

Cultivo de células madre limbares en membrana amniótica para reconstrucción de tejido corneal: una comparación de dos métodos

Limbal stem cell culture on amniotic membrane for corneal tissue reconstruction: a comparison of techniques

Cultivo de células mãe limbares em membrana amniótica para reconstrução de tecido corneano: uma comparação de dois métodos

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RESUMEN

Objetivo: Obtener un cultivo celular de células del limbus corneal en membrana amniótica como soporte por dos métodos diferentes, suspensión y explante, para obtener datos de confluencia, viabilidad y diferenciación en ambos métodos.

Métodología: Muestras de LSC (limbal stem cells, por sus siglas en inglés) fueron obtenidas de 16 donantes cadavéricos. Se realizaron seis cultivos de cada muestra, 3 por el método de suspensión y 3 por el método de explante, todo por triplicado. Como control negativo se utilizó membrana amniótica sin LSCs. Las células fueron cultivadas con SHEM, 37°C, 5% CO₂ y 95% de humedad. Los cultivos fueron mantenidos por 14 días con un recambio de medio cada 3 días. El día 14 los cultivos fueron analizados para evaluar viabilidad con azul de tripano, confluencia por microscopio y la diferenciación celular con Inmunohistoquímica para detectar citoqueratina 3/12.

Resultados: Como resultado de los experimentos mencionados anteriormente, para los métodos de suspensión y explante, se encontró una viabilidad de 98.92% y 98.32%, una confluencia de 55.95% y 48.27%, una concentración celular de 38.83x10⁴ células/ml y 36.484x10⁴ células/ml, y una diferenciación de 22.93% y 16.55%, respectivamente.

Conclusiones: Aunque éste estudio compare dos métodos, cultivos en suspensión de LSC y cultivo en explante de LSC, ambos con membrana amniótica como soporte, nosotros encontramos una pequeña diferencia en ellos. Como resultado, el cultivo en suspensión fue mejor que el explante, sin embargo los resultados no son conclusivos, por lo tanto es necesario realizar más investigaciones en este campo para lograr una conclusión con respecto al desempeño de estos dos métodos.

Palabras clave: amnios; técnicas de cultivo de célula; enfermedades de la córnea; células madre.

ABSTRACT

Objective: To grow cells on amniotic membrane corneal limbus with two different methods to assess the confluence, viability and differentiation of both.

Methods: LSC samples were obtained from 16 deceased donors. Additionally, six cultures were made from each sample, 3 by LSC suspension method and 3 by LSC explant method, all in triplicate. Furthermore, AMs without oral mucosal cells were used as negative control cultures. The cells were seeded with SHEM, 37°C, 5% CO₂, 95% humidity. Moreover, the cultures

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were maintained for 14 days and the culture medium was changed every other day or every 3 days. Also, on the 14th day, the cultures were analyzed to evaluate viability with trypan blue, confluence with microscopy and cell differentiation with immunohistochemistry to detect cytokeratin 3/12.

Results: As a result of the experiments mentioned above, for suspension and explant cultures, we found a viability of 98.92% and 98.32%, a confluence of 55.95% and 48.27%, a final cellular concentration of 38.83×10^4 cells/ml and 36.484×10^4 cells/ml, and a differentiation of 22.93% and 16.55%, respectively.

Conclusions: Although this study compared two methods, LSC suspension culture and LSC explant culture, both with AM as scaffold, we did find a slight difference between them. As a result, the suspension culture was better than the LSC explant culture, but the results are not conclusive. It is necessary to conduct more research in this field to reach a conclusion regarding the behavior of these two methods.

Keywords: amnion; cell culture techniques; corneal diseases; stem cells.

RESUMO

Objetivo: Obter um cultivo celular de células do limbus corneano em membrana amniótica como suporte por dois métodos diferentes, suspensão e explante, para obter dados de confluência, viabilidade e diferenciação em ambos métodos.

