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Evaluation of the Human Amniotic Membrane as a Scaffold for Periodontal Ligament Fibroblast Attachment and Proliferation

(Penilaian Membran Amnion Manusia sebagai Perancah untuk Pelekatan dan Pengembangbiakan Fibroblas Ligamen Periodontal)

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ABSTRACT

*This study was aimed at evaluating the ability of the human amniotic membrane (HAM) to act as a scaffold for the growth of the main cells in periodontal regeneration, human periodontal ligament fibroblasts (HPDLFs). The HAM has many biological properties that are suitable for periodontal tissue regeneration such as low immunogenicity, anti-fibrosis, anti-inflammation, and a rich extracellular matrix component. Commercially available HPDLFs were seeded onto the HAM, and the attachment and proliferation of the cells were observed through scanning electron microscopy (SEM) and histological analysis. Cell viability was assessed using the alamarBlue® proliferation assay at days 1, 3, 7, 14 and 21. Histologically, the HPDLFs showed a monolayer to multilayer attachment onto the HAM from day 1 to day 7. The SEM analysis demonstrated that the HPDLFs had attached appropriately onto the HAM surface at day 1 to day 3, and began overlapping at day 7, while maintaining their flat shape. However, by days 14 and 21, there was an alteration in the morphology of the cells, where they later became rounded. The proliferation assay showed that the viability of the HPDLFs on the HAM had increased significantly from day 1 to day 7 (*p*=0.012), but later showed significant reduction at day 14 (*p*=0.002) and day 21 (*p*=0.005). In conclusion, this study showed that the HAM was able to function well as a scaffold for HPDLFs within 7 days, and thus, it can be a promising scaffold for periodontal regeneration. However, the behaviour of the cells in relation to the membrane over longer culture duration warrants further investigation.*

Keywords: Amniotic membrane; periodontal fibroblasts; periodontal regeneration; scaffold

ABSTRAK

*Kajian ini bertujuan untuk menilai keupayaan membran amniotik manusia (HAM) untuk bertindak sebagai perancah bagi pertumbuhan sel-sel utama dalam regenerasi periodontal iaitu sel fibroblas ligamen periodontal manusia (HPDLFs). HAM mempunyai banyak ciri biologi yang sesuai untuk pertumbuhan semula tisu periodontal seperti keimunogenan yang rendah, anti-fibrosis, anti-keradangan dan kaya dengan komponen matriks luar sel. HPDLFs yang tersedia secara komersial telah dibiakkan ke atas HAM, dan pelekatan serta pengembangbiakan HPDLFs diperhatikan melalui pemeriksaan mikroskop imbasan elektron (SEM) dan analisis histologi. Daya tahan sel telah dinilai menggunakan ujian percambahan alamarBlue® pada hari 1, 3, 7, 14 dan 21. Secara histologi, HPDLFs menunjukkan pelekatan ekalapisan kepada beberapa lapisan ke atas HAM daripada hari pertama hingga ke-7. Analisis SEM menunjukkan bahawa HPDLFs telah melekat dengan baik pada permukaan HAM pada hari pertama hingga hari ke-3 dan mula bertindih pada hari ke-7, sambil mengekalkan bentuknya yang rata. Bagaimanapun, pada hari ke-14 dan ke-21, terdapat perubahan pada morfologi sel, dengan mereka kemudiannya menjadi bulat. Ujian percambahan menunjukkan bahawa daya tahan sel HPDLFs pada HAM meningkat dengan ketara daripada hari pertama hingga hari ke-7 (*p*=0.012) namun kemudiannya menunjukkan penurunan yang ketara pada hari ke-14 (*p*=0.002) dan hari ke-21 (*p*=0.005). Kesimpulannya, kajian ini menunjukkan bahawa HAM dapat berfungsi dengan baik sebagai perancah untuk HPDLFs dalam tempoh 7 hari, dengan itu mampu menjadi perancah untuk regenerasi periodontal. Walau bagaimanapun, tingkah laku sel berhubung dengan membran ini dalam tempoh yang lebih lama masih memerlukan kajian lanjut.*

Kata kunci: Fibroblas periodontal; membran amnion; perancah; pertumbuhan semula periodontal

