Composite characterization of freeze-dried human amnion membrane and human adipose tissue derived stromal cells for soft tissue engineering

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INTRODUCTION

The innermost layer of the fetal placenta, the freeze-dried human amnion membrane (FdHAM), envelopes and protects the developing fetus. In addition to its physical and biological qualities, its distinctive structure makes it a beneficial chemical in numerous regenerative medicine applications. The biological properties of FdHAM include an anti-inflammatory effect, antibacterial and antiviral effect, low antigenicity and non-immunogenicity, anti-scarring and antiadhesive effect in wound healing, angiogenesis and anti-angiogenesis properties (surface dependent), an anticancer agent with low anti-angiogenesis properties, promotes epithelialization, pain reliever, support cell adhesion, and growth. FdHAM processing and gamma irradiation sterilization with doses of 15 kGy and 25 kGy will changes in the biological properties, affecting the growth factor levels in the amnion membrane's final form.

In cell-based therapy, human adipose-derived stromal cells (hADSCs) are frequently the cell source of choice for transplantation. Clinical trials for regenerative medicine are increasingly using a lot more hADSCs. The robustness of hADSCs biological functions, which calls for regulating source heterogeneity and production processes and developing biomarkers that foretell desirable responses, maybe a determining factor in the success of hADSCs therapy. Several studies have specifically looked at how hADSCs affect pain relief, wound healing, and innervation.

Scaffold options for augmentation in the treatment of musculoskeletal injuries, including xenografts, allografts, and synthetic matrices, can facilitate cellular growth and collagen deposition. The amnion membrane has low immunogenicity and is readily available. Minimum ethical restrictions on its use. One of the properties of the amnion membrane is that it stimulates wound healing. The amnion membrane is a good material for use in various clinical applications in cell therapy and regenerative medicine. Previous studies have shown that the combination of MSCs and epithelial cells on the amnion membrane scaffold has high interconnection and impacts the tissue.

ABSTRACT

Background: Soft tissue engineering has widely explored freeze-dried human amniotic membrane (FdHAM) and human adipose-derived stromal cells (hADSC) as separate components. In this study, the composite of FdHAM and hADSC combination was investigated regarding cell morphology and density, constituent components, and cytotoxicity evaluation.

Methods: In vitro experimental laboratory research was conducted to analysis of the FdHAM and hADSC composites. Evaluation of cell viability and CD105 for hADSC, evaluation of pore diameter for amniotic membrane and assessment of composite cell morphology, constituent components, evaluation of cytotoxicity, and cell density was conducted.

Results: Our morphology data indicated that clusters of hADSC with homogenous distribution on FdHAM. The viability test showed that the hADSCs were still dominantly alive with an average percentage of 69.3% with the mean cell concentration being 1.53 x 10⁶/mL. The average FdHAM pore diameter was 1.90±0.30 µM. Our data suggested that FdHAM was nontoxic with good cell survivability after exposure to FdHAM.

Conclusions: The structure of FdHAM and hADSC has excellent basic properties and therefore suitable for use as carriers of hADSC or in soft tissue engineering. Compared to the available literature, the characteristic of the FdHAM and hADSC composite is ideal and may be used as an end-product for use in humans.

Keywords: Freeze-dried human amniotic membrane, human adipose-derived stromal cells, mesenchymal stem cell, stem cell engineering.

that the composite is trying to help with. However, these composites' compatibility tests and characteristics are not well known. This study was conducted to characterize FdHAM and hADSC in terms of MSC cell viability, scaffold pore diameter, composite morphology, CD 105 expression, composite components, and amnion membrane cytotoxicity to MSCs.

METHODS

Study design
This study was an experimental laboratory study with an in vitro post-test design with FdHAM and hADSC as materials. The experiment was carried out three times for each examination. The data obtained was analyzed qualitatively and quantitatively. Quantitative data are presented with mean ± standard deviation (SD).

