A Preliminary Study of Human Amniotic Membrane as a Potential Chondrocyte Carrier

L Boo, BSc, **S Sofiah*,** MMed (O & G), **L Selvaratnam**,** PhD, **CC Tai,** FRCS, **Pingguan-Murphy B***,** PhD, **T Kamarul,** MS Ortho

Department of Orthopaedic Surgery, University of Malaya, Kuala Lumpur, Malaysia
*Department of Obstetric and Gynaecology, University Malaya Medical Centre, Kuala Lumpur, Malaysia
**School of Medicine and Health Sciences, Monash University, Selangor, Malaysia
***Department of Biomedical Engineering, University of Malaya, Kuala Lumpur, Malaysia

ABSTRACT

Purpose: To investigate the feasibility of using processed human amniotic membrane (HAM) to support the attachment and proliferation of chondrocytes *in vitro* which in turn can be utilised as a cell delivery vehicle in tissue engineering applications. ***Methods:*** Fresh HAM obtained from patients undergoing routine elective caesarean sections was harvested, processed and dried using either freeze drying (FD) or air drying (AD) methods prior to sterilisation by gamma irradiation. Isolated, processed and characterised rabbit autologous chondrocytes were seeded on processed HAM and cultured for up to three weeks. Cell attachment and proliferation were examined qualitatively using inverted brightfield microscopy. ***Results:*** Processed HAM appeared to allow cell attachment when implanted with chondrocytes. Although cells seeded on AD and FD HAM did not appear to attach as strongly as those seeded on glycerol preserved intact human amniotic membrane, these cells to be proliferated in cell culture conditions. ***Conclusion:*** Preliminary results show that processed HAM promotes chondrocyte attachment and proliferation.

Key Words:
Amnion, Chondrocytes, Tissue Engineering, Cell Carrier

INTRODUCTION

Articular cartilage is a highly organised connective tissue located on surfaces of diarthroidal joints designed to absorb and distribute high amounts of mechanical loads [1]. Injury to articular cartilage is therapeutically irreversible due to the largely avascular nature of this tissue and the self-limited regenerative ability of the cartilage cells [2, 3]. Physically mediated cartilage degeneration is often the result of both chronic and acute insults to the cartilage surface, leading to osteoarthritis, which is currently a major health problem worldwide [4, 5]. It is well known that osteoarthritis of the knee joints impedes mobility and negatively impacts upon quality of life resulting in a major financial healthcare burden [6, 7]. In trying to reduce the progression of the disease in the relatively young patient with established cartilage damage, a number of surgical options have been advocated to aid in early tissue repair. These include microfracture, athroscopic lavage, abrasion, and sub-chondral drilling [8, 9]. However, these procedures have been reported to have inconsistent short to midterm clinical outcomes, with most clinicians reporting that the majority of their patients did not have substantial improvements [10-14].

The emergence of cell-based therapies offers the prospect of a more effective therapeutic treatment which involves the regeneration of injured articular cartilage. One of the most successful methods is the autologous chondrocyte implantation (ACI) [15] technique which has gained popularity over conventional methods over the past decade [16]. ACI, which is based on a tissue engineering concept, has enabled the regeneration of hyaline-like cartilage upon it successful transplantation in cartilage defects as compared to the production of fibrocartilage tissue in the repaired sites using other regular techniques; which, if remain uncorrected, will result in detrimental outcomes to the joint. However, the benefits of ACI are offset by a number of limitations which include incomplete regeneration of the defective cartilage, periosteal hyperthrophy, the limited availability of autologous chondrocyte, and the reduced proliferative activity of cells obtained from the more elderly patients [17-19]. These limitations have driven the need for a cell delivery vehicle which combines both cell and scaffolding materials for transplanted chondrocytes to proliferate and retain their native phenotypic characteristics within the defect sites. This in turn will allow effective repair to take place within these sites and produce repair tissue which mimics the surrounding native cartilage. Cell scaffolding provides at least two functions in cell transplant therapies. Its designed structure not only assists in the integration of transplanted cells to the host tissue; but it also supports cell attachment and enhances cell proliferation. Their role as one of the key components in tissue engineering is reflected by an ever increasing number of published works over the past decade [20-23]. To ensure that

Corresponding Author: Boo Lily, Department of Orthopaedic Surgery, Faculty of Medicine, University of Malaya, 50603 Kuala Lumpur, Malaysia Email: boolily83@gmail.com

effective repair and regeneration of injured tissues and organs can take place, seeding of cells onto the three-dimensional scaffold is important as cell-matrix interaction that occurs in these constructs helps to provide better cell expressions [24-27]. However, one of the major limitations of clinical application of scaffolding is the availability of a material which is both biocompatible and biodegradable.

