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VIABILITY OF ADIPOSE-DERIVED STEM CELLS (ASCs) ON POROUS CHITOSAN SCAFFOLD

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ABSTRACT: Background: Stem cells have great potential in regenerative medicine due to their multipotentiality. Treatments involving cell sheets for tissue reconstruction require a large number of cells to repair the tissue damage, and the cells are fragile. Combined with a porous chitosan scaffold (PCS), adipose-derived stem cells (ASCs), which are ubiquitous, can be delivered to a target tissue in vivo. This approach provides mechanical support to the skin until the normal tissue is regenerated. In the present study, ASCs were characterized and tested for their viability on a PCS in vitro prior to in vivo application. **Results:** The ASCs expressed CD29, CD73, CD90 and CD105 but did not express CD34, as demonstrated by immunocytochemistry. Most of the ASCs were found to be viable on the PCS at 24 and 72 hours of culture. These results suggest that ASCs seeded on a PCS have high viability, allowing ASCs to be enriched for therapeutic applications. **Conclusions:** This qualitative study has proven ASCs were able to survive on PCS as indicated by the number of live cells exceeded the number of dead cells as demonstrated by live/dead assay.

INTRODUCTION: Autologous transplantation of bone marrow-derived stem cells has been used to treat various abnormalities and skin lesions. In vitro studies and animal models have demonstrated that these bone marrow-derived stem cells have the potential to reconstitute tissue vascularization and to restore normal function [1, 2, 3]. Adipose-derived stem cells (ASCs) have similar biological properties as bone marrow-derived cells and can be induced to differentiate into different cell lineages. Adipose tissue is a potential source of adult and somatic stem cells and has a wide range of clinical and therapeutic applications.

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In addition, adipose tissue is an abundant source of various growth factors and cytokines that stimulate the growth, proliferation and differentiation of ASCs [4, 5]. Recent studies have proven that ASCs play a significant role in tissue regeneration. To evaluate the possibility of improving tissue regeneration, the ASCs have been characterized in this study.

Reconstruction of skin after injury involves different types of wound healing, depending on the wound's classification. Over several decades, studies have been performed on different reconstructs seeded with ASCs. Skin reconstructs create the optimal conditions for accelerating wound healing, are easily handled for transplantation and may replace the use of skin grafts [6]. Most of the biomaterials that are available on the market lack these properties, and therefore, the demand for perfect skin reconstructs is high.

The remarkable properties of chitosan offer unique opportunities for the development of biomedical applications. However, to date, no study has examined the viability of human ASCs on a porous chitosan scaffold (PCS). The present study reveals the greater viability of ASCs on a PCS, given that the chitosan scaffold allows greater cell attachment and proliferation when the cells are seeded on it.

MATERIALS AND METHODS:

Materials:

Adipose tissue:

Adipose tissue was obtained from individuals who had provided informed consents and who had undergone elective surgery at Hospital Universiti Sains Malaysia, Kelantan, as approved by the Human Research Ethics Committee of Universiti Sains Malaysia (USMKK/PPP/JEPeM (236.3[16])).

Porous chitosan scaffold (PCS):

Chitosan scaffolds were obtained from Advanced Materials Research Centre, Standards and Industrial Research Institute of Malaysia (SIRIM) Berhad in Malaysia [7]. The thickness of the PCS was 2 mm, and the diameter was 5 mm. These scaffolds were sterilized with ethylene oxide (EO) gas and stored at room temperature for one month in a dark and dry place prior to use.

Methods:

Derivation and culture of ASCs:

The tissue obtained was washed with Dulbecco's phosphate-buffered saline (DPBS) (Gibco®, USA) with 1% antibiotic-antimycotic. The tissue was then digested with collagenase type I (200 U/mL) (Worthington, NJ) overnight at 37°C. The cells were passed through a 70 µm cell strainer, washed with DPBS and centrifuged at 805g for 5 minutes. The cells were then cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco®, Life Technologies, USA) 10% mesenchymal stem cell (MSC)-qualified fetal bovine serum (Gibco®, Mexico) and 1% antibiotic-antimycotic. The cells were seeded at a density of $4x10^4/cm^2$ in T25 culture flasks and incubated at 37°C with 5% CO_2.