Isolation and preparation of FdHAM
Fresh amnion was obtained from donor and washed with NaCl, and afterward the amnion was washed with phosphate buffer saline (PBS), and the chorion was separated bluntly from the amnion. After washed with PBS, the amnion was stretched in a template container and frozen at -80°C for 24 hours. Subsequently, it was dried in a laminar flow. Then the amnion was sterilized with 25kGy gamma radiation.

Isolation, culture, and seeding of hADSCs
An adipose tissue sample was taken from transport medium and washed with PBS to eliminate contaminating debris and red blood cells and then was mixed with collagenase enzyme and poured into magnetic stirrer chamber. The sample was then incubated on a hot plate at 37°C for 30 min until fully dispersed. It was then added with a medium stopper, and further incubated for 10 min until a homogeneous solution achieved. Then the solution was poured into a 50cc conical tube and filtered and centrifuged at 3000 rpm for 5 min. Pellets that formed were resuspended with alfa MEM into homogenous solution and then cultured in a Petri dish inside the CO₂ incubator for 24 h minimum. The cells were then cultured in a Petri dish until passage 5th and ready to seed. The confluent cells were then harvested and washed with PBS and TrypLE™ Express Enzyme (Life Technologies Corporation, Carlsbad, CA, USA) was added to release adherent cells and pipetted for single cell isolation and centrifuged at 3500 rpm for 5 min. The pellets were added with alfa MEM 1-2 cc and homogenized.

FdHAM was placed in petri dish and the hADSC suspension was added on the top. The prepared plate was gently transferred to an incubator with 37°C, 98% humidity and 5% CO₂ for a minimum of 24 h.

Assessment of cell viability and surface marker of hADSC
Cell viability was evaluated using a Cell Counter (Invitrogen Countess 3 Automated Cell Counter, Thermo-fisher). Briefly, as much as 10,000 hADSC cultured inside the chamber slide. Afterward, cells were washed with PBS twice and stained with calcein/AM and ethidium homodimer-1 and were evaluated using a fluorescent microscope. The result was evaluated using the Cell Counter of which alive cells indicated as green while or dead cells in red.

The cell surface marker expression for CD105 on was evaluated using immunohistochemistry (IHC) approach. The cells were added with H₂O₂ 3% and incubated at 37°C for 10 min and then washed with PBS. Then trypsin 0.025% was added and incubated in 37°C for 6 min. Ultra V block was added and incubated further for 5 min 37°C temperature and washed again with PBS. The cells were mixed with 1:100 diluted CD105 antibody (Abcam Anti-CD105 antibody), incubated for 30 min and washed with PBS. HRP polymer was added and incubated for 10 min and then rinsed. Then DAB chromogen (20μl/1 ml substrate) was added, incubated for 15 min in a dark room, and washed. The cells were then stained with Meyer Hematoxylin, incubated for 10 min at 37°C, washed and room dried. The expression was evaluated under the microscope (Leica DM750) of which the cells expressing CD105 had brown stained expression of the cell nucleus and cytoplasm.

Morphology, cell distribution and pore diameter of the composites
The morphology and cell distribution of the composites were qualitatively evaluated using a scanning electron microscope (SEM, HITACHI FlexSEM 1000). Three composite samples and assessed them from the image taken by the SEM. The pore diameter was also evaluated in three samples using SEM. Three scaffold pores were selected for each sample, and the average pore diameter was measured. The poor diameter was measured by calculating the diameter of the inner diameter of at least five pores with 20.000x magnification. If the pore is ovoid, the longest diameter was measured.

Constituent component of the composite
The composite components were evaluated by Energy Dispersive X-ray Spectroscopy (EDX/EDS) with HITACHI FlexSEM 1000 integrated into the SEM. The evaluation was conducted in three samples composites. The weakness in this evaluation was that EDX might unable to detect elements whose atomic number was below eleven accurately.