In an attempt to discover a suitable material which has both of these properties, human amniotic membranes (HAM) offered potential owing to its many desirable properties. HAM is inert, biocompatible, biodegradable, and more importantly has been used successfully in the past for tissue transplantation. Since initial surgical use as a skin graft material [28], HAM has proven to be an excellent tissue transplantation material with clinical applications being reported with good to excellent outcomes for a variety of diseases [29, 30]. However, the use of HAM in tissue engineering has not been widely reported [31, 32]. This study was therefore conducted to investigate the feasibility of processed HAM as a cell carrier for cultivation of chondrocytes.

MATERIALS AND METHODS

Procurement and processing of human amniotic membranes (HAM)

Amniotic membranes (n=3) from placentas were collected and processed in accordance to the approval from the Medical Ethics Committee, University of Malaya. Human placentas were obtained by the operating obstetricians from elective Caesarean-sectioned mothers who were seronegative for hepatitis B and C, syphilis, and human immunodeficiency virus. Informed consent was obtained from all donors. Following the delivery of the placenta, the amnion membrane was carefully peeled off from the placental mass and washed with running water. Blood clots present on the surface were carefully removed. The amniotic membrane was then placed in a bottle containing sterile normal saline and stored at 4°C before further processing. The following day, the amniotic membrane was transferred into another sterile bottle containing sterile distilled water. The amnion was then washed for 10 minutes in a shaker before being transferred into another bottle containing 0.05% (v/v) sodium hypochlorite and slowly agitated for another 10 minutes. Three subsequent treatments consisting of washing with normal saline and agitation for 20 minutes were performed. Each donor membrane was then cut in half and processed by 2 different methods: air dried (AD) or freeze dried (FD). For the AD group, the cleaned amniotic membrane was spread and dried under laminar flow hood overnight. For the FD group, the stretched amniotic membrane was placed in plastic bags in the freeze dryer for 6 hours. The AD and FD membranes were then trimmed to size (2cm x 2cm) before being packed into vacuum sealed packages. These processes were all performed within the laminar flow hood environment to reduce possible contamination. The packed clean amniotic membranes were then delivered for sterilisation using gamma rays (Cobalt 60) at 25 kGy for how long. Following irradiation, the packed HAMs were kept in a dry cabinet away from direct sunlight until used for further analysis. The sterility and efficacy of processing amnion has been described previously [33].

Characterisation of HAM before cell seeding

To investigate the structure of the processed HAM after the air drying and freeze drying techniques, the processed HAM was subjected to histological examination. Both FD and AD HAM were fixed by immersing HAM in 10% buffered formalin solution overnight and subjecting to serial dehydration in ethanol 70% twice, 95% twice, 100% thrice, toluene thrice, and wax thrice for one hour each. The processed HAM was embedded in paraffin, and then sectioned into 5μm thicknesses using a microtome. Hematoxylin and Eosin staining was performed to differentiate the two main layers of the processed HAM, namely, epithelial and basement layers. For experimental control, we used commercially available glycerol preserved intact human amniotic membranes (GPHAM). The GPHAM was also fixed, embedded and subjected to histological examination as described above in order to perform similar characterisation process.

Isolation and culture of rabbit autologous chondrocytes (RAC)

Regulations on animal experimentation were strictly adhered to throughout the study as determined by the University of Malaya animal ethics approval. New Zealand white rabbits (n=3) aged between 3 to 4 months and weighing approximately 2.5 kg were used for this study. The rabbits were sacrificed by an overdose intravenous injection of Pentobarbital. Articular cartilage from the shoulders, hips and knees was harvested under sterile conditions as described previously [34]. Once chondrocyte confluence was achieved, cells were trypsinised and suspended for further analysis in the study. Cell number and viability were determined using the Trypan blue dye exclusion method.

Characterisation of cultured RAC

To ensure that the cells isolated consisted of homogeneous populations of autologous chondrocytes, cell characterisations were performed. Cell detachment from culture vessels at passages 1 (P1) and 2 (P2) was followed by seeding at 10, 000 cells per chamber in the four well chamber slides. After 3-4 days, the slides underwent Hematoxylin and Eosin (H&E) staining for morphological examination and Safranin-O/fast green staining to determine matrix proteoglycan depositions. Immunocytochemistry staining was performed using a Dako immunostaining kit (DakoCytomation, USA) to detect the presence of collagen type II. Slides were fixed in methanol for 15 minutes before being rinsed with Tris-buffered saline (TBS) and further treated with 0.03% hydrogen peroxide containing sodium

azide. These slides were then incubated with primary antibody against type II collagen (mice antibody) for 30 minutes before being further incubated with peroxidase labelled polymer conjugated to goat anti-mouse immunoglobulins for 30 minutes. The resulting slides were then stained with liquid diaminobenzidine (DAB kit, DakoCytomation, USA) for visualisation and observed using the light microscope to verify the presence of type II collagen.