INTRODUCTION

Recent advancements in scaffold designs and biomaterials have enhanced the current capabilities of scaffolds to recapitulate the natural extracellular functions of the matrix both temporally and spatially. Many natural, synthetic, semi-synthetic and hybrid scaffolds have been constructed and applied for tissue regeneration (Causa et al. 2007). The human amniotic membrane (HAM) has proven to be suitable for application in various medical procedures such as dressings for burns, treatment of the conjunctiva, and epithelial regeneration (Barabino et al. 2003; Cooper et al. 2005; Kruse et al. 2000; Malhotra & Jain 2014). The HAM is easily available, cheap and possesses exceptional biological properties such as

anti-inflammatory, anti-scarring, anti-microbial (Chopra & Thomas 2013), anti-angiogenic, epithelialization promotion (Mamede et al. 2012) and anti-carcinogenic properties (Niknejad & Yazdanpanah 2014). Over the past few decades, with advancements in tissue engineering, biomaterial science and scaffold design, this 'miracle' membrane has been introduced into periodontal tissue engineering (Litwiniuk & Grzela 2014).

Adachi et al. (2014) demonstrated that periodontal cells cultured on amniotic membrane substrates express essential proteins for cell-substrate adhesion and the maintenance of tissue integrity. The HAM has also been shown to have good adhesion with periosteal-derived cell sheets and dental pulp-derived cell sheets (Amemiya et al. 2014; Honjo et al. 2014). Iwasaki et al. (2014) demonstrated that enhanced periodontal regeneration occurred after a periodontal ligament stem cell (PDLSC)-associated amniotic membrane was transplanted into surgically-created periodontal defects at the first maxillary molars of rats.

Human periodontal ligament fibroblasts (HPDLFs) are fibroblasts that reside in the periodontal ligament surrounding the root of the tooth and are of mesenchymal origin. They are large spindle-shaped or stellate cells with an oval nucleus (Nanci & Ten Cate 2013). They have an extensive cytoplasm with an abundance of organelles associated with the synthesis and secretion of protein. HPDLFs possess a well-developed cytoskeleton with adherens and gap junctions (Nanci & Bosshardt 2006). They are characterized by a rapid turnover, high level of remodelling, and a remarkable capacity to renew and repair periodontal tissues. Therefore, these cells have a high potential to be used for the regeneration of oral tissues, specifically the periodontal tissue itself.

Despite advances in tissue engineering, an ideal scaffold that is cheap, easily available and possesses all the biophysical and biochemical properties necessary for periodontal reconstruction has yet to be established. Therefore, this study evaluated the efficacy of the HAM as a scaffold for the growth of human periodontal ligament fibroblasts (HPDLFs) *in vitro*. To date, there is very little data available on the efficacy of HAM as a scaffold for HPDLFs. This assessment was done by means of histological and SEM analyses as well as a cell viability analysis.

Materials and Methods

HAM procurement and decellularization

Glycerol-preserved HAM was purchased from the Tissue Bank of Universiti Sains Malaysia, Malaysia. The HAM was processed further for the purpose of the experiment. It was washed in phosphate buffered saline (PBS) to remove glycerol. Next, it was placed in 12.5 µg of thermolysin (Nacalai Tesque, Japan) and mixed with 10 mL of PBS for 25 min in a shaker, before being washed again in PBS. Later, it was mechanically scraped to remove the epithelial layer. It was then soaked in the complete growth medium for one hour.

Seeding of HPDLFs on HAM

Commercially available HPDLFs [item code: CC-7049 (Lonza, USA)] harvested from passage 6 were used in this study. The HAM was cut into a few 1 cm^2 pieces, which were then placed on top of the cover slides of 6 multi-well culture plates with the stromal side facing upwards. A sterile brass ring (with a diameter of 1 cm) was placed on top of it to stabilize the HAM in the medium. The cells were then seeded onto the HAM at a cell density of 5×10^4 cells. Non-epithelialized HAM (HAM-E), de-epithelialized HAM (HAM-DE), and HPDLFs only were also cultured on cover slides and prepared according to each analysis (Kiernan 2000; Murtey & Ramasamy 2016; Sittampalam et al. 2004). The membranes were kept in an incubator for 4 h at 37°C and 5% CO_2. After that, 2 mL of the growth medium was added to each sample. The medium was changed every alternate day until the day of harvesting/observing the sample for further analysis.

