

Dehydrated Amniotic Allograft Seeded with Stem Cells: The Clinical Perspectives for Wound Healing

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Abstract

Amniotic membrane is the inner most layer of the placenta; it has unique properties of the smooth proliferation of epithelial cells and contains many different growth factors and cytokines. Amniotic membrane transformation is currently being used for continuously widening the spectrum of regenerative medicine. It gets wide spread attention as an effective method of reconstruction and rejuvenation. Amniotic membrane can be processed to produce dehydrated human amnion membrane allograft (dHAM). This allograft has shown its efficacy in wound healing due to its inherited properties and balanced biological structure. It is a very easy to use and less painful supplement to the traditional wound healing procedure. In the present study, the dHAM structure, primary cytokines functions, viability of seeded WJ-MSCs on dHAM are evaluated. Besides, the competence compared with fresh amniotic membrane and the current clinical usages in wound healing applications are summarized.

Keywords: Amniotic membrane (AM); Dehydrated human amnion membrane (dHAM); Mesenchymal stem cells (MSCs); Wound healing; Cytokines; Growth factors.

Introduction

Skin injury incidences due to burns and trauma are worrying concern all over the world. Various treatment strategies such as autograft, allograft, xenograft, tissue-engineered skin substitutes, etc. are being used to treat wounds for a long time. The gold standard for a grave wound/ injury is the autologous skin graft in which skin from a patient is taken and transplanted to the affected area on the same individual^[1]. These treatments exhibit several disadvantages such as autografts are a painful procedure and not possible in case of extensive burns. On the other hand, allografts and xenografts involve the transfer of skin tissue between species; however, have limitations of graft rejection, immune reaction, lack of donor, etc^[2,3]. Commercial available synthetic grafts have problems of reduced vascularization, wound contraction, poor integration with the host tissues, and scarring, which encouraged the researchers to develop new wound care product^[4,5].

For more than a decades, amniotic membrane (AM) transplantation has widely used in wound care and various clinical applications. Mainly, there are two different ways to transplant AM either it can apply as a permanent graft in which it functions as a substrate for cells to grow and temporary bandage or patch in which it acts as a cover. Amniotic membrane has gained much popularity in wound healing treatments due to its distinctive properties, including reduce scarring, anti-inflammation effects and enhance epithelialization and treating a different type of wounds such as diabetic foot ulcer, venous leg ulcer,

neuropathic foot ulcers, varicose ulcer, etc^[6-9]. In our previous publication, various clinical studies significantly summarized the potential of amniotic membrane for wound healing applications^[10]. The AM graft can provide a pathophysiological micro environment closer to the wound autografts, providing an in-vivo environment suitable for skin regeneration.

Dehydrated human amniotic membrane (dHAM) is an innovative technology that is considerably showing its efficacy in regenerative medicine, especially in wound healing. The presence of regenerative growth factors, cytokines and chemokines all together balance the biological activity and promotes a wound healing environment. dHAM structurally support promoting cellular differentiation and adhesion, infection suppression, pain suppression, epithelialization, and anti-scarring effects. Dehydrated human amniotic membrane is a very simple graft and can store in room temperature for the shelf life of around five years. dHAM allograft can be used in both ways as

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a therapy to decrease pain and reduce fibrous tissue formation or as a supplement to current painful and traditional procedures used. Since release, the application has been used in surgical settings and have no adverse events associated with various fields and applications. One hundred thirty thousand allografts have been successfully released for human implantation in various surgical forms, such as orthopedics, gynecology, trauma, and burn units. No adverse event is reported associated with these 130,000 dispensed grafts^[11].

In this study, we used the dehydrated and fresh human amniotic membrane seeded with Wharton's jelly (WJ) derived mesenchymal stem cells because AM has been used as a natural scaffold for the repair of various injuries and provide mechanical support for MSCs growth. This study explores the potential of amniotic membrane as a scaffold for cell adhesion to offer alternative sources for transplantations to bridge this gap between the need and lack of tissue graft. These findings also suggest that both fresh and dehydrated amniotic membrane with mesenchymal stem cells can be used as an overpass to guide skin growth to a certain extent.

Material and Methods

The study conducted in house R&D, recognized by Department of Scientific and Industrial Research (DSIR), CelluGen Biotech, Gurgaon, India, after obtaining prior approval of the study protocol by the Institutional Committee for Stem Cell Research (IC-SCR). This study supported by a Small Business Innovation Research Initiative (SBIRI) funded by the Biotechnology Industry Research Assistance Council (BIRAC), India.

Procurement of Raw Material

The Umbilical cord (UC) tissue was collected, after caesarean section, cleaned with a swab and was put into a falcon tube containing transport media. For amniotic membrane collection, the junction of the cord with placenta separated under sterile conditions, and amniotic membrane of approximately 250 mm x 250 mm was obtained by manual dissection and placed into a sterile 50 ml screw-capped reagent tube (Falcon) containing transport medium^[10]. The medium contains balanced phosphate buffer saline (PBS; Himedia) solution along with 10 µl/mL Antibiotic-Antimycotic solution (Gibco) used for transportation. The collection tubes wiped with ethanol, sealed and placed in the collection kit box and transported to the laboratory within 48 hours between 10-15°C.