Seeding of RAC on HAM substrates

The primary culture of RAC was routinely maintained, expanded, and passaged. The second passage (P2) of the cells was subsequently detached and seeded onto the three different substrates. Cell counts were performed prior to seeding to ensure consistency of seeding density. The cells were in culture for about three weeks and subject to microscopic observation. Samples of the membranes (n=3) of processed HAM (AD or FD) and GPHAM approximately 4cm^2 were placed individually into a small sterile 35mm ultra low attachment culture dish (Corning Inc., Corning, NY). In each of the culture dishes, an autoclavable stainless steel ring was placed on top of the amniotic membrane to prevent curling up of the membrane upon addition of culture medium. The amniotic membrane was spread uniformly, basement membrane-side up, onto the bottom of the dish with sterile forceps before cells were seeded. RAC at passage 2 (P2) were seeded at 4 x 10^5 cells per membrane. Cultures were cultivated in growth medium at 37°C in a humidified incubator with 5% carbon dioxide supply with medium changed three times a week. Examination using inverted brightfield microscopy of the cultures was carried out throughout the three week culture.

Characterisation of HAM after cell seeding

To observe the attachment patterns of the seeded chondrocytes on the different HAM substrates, the samples were subjected to histology staining after 21 days. The resultant slides were then observed using light microscopy and photographed using Infinity 2.0 camera and software.

RESULTS

Characterisation of RAC

Primary culture of RAC was established using the enzymatic digestion method, forming a monolayer which attained confluence at day 10. Using inverted brightfield microscopy, once confluence is reached, these cells appeared flattened and almost spherical in shape. Staining with H&E demonstrated multipolar and bipolar morphology of chondrocytes with rounded bluish-black nuclei and pink cytoplasm in the monolayer arrangement (Figs 1a, b). Immunohistochemistry of monolayer chondrocytes cultured on chamber slides with Safranin-O/fast green staining showed positive for the presence of proteoglycans in the extracellular matrix (Fig 1c). The presence of proteoglycans (stained red) was most evident in the territorial and interterritorial areas of the cells (Fig. 1d). Immunocytochemistry of the chondrocytes in monolayer on chamber slides was positive for type II collagen in the pericellular and intracellular regions (Figs 1e, f).

Morphologic characterisation of the HAM substrates before cell seeding

Gross appearance did not reveal any significant morphological differences between the unprocessed (control) and processed HAM. HAM appeared to be light, transparent, thin, easy to handle and curvy upon hydration. Examination of the GPHAM using light microscopy revealed a continuous strata layer of epithelial cells with cuboidal and polygonal cells (Fig. 2a). The nuclei of both types appeared large, dense, ovoid and centrally located in the cytoplasm. The underlying layers were evidently intact to the extent of the epithelial layer being observed to include a smooth layer of fibrous collagen stroma. In contrast, H&E stained slides from the FD group revealed an indistinct layer of epithelium and basement membrane due to the physical alterations upon freeze drying treatment (Fig. 2b). The epithelial layers had collapsed and become indistinct from the underlying membranes. However, the network of collagen fibrils was still clearly present and remained intact to the membranes. In the AD group, the H&E staining revealed tight adherence and alignment of the epithelial cells to the surface of the basement membrane (Fig. 2c).

Observation of cells seeded on HAM substrates

Chondrocytes appeared in single and aggregate following 30 minutes of seeding on all substrates (Figs 3a, d, g). The seeded chondrocytes appeared rounded and refractive indicating that the cells were viable. After one hour, the majority of single chondrocytes remained in suspension whilst smaller aggregated chondrocytes had attached to the substrates (Fig. 3d). Attachment of chondrocytes became stable within the first day following seeding and occurred at the same rate on all other substrates (replicates). Chondrocytes which had initially been in aggregate remained attached and showed signs of proliferation. By the third day of culture, protrusion and outgrowth of flattened cells from the peripheral regions of cell colonies demonstrated clear proliferation on all substrates (Figs 3b, e, and h). On ADHAM and FDHAM substrates, upon changing of culture medium, some of the attaching cells appeared to have detached from the surfaces possibly due to accidental mechanical agitation or fluid shear forces. In contrast, almost all of the cells which were attached on GPHAM from Day-1 appeared to have survived, underwent morphologic changes, and remained attached to the membrane until day 21. The chondrocytes were fairly uniform in size and in close contact with one another. Rather than forming a monolayer, the chondrocytes infiltrated into the extracellular matrix of the basement membrane forming a clump-like structure as shown in the paraffin sections (Figs 4a and b). In the