Verification of adipose-derived stem cell (ASC) phenotype by immunocytochemistry: This experiment was performed using an IHC Select® HRP/DAB Kit (DAB150, Chemicon) according to the instructions given by the manufacturer, with certain modifications. The staining was performed in a 24-well plate.

The culture medium was discarded, and the cells were washed with PBS and fixed in cold methanol for 15 minutes at 4°C. Blocking reagent was added and incubated at room temperature for 30 minutes. The cells were incubated with optimized dilutions of mouse anti-human IgG1 monoclonal antibodies against CD29 (Abcam, clone number 12G10) (1:1000), CD34 (Abcam, clone number BI-03C5) (1:1000), CD105 (Abcam, clone number SN6) (1:1000), CD73 (BD Pharmingen™, clone AD2, 550256) (1:1000), and CD90 (BD Pharmingen™, clone 5E10, 555593) at 4°C for one hour.

After washing with PBS, the cells were incubated with biotinylated goat anti-mouse IgG at 4°C for 15 minutes. Streptavidin-horseradish peroxidase was added to the wells, incubated for 10 minutes and washed away with PBS. The cells were incubated in diluted Chromogen A+B (1:25) for 10 minutes and, after washing, counterstained with hematoxylin for 5 minutes. The staining was observed under an inverted phase-contrast microscope.

Seeding of ASCs on PCS:

Cells at passage three were detached by incubation in TrypLE Express for 10 minutes and centrifugation at 805g for 5 minutes. A cell suspension with a seeding density of $2.5x10^4$ cells was seeded onto PCS within the wells of a 96-well plate and incubated at 4°C for 30 minutes. Next, 50 µL of media was added to each well. The constructs were incubated in low-glucose DMEM, GlutaMAX-I and 10% MSC-qualified fetal bovine serum at 37°C and 5% CO_2 incubator. The growth media were changed daily.

Viability of ASCs, assessed using LIVE/DEAD® Viability/Cytotoxicity Kit (Molecular Probes):

The constructs were washed in DPBS for 5 minutes. The constructs were then incubated in 100 µL of LIVE/DEAD reagent for 30 minutes at room temperature. The constructs were observed and image were captured using a confocal laser scanning microscope (CLSM) (Leica Microsystems

Heide GmbH**)** with an excitation wavelength of 488 nm and an emission wavelength of 534 nm.

RESULTS:

Immunocytochemical analysis of cultured ASC:

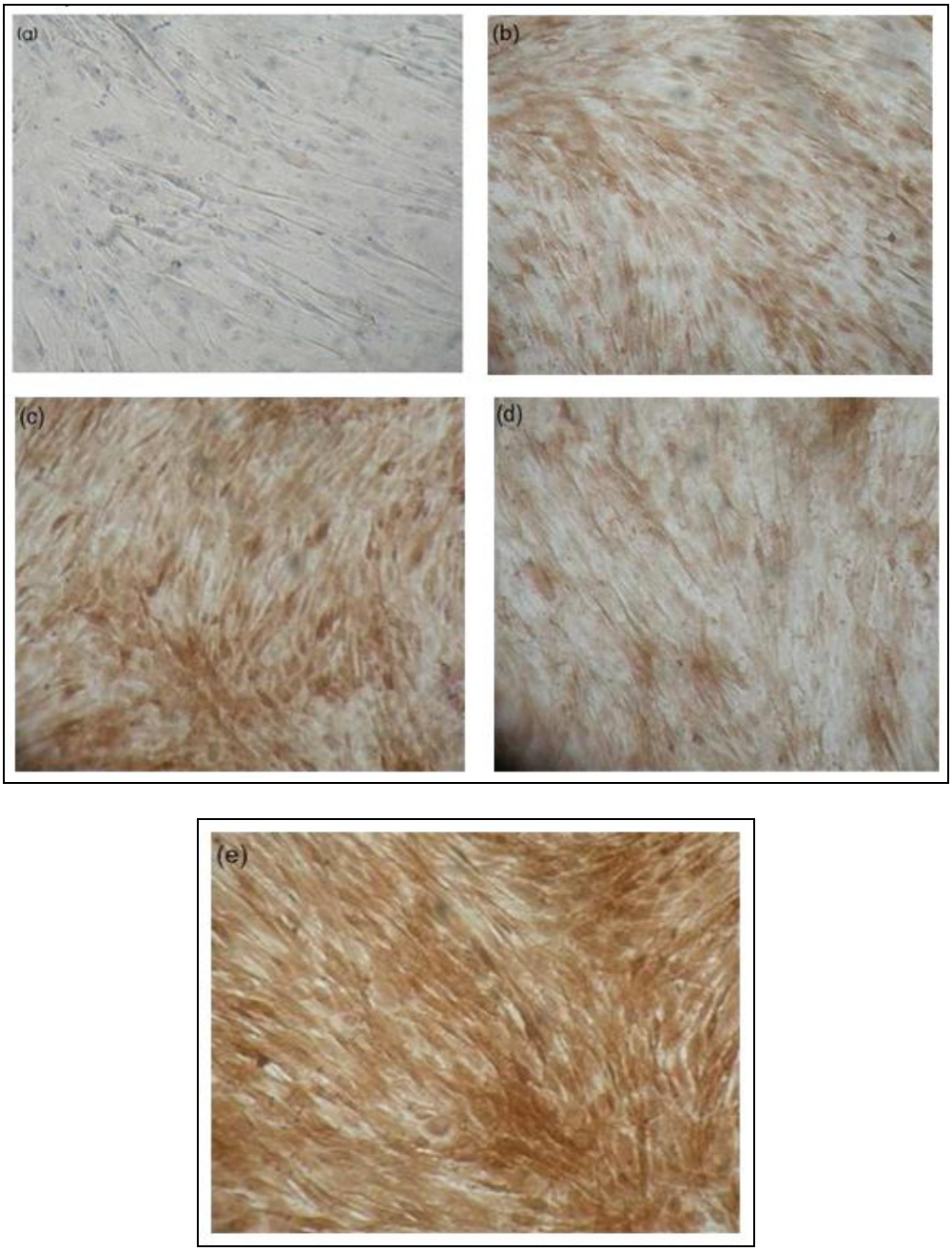
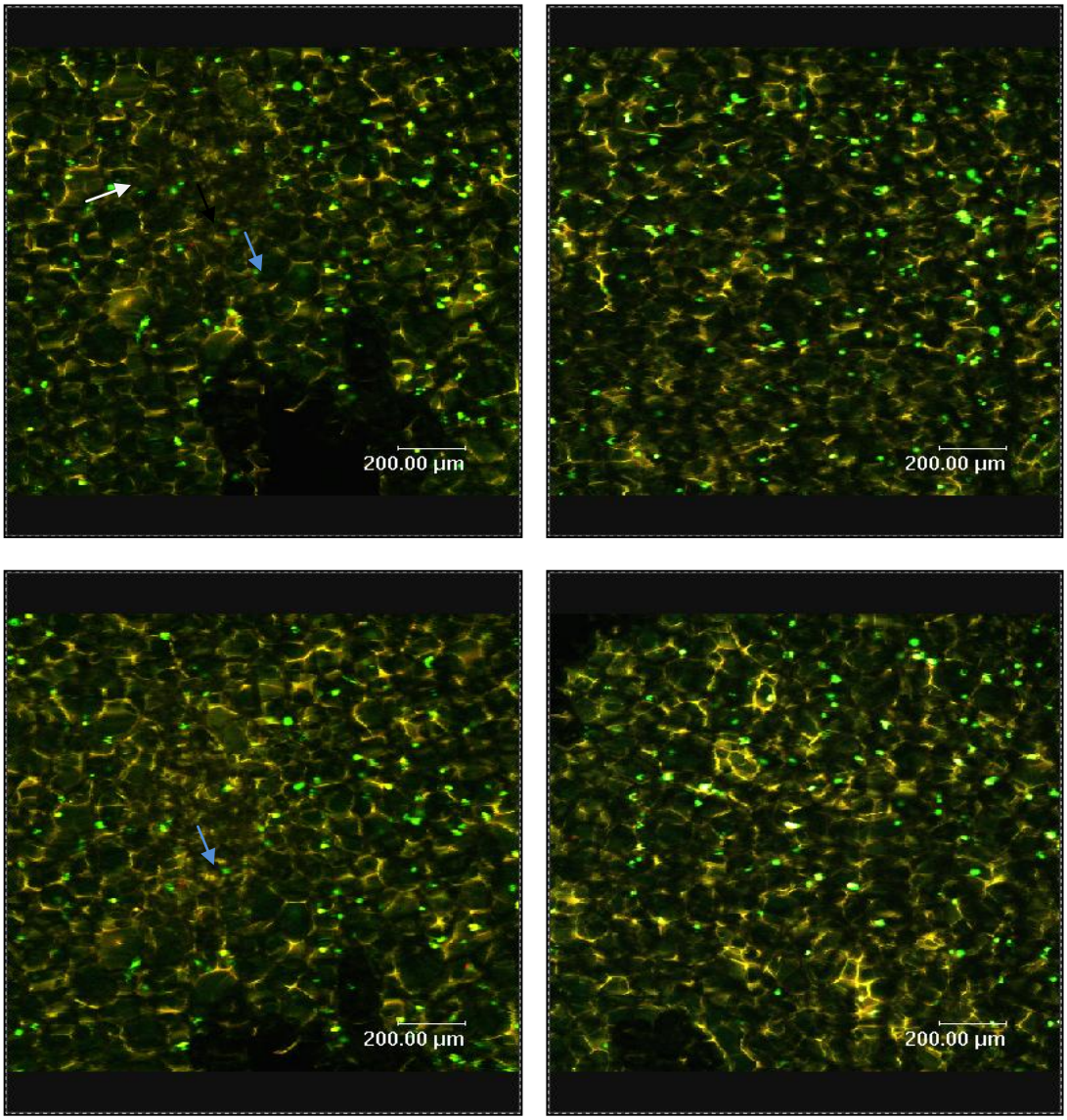