Métodos: Mostras de LSC (limbal stem cells, por suas siglas em inglês) foram obtidas de 16 doadores cadavéricos. Realizaram-se seis cultivos de cada mostra, 3 pelo método de suspensão e 3 pelo método de explante, tudo por triplicado. Como controle negativo se utilizou membrana amniótica sem LSCs. As células foram cultivadas com SHEM, 37°C, 5% CO₂ e 95% de umidade. Os cultivos foram mantidos por 14 dias com uma troca de meio cada 3 dias. O dia 14 os cultivos foram analisados para avaliar viabilidade com azul de tripan, confluência por microscópio e a diferenciação celular com Imunoistoquímica para detectar citoqueratina 3/12.

Resultados: Como resultado dos experimentos mencionados anteriormente, para os métodos de suspensão e explante, encontrou-se uma viabilidade de 98.92% e 98.32%, uma confluência de 55.95% e 48.27%, uma concentração celular de 38.83×10^4 células/ml e 36.484×10^4 células/ml, e uma diferenciação de 22.935% e 16.558%, respectivamente.

Conclusão: Ainda que este estudo compare dois métodos, cultivos em suspensão de LSC e cultivo em explante de LSC, ambos com membrana amniótica como suporte, nós encontramos uma pequena diferença neles. Como resultado, o cultivo em suspensão foi melhor do que o explante, no entanto os resultados não são conclusivos, portanto é necessário realizar mais investigações neste campo para conseguir uma conclusão com respeito ao desempenho destes dois métodos.

Palavras chave: âmnio; técnicas de cultura de células; doenças da córnea; células-tronco.

INTRODUCTION

The cornea is an avascular tissue of the eye that has two specialized functions: to form a protective barrier and to act as the main refractive element of the visual system¹. It is composed of layers organized as follows: epithelium and its basement membrane, Bowman's membrane, stroma, Descemet's membrane and endothelium². The corneal epithelium is a tissue composed of 5 to 7 layers of stratified tissue. Like other epithelial barriers in the human body, it is continually subjected to physical, chemical and biological processes and, in some cases, to the loss of its functions. Adequate compensation for damage to the corneal epithelium is vital for maintaining a clear, healthy cornea and

preserving vision. The corneal epithelium and other epithelia respond rapidly to injury by cell migration, covering the defect to restore the barrier. For this healing to be successful, it must involve a series of processes including cell migration, cell proliferation, re-stratification, cellular matrix deposition and tissue remodeling¹, all of which are complex interactions.

This process of cell regeneration is carried out by a small population of limbal stem cells (LSCs limbal stem cells) located in the basal layer of the limbal epithelium, which plays a key role in regeneration and repair of damage to the corneal tissue. The deficiency of limbal stem cells is associated with a loss of limbal palisades, where LSCs can be found^{3,4}.

A decrease in cell population or a functional abnormality of the LSCs, leads to abnormal cell repopulation of the epithelium and the loss of margin between the conjunctiva and cornea, producing corneal conjunctivalization⁵. Limbal cell deficiencies can be classified as partial or total, according to their extension^{6,7}. In partial deficiency, there is a lack of LSCs located in parts of the corneo-scleral limbus, while populations of intact LSCs can be found in other areas, maintaining a healthy corneal epithelium or generating a repopulation of the conjunctival epithelium in areas in which there is absence of LSCs. In total LSC deficiency, there is a dysfunction or destruction of the population of LSCs showing a complete cornea-conjunctivalization⁷.

Current conservative treatments available for patients with limbal cell deficiency include ocular lubricants, therapeutic contact lenses and topical autologous serum⁸⁻¹⁰. In the case of partial LSC deficiency, it has been suggested that the continuous migration debridement of conjunctival epithelium in the acute phase after injury, known as a sequential conjunctival epitheliectomy can reduce or prevent conjunctival re-epithelialization¹¹. There are several techniques for treating these diseases, including LSC transplant. Among the techniques used are: the lamellar keratolimbal allograft, limbal-conjunctival autograft, and conjunctival-limbal allograft from a living, HLA-matched donor⁵.