Infectious screening and selection criteria

Eligible amnion donors are living mothers that have delivered a live birth through caesarean section. All tissues recovered under full informed consent of the donor. Each donor then answered a series of questions to ensure the donor has not engaged in behaviours to place her at an increased risk for the transmission of infectious diseases and to ensure the donor has not shown signs or symptoms of illnesses. Donor procurement was pre-screened to prevent the transmission of infectious diseases from donors to recipients of the material. The amniotic membrane in a fresh form collected from the tie-up hospitals across pan India. Mothers carrying infections like HIV, Hepatitis B, Hepatitis C, Syphilis, CMV, and malaria before childbirth were not included. Donors with a history of antenatal problems, e.g. gestational dia-

betes or placenta Previa excluded from the study. For HIV, Hepatitis B, Hepatitis C, malaria, CMV, HTLV and syphilis infections, serological tests of Anti-HIV 1 and 2, HBsAg, Anti-HCV, PF/PV-ICT, CMV IgM and IgG, RPR, HTLV 1 and 2, and RPR carried out respectively.

Preparation of dHAM

The amnion was carefully separated from the rest of the chorion by blunt dissection and using round-ended forceps under the strict sterile environment in Class II Type A2 biosafety cabinet (Thermo Fisher Scientific, USA). The peeled chorion was discarded, and amnion was washed thrice with washing solution that contains balanced PBS solution along 10 µl/mL antibiotic-antimycotic solution to remove blood and mucus. The amniotic membrane then incubated in Dulbecco's modified Eagle Medium-Nutrient Mixture Ham's F-12 (1:1) (DMEM F12) medium with 10 µl/mL antibiotic-antimycotic solution for 1 hour at room temperature. The amniotic membrane was then uniformly flattened without folds or tears onto individually sterilized 0.22 micron, 47 mm diameter Nitrocellulose membrane (Himedia), with the epithelium/basement membrane surface up. The membrane was allowed to adhere to the nitrocellulose membrane for 30 minutes and was then cut into different dimensions (a) 50 mm x 50 mm (b) 100 mm x 100 mm (c) 200 mm x 200 mm pieces correspondingly as per the necessity^[10] and preserved by the freeze-drying technique.

Wrapping for Clinical Studies

The processed amniotic membrane allografts are packaged aseptically into an inner peel pouch and sealed with an outer peel pouch system within a clean room environment. The outer peel pouch is not considered sterile. The inner-pouch, which contains the graft, is deemed to be pure unless damaged or compromised and then sterilized by γ irradiation for further clinical studies (data not disclosed).

Preparation for Treatment and Administration Techniques

The graft placed in a respective inner pouch of the sterile pack. Toward verify for further suitability of amniotic membrane, examined for different testing parameters such as holes, broken seals, tears, contamination, or other physical defects, uniformity of appearance, including the absence of spots or discoloration as per our earlier publication^[10]. The extent possible, oxygen was removed from the inner pouch and sealed. Each internal bag separately packed in an outer pouch for further protection, storage, and shipment. The final inspection was made of both the inner and outer pouches to ensure that the amniotic membrane contained there in matches the product specifications, such as shape, size, thickness, and tissue type.

In-vitro studies

Preparation of dHAM homogenized extracts

dHAM of individual donors was homogenized in a mortar and pestle and extracted in phosphate-buffered saline (PBS) at a concentration of 15 mg tissue/ml. The crude homogenates were centrifuged at 5000 x g, 4°C for 20 min, supernatant collected and sterile filtered before using in tissue culture. For heat-inactivation experiments, dHAM homogenate was boiled at 95°C for 5 min and subsequently used for analyses. For thermal precondi-

tioning, dHAM was dropped at the indicated temperature for 48 hours before homogenization.

Analyses of Growth Factors, Cytokines, Proteases, and Inhibitors

Growth factors, interleukins (ILs), Chemokines, and tissue inhibitors of metalloproteinases (TIMPs) were measured in samples of processed, dehydrated human amnion grafts with standard enzyme-linked immunosorbent assays (Bioassay Technology Laboratory). The weighed, minced samples were placed in lysis buffer containing protease inhibitors for 24 hours at 4°C. Tissues were then homogenized, centrifuged to remove tissue residue, and the amount of each factor in the lysis buffer was measured in diluted aliquots with standard ELISA assays.

Characterization of the MSCs seeded scaffolds

Cytotoxicity evaluation of dHAM

The cytotoxicity analysis for fresh, dehydrate, and MSCs on AM was studied by MTT (3-(4, 5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay after 64 hours^[12]. WJ-MSCs (n=5) were grown to 85% confluency, passaged, and re-suspended in a cell culture medium. $5 \times 10^4/10 \mu\text{l}$ and $10 \times 10^4/10 \mu\text{l}$ were seeded dropwise onto amniotic membrane placed in a six-well plate (3516, Costar) and incubated at 37°C for 3 hours to allow the cells to adhere, then 2 ml of medium was added to each well. The seeded AM were incubated for 64 hours, and the medium removed from well, 200 μL of MTT (TC191, Himedia; 5 mg/mL of PBS) was added to 6 well plates and incubated for four hours at 37°C in a 5% CO₂ incubator. Following incubation, 1.8 ml of DMSO (CP100, OriGen) was added to dissolve the formazan crystals produced by the activity of live cells, and the colored supernatant was read at 595 nm using plate reader (Bio-Rad, USA). For measure absorbance, 100 μL solution as transferred to 96 well plates (TPG96, Himedia) and absorbance measured with the plate reader. After that, the standard graph and standard deviation were plotted using the known absorbance values, and cytotoxicity of the allograft was determined based on the viability of the cells.

Cell attachment and Cell proliferation

Cell proliferation was evaluated by using the Alamar Blue (AB) assay. This assay based