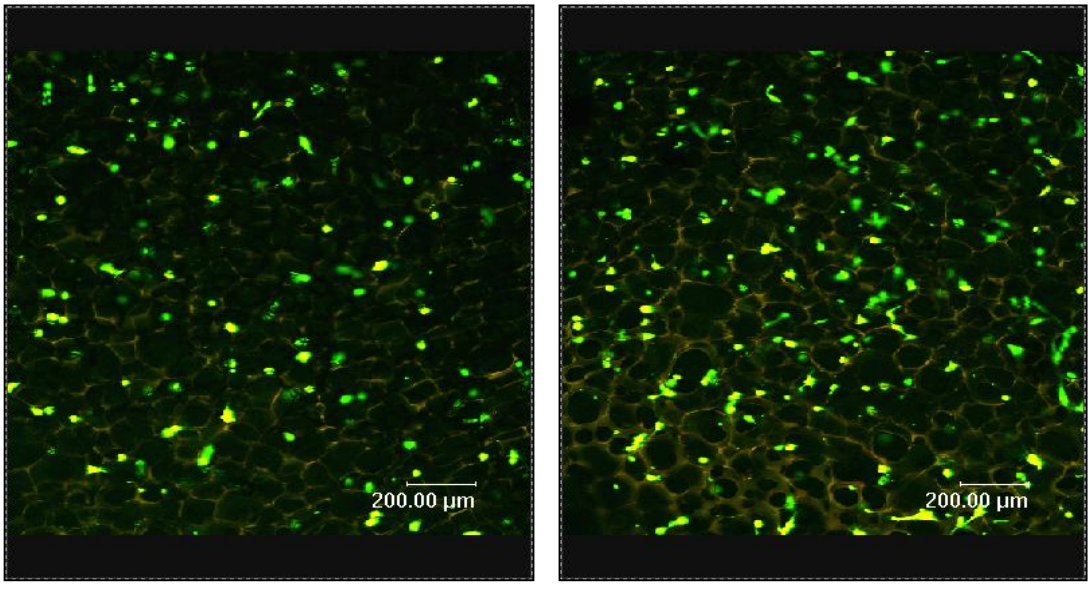


