultures (c). Once 90-100 % of cellular confluence was reached, transfers (T1 and T2) were made to culture flasks with a volume of 75 mL. A smoothing line with probability bands (0.95) is present on each plot to show data trends. Outliers are shown as grey circles.

By day 14, cell length (132 - 188 μm) and area (3,145 - 8,123 μm²), projection lengths (33 to 80 μm), and nucleus diameter (22 ± 7 μm) had increased (p < 0.05). At this time, a single (occasionally > 2) centrally positioned nucleus, with 1 or 4 nucleoli, was observed per cell (Fig. 2E). By day 16, cell width and area decreased (p < 0.05), coinciding with a cellular confluence near 100 %. At this point, cultures were transferred, and cell size was observed to progressively increase until day 23 (p < 0.05).

On day 23, the maximum values for cell length (110 - 262 μm) and area (2,642 - 9,480 μm²), projection lengths (45 - 127 μm), and nucleus diameter (28 ± 11 μm) were recorded (p < 0.05). At this time, a single (occasionally 2 - 3) centrally positioned nucleus, with nucleoli numbers ranging from 1 to 5, was observed. From day 28, and prior to day 39 when transfer took place, cell sizes progressively decreased (p < 0.05), while cellular confluence increased, until relatively low values were observed for cell length (67 - 146 μm), area (1,129 - 2,107 μm²), projection lengths (31 - 47 μm), and nucleus diameter (11 ± 5 μm).

Myoblast morphology. The registered cell forms observed in the present morphological analysis (spherical, irregular outline, triangular, and spindle/fusiform), are representatives of the morphological variations observed in developing myoblasts under culture conditions (Fig. 3). The spherical cells were from 28 to 44 μm in diameter (37 ± 15 μm) and, during development, some were observed floating as they became detached near the maximum cellular confluence.
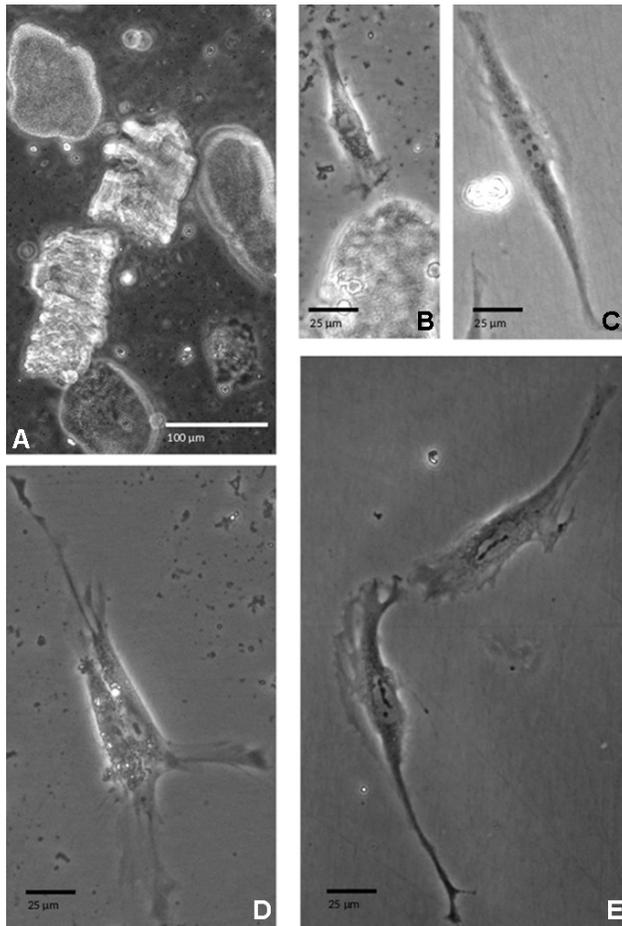
Fig. 2. (A) Appearance of human skeletal muscle cells under primary culture on the first day of seeding. Details on cells observed (B) on day 2, and (C) on day 8 of culture. (D) Cells resulting from mitotic division between days 9 and 13 of culture. (E) Cells observed on day 14 of culture. All photomicrographs are of live cells using phase contrast without staining.
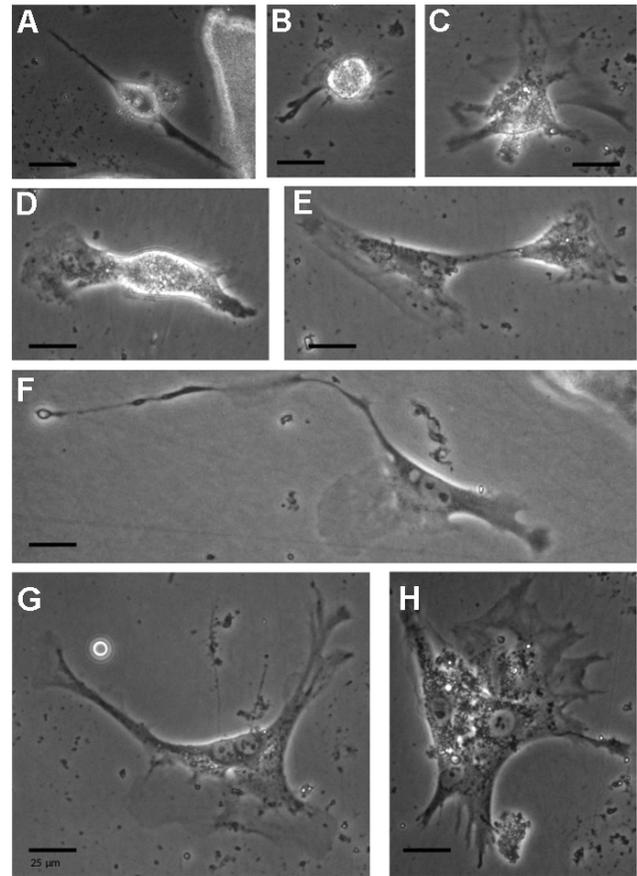