Cytotoxicity evaluation
After a 24- or 72-h incubation, 20 μL of a 5 mg/ml MTT solution ((3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide) was added to each well and the plate was further incubated at 37°C for 4 h. The medium was then aspirated and the wells were washed with PBS, allowed to dry for approximately 2 h of after which and 200 μL of dimethyl sulfoxide (DMSO) was added to each well. The microtiter plate was placed on a shaker to dissolve the dye. After the formazan crystals had dissolved, the absorbance was determined spectrophotometrically at 570 nm using a reference wavelength of 630 nm with an ELX800 UV universal microplate reader (Bio-Tek Instruments Inc., Vermont, USA) for sample optical density that interprets as cytotoxicity and proliferation of cells.

RESULTS

Cell viability
A total of 10,000 MSCs were cultured on a chamber slide. After that, the cells
Table 1.  **Cell viability analysis in three samples.**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Concentration</th>
<th>Live cells (%)</th>
<th>Dead cells (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample 1</td>
<td>1.68 x 10^6/mL</td>
<td>72%</td>
<td>28%</td>
</tr>
<tr>
<td>Sample 2</td>
<td>1.44 x 10^6/mL</td>
<td>67%</td>
<td>33%</td>
</tr>
<tr>
<td>Sample 3</td>
<td>1.68 x 10^6/mL</td>
<td>69%</td>
<td>31%</td>
</tr>
<tr>
<td>Mean</td>
<td>1.53 x 10^6/mL</td>
<td>69.3%</td>
<td>30.7%</td>
</tr>
</tbody>
</table>

were rinsed with PBS twice and then stained with calcein/AM and ethidium homodimer-1. The final results were evaluated using a fluorescent microscope to determine the number of live (green) and dead (red) cells (Figure 1). The results of the cell viability test showed that after various processes passed by the hADSC, the cells were still dominantly alive with an average percentage of 69.3%. The average cell concentration was at 1.53 x 10^6 mL (Table 1).

**hADSC surface cell marker**
The cells expressing CD105 were stained brown and were observed predominantly around the cell nucleus. Some cells express large quantities to fill the cytoplasm while others are present only in the nucleus. Our data suggested >50% of cells express CD105 (Figure 2).

**Composite cell morphology**
The representative cell morphology analyses using SEM of sample 1 are presenting Figure 3. MSCs were more evenly distributed along the scaffold area. In almost the entire visual field with 5,000x magnification, the cells were evenly distributed with not too large and even gaps between the cells. At a higher magnification (50,000x), it was seen that the MSCs were neatly arranged and stratified. Cells are connected by connecting nets, making MSCs connected neatly.

**Composite distribution cells**
In evaluating the cell density in the composite, a qualitative evaluation was carried out on the scaffold by looking at the cell density and distribution at the edge of the scaffold after seeding. For all three samples, two analysis points were evaluated using SEM: (1) the top left edge of the scaffold and (2) the bottom right edge of the scaffold with two types of magnification: 5000x and 10,000x.

The condition of the first sample from two locations using two magnifications is presented in Figure 4. In small or large magnification, the distribution of cells on the surface of the scaffold was very good, where all cells adhered to all membrane layers well and evenly without any clusters of cells clustered apart and there was no empty area without cells (Figure 4). It appears that the hADSC was attached to the edge of the scaffold on both the top left and bottom right of the scaffold. The proliferation of hADSC was good and solid.

**Composite cell pore diameter**
Pore diameters were analyzed based on the obtained SEM images and averaged from various points of the two network images. Detailed data are presented in Table 2. It was found that the average pore diameter of the scaffold was 1.9±0.3 μM.

**Composite component constituent**
In terms of composition, the carbon and sodium content was analyzed using...
the amnion membrane scaffold live well and develop after being implanted in the amnion. In addition, these results also indicate that the amnion membrane was not cytotoxic considering the increasing number of cells and not decreasing. Quantitatively, there was an increase in the number of cells up to five times compared to before the MSC was implanted (Table 4).