Histological analysis

The cell-seeded HAMs were retrieved at days 1, 3, 7, 14, and 21 for haematoxylin and eosin (H&E) staining. An unseeded HAM with a similar size was also stained and used as a negative control at the end of day 21. The samples were processed by means of formalin, xylene and wax using the Excelsior ES System (Thermo Scientific, UK). The samples were mounted on wax blocks and sectioned using a rotary microtome HM 355 (MICROM International GmbH, Germany), with each section having a thickness of 5 µm. The selected slides were dewaxed over a lab-line slide warmer (Branstead, USA) and stained with the nuclear dye, haematoxylin (Merck, Germany). They were then rinsed, counter stained with eosin (Merck, Germany), dehydrated and mounted with a DPX mountant (BDH Lab, UK). The stained slides were air dried and viewed under a digital histological slide analysis system known as Mirax Desk (Zeiss, Germany).

Scanning electron microscopy profile analysis of HPDLFs/HAM

Investigations using scanning electron microscopy (SEM) were carried out on days 1, 3, 7, 14, and 21. The samples, including HAM-E and HAM-DE, were rinsed using PBS, while the HAM basement membrane surface (HAM-BS) and HAM stromal surface (HAM-SS) were kept facing upwards. Then, the samples were fixed using 4% paraformaldehyde in 0.1 M PBS at pH7.4 for 2 h at room temperature. Later, the samples were incubated at 4°C for 2 days with 8% formaldehyde (Kiernan 2000) prior to being dehydrated with a series of ethanol solutions of increasing concentrations (30%, 50%, 70%, 80%, 90%, and 100%) for 10 min each. The membranes were soaked

in hexamethyldisilazane (HDMS) (Sigma, USA) for 10 min and finally, air dried. The dried membranes were then attached to a glass slab with the help of double-sided tape. The samples were gold-sputtered using a sputter coater, Leica SCD 005 (Leica microsystems, Germany), prior to viewing under ×1000 and ×3000 magnifications using a scanning electron microscope, Quanta FEG 450 (FIE, Netherlands).

CELL PROLIFERATION ANALYSIS USING alamarBlue® ASSAY

The HAM that had been seeded with HPDLFs at cell density of 3500 cells per 1 cm^2, and the unseeded HAM-E and HPDLFs only were retrieved after days 1, 3, 7, 14, and 21. All the samples were incubated at 37°C with 5% CO_2 and 1 mL of 10% alamarBlue® in a growth medium for 4 h. The samples were kept under dark conditions by wrapping the 6-well plate with aluminium foil. After incubation, the test reagent was transferred into a 1-mL micro centrifuge tube, wrapped in aluminium foil, and labelled and stored at -20°C under dark conditions. Then, 100 µL of the test reagent of each sample was transferred to a 96-well plate along with a negative control (medium only) and a positive control (100% reduced alamarBlue® prepared by autoclaving alamarBlue® for 10 min in distilled water). Finally, the fluorescence intensity was read by a VarioskanFlash (Thermofisher, Finland) using an excitation wavelength of 540 nm and an emission wavelength of 590 nm. The results were given in tabulated form, and then converted into graphs.

STATISTICAL ANALYSIS

The SPSS version 22.0 software was used for the data analysis. A comparison of the proliferation of the HPDLFs was done using Friedman's two-way analysis of variance by ranks. A pairwise comparison was then conducted to assess the significance within the groups. The comparison with negative and positive controls was done by using the Kruskal Wallis test. A value of $p<0.05$ was considered as significant. The histological analysis and SEM analysis were done qualitatively by observing the morphology and attachment of the HPDLFs on the amniotic membrane.

RESULTS AND DISCUSSION

HISTOLOGICAL ANALYSIS OF CELL MORPHOLOGY

The HAM-E showed a monolayer of cuboidal epithelial cells at the basement side of the HAM. No epithelial layer was observed in the HAM-DE sample as the layer had been removed. The different layers of HAM, characterized by a basement membrane layer, a compact layer, a fibroblast layer and a spongy layer, were evident in the histological staining (Figure 1).