Fig. 3. Some of the morphological variations observed in the development of muscle cells under culture conditions. (A & B) Spherical form shortly after adhering to the substrate, and (C) the posterior spreading of projections resulting in elongation. (E & F) Triangular shape with projections of different sizes. (G & H) Multinucleated cells. All photomicrographs are of live cells using in phase contrast without staining.

This myoblast form displayed a bright appearance, due to the phase contrast illumination, and it was not possible to define the nucleus. It should be noted that the myoblasts also acquired this spherical form during the transfer process. Following trypsinization, the adhered myoblasts detached and took on a spherical form while in suspension; this form was maintained until they were transferred into culture flasks. A few hours post-inoculation, the previously suspended spherical myoblasts were adhered to the flasks and presented an irregular outline (Figs. 3A-D).

Throughout development in culture, myoblasts displayed an irregular outline, although this was principally observed in conditions of low cellular confluence (< 50 – 60 %). This form was characterized by the presence of numerous projections of variable lengths (19 - 61 µm) which modified the cellular contour to create an irregular geometric appearance, variations in cell length (62 - 159 µm), and area (1,747 - 5,422 µm^2). Although the myoblasts showed a dynamic development of projections and changes in shape, cells with three projections in opposite positions were frequently observed, creating a triangular configuration (Figs. 3E-F). The cell length (96 - 186 µm), projection lengths (28 - 72 µm), and area (1,839 - 5,094 µm^2) of this shape did not show significant differences with respect to the irregular form (p > 0.05).

As cellular confluence increased (70 - 100 %), the myoblasts generally displayed two opposing projections, generating a spindle/fusiform form. The cell length (92 - 177 µm), projection lengths (22 - 59 µm), and area (1,454 - 3,492 µm^2) of this shape did not show significant differences with respect to the triangular form (p > 0.05). However, cell width (16 - 28 µm) and nucleus diameter (18 ± 6 µm) in the

spindle/fusiform form were smaller than cell width (27 - 51 µm) and nucleus diameter (20 ± 7 µm) observed in the other forms (p < 0.05).

Although the majority of cell forms (spherical, irregular outline, triangular, and spindle/fusiform) showed one centrally positioned circular to semi-circular nucleus with variable numbers (1 - 7) of small nucleoli (5 ± 2 µm), after the first transfer on day 17, some cells (n = 25) were observed to contain rounded nuclei (3 - 5; 15 ± 6 µm in diameter) dispersed throughout the cell. It is important to note that cell length (155 - 275 µm, 212 ± 78 µm), width (22 - 46 µm, 37 ± 26 µm), area (3,525 - 7,803 µm², 5,995 ± 3,468 µm²), and projection lengths (47 - 110 µm, 75 ± 41 µm) displayed wide ranges of variation (Figs. 3G-H and 4).

