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Morphology of Adherent Cells of the Line Vero Cultivated in a Three-Dimensional Environment inside a Microfluidic Device Differs from their Morphology when Cultivated in Monolayers

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Traditional *in vitro* culture models have significant limitations in mimicking important physiological interactions, such as cell-cell interactions, cell-extracellular matrix interactions, and the three-dimensional morphology of cells. In contrast, 3D culture models have the ability to replicate the natural three-dimensional environment of cells.

Aims: The objective of this study is to evaluate the morphology of adherent Vero cells grown in two-dimensional (2D) and three-dimensional (3D) culture models.

Methodology: For the 2D culture model, Vero cells were thawed and grown in culture flasks in nutrient-rich culture medium. As for the 3D culture, a collagen hydrogel solution was prepared to mimic the extracellular matrix and injected into the central microchamber of the microfluidic device along with the Vero cells. To compare the morphological differences between the two culture models, measurements of the shortest and longest axes of the cells were performed, and the proportion of the cell axes in the two types of culture was compared.

Results: The results indicated that in both 2D and 3D cultures, the minor axis of the cells has similar sizes, being 106 ± 22.7 and 109.9 ± 35.8 µm, respectively. However, the major axis of the cells in 3D culture was significantly larger, compared to 2D, with values of 154.8 ± 11.96 and 114.1 ± 6.25, respectively. Similarly, Vero cells had a higher proportion value, being 1.08 ± 0.11 and 1.48 ± 0.39, respectively for 2D and 3D cultures.

Conclusion: We conclude that Vero cells in a 3D environment have a different morphology than cells cultured in 2D. One of the main differences is related to the size of the largest cell axis and consequently the proportion of the axes. The data suggest that in 3D cultures, cells are more elongated, with filopodia involved in cell-cell and cell-ECM interactions.

Keywords: Two-dimensional culture; three-dimensional culture; Vero cell line; cellular morphology.

1. INTRODUCTION

Bioassays constitute an important step in evaluating the pharmacological actions of new drugs. In the initial phases, *in vitro* tests take precedence over *in vivo* studies for economic, ethical, and scientific reasons [1]. Cell culture is a technique applied for studying living cells outside the organism. *In vitro* studies are useful for analyzing disease mechanisms, drug actions, protein production, and the development of tissue engineering [2,3]. In cell cultures, environmental conditions such as temperature, osmolarity, and pH, as well as nutrient availability, can be controlled to ensure cell survival and development in the microenvironment [4]. Cultures can be carried out under adherent conditions where cells are deposited in flasks, tubes, or plates made of materials such as polypropylene and polystyrene, or they can be placed in suspension in the presence of culture medium [5]. However, these two-dimensional monolayer cultures (2D) often fail to mimic the cellular environment, resulting in inconsistent outcomes compared to *in vivo* results [6].

In this regard, three-dimensional (3D) models have been developed, in which cells are cultivated on a substrate that mimics the extracellular matrix (ECM) such as hydrogels containing collagen and matrigel [7,8]. 3D cell cultures differ from 2D ones on different aspects: cell-cell interaction, cell-ECM interaction, cellular organization of cytoskeleton, and nutrient access. Thus, it is expected a higher degree of confidence in developed tests in 3D cultures [9-11]. The 3D model allows cells to organize into complex histological structures. 3D cultures can use microfluidic devices, typically microfabricated of polydimethylsiloxane (PDMS) [12,13]. Given the more precise mimicry of the 3D culture, cells exhibit different behaviors in terms of cell proliferation, differentiation, apoptosis, cell movement and also in morphology.

The three-dimensional (3D) culture model in microfluidic devices is widely used as it provides a more accurate representation of the in vivo environment. Unlike 3D scaffolds, where cells are fixed to a rigid surface, in the microfluidic model, cells can interact freely with the extracellular matrix and other cells. This allows for a more realistic observation of biological processes [14-16]. For this reason, this study utilized this technology.

The Vero cell line is widely used in studies on vaccines and viral diseases [17-19]. However, the authors did not find records of previous

studies using Vero cells in microfluidic devices or analyzing their morphology in such cultures. Therefore, mimicking the physiological conditions of Vero cells in microfluidic devices provides an opportunity to recreate their morphology in a more realistic environment. Analyzing this morphology is of great scientific relevance, as cellular morphology is closely linked to its functionality.