The LSCs plated in amniotic membrane (AM) as a scaffold has been widely used as a new treatment. Other researchers have investigated different materials as scaffolds with better optical characteristics such as biopolymers, fibrin substratum¹², temperature sensitive scaffolds¹³. Furthermore, other cell sources have been investigated such as mesenchymal stem cells, oral mucosal cells, nasal mucosal cells and embryonic stem cells¹⁴. In combination with the aforementioned cellular sources and various scaffolds, there are two types of culture medium: explant and suspension cultures.

The method chosen for this study was to seed LSCs from deceased donors over AM, since the LSC cultures and AMs as a scaffold have been the most widely used methods due to immunologic and inflammatory characteristics^{3,14-19}; and explant and suspension cultures were compared to assess the confluence, viability and differentiation of both.

METHODS

Subjects and samples

In accordance with the tenets of the Declaration of Helsinki, the study was approved by the Ethics Committee at Hospital Pablo Tobón Uribe. The study consisted of 16 LSC samples of deceased donors without known history of a LSC deficiency or any ocular disease. After providing informed consent, the AMs were obtained from a healthy patient at the time of cesarean section. All tests were conducted in triplicate inter- and intra assay.

Preparation of Human Amniotic Membrane

The AMs were obtained at the time of cesarean section in a sterile container with phosphate-buffered saline containing antibiotics (400µl of 50mg/ml gentamicin and 20µl of 50U/ml penicillin) and transported to the laboratory under sterile conditions in a cooler. The AMs were washed several times until all blood was removed and cut into pieces measuring approximately 4x3 cm and stored at -80°C in phosphate-buffered saline and glycerol at a ratio of 1:1 (vol/vol). Immediately before use, the AMs were thawed, washed three times with sterile phosphate-buffered saline, and cut into pieces measuring approximately 1.5 cm in diameter. Membranes were then deprived of their amniotic epithelial cells by incubation with 0.2% ethylene diamine tetra acetic acid (Sigma-Aldrich) at 37°C for 15 minutes to loosen cellular adhesion, followed by gentle scraping with a cell scraper. Finally, the culture wells were covered with the AMs²⁰.

Preparation of Limbal Stem Cells

Sixteen biopsies were obtained from corneal limbus of deceased donors, some of which were contaminated with fungi or bacteria and therefore 16 LSC explant cultures and 13 LSC suspension cultures were analyzed.

The samples were transported in a cooler and arrived at the molecular biology laboratory in a preservation medium called EUSOL-C, where they were processed as shown in Figure 1. Under a laminar flow cabinet, the samples were taken from their container and washed with PBS and cut into pieces measuring 1x1mm². Then they were divided with the purpose of being plated using two different methods: explant and suspension. As a negative control cell-free amniotic membranes remained with the same conditions as the cultures of LSCs.

Briefly, the specimens chosen to be plated by explant method were cut into pieces measuring 1x1mm² and plated into the culture wells previously covered with AMs. They were then incubated with supplemental hormonal epithelial medium (SHEM)²¹ (5% fetal bovine serum, 2mg/ml epidermal growth factor (Sigma-Aldrich E9644), 5µg/ml insulin, transferrin, sodium selenite (Sigma-Aldrich, I1884), 0.5µg/ml hydrocortisone (Sigma-Aldrich, H6909), 50µg/ml gentamicin (Sigma-Aldrich, G1397), 100UI/ml penicillin, 0.5% dimethyl sulfoxide (Sigma-Aldrich, D8414), in DMEM/F12) at 37°C under 5% carbon dioxide for 14 days, and the medium was changed every 2 or 3 days²². The specimens chosen to be treated as LSCs in suspension were collected by removing all epithelial layers after treatment with 0.25% of trypsin and 0.53mM ethylene diamine tetra acetic acid (Sigma-Aldrich) at 37°C for 20 minutes. The supernatant was centrifuged at 100rpm at 4°C for 3 minutes to obtain a single cell suspension. The process was performed three times²³. The cells were plated at 1.5x10⁴cells/ml per culture well (Falcon 353047) at 37°C under 5% carbon dioxide for 14 days, with medium change every 2 or 3 days^{21,24}.