DISCUSSION

Morphometry is based on solid theoretical concepts that have led to the development of diverse techniques for the management of informational data (Rohlf, 1990). Establishing simplified models allows for studying the effects and interaction of intrinsic (genetic variation, phenotypic plasticity) and extrinsic (environmental factors, random variation in development) factors, as well as the morphological manifestation in the possible scenarios (Klingenberg, 2019). Variables such as donor age, endocrine condition, and presence of lesions prior to biopsy (Allen *et al*., 1997), as well as the origin and type of muscle collected (Yablonka-Reuveni) can affect the development and behavior of myoblasts in culture.
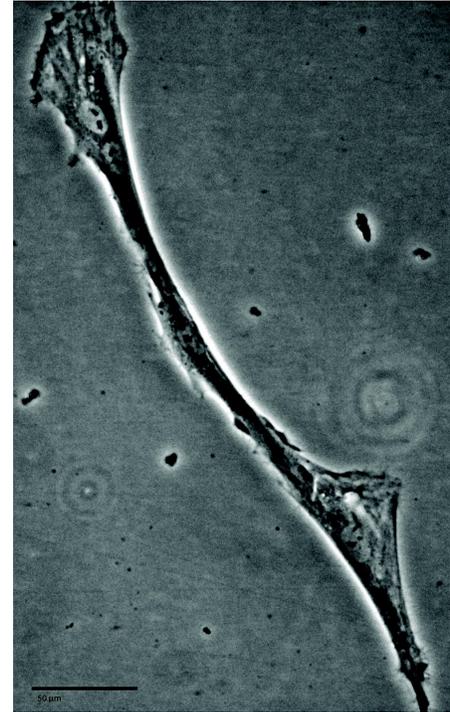

Fig. 4. Multinucleated cell, possibly the initial step in the development of a myocyte, observed on day 23 of culture. Photomicrographs are of live cells using phase contrast without staining.

In this study, skeletal muscle cells isolated from the rectus abdominis of healthy women, during programmed caesarean section, were cultured and morphometric data was recorded and analyzed by means of photographic monitoring. The presence of spindle/fusiform myoblasts from the second day of primary culture, their proliferation in the first 10 days, as well as the presence of cells in spherical and irregular outlines are described and coincide with reports by other authors (Godman, 1957; Blau & Webster, 1981; Scharner & Zammit, 2011).

Myoblast migration from muscle fibers and their capacities of adhesion, displacement, and form change (from spherical to spindle/fusiform) have been reported since the first *in vivo* and *in vitro* investigations of muscular regeneration (Godman). Mauro (1961) described spherical and spindle/fusiform myoblasts (as "free round cells" and "fusiform cells," respectively), and associated them with the muscular regeneration described during the first half of the 20th century (Clark, 1946; Pogogeff & Murray, 1946). These cells were observed in the striated muscle of rabbits 48 hours after sustaining trauma and in the striated muscle of rats between days 3 and 10 of culture (Godman). In his observations of cell cultures, Godman described a large

quantity of refractory "round or corpuscular cells" originating from within the interior of muscular tissue fragments or migrating from within the sarcolemma of isolated muscle fibers. These cells demonstrated a surprising capacity to adhere to the surface of the medium and endowed with mobility to invade adjacent zones (described as "macrophagic capacity"); these cells also demonstrated notable form change, and with time, they became spindle/fusiform-shaped or had multiple projections (Godman).

The round cells described and photographed by Godman coincide with the spherical cells observed in the present study (Figs. 3A-C). Furthermore, the adhesion capacity, mobility, and transformation process to spindle/fusiform-shaped cells were also consistent (Figs. 2C-E). Following the isolation of satellite cells from muscle fibers, it is possible to obtain and grow myoblasts in culture (Yablonka-Reuveni). Under these conditions, myoblasts arise following a general process that includes an inactive (quiescent) state, migration, proliferation, and differentiation, or the return to inactivity (Allen *et al*.). In each of these stages, the cells experience morphological changes, and as a consequence, morphometric changes occur. In this study, cyclic changes in cell size and interspersed periods of

increases and decreases in myoblast length, width, and area were observed (Fig. 1).