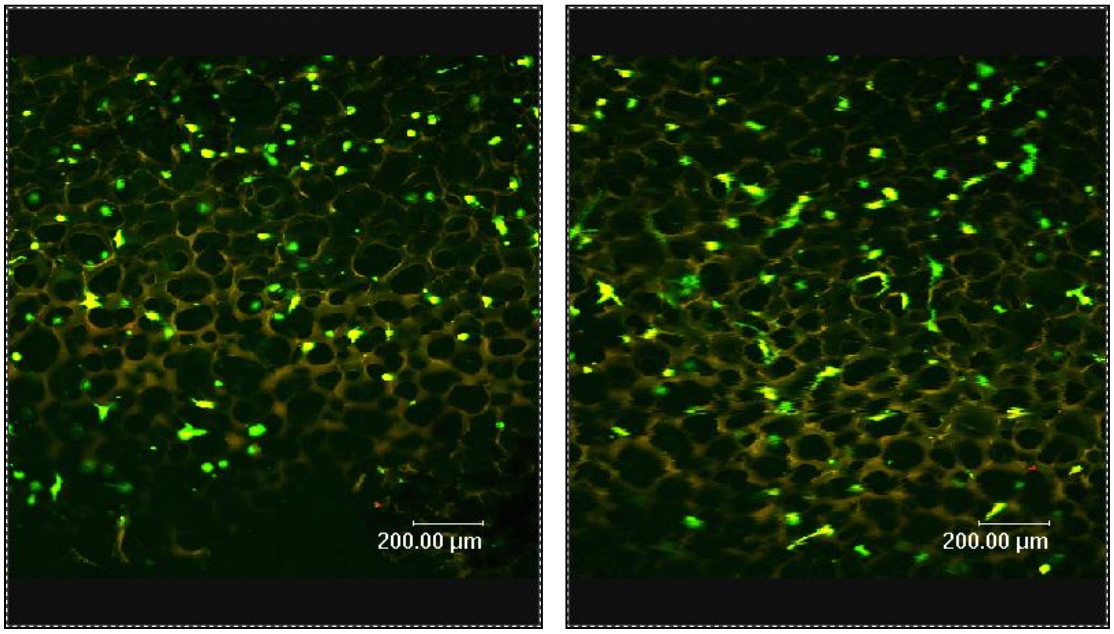
FIG.1: SELECTED IMAGES OF IMMUNOCYTOCHEMICAL STAINING OF CULTURED ASCs POSITIVE FOR (b) CD29, (c) CD105, (d) CD73 AND (e) CD90, WHEREAS ASCs DID NOT EXPRESSED CD34 (a) AS OBSERVED UNDER A PHASE-CONTRAST MICROSCOPE. MAGNIFICATION (10x). THE POSITIVE EXPRESSION OF MARKERS IS INDICATED BY BROWN COLOR AT PROTEIN MEMBRANE OF ASCs. THE NEGATIVE EXPRESSION OF MARKERS IS INDICATED BY AN ABSENCE OF BROWN COLOR AT PROTEIN MEMBRANE OF ASCs