Controls

All cultures were done in triplicate; AMs were plated without LSCs as negative control under the same conditions.

For sterility control, we analyzed all specimens before proceeding to ensure quality and the absence of microbiology pathogens.

Cellular viability

The epithelial cells in suspension were analyzed by trypan blue (Sigma-Aldrich, T6146) and counted using a Neubauer camera. On the other hand, for the explant cultures, there was no available method to count cells in the tissue without detaching cells from it.

Confluence

The confluence was evaluated with a contrast using trypan blue to observe the cells in microscope 10X to determine their growth.

Immunohistochemistry

All cultures were embedded in optimal cutting temperature compound (OCT, Thermo scientific 6769006) and snap frozen in liquid nitrogen. Tissues were cryosectioned at a thickness of 4 µm, and four slices were mounted per slide. The slices obtained from all LSC cultures were stained with antibody (cytokeratin 3/12, Fitzgerald), and each staining was performed in triplicate. A negative control (primary antibody omitted) was included on every slide. The tissue was fixed with cold acetone for 10 minutes and rinsed in Tris-buffered saline (TBS, 100mM TrisCl, pH7.5). The slices were encircled with PAP pens (Ted Pella, 2311) incubated with skim milk powder at 5% to reduce nonspecific binding, and rinsed in TBS. The slices were incubated with the primary antibody (cytokeratin 3/12) diluted in phosphate buffered saline (PBS) 1:500 containing 1% bovine serum albumin for 1 hour at room temperature.