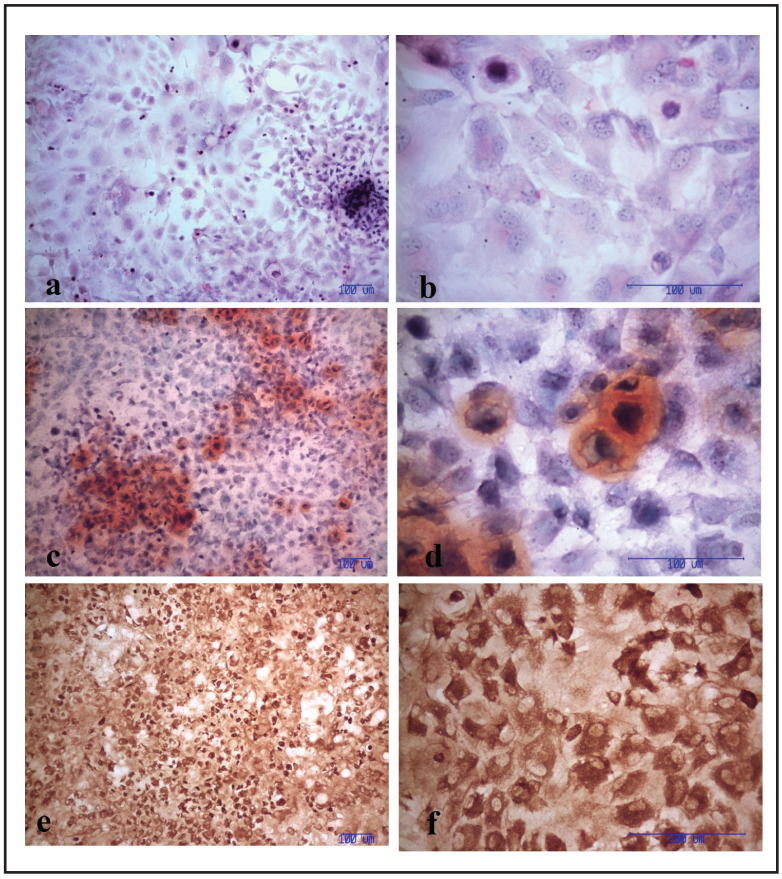

Fig. 1 Characterisation of RAC using morphological and immunohistochemical staining. (a, b) hematoxylin and eosin , (c, d) Safranin-O/Fast green, (e, f) Type II collagen. (X100 and X400 magnification).

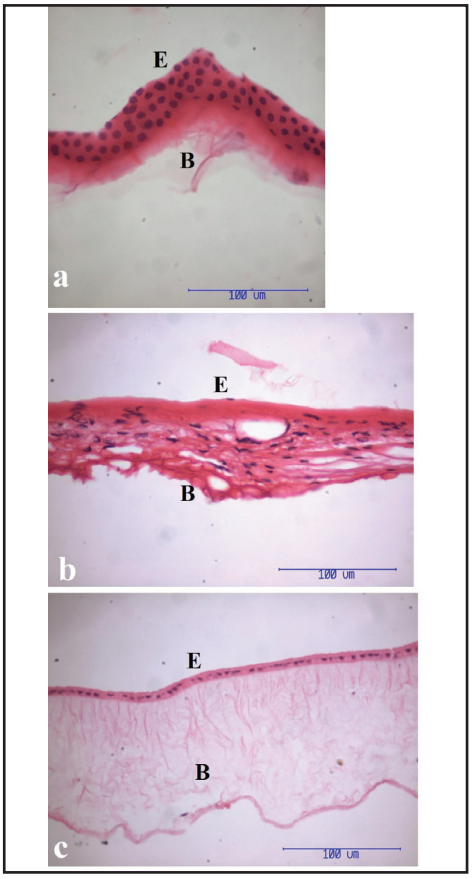

Fig. 2 Cross-sections of the HAM, stained with hematoxylin and eosin. (a) GPHAM, (b) FDHAM, (c) ADHAM. (E- Epithelium and B-basement membrane) (X400 magnification).

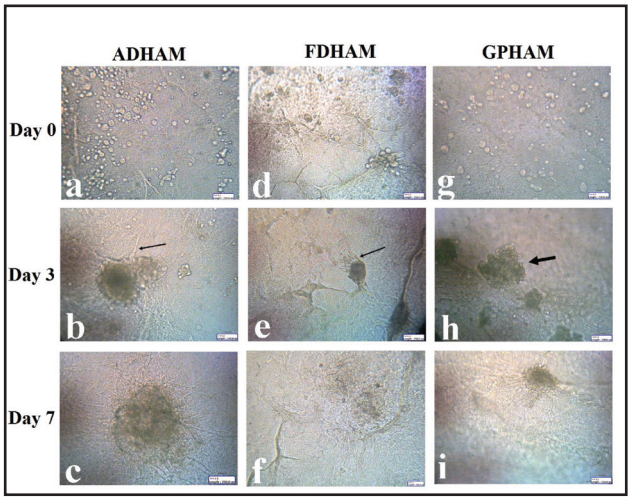

Fig. 3 Inverted brightfield microscopy images of the RAC seeded on different substrates. Cell proliferation on the ADHAM and FDHAM was indicated by the outgrowth of the cells *(thin arrows)*. Cell colonies attached to the GPHAM *(thick arrow)* (X200 magnification).