This study aimed to compare morphological parameters of Vero cells cultivated in polystyrene flasks, referred as 2D culture, and the same kind of cells cultivated in microfluidic devices made of lipophobic thermoplastic materials, referred as 3D culture, both of them evaluated using an optical microscope (Zeiss LSM 700®).

2. METHODOLOGY

2.1 Cell Culture

The cell line used in this study was Vero cells, which consists of adherent epithelial cells derived from monkey kidney (ATCC CCL-81). To remove the cryoprotectant agent used during the freezing process, the cells were taken from the freezer at -86°C and the cryotube was shaken in a water bath for 2 minutes. Next, the cell suspension was centrifuged at 240 g for 7 minutes. The supernatant was then discarded, and the cells resuspended in growth medium were added to a 25 cm² culture flask (Kasvi®; model: K11-1050). The flask was subsequently incubated at 37°C with 5% CO_2.

2.2 Cell Culture in a Two-Dimensional Environment

The thawed cells were cultured in Dulbecco's Modified Eagle Medium (DMEM; Ref: 12100-046; Gibco®, Invitrogen Corporation, New York, USA) culture medium supplemented with 10% inactivated fetal bovine serum (FBS; Gibco®, Invitrogen Corporation, New York, USA), 1% L-glutamine (Sigma®, St. Louis, USA), and 1% antibiotic (100 U/mL penicillin and 100 ng/mL streptomycin Sigma®, St. Louis, USA), maintained at 37°C in a humidified atmosphere containing 5% CO_2. When the cells occupied approximately 80% of the available adhesion area in the culture flask, a cell passage process was initiated. For this, the flask was washed twice with phosphate-buffered saline (PBS), the cells were then detached using trypsin-EDTA, homogenized in culture medium, and a proportion equivalent to one-third of the original cell density was maintained in the flask for continued cultivation.

2.3 Cell Culture in a Three-Dimensional Environment

To mimic the extracellular matrix, a hydrogel solution of type I collagen from rat tail was prepared. The hydrogel was composed of 100 μL of type I collagen (2.0 mg/mL), Dulbecco's Phosphate Buffered Saline (DPBS), 10X, with phenol red, 71.8 μL of Milli-q water, and approximately 8.2 μL of 0.5M NaOH. 10 μL of the cell suspension at a concentration of 1.0×10^7 cells/mL were homogenized with the collagen hydrogel before being injected into the device. The entire procedure was carried out keeping the reagents on ice.

Using a micropipette, the prepared hydrogel mixture was injected into the central microchamber of the Be-Gradient microfluidic device (BeOnChip®; Zaragoza, Spain). Fig. 1 illustrates the design of the microfluidic device. A drop of the hydrogel mixture was placed at the entrance of the central microchamber to prevent evaporation. Culture medium was perfused into the side microchannels to provide nutrients to the cells. The microfluidic device was then incubated at 37°C with 5% CO_2 to allow collagen polymerization. The cells were monitored using an optical microscope (Zeiss LSM 700®), with observations made after 24 hours of incubation.

2.4 Measurements and Image Acquisition

Images were obtained using optical microscopy (Zeiss LSM 700®). Measurements were conducted with the Fiji application. Images were post-processed to equalize illumination and improve contrast. Twelve cells were measured in each culture to determine the largest and smallest axes of each observed cell. Initially, the smallest rectangle that limited the edges of the cells was overlaid, allowing it to be in any orientation, meaning the rectangle could be in any orientation.The length of the longer side of the rectangle was considered the major axis of the cell, while the length of the shorter side was considered the minor axis.Values were expressed as mean ± standard deviation. Data was evaluated by the Shapiro-Wilk test, followed by the t-test. A significance level of $P \leq 0.05$ was adopted. The software used for the analyses was GraphPad Prism 5® (GraphPad Software Inc., San Diego, CA, USA).