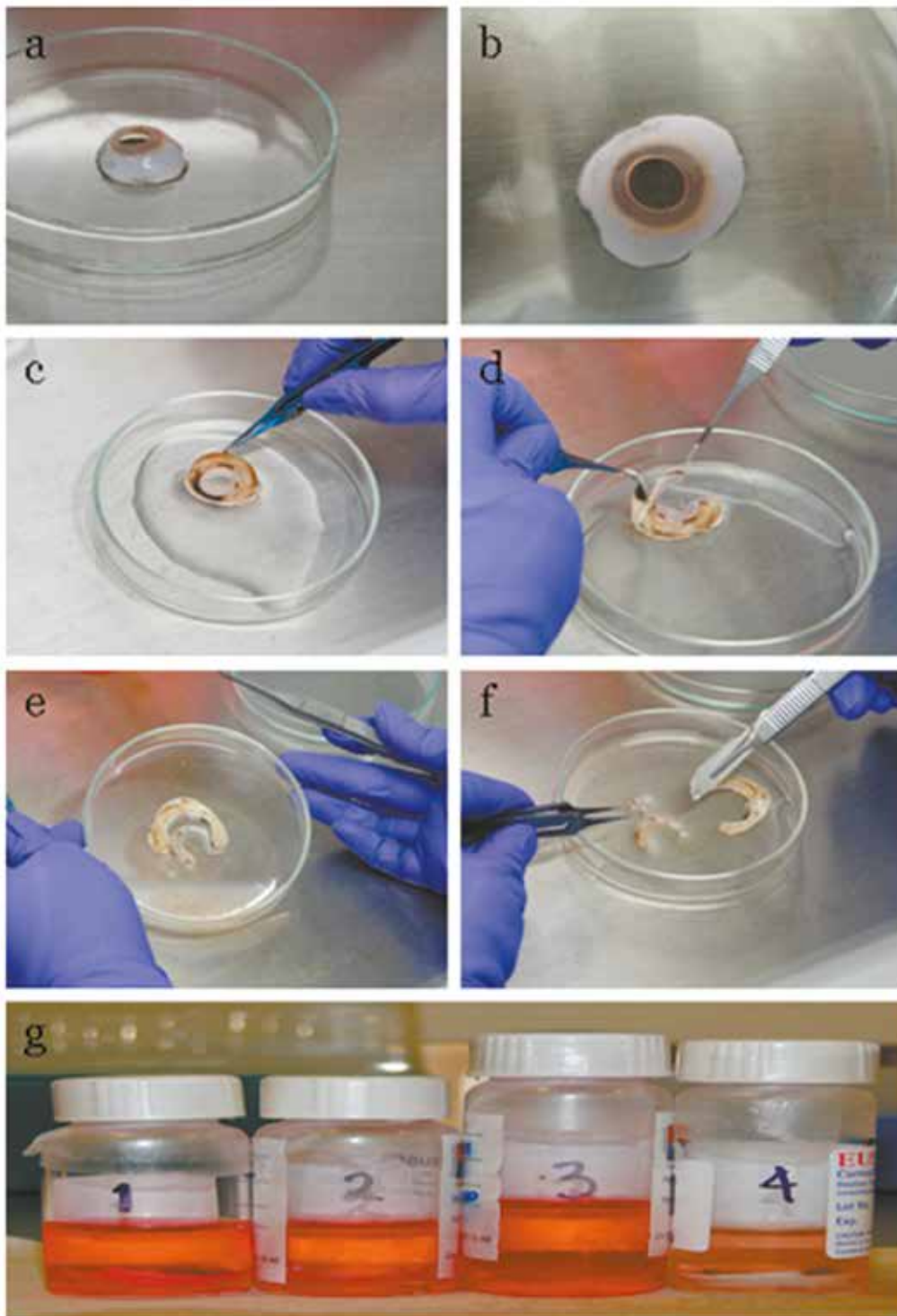


Figura 1. Diagnósticos en el momento del ingreso al programa. BPEG: Bajo peso para la edad gestacional. RCIU: Restricción del crecimiento intrauterino. RNPT: Recién nacido pretérmino.

The specimens were washed with TBS and incubated with the second antibody (Alkaline phosphatase-conjugated Affini Pure Goat Anti-Mouse IgG (H+L))²⁵ (1:2500) for 1 hour at room temperature. After rinsing in TBS, the slices were mounted with Fast Red/Naphtol (SIGMAFAST Fast Red/Naphtol AS-MX, Sigma-Aldrich). To counterstain, the slices were mounted in fast hematoxylin. Then, the slides were mounted and examined by microscopy using an Olympus BX41 (U-TV1X-2) at a magnification of 40X. Images were taken using an Olympus 5060-ADV camera. Each image was processed by ImageJ, NIH, to delimit the positive area (red) and determine the percentage of positive cells. One slice per culture was mounted in hematoxylin-eosin.

Statistical analysis

Data was analyzed using GRAPHPAD PRISM version 5.0 for Windows, GraphPad Software, San Diego, CA (www.graphpad.com). The results were expressed as the mean \pm standard error of the mean (SEM).

RESULTS

Limbal corneal tissues were excised successfully. In order to verify the behavior of the LSC explant and LSC suspension cultures, all cultures were assessed for confluence, viability, cellular differentiation and histology after 14 \pm 2 days (Table 1). Sixteen LSC explant cultures and 13 LSC suspension cultures were analyzed in triplicate for the confluence, viability, final cellular concentration and histology (Figure 2). For cellular differentiation, three LSC suspension

cultures and two LSC explant cultures were analyzed. The differences between the numbers of samples are due to culture contamination.

The hematoxylin-eosin staining was performed in order to evaluate tissue morphology (Figure 3). The LSC explant and suspension cultures showed an epithelium with two to five cellular layers (Figure 3 a, b). Figure 3 (a and b) shows epithelium (E), Bowman membrane (B), substantia propia (P), consistent with the corneal tissue. The negative controls, AM cultures with only SHEM show a low mono-layer cell formation with both types of cultures (Figure 3, c).