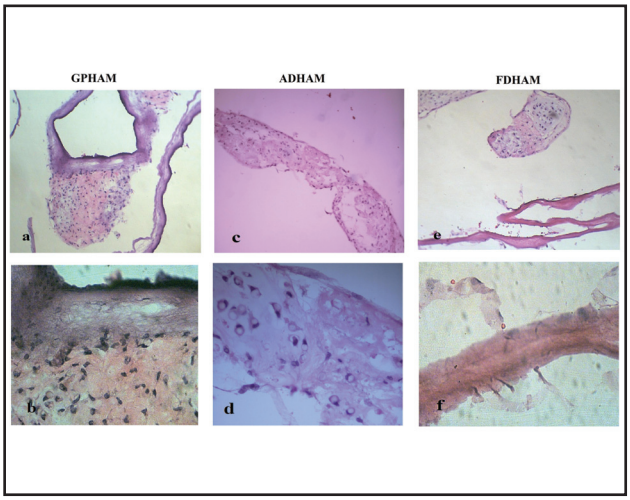

Fig. 4 Cross sections of the HAM seeded with cells stained with hematoxylin and eosin. (a,c,e) X100 magnification. (b,d,f) X400 magnification.

ADHAM section, chondrocytes attached along the basement membrane but not in close contact with one another (Figs 4c and d). Comparatively, chondrocytes seeded on FDHAM appears to have attached less firmly to the membrane and were removed during the tissue processing (Figs 4e and f). This suggests that although both ADHAM and FDHAM appear to promote cell proliferation, the amount differs with the different preparations.

DISCUSSION

The choice of a cell carrier is an important aspect of tissue engineering as it can greatly influence cell attachment, migration, differentiation, mass transport, mechanical integrity of tissue constructs and their subsequent integration in vivo [35, 36]. Ideally, a scaffold used as a cell delivery vehicle should be reproducible, of three-dimensional form, have a high porosity so that cell distribution during cell seeding can be uniform, be able to minimise diffusional constraints during *in vitro* cultivation, and be able to provide controlled biodegradation for long-term gradual creeping substitution [24, 25]. The success of tissue engineered constructs for cartilage repair and regeneration depends on the biocompability and biodegradability of the appropriate scaffold materials.

A wide range of scaffolds, either synthetic or natural polymers, has been developed and investigated for the cartilage repair and regeneration. The most widely used synthetic scaffold materials are FDA-approved polyglycolic acid (PGA) and poly L-lactic acid (PLLA) [37, 38]. Both PGA and PLLA are biodegradable polymers often used in orthopaedics. Conversely, naturally derived polymers such as collagen, alginate gels and injectable hydrogels have also been extensively studied [39-43]. Nevertheless, both types of scaffold material are financially prohibitive, and require extensive processing before they can be fully utilised. The unique characteristics of HAM such as availability, cost-effectiveness, and present of various growth factors make it a valuable biological matrix with the potential of delivering tissue engineering solutions at a lower cost and with fewer processing requirements [30, 31, 44, 45].

Among the five layers, the basement membrane of the amniotic membrane is of great interest in our study as it is a membrane made of collagen fibers, fibronectin and laminin [46]. The basement membrane is a thin layer composed of reticular fibers adhered to the base of the amniotic epithelium; the adherence of the basement membrane to the other layers is firm and separation of the layers is not easily achieved [47]. However, in our study, following air or freeze drying and later exposed to gamma irradiation, the basement membrane was separated from the underlying layers but remained adherent to the epithelial layer with an intact collagen structure (Figs 2a, b, and c). The basement membranes were used for seeding throughout the study. In addition, to minimise the manipulation of HAM, we did not denude the epithelial cells from the basement membrane. It is noted that denudation of the epithelial layer was performed in previous studies, e.g. Nakamura *et al.* [44]. which demonstrated cultivation and transplantation of non-ocular surface origin cells (oral epithelial cells) on the denuded HAM carrier. Denuded human amniotic membrane that did not possess cells or residual nucleic acid was thought to be important as it could minimise the cell-mediated immune response following clinical implantation [30, 48]. Limbal cells grown on denuded amniotic membrane appeared well stratified and differentiated, and was preferred to the results attained from monolayer cells grown on the intact amniotic membrane [49]. Denudation of HAM involves the treatment of the intact HAM with EDTA at 37 °C and the scraping out of the epithelial cells under a microscope. However, the usage of denuded or intact HAM is largely dependent on the type of cell to be implanted and, more importantly, its final clinical application. In the present study, the denudation of the epithelium cells from the basement membrane was irrelevant as the aim was to seed the chondrocytes on the basement membrane and not on the epithelial layer.