At day 1, the HAM-seeded HPDLFs showed a layer of spindle-shaped and rounded nuclei of HPDLFs, and this was seen to be attached across the HAM surface (Figure 2). On the other hand, the HAM-E showed cuboidal-shaped epithelial cells. The cells remained attached to the membrane at day 3, and increased to a thickness of 2 or 3 layers at day 7, whereas the cells became flatter and were reduced to a single layer at day 14 to day 21.

SEM ANALYSIS OF HPDLFS/HAM PROFILE

The HAM-E displayed compact epithelial cells with hexagonal or pentagonal (polygonal) borders. The HAM-BS showed more compact filaments compared to the HAM-SS (Figure 3). At day 1, the HPDLFs were seen to be firmly attached to the HAM surface. They were flattened in shape and covered the entire surface of the membrane. Small blebs were observed over the cell bodies. On day 3, the cells remained flattened and appeared to be overlapping, and this continued to increase until day 7. Later, the cells began to lose their flat shape at day 14 and day 21. Only a few cells remained flat, and these were seen to have stretched out to form filopodia at the edges of the cells (Figure 4).

PROLIFERATION OF HPDLFS ON HAM

The bar chart in Figure 5 shows a comparison of the proliferation rate of HPDLFs on the HAM on days 1, 3, 7, 14, and 21. The unseeded HAM and HPDLFs alone were used as the negative and positive controls, respectively. A comparison of the days showed that the mean percentage of cell proliferation increased significantly from day 1 to day 7 ($p=0.012$). However, beginning from day 14, the proliferation rate was reduced significantly ($p=0.002$)

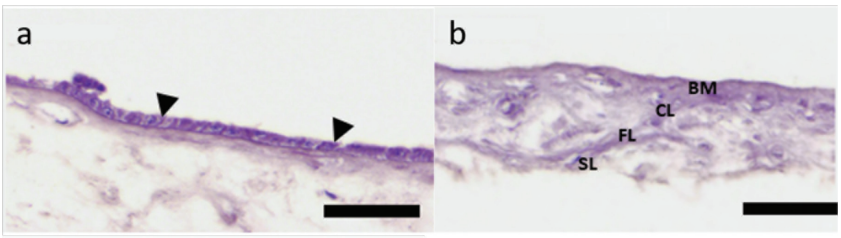

FIGURE 1. Histological section of human amniotic membrane: a) HAM with epithelium (HAM-E), and b) HAM after de-epithelialized (HAM-DE). A single layer of cuboidal epithelial cells (arrow heads) are present on basement membrane side of the HAM-E. No epithelial seen on HAM-DE. BM=Basement membrane; CL=Compact layer; FL=Fibroblast layer; SL=Spongy layer. Magnification, scale bar: ×40, 50 µm

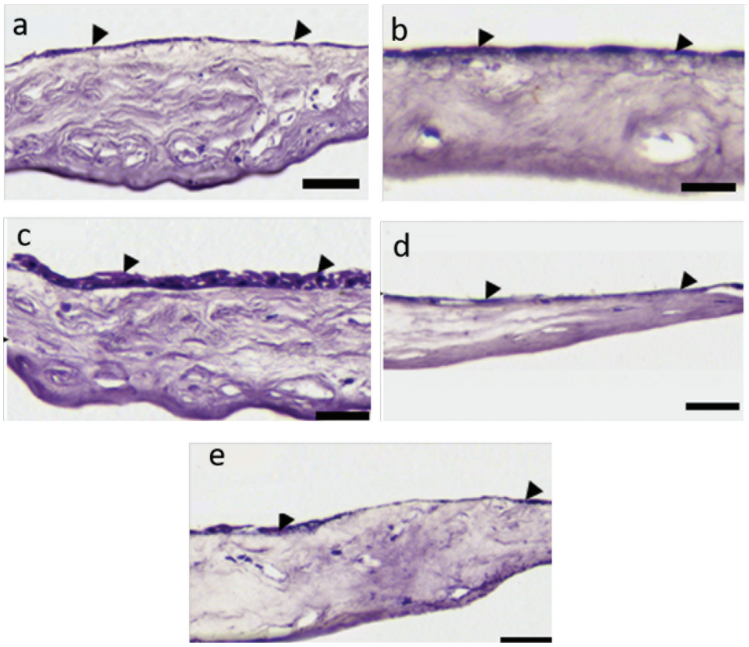

FIGURE 2. Histological section of human amniotic membrane (HAM) seeded with human periodontal ligament fibroblasts (HPDLFs) at day 1 to day 21. HPDLFs are seen attached on the surface of HAM (arrow heads): a) Day1 b) Day 3 c) Day 7 d) Day 14, and e) Day 21. Magnification, scale bar: ×40, 50 μm