In order to compare the confluence between LSC explant and LSC suspension cultures, we measured the cell population in microscopy (Figure 4). The LSC explant cultures (Figure 4 a, b), LSC suspension cultures (Figure 4 c, d) and AM without cells as negative controls (Figure 4 e (explant), f (suspension)). The results show a confluence (% \pm SEM) of 55.95 \pm 11.869 in LSC suspension cultures and 48.27 \pm 9.1651 in LSC explant cultures. The viability (% \pm SEM) in LSC suspension cultures was of 98.92 \pm 0.56 and 98.32 \pm 0.58 in LSC explant cultures. The cellular final mean (x10⁴cells/ml mean \pm SEM) obtained for OMC suspension cultures was 38.83 \pm 10.923 and 36.484 \pm 11.863 for LSC explant cultures. The negative controls, AM cultures with only SHEM show no confluence and a cellular final mean (x10⁴cells/ml mean \pm SEM) of 0.40 \pm 0.4 for OMC suspension cultures and of 0.17 \pm 0.1 for OMC explant cultures (Data not shown).

With the aim of achieving cellular differentiation toward corneal epithelial cells,

Table 1. Results from two assessed methods mean \pm standard error of the mean (SEM).

Variables assessed	LSC suspension cultures	LSC explant cultures	N
Viability, % \pm SEM	98.92 \pm 0.56	98.32 \pm 0.58	13/16
Confluence, % \pm SEM	55.95 \pm 11.869	48.27 \pm 9.1651	13/16
Final cellular concentration (x10 ⁴ cells/ml) \pm SEM	38.83 \pm 10.923	36.484 \pm 11.863	13/16
Immunohistochemistry positive, % \pm SEM	22.93 \pm 5.485	16.55 \pm 1.056	3/2

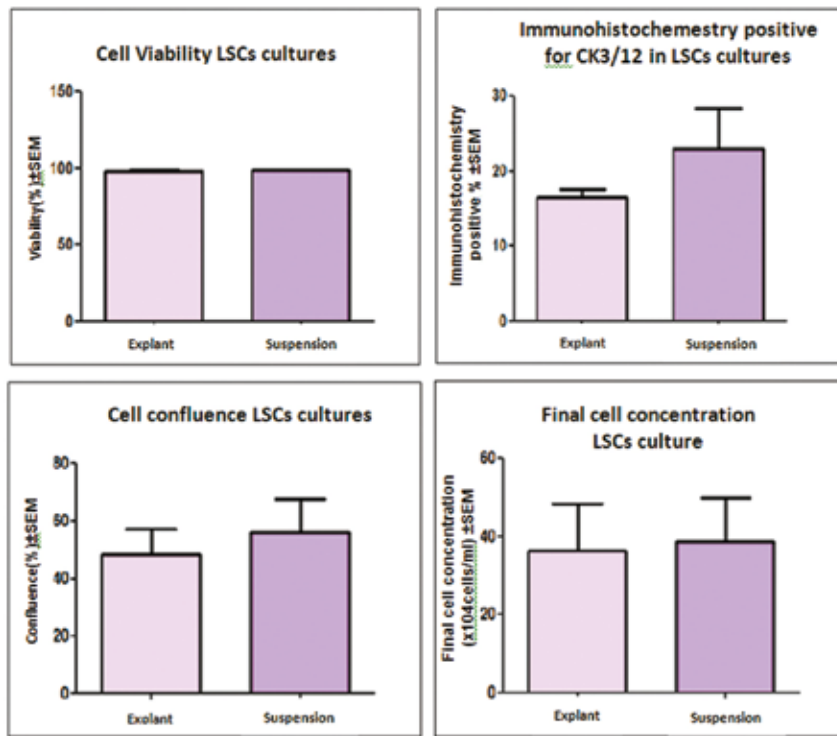


Figure 2. Comparison between two assessed methods mean ± standard error of the mean (SEM).