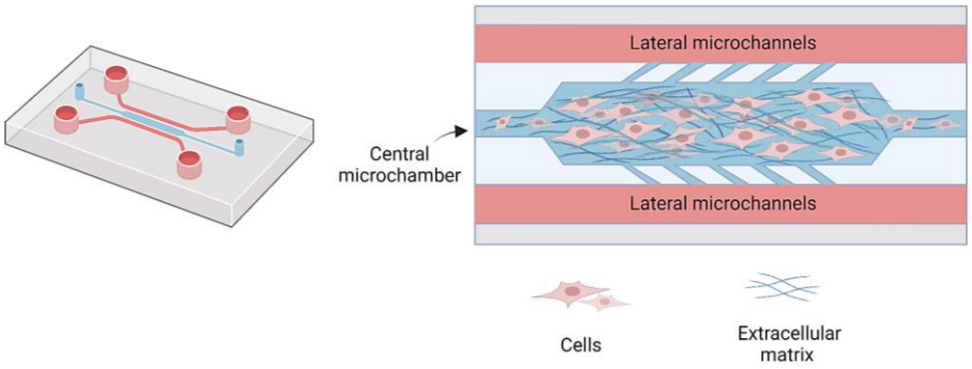

Fig. 1. Schematic representation of the microfluidic device used for the three-dimensional cell culture. The chip consists of a central microchamber, where Vero cells are combined with collagen hydrogel, and lateral microchannels that distribute nutrient-rich culture medium to the cells. Created with BioRender.com

3. RESULTS AND DISCUSSION

Cells cultured *in vitro* can alter their morphology according to the conditions in which they are cultured [20]. In this study, it was possible to visualize Vero cells individually adhered in a monolayer in a culture flask (2D) and cells interacting cell-to-cell and cell-to-extracellular matrix in a microfluidic device (3D) using optical microscopy (Zeiss LSM 700®). Fig. 2.A shows 3D cultured cells extending their filopodia, while Fig. 2.B shows 2D cultured cells with shorter projections. In the 2D culture, cells are flattened and adhered to the bottom of the flask, predominantly interacting with the flask surface, giving them a two-dimensional morphology. The presence of collagen hydrogel to simulate the extracellular matrix in the 3D culture allows cells to interact with the extracellular matrix, giving to them a three-dimensional morphology more similar to the one that occurs *in vivo*. The cellular projections towards the ECM or another cell make the cells larger in at least one of their axes, and these projections cause morphological changes indicating possible cell-cell and cell-ECM interactions with cellular communication and signaling.

It is important to point out that 3D interactions are important in order to reproduce the actual environment of the cell. In a study in which mammary cancer spheroids (MCF-7) were mimicked in a microfluidic device, it was shown that the relationship of cells with the ECM was crucial to evaluate the ability of nano formulations to cross the tumor tissue barrier [7].

Now, considering the objective of the present study, the morphology of Vero cells is a complementary analysis to indicate their physiological state [21]. As previously mentioned, the author did not find any previous study reporting morphology of Vero cells in a microfluidic device.

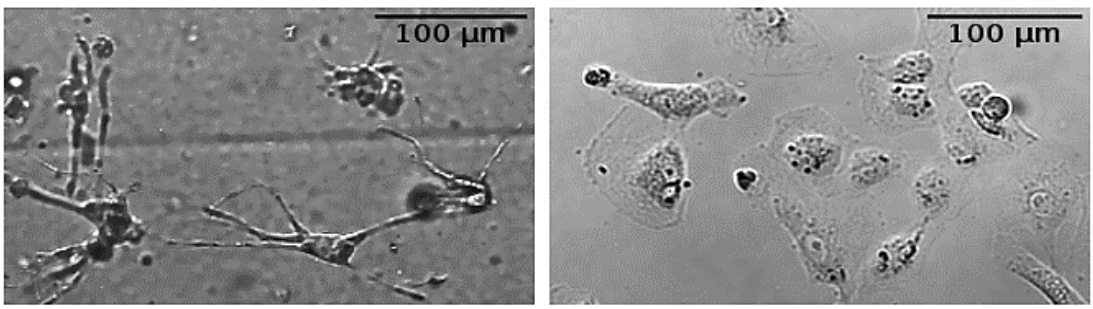

Fig. 2. Morphological differences in Vero cell cultures cultivated in different models. (A) Micrographs of 3D culture in microchip after 3 days of cell culture. (B) Micrographs of 2D culture in polystyrene flasks after 1 day of cell culture. The images obtained by optical microscope (Zeiss LSM 700®)