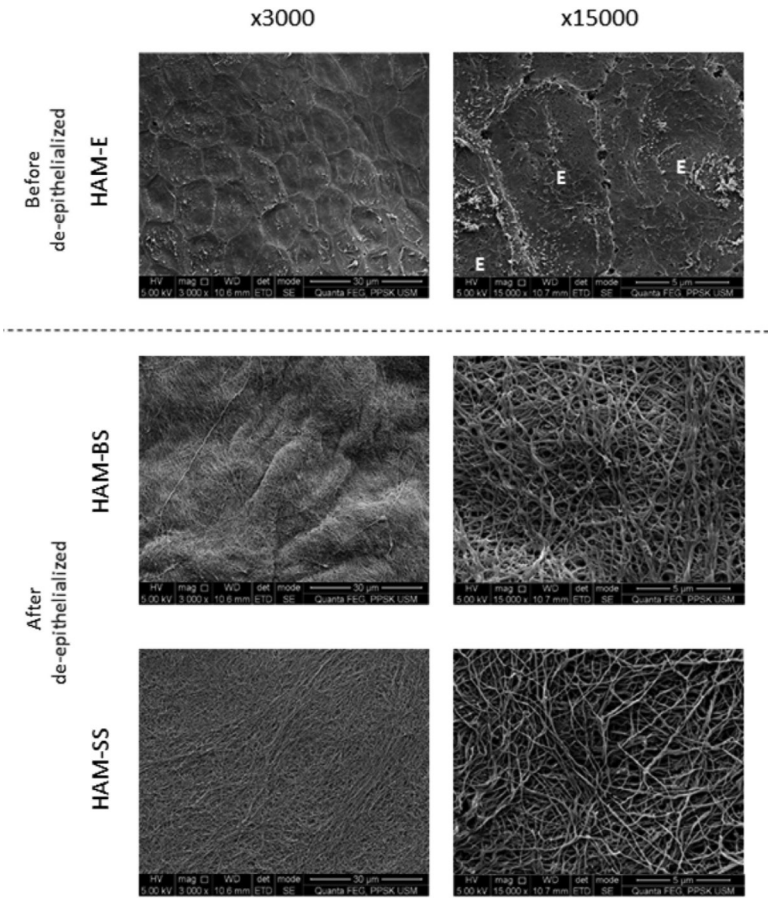

FIGURE 3. Scanning electron microscopy of HAM-E and HAM-DE. Epithelial cells (E) can be seen on the surface of HAM before de-epithelialized. Basement surface of HAM (HAM-BS) and stromal surface of HAM (HAM-SS) after de-epithelialized showing small interfibrous spaces (pores). Magnification, scale bar: HAM-E= ×3000, 50 μm; ×15000, 10 μm. HAM-BS and HAM-SS= ×3000, 10 μm; ×15000, 2 μm