Satellite cells are described as "free" mononucleated cells precisely located between the sarcolemma and the external membrane (basal lamina) that surrounds myocytes (Laguens, 1963; Lee, 1965). Satellite cells can divide and act as a reservoir of new nuclei (stem cells) for muscle regeneration and repair. Moss & Leblond (1971) estimated that there are around 81 nuclei per mm of muscular fibre present in the tibialis anterior of 14 to 17 days old Sherman rats, of which, 88 % correspond to "true" nuclei of the muscle fibre, while 12 % correspond to satellite cell nuclei. Furthermore, it is estimated that satellite cell numbers double within 24 hours during muscle development (from 103 to 208 nuclei per mm of muscle fiber); half of which become incorporated in muscle fibers as nuclei, while the other half remain as stem cells (Moss & Leblond). With enzymatic isolation and cell culture techniques, it was estimated that, for each gram of rat muscle, up to 2.5×10^5 satellite cells could be obtained (Bischoff). Similarly, Blau & Webster calculated that each human satellite cell could generate at least 1×10^7 new muscle cells, and that from a 0.1 cm^3 biopsy, up to 5×10^3 viable satellite cells could be obtained and cultured for the development of myoblasts, myocytes, and myofibers. This proliferation capacity was exploited in the 1980s to optimize human muscle fibre culture techniques, giving rise to numerous investigations with diverse approaches and objectives (Yablonka-Reuveni).

Advances have been made regarding molecular aspects of muscle cells (Yablonka-Reuveni & Lepper, 2020). However, classical aspects of cell biology need to be investigated (Yablonka-Reuveni). Information related to cell length, width, perimeter, area, diameter, or volume, as well as morphological patterns during cell development and differentiation in culture can contribute to the interpretation of cellular responses under experimental or pathological conditions (Bishop & Drummond, 1979; Wahab *et al.*, 2018). For example, the multinucleated cells observed in this study (Fig. 4) represent an initial step in myocyte development and when their length (212 ± 78 µm) and width (37 ± 26 µm) were compared with measurements of myocytes from patients with Duchenne muscular dystrophy (length, 151 ± 20 µm; width, 8.2 ± 0.9 µm), it was possible to confirm the morphometric differences found by Delaporte *et al*. The photographic analysis and results obtained in this study provide information on the forms and morphometry adopted by myoblasts during cell culture, from their differentiation from satellite cells to their maturation into myocytes.

A brief historic summary highlights the practical potential of applying cell culture techniques in conjunction with a morphological approach. In pathological studies, morphometry and image analysis require a considerable amount of time, but this disadvantage is compensated for by the fact that the application is relatively simple, economical, and efficient (Laishram, 2017).

CONCLUSIONS

The cell growth dynamics, the proliferative capacity, and culture longevity indicate that the inoculation and maintenance procedures used in this study were pertinent. The observed morphology was comparable to other investigations. As such, we consider that the analyzed variables contribute to the description of myoblasts maintained under standard cell culture conditions. In particular, we consider that cell length, width, and area are fitting characteristics that describe the development of myoblasts and that can be used for the routine monitoring of cell cultures.

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RESUMEN: A partir de células madre musculares, surgen los mioblastos que se dividen y fusionan entre sí para formar a los miocitos. Estas células ya diferenciadas son precursoras de miocitos que maduran en fibras musculares y posteriormente forman los músculos. La implementación de cultivos celulares de mioblastos ha permitido obtener conocimiento detallado del tejido muscular. Particularmente, algunas de las aportaciones morfológicas fueron el punto de partida de técnicas clínicas, terapias o investigaciones biomédicas. Sin embargo, los estudios morfométricos en condiciones de cultivo celular son escasos. Por lo cual, realizamos seguimientos fotográficos a cultivos desarrollados bajo condiciones estándar, registramos datos para nueve características celulares y aplicamos técnicas morfométricas para

analizar estas células (n = 559). Se caracterizaron cuatro formas celulares adoptadas por los mioblastos (esférica, irregular, triangular y huso) y se registraron intervalos amplios de variación en los caracteres. Hacia el día 23 de cultivo se presentaron los valores máximos en la longitud (110–262 µm), el ancho (35–66 µm) y el área celular (2,642–9,480 µm^2), así como en el tamaño máximo de las proyecciones celulares (45–127 µm) y el diámetro del núcleo (28±11 µm). El núcleo se observó como único y en posición central; los nucleolos variaron poco en diámetro (5±2 µm), aunque no en número (1 a 5). En términos generales, se identificaron cambios cíclicos en la talla de las células durante los cultivos, esto es, períodos intercalados de incremento y decremento en el largo, ancho y área celular. Debido a que estas características reflejaron los cambios generales sufridos por los mioblastos durante el cultivo, se proponen para monitorear sus etapas de desarrollo en cultivo.

PALABRAS CLAVE: Músculo; Morfología; Histología; Biología cellular.

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