**DISCUSSION**

This study characterizes hADSCS affixed to the FdHAM to assess the possibility of being used directly as therapeutic agents in humans. hADSCs can be extracted from bone marrow or adipose tissue and are capable of helping tissue regeneration in both animal and human models. Previous studies have found a therapeutic effect on cells transplanted into the fetus, including epithelial cells from the amniotic and fluid membranes. A well-designed tissue engineering product consisting of a biomimetic scaffold combined with cells is an essential prerequisite for successful bone tissue engineering. Ideally, materials that mimic natural extracellular matrix (ECM) should be used. This is important because in order for a final tissue in the target organ to be formed, the composite must have adequate characteristics to become a good home for stem cells.

Assessment of the distribution and cell adhesion suggested that after the attempt to dislodge the composite, the cells remained firmly attached to the scaffold. The distribution of cells was even without being too clumped on one side. Although the method used in this study is one of the simplest methods, it is the most accurate method. The diameter of the composite was also assessed since it is critical and without a proper diameter, there can be no circulation, both nutritionally and new and old cells that come in and out of this scaffold. Comparing hADSCs implanted in scaffolds of different diameters showed that the larger pores enhance cell-scaffold interactions, increase osteogenic potential, and maximize cell adhesion to the scaffold. Previous studies agree that a diameter of 500-1000 μm is the most optimal compared to other pore sizes.

Energy Dispersive X-ray spectroscopy and the results presented in Figure 5. On average, the carbon content was 64.98% while the sodium was 35.01% as detailed in Table 3.

**Composites cytotoxicity evaluation**

The optical density (OD) of the amnion membrane seeded with MSC (mean OD = 1.008) was much greater than that of the control alone (mean OD = 0.201). This shows that the cells implanted in the amnion membrane scaffold live well and develop after being implanted in the amnion. In addition, these results also indicate that the amnion membrane was not cytotoxic considering the increasing number of cells and not decreasing. Quantitatively, there was an increase in the number of cells up to five times compared to before the MSC was implanted (Table 4).

**Figure 3.** Representative image of sample viewed with SEM at (A) 5,000x, (B) 10,000x, (C) 20,000x, and (D) 50,000x magnification.

**Figure 4.** Representative images of cell distribution in sample from two locations of the scaffold (the top left edge and bottom right edge of the scaffold) using two different magnifications (5,000x and 10,000x).
MTT assay is commonly used to test whether the exposure to certain material would reduce or retain similar number of living cells after exposure or cytotoxicity evaluation.\textsuperscript{15-17} In this study, within a period of 3 hours during which hADSCs were implanted in the composite, it was found that there was a significant increase in the number of cells suggesting that the implanted cells were viable and alive in the composite. Furthermore, the similar OD of the three samples also indicated that our finding is not incidental, but consistent on different samples. This suggests that FdHAM could be used as a host for hADSC and is not cytotoxic to hADSC.

**CONCLUSIONS**

Our study found that FdHAM and hADSC composites have adequate pore diameter, good cell viability, and has no cytotoxicity. This suggests that the composite has good basic properties and is suitable for use as a carrier of hADSC.

**ETHICAL APPROVAL**

The ethical approval for this study was obtained from the Ethical Clearance Committee of Faculty of Medicine, Universitas Airlangga - Dr. Soetomo General Academic Hospital, (Reference number: 0369/107/2/XI/2020).

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**DISCLOSURE OF CONFLICTS OF INTEREST**

The authors declare have no conflict of interest. Each author has no commercial associations (e.g., consultancies, stock ownership, equity interest, or patent/licensing arrangements) that might pose a conflict of interest in connection with the submitted article.
ORIGINAL ARTICLE

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AUTHORS CONTRIBUTION
The authors contributed to the research process, including preparation, conceptualization, data collection and analysis, drafting, and publishing approval.

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