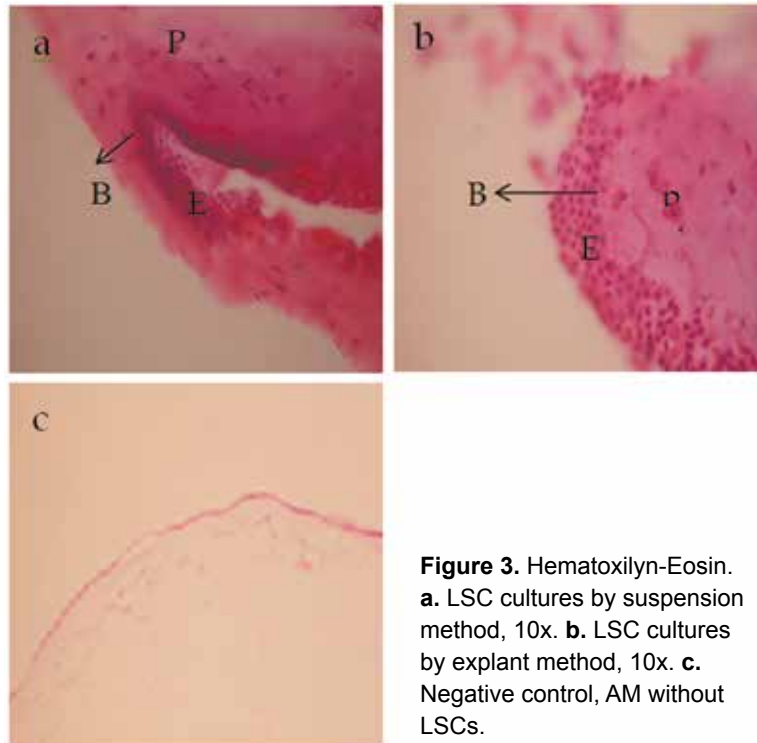


Figure 3. Hematoxylin-Eosin. **a.** LSC cultures by suspension method, 10x. **b.** LSC cultures by explant method, 10x. **c.** Negative control, AM without LSCs.

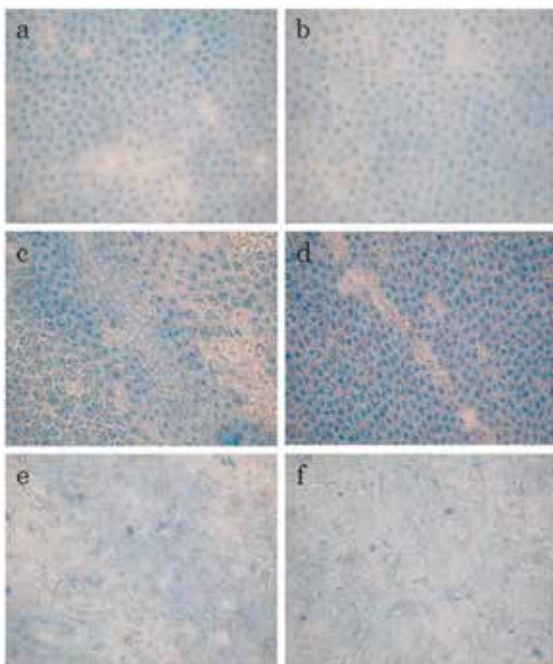


Figure 4. Cell confluence. **a** and **b**, LSC culture by explant method, 10x. **c** and **d**, LSC cultures by suspension method, 10x. **e** and **f**, negative control, AM without LSCs.

a differentiation of 22.935% and 16.558%, respectively. As a conclusion, we can deduce that the results show almost the same viability percentage for the suspension cultures as for the explant cultures. Nevertheless, the suspension cultures show more final cell concentration and confluence than the explant cultures.