Measurements of the major and minor axes of Vero cells, using rectangles (Fig. 3), revealed a different morphology in cells cultured in 3D compared to those cultured in a 2D environment. As observed in Fig. 4, the results indicated that in both 2D and 3D cultures, the minor axis of the cells has similar sizes, being 106 ± 22.7 and 109.9 ± 35.8 µm, respectively. However, the major axis of the cells in 3D culture was significantly larger, with values of 154.8 ± 11.96 and 114.1 ± 6.25, respectively ($P = 0.006$), showing that in three-dimensional cultures conducted in microfluidic devices, the cells can acquire a more extended morphology due to cellular projections likely seeking interactions that mimic cells in real tissue. In addition to measuring the axes, an analysis of the ratio of the major to the minor axis was conducted. Similarly, Vero cells had a higher proportion value, being 1.08 ± 0.11 and 1.48 ± 0.39, respectively for 2D and 3D cultures, $P = 0.002$ (Fig. 5). These data indicate that the cell in 2D culture has axes of similar sizes, while the 3D cell is a more extended cell, which may reflect a cell with greater migratory capacity and also a cell capable of making more contact interactions, mimicking cells in tissue.

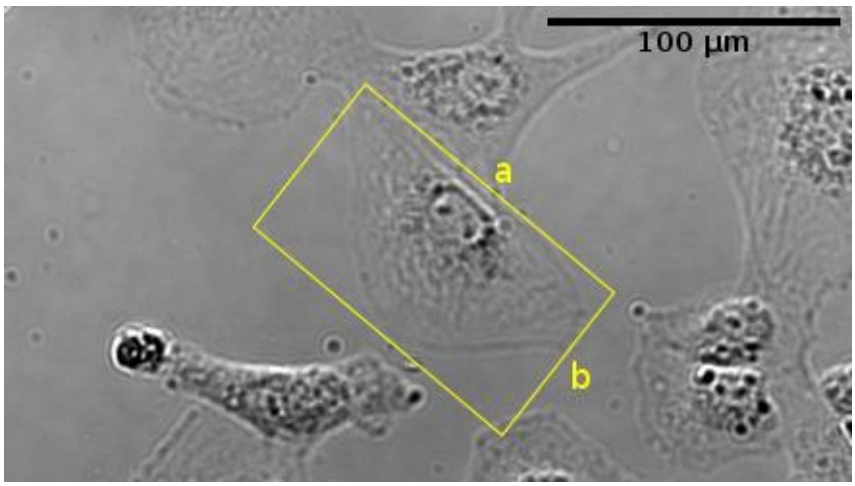

Fig. 3. Actual representation of the measurements of the Vero cell axes. The length of the longer side of the rectangle was considered the major axis of the cell, denoted by the letter 'a', while the length of the shorter side was considered the minor axis, denoted by the letter 'b'. The images were obtained using an optical microscope (Zeiss LSM 700®)