All substrates (GPHAM, ADHAM and FDHAM) appeared to have supported the attachment and proliferation of cells on its surfaces. The attachment of cells to scaffolds was largely affected by the components of the scaffold's extracellular matrix components and the length of time the cells were left to rest on the surfaces. This was due to the presence of the extracellular matrix (ECM) components such as collagen, laminin, fibronectin, and vitronectin within the basement membrane of the intact HAM providing a favourable environment for cell attachment [32]. In our results, although the attachment rate was not quantified, we observed that cell aggregates had a higher attachment ability compared to single cells, an effect which might be explained by the secretion of proteoglycans and type II collagen stimulated by cell-to-cell interaction in these aggregates. These extracellular matrix proteins may help the cell aggregates to have a better attachment on the basement membrane, which is also predominantly high in collagen. These chondrocyte-matrix interactions may enhance the secretion of extracellular matrix components and other trophic factors from implanted chondrocytes. This, in turn, will further assist in cell attachment, proliferation and maintenance in cell culture systems. However, the detachment of some of the cells from the ADHAM and FDHAM cultures after one week of culture demonstrated unstable attachment of extracellular matrix components of the cell-matrix interaction. The weak attachment together with the shear forces during medium changes accounts for cell loss on these substrates. In contrast, cells seeded with GPHAM tended to do better with cells attaching firmly up to 3 weeks in cell culture environment, possibly due to secretion of certain growth factors in the preserved amnion membranes compared to the processed membranes. However this may be due to the fact that the number of cells was fewer on the AD and FD HAM due to cell detachments during the change of culture medium.

Despite the relatively inferior attachment and proliferation of cells on the processed HAM (ADHAM and FDHAM) as compared to GPHAM in prolonged cell culture environment, the strong and early attachment of cells on these substrates and the successful maintenance of cultured cell on GPHAM substrates for prolonged periods indicate promising clinical potential for HAM in general to be used as a cell carrier.

Moreover, a more recent study has demonstrated the ability of denuded HAM, especially the stromal side, to support chondrocyte proliferation and maintenance of phenotype in vitro and regeneration of hyaline cartilage *in vivo* [50]. However, in our study, the results of the ADHAM, FDHAM and GPHAM could not be compared because the structure of the processed HAM was different from the denuded HAM. Although, in this preliminary report, HAM appears to demonstrate positive results which supports our hypothesis that HAM may be useful as a cell carrier for tissue engineering application, further assessment is required before any conclusive use for practical application can be undertaken. Further characterisation of the three different substrates in terms of their biochemical compositions, the specific collagen fibres which account for the different attachment patterns, and the distribution and proliferation of the chondrocytes will enable a more concrete assessment of the properties of processed HAM. This would also provide the necessary knowledge on how to improve the properties of HAM in order to promote enhanced cell proliferation and expression. Further biomechanical analysis to measure the strength of the membranes, scanning electron microscopy to visualise the infiltration and proliferation of the cells, and in vivo animal studies are also needed to fully assess the suitability of these substrates in cartilage regeneration.

CONCLUSION

These preliminary results show that AD and FD HAM may support a degree of chondrocyte proliferation. However, the processing methods described here do not appear to have provided better cell attachments when compared to GPHAM, thus warranting the need to further improve current protocols. In general, HAM appears to provide a suitable environment for cell attachment and proliferation. In light of its successful history in tissue transplantations, HAM may become the much needed biological cell carrier for tissue engineering purposes.

ACKNOWLEDGEMENTS

We thank the doctors, staffs and nurses at the Maternity Ward One, University Malaya Medical Centre, Kuala Lumpur for their help in getting the amniotic membranes. We would also like to thank Dr. Norimah and staff of the gamma irradiation unit at the Malaysia Institute for Nuclear Technology (MINT) Research facility in Bangi, Selangor. This work was supported by National Biotechnology Directorate, Ministry of Science and Technology of Malaysia (Research grant number: FGRS FQ002/2007A).