Several studies have been carried out to cultivate LSCs in AM, either by the explant or suspension method, but few compare both methods under the same conditions, and the results are inconclusive, since they contradict each other. In Kollis' group, they showed that the growth in LSC cultures obtained by the explant method on AM as a scaffold is faster than the growth of LSC culture produced by the suspension method conserving the same phenotype¹⁶. Contrariwise, Kim's group showed that the suspension method promotes a faster epithelial growth than by the explant method, and both retain the same phenotype²⁶.

immunohistochemistry was performed to evaluate the presence of cytokeratin 3/12 characteristic of corneal epithelium in the LSC culture for both methods. Despite the contamination of cultures and the difficulty to manipulate the tissue morphology obtained in the cultures, the presence of cytokeratin 3/12 (red staining, Figure 5) was found after the analysis with Image J in a percentage of 22.935 ± 5.485 of red pixels or positive cells for cytokeratin 3/12 in LSC suspension cultures and 16.558 ± 1.056 in LSC explant cultures.

DISCUSSION

As we have been discussing, we found a viability of 98.92% and 98.32%, a confluence of 55.95% and 48.27%, a final cellular concentration of 38.83×10^4 cells/ml and 36.484×10^4 cells/ml, and

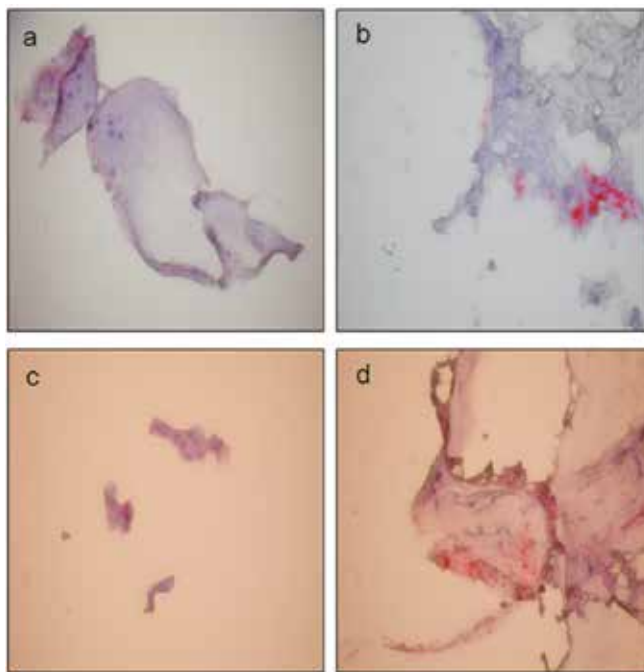


Figure 5. Immunohistochemistry. **a** and **c**, LSC suspension cultures. **b** and **d**, LSC explant cultures. For both methods CK3/12 positive in red.

This study had a similar behavior in both culture methods. The LSC suspension cultures had slightly better results than LSC explant cultures in the confluence and final cell concentration, but they were very similar in viability.

The tissue phenotype of both types of cultures, suspension and explant, do not have differences. Both types of cultures showed a similar corneal phenotype (2 to 5 epithelial cell layers, Bowman's membrane, and substantia propria).

The cultures were analyzed by immunohistochemistry for the presence of cytokeratin 3/12. They showed mostly a cell confluence or cell stratification, where only 3 LSC suspension cultures and 2 LSC explant cultures were tested as shown on the graphs and statistical data in Figure 5.

Even though there is just a slight difference between the two methods, our results are similar to those found in the study by Kim H²⁶.

The results found in the present study are promising since they show a good behavior in both culture types. Furthermore, it is indispensable to conduct more assays to evaluate the differentiation, and more passages are required in order to obtain an ideal tissue differentiation. It is also necessary to evaluate the differentiation stage in each passage.

In summary, more studies are needed in order to confirm the behavior of both culture types so as to guarantee the best treatment for patients with LSC deficiencies.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

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