Viability of ASCs

Table 1: Selected CLSM images of ASCs at 24 and 72 hours post-seeding (magnification: 10x). The images were taken at four different parts of a PCS. The green dots represent live cells, whereas the red dots are dead cells as shown by white and blue arrows respectively. Numerous live ASCs can be observed lining the interconnected pores of the PCS (yellow color) compared with dead ASCs.

TABLE 1: SELECTED CLSM IMAGES OF ASCS AT 24 AND 72 HOURS POST-SEEDING (MAGNIFICATION: 10X)

POST-SEEDING	CLSM IMAGE
24 hours	
72 hours	



DISCUSSION: Tissue engineering involves the combination of cells with a scaffold to enhance the basic functions of the cells, such as attachment, viability, proliferation, migration and differentiation [8]. Human ASCs have been shown to exhibit great proliferative potential and the ability to differentiate into different cell lineages [9]. Although these ASCs have various preclinical and clinical applications, their use in wound repair and regeneration is more promising[10]. Chitosan, a partially deacetylated form of chitin composed of glucosamine and N-acetylglucosamine, has been tested for its effects on the growth of various types of cells, such as bone-marrow-derived stem cells [11], embryonic stem cells [12], and fibroblasts and keratinocytes [13, 14]. Chitosan was chosen due to its interesting biological properties, such as wound healing acceleration [15], drug delivery potential [16] and antibacterial activities [17]. In addition, chitosan has been proven to be nontoxic, biodegradable into a harmless product and biocompatible[18] . The glycosaminoglycans present in chitosan form a structure analogous to the extracellular matrix, to which cells can adhere while proliferating to generate a functional tissue or organ.

Immunocytochemistry is a technique in which antibodies are used to detect the presence of antigens in cells [19]. The interpretation of immunocytochemistry is qualitative, usually based on observation by personnel, and this technique indicates whether a marker is expressed. In the current study, ASCs were characterized based on their expression of the MSC markers CD73, CD90, and CD105; the adhesion molecule CD29; and the hematopoietic stem cell marker CD34. ASCs were found to be positive for CD29, CD73, CD90 and CD105, as demonstrated by the precipitation of brown deposits in the cells as shown in **Fig. 1(b), (c), (d), (e)**.

This phenomenon was due to the reaction of the horseradish peroxidase with the DAB substrate, which resulted in the precipitation of brown-to-black deposits at the antigenic sites (membrane) where antigen-antibody complexes formed. Our findings showed an absence of brown color at the membrane of cells for CD34, indicating a lack of expression this marker (Figure 1a). The findings were in accordance with previous studies, which suggested expression of CD73, CD90 and CD105 [20, 21, 22], but not expression of CD34 [20].

Recent studies have shown that ASCs seeded on a silk fibroin-chitosan scaffold enhance wound healing [23]. Studies have been performed on ASCs seeded on chitosan particle-agglomerated scaffolds for cartilage and osteochondral tissue engineering [24]. ASCs derived from rats and seeded on chitosan film differentiated into osteogenic cell lineages [11]. However, studies based on human ASCs seeded on a PCS have not been well established. Therefore, in the present study, ASCs were seeded on a PCS, and the viability of the ASCs was tested. This viability

analysis enhances understanding of the growth and proliferation of ASCs.

The viability of cells and their proliferation are essential to produce a functional tissue. The viability of cells within a scaffold reflects the cells' enzymatic activity. The calcein-AM in live/dead assay kits enters live cells, which have intact membranes and converting the calcein-AM into green fluorescence. The ethidium homodimer binds to DNA following the membrane rupture of dead cells, thus resulting in red fluorescence. This *in vitro* test was performed to evaluate the viability of ASCs on a PCS 24 and 72 hours post-seeding.

Table 1 shows images of the green fluorescence and red fluorescence as dots on the chitosan. Post-seeding, after 24 and 72 hours, rounded green dots indicated that the live cells were scattered, lining the interconnected pores of the chitosan. The number of live ASCs within the chitosan was higher compared with the number of dead cells.

This study proved that ASCs are able to grow on a PCS for 72 hours. The cells either can be differentiated into desired cell lineages or can deliver growth factors upon transplantation into damaged tissue. Therefore, this finding is relevant to engineering ASCs on PCS.

CONCLUSIONS: This qualitative study has proven ASCs were able to survive on PCS as indicated by the number of live cells exceeded the number of dead cells as demonstrated by live/dead assay. The high potential of viable ASC growth on PCS could be applied for tissue engineering purposes. This study only highlighted the viability of human ASCs seeded on a PCS. Further investigations on the proliferation rate and differentiation of ASCs into desired lineages on PCS should be explored.

COMPETING INTERESTS: The authors declare that they have no competing of interest.

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