REFERENCES

1. Poole AR, Kojima T, Yasuda T, Mwale F, Kobayashi M, Laverty S. Composition and structure of articular cartilage: A template for tissue repair. *Clin Orthop Relat Res,* 2001; 391 Suppl: 26-33.
2. Aigner T, Söder S, Gebhard PM, McAlinden A, Haag J. Mechanisms of disease: Role of chondrocytes in the pathogenesis of osteoarthritis—structure, chaos and senescence. *Nat Clin Pract Rheumatol,* 2007; 3(7): 391-9.
3. Eyre DR, Weis MA, Wu JJ. Articular cartilage collagen: An irreplaceable framework? *Eur Cell Mater,* 2006; 12: 57-63.
4. Lawrence RC, Helmick CG, Arnett FC, Deyo RA, Felson DT, Giannini EH, *et al.* Estimates of the prevalence of arthritis and selected musculoskeletal disorders in the United States. *Arthritis Rheum,* 1998; 41(5): 778-99.
5. Quintana JM, Arostegui I, Escobar A, Azkarate J, Goenaga JI, Lafuente I. Prevalence of knee and hip osteoarthritis and the appropriateness of joint replacement in an older population. *Arch Intern Med,* 2008; 168(14): 1576-84.
6. Magne D, Vinatier C, Julien M, Weiss P, Guicheux J. Mesenchymal stem cell therapy to rebuild cartilage. *Trends Mol Med,* 2005; 11(11): 519-26.
7. Hunter DJ, Felson DT. Osteoarthritis. *BMJ,* 2006; 332(7542): 639-42.
8. Redman SN, Oldfield SF, Archer CW. Current strategies for articular cartilage repair. *Eur Cell Mater,* 2005; 9: 23-32.
9. Browne JE, Branch TP. Surgical alternatives for treatment of articular cartilage lesions. *J Am Acad Orthop Surg,* 2000; 8(3): 180-9.
10. Knutsen G, Engebretsen L, Ludvigsen TC, Drogset JO, Grøntvedt T, Solheim E, *et al.* Autologous chondrocyte implantation compared with microfracture in the knee. A randomized trial. *J Bone Joint Surg Am,* 2004; 86(3): 455-64.
11. Tuli R, Li WJ, Tuan RS. Current state of cartilage tissue engineering. *Arthritis Res Ther,* 2003; 5(5): 235-8.
12. Bentley G, Biant LC, Carrington RWJ, Akmal M, Goldberg A, Williams AM, *et al.* A prospective, randomised comparison of autologous chondrocyte implantation versus mosaicplasty for osteochondral defects in the knee. *J Bone Joint Surg Br,* 2003; 85(2): 223-30.
13. Steadman JR, Briggs KK, Rodrigo JJ, Kocher MS, Gill TJ, Rodkey WG. Outcomes of microfracture for traumatic chondral defects of the knee: Average 11-year follow-up. *Arthroscopy,* 2003; 19(5): 477-84.
14. Simon TM, Jackson DW. Articular cartilage: Injury pathways and treatment options. *Sports Med Arthrosc,* 2006;14(3): 146-54.
15. Brittberg M, Lindahl A, Nilsson A, Ohlsson C, Isaksson O, Peterson L. Treatment of deep cartilage defects in the knee with autologous chondrocyte transplantation. *N Engl J Med,* 1994; 331(14): 889-95.
16. Verdonk R, Steinwachs M, Vanlauwe J, Engebretsen L. Current opinion of orthopaedic surgeons on cartilage repair: The results of a global web-based survey. Trends in cartilage repair, 2006; 3-14.
17. Bachmann G, Basad E, Lommel D, Steinmeyer J. MRI in the follow-up of matrix-supported autologous chondrocyte transplantation (MACI) and microfracture. *Radiologe,* 2004; 44(8):773-82.
18. Browne JE, Anderson AF, Arcierco R, Mandelbaum B, Moseley JB Jr, Micheli LJ, *et al.* Clinical outcome of autologous chondrocyte implantation at 5 years in US subjects. *Clin Orthop Relat Res,* 2005; 436: 237-45.
19. Horas U, Pelinkovic D, Herr G, Aigner T, Schnettler R. Autologous chondrocyte implantation and osteochondral cylinder transplantation in cartilage repair of the knee joint: A prospective, comparative trial. *J Bone Joint Surg Am,* 2003; 85(2): 185-92.
20. Tuli R, Seghatoleslami MR, Tuli S, Wang ML, Hozack WJ, Manner PA, *et al.* A simple, high-yield method for obtaining multipotential mesenchymal progenitor cells from trabecular bone. *Mol Biotechnol,* 2003; 23(1): 37-49.
21. Schaefer D, Martin I, Jundt G, Seidel J, Heberer M, Grodzinsky A, *et al.* Tissue-engineered composites for the repair of large osteochondral defects. *Arthritis Rheum,* 2002; 46(9): 2524-34.
22. Vunjak-Novakovic G, Obradovic B, Martin I, Bursac PM, Langer R, Freed LE. Dynamic cell seeding of polymer scaffolds for cartilage tissue engineering. *Biotechnol Prog,* 1998; 14(2): 193-202.
23. Schaefer D, Martin I, Shastri P, Padera RF, Langer R, Freed LE, *et al. In vitro* generation of osteochondral composites. *Biomaterials,* 2000; 21(24): 2599-606.
24. Lu L, Mikos AG. Biodegradable polymers for cell transplantation. Sci Med, 1999; 6:6-7.
25. Martin I, Miot S, Barbero A, Jakob M, Wendt D. Osteochondral tissue engineering. *J Biomech,* 2007; 40(4): 750-65.
26. Walgenbach KJ, Voigt M, Riabikhin AW, Andree C, Schaefer DJ, Galla TJ, *et al.* Tissue engineering in plastic reconstructive surgery. *Anat Rec A Discov Mol Cell Evol Biol,* 2001; 263(4): 372-8.