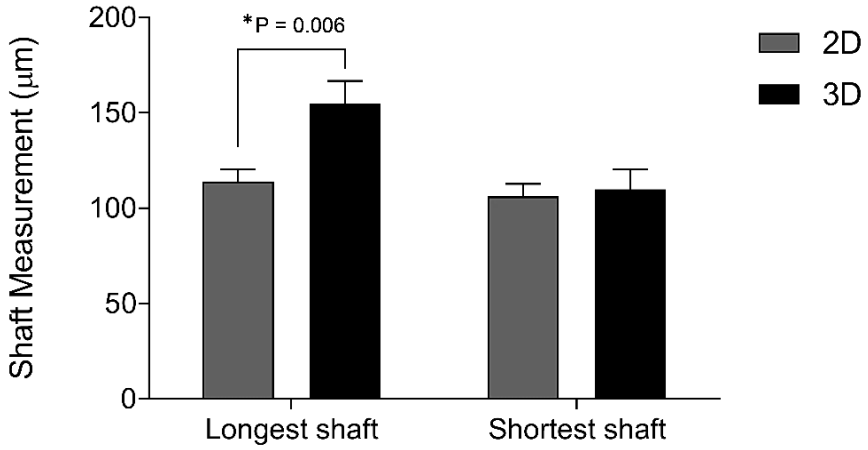

Fig. 4. Analysis of the axes of cells cultured *in vitro* in 2D and 3D models. Measurements were taken from 12 cells in each culture using an optical microscope (Zeiss LSM 700®), measurements performed in µm. Results are reported as means ± SEM. Data was evaluated by Shapiro-Wilk test, followed by t-test. A significance level of *P ≤ 0.05* was adopted. The software used for analysis was GraphPad Prism 5 (GraphPad Software Inc., San Diego, CA, USA)

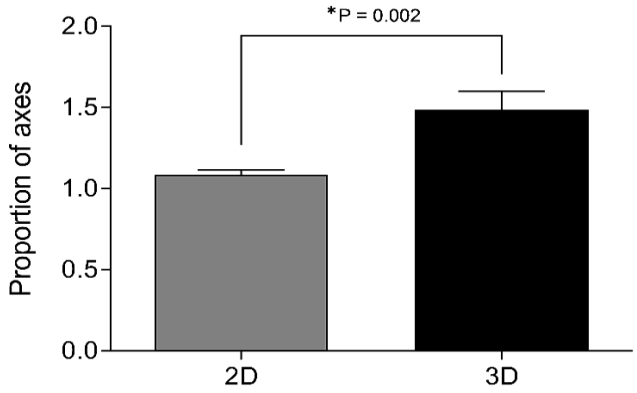

Fig. 5. Analysis of the axis ratio of cells cultured *in vitro* in 2D and 3D models. Measurements were taken from 12 cells in each culture using an optical microscope (Zeiss LSM 700®), measurements performed in µm. Results are reported as means ± SEM. Data was evaluated by Shapiro-Wilk test, followed by t-test. A significance level of *P ≤ 0.05* was adopted. The software used for analysis was GraphPad Prism 5 (GraphPad Software Inc., San Diego, CA, USA)

Previous studies have also reported changes in morphology in 2D and 3D cultures. In a study analyzing human breast cancer cells (MCF-7), the authors noted that after growth in 2D, the cells exhibited predominantly flat morphologies, with trigonal or polygonal shapes resembling leaves [22]. However, when cultured in a collagen structure, these cells showed a wide variety of morphologies, including more rounded, spread-out, and elongated forms. In another study, researchers investigated morphological differences between triple-negative breast cancer cells (MDA-MB-231) cultured in 2D and 3D environments [23]. The study observed notable changes in morphology, especially in cell size. Using nuclear and actin filament markers, the researchers demonstrated that MDA-MB-231 cells cultured on 2D plastic culture plates had a flattened shape. Some exhibited cytoplasmic extensions, while others had a rounded shape with predominantly ellipsoidal nuclei. On the other hand, in the 3D environment, cells cultured on poly (ε-caprolactone) (PCL) scaffolds with acetone (15% w/v) showed more elongated cellular projections compared to those cultured in 2D. To quantify the morphological differences between the two culture types, researchers measured cytoplasmic elongation. The results indicated that cells cultured on 15% PCL scaffolds exhibited a significantly higher elongation factor compared to those cultured in 2D.

4. CONCLUSION

The data presented here allow us to conclude that in three-dimensional cultures using microfluidic devices, cells from the Vero line exhibit a morphology distinct from cells in 2D culture. One of the main differences are related to the size of the major cellular axis and consequently in the ratio of the axes. The data suggest that in 3D cultures, cells are more elongated, with filopodia engaging in cell-cell and cell-ECM interactions. Therefore, three-dimensional cultures mimic the aspects of cells in tissue. These findings highlight the importance of using three-dimensional culture models to better simulate cellular behaviors and interactions *in vivo*. Such models provide information about how cells respond to their microenvironment and can improve our understanding of physiological and pathological processes.

DISCLAIMER (ARTIFICIAL INTELLIGENCE)

Author(s) hereby declare that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc) and text-to-image generators have been used during writing or editing of manuscripts.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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