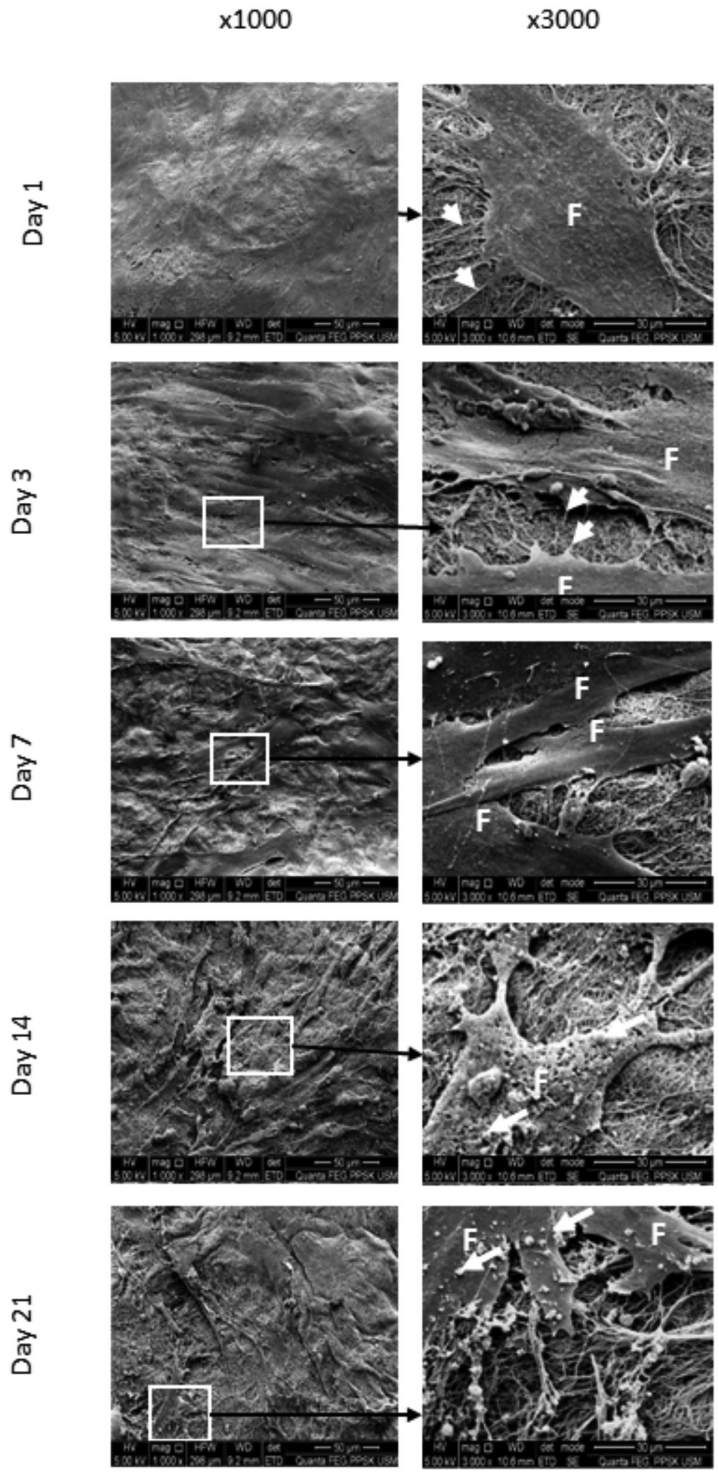

FIGURE 4. Scanning electron microscopy (SEM) observation of human amniotic membrane (HAM) seeded with human periodontal ligament fibroblasts (HPDLFs). On day 1, flattened HPDLFs (F) are seen attached to HAM with their lamellipodia (white arrowheads). On day 7, fibroblasts show over lapping. Blebs can be seen on day 14 and 21 (white arrows). Magnification, scale bar: ×1000, 50 µm; ×3000, 30 µm

till day 21 (p=0.005). Meanwhile, a comparison with the negative control showed that the percentage of cells on the HAM was significantly higher at day 7 (p=0.004) but significantly lower than the positive control for each day (p<0.05).

DISCUSSION

This present study was aimed at assessing the ability of the HAM to act as a scaffold for the basic, yet most abundant, cell population in the periodontal structure known as HPDLFs. In order to characterize the HAM as a

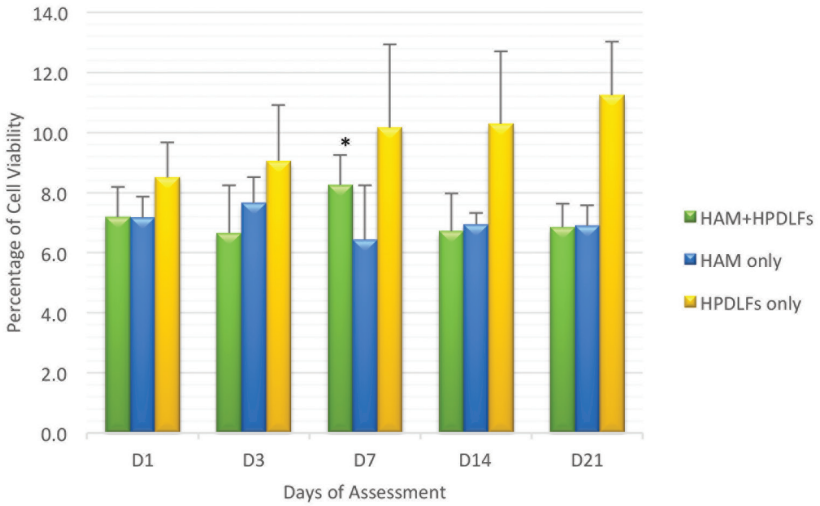

FIGURE 5. Quantification of proliferation of human periodontal ligament fibroblasts on human amniotic membrane using alamarBlue® assay. *HPDLFs viability on HAM is significantly higher at day 7 compared to day 3 and day 1 (p=0.003 and p=0.012, respectively) but significantly decline at day 14 and day 21 (p=0.002 and p=0.005, respectively). Cell viability increased in positive control (HPDLFs only) whereas no significant changes for negative control (HAM only).

scaffold for future use in periodontal defects, the surface architecture and proliferation ability of HPDLFs over the HAM should be evaluated. The role of a scaffold is to mimic the natural ECM of the host structure and to integrate the *ex vivo* cultured cells into its matrix (Garg et al. 2012). This is translated by the physical cues of the micro/nano structure of the membrane such as the pore size and porosity (Shimauchi et al. 2013). When observed under SEM, the pore size of the stromal surface of the HAM appeared larger than that of the surface of the basement membrane seen after de-epithelialization (Figure 3). This was evident as the filaments on the basement side were more compact than those on the stromal side of the HAM. Adachi et al. (2014) demonstrated that the stromal surface showed a hemi-desmosomal attachment between the cell-membrane interface, while only physical interlocking was seen on the basement membrane side of the HAM, as evident from both the transverse electron microscopy and immunohistochemistry.

The results of this study demonstrated that the HAM was able to act as a scaffold for HPDLFs within 7 days. Both the histological and SEM analyses were consistent in demonstrating the attachment of the HPDLFs for up to 7 days of observation. The cells were attached and proliferated over the HAM and displayed the morphology of normal cells. This was evident on day 1 in the SEM images (Figure 4), where the cells appeared flat with filopodia extending from the cells and attaching to the HAM filaments. The histological sections also showed a continuous layer of cells on the HAM surface, with their round to oval nucleus and spindle cell morphology. Similar results were seen in the study by Adachi et al. (2014), where periodontal ligament cell sheets were cultured on HAM. Their findings were based on histological staining, immunohistochemistry and SEM.

The density of the cells increased from day 3 and became more prominent by day 7, thereby indicating cell growth, as seen in the SEM and cell viability analysis. Adachi et al. (2014) also showed that there were up to 5-7 stratified layers of cells. Other studies on HAM cultured with human adipose tissue-derived stromal cells (hADSC) and human dental apical papilla cells (APCs) demonstrated the proliferation of cells for up to 7 days using MTT [3-(4,5-dimethyhhiazol-2-yl)-2,5-diphenyl tetrazolium bromide] assay (Chen et al. 2012; Gholipourmalekabadi et al. 2016).

On the other hand, the HPDLFs seemed to reduce their metabolic activity after day 7, as shown by the changes in the cell morphology and viability at days 14 and 21. In the histological sections, the HPDLFS appeared to be flatter, with longer spindle-shaped cells and with the cell layers being reduced up to a single layer. In contrast, Taghiabadi et al. (2015) showed that there was significant metabolic activity of foetal fibroblasts in the 3D spongy HAM scaffolds for up to 21 days using MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] assay. However, in their study, the cross-linker agent, 1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide hydrochloride (EDC)/N-hydroxysuccinimide (NHS), was used to improve the stability of the HAM, and this can be considered in future studies.

The decline in cell density could have been due to a number of factors. Since there was a confluence of cells on day 1, as seen in the SEM images, the 'density-dependent inhibition of cells' could have been one of the reasons for the decline in cell density when the cells became over-confluent by day 7 (Oren & Kohn 1969). According to this phenomenon, the reduction in cell density was due to the retardation of DNA synthesis in the cells in the S phase of

the cell cycle. In such a case, due attention must be given to the cell replication rate, and the cell seeding density has to be modified accordingly.

Another explanation for the detachment of cells from the substrate could be the modification of the substrate by the cells. The role of fibroblasts is not only to secrete the components of the extracellular matrix (ECM) such as collagen, fibronectin and proteoglycan (Graham et al. 2008; Spinale 2007), but also to secrete ECM-degrading enzymes called matrix metalloproteases (MMPs) (Kukacka et al. 2005; Lindner et al. 2012). In the case of periodontal fibroblasts, it is known that they release MMP-2, 8 and 9 (Kim et al. 2012; Lisboa et al. 2009; Rai et al. 2010). The MMPs may degrade the collagen of the membrane, resulting in a lack of attachment cues for the cells, thereby promoting cellular detachment and eventually, cellular death.

The reduction in the proliferation of HPDLFs can also be due to foetal hyaluronic acid, which is one of the components of HAM. Foetal hyaluronic acid suppresses the transforming growth factor-beta (TGF-β) signalling by reducing the expression of TGF β-1, β-2 and β-3 isoforms and the expression of the TGF receptor, which has an inhibiting effect on the proliferation of fibroblasts (Lee et al. 2000; Paolin et al. 2016). This is also the main mechanism behind its anti-scarring properties (Tseng et al. 1999). Therefore, the extent of the reduction in the proliferation of fibroblasts due to the foetal hyaluronic acid of the amniotic membrane needs to be further investigated.

Keeping under consideration of the density-dependent inhibition of cells factor, the cells were already seeded at the optimal density recommended by the protocol. Therefore, a sub-optimal density would have affected the cell growth. However, with regard to the modification of the substrate, it is necessary that a further evaluation by proteomic analysis and an investigation into the release of MMPs from HPDLFs and its effect on the degradation of the HAM be carried out. Certain strategies have already been adopted to increase the stability of the HAM, as it has been seen that fresh HAM dissolves in one week and cryopreserved HAM dissolves in 2-3 weeks, depending on the storage conditions and inflammation of the host site. This is probably due to the endogenous enzymatic degradation of the HAM matrix (Spoerl et al. 2004). It was also seen in the current study that the HAM should be reinforced to improve its stability both *in vitro* and *in vivo* by the bridging/cross-linking of its collagen chains. This can be done through physical treatments such as ultraviolet (UV), gamma ray and electron beam irradiation (Fujisato et al. 1999; Lai 2014; Spira et al. 1994;), and chemical treatments such as glutaraldehyde (Lai & Ma 2013; Spoerl et al. 2004), tissue transglutaminase (TG-2) (Chau et al. 2012) and carbodiimide (Lai et al. 2014, 2013; Ma et al. 2010). The stability of the HAM is enhanced as it cleaves the triple helical structure of collagen, which is supported by the hydrogen bonding and Van der Waal's attractions, to form random coils. This in turn will increase the resistance to protease digestion and will make the HAM more hydrothermally stable by preventing thermal denaturation (Lai et al. 2014; Lai & Ma 2013; Ma et al. 2010). Cross-linking will also alter the fibre dimensions (Lai et al. 2013). Usually, fibres with smaller dimensions are better for cell differentiation (Sisson et al. 2010). Hence, membrane cross-linking can be used, depending upon the kind of cells that are being cultured and the desired outcome.

Conclusion

This present study showed that HPDLFs are able to attach, proliferate and show signs of integration with the HAM within a short period of time. Since fibroblasts are the predominant cell type in the periodontium, this study demonstrated the biocompatibility of the HAM with the HPDLFs, and provided a deeper understanding of this membrane and its interaction with its natural niche. The results of the study suggest that the HAM can be used as a scaffold for periodontal tissue engineering, provided further characterization studies are undertaken. The behaviour of the cells in relation to the HAM within a short duration showed promising biocompatibility.

Acknowledgements

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