27. Lu L, Valenzuela RG, Yaszemski MJ. Articular cartilage tissue engineering. E-biomed: *J Tissue Eng Regen Med,* 2000; 1(7): 99-114.
28. Davis JW. Skin transplantation with a review of 550 cases at the Johns Hopkins Hospital. *Johns Hopkins Med J.* 1910; 15: 307-96.
29. Fernandes M, Sridhar MS, Sangwan VS, Rao GN. Amniotic membrane transplantation for ocular surface reconstruction. *Cornea.* 2005; 24(6): 643-53.
30. Wilshaw SP, Kearney JN, Fisher J, Ingham E. Production of an acellular amniotic membrane matrix for use in tissue engineering. *Tissue Eng,* 2006; 12(8): 2117-29.
31. Hodde J. Naturally occurring scaffolds for soft tissue repair and regeneration. *Tissue Eng.* 2002; 8(2): 295-308.
32. Niknejad H, Peirovi H, Jorjani M, Ahmadiani A, Ghanavi J, Seifalian AM. Properties of the amniotic membrane for potential use in tissue engineering. *Eur Cell Mater,* 2008; 15: 88-99.
33. Chong PP, Kamarul T, Jalil K, Bakar NA, Ng LL. Processing and bioburden analysis of human amniotic membrane for biological and tissue engineering application –a preliminary report. *J Asean Orthop Assoc,* 2008; 19(1): 28-34.
34. Selvaratnam L, Abd Rahim S, Kamarul T, Chan KY, Sureshan S, Penafort R, *et al.* Colonies in engineered articular cartilage express superior differentiation. *Med J Malaysia,* 2005; 60(Suppl C): 49-52.
35. Freed LE, Guilak F, Guo XE, Gray ML, Tranquillo R, Holmes JW, *et al.* Advanced tools for tissue engineering: Scaffolds, bioreactors, and signaling. *Tissue Eng,* 2006 Dec; 12(12): 3285-305.
36. Nesic D, Whiteside R, Brittberg M, Wendt D, Martin I, Mainil-Varlet P. Cartilage tissue engineering for degenerative joint disease. *Adv Drug Deliv Rev,* 2006; 58(2): 300-22.
37. Allen KD, Athanasiou KA. Scaffold and growth factor selection in temporomandibular joint disc engineering. *J Dent Res,* 2008; 87(2): 180-5.
38. Chen DC, Avansino JR, Agopian VG, Hoagland VD, Woolman JD, Pan S, *et al.* Comparison of polyester scaffolds for bioengineered intestinal mucosa. *Cells Tissues Organs,* 2006; 184(3-4): 154-65.
39. Lemare F, Steimberg N, Le Griel C, Demignot S, Adolphe M. Dedifferentiated chondrocytes cultured in alginate beads: Restoration of the differentiated phenotype and of the metabolic responses to Interleukin-1,. *J Cell Physiol*, 1998; 176(2): 303-13.
40. Passaretti D, Silverman RP, Huang W, Kirchhoff CH, Ashiku S, Randolph MA, *et al.* Cultured chondrocytes produce injectable tissue-engineered cartilage in hydrogel polymer. *Tissue Eng,* 2001; 7(6): 805-15.
41. Stone KR, Steadman JR, Rodkey WG, Li S-T. Regeneration of meniscal cartilage with use of a collagen scaffold. Analysis of preliminary data. *J Bone Joint Surg Am,* 1997; 79(12): 1770-7.
42. Chung C, Erickson IE, Mauck RL, Burdick JA. Differential behavior of auricular and articular chondrocytes in hyaluronic acid hydrogels. *Tissue Eng Part A,* 2008; 14(7): 1121-31.
43. Yang XB, Bhatnagar RS, Li S, Oreffo RO. Biomimetic collagen scaffolds for human bone cell growth and differentiation. *Tissue Eng*, 2004; 10(7-8): 1148-59.
44. Nakamura T, Endo K, Cooper LJ, Fullwood NJ, Tanifuji N, Tsuzuki M, *et al.* The successful culture and autologous transplantation of rabbit oral mucosal epithelial cells on amniotic membrane. *Invest Ophthalmol Vis Sci,* 2003; 44(1): 106-16.
45. Nakamura T, Koizumi N, Tsuzuki M, Inoki K, Sano Y, Sotozono C, *et al.* Successful regrafting of cultivated corneal epithelium using amniotic membrane as a carrier in severe ocular surface disease. *Cornea,* 2003; 22(1): 70-1.
46. Dua HS. Amniotic membrane transplantation. *Br J Ophthalmol,* 1999; 83(6): 748-52.
47. Farazdaghi M. Electron microscopy of human amniotic membrane. New York: World Scientific Publishing Co. Ltd; 2001.
48. Yang L, Shirakata Y, Shudou M, Dai X, Tokumaru S, Hirakawa S, *et al.* New skin-equivalent model from de-epithelialized amnion membrane. *Cell Tissue Res,* 2006; 326(1): 69-77.
49. Koizumi N, Rigby H, Fullwood NJ, Kawasaki S, Tanioka H, Koizumi K, *et al.* Comparison of intact and denuded amniotic membrane as a substrate for cell-suspension culture of human limbal epithelial cellls. *Graefes Arch Clin Exp Ophthalmol,* 2007; 245(1): 123-34.
50. Jin CZ, Park SR, Choi BH, Lee KY, Kang CK, Min BH. Human amniotic membrane as a delivery matrix for articular cartilage repair. *Tissue Eng,* 2007; 13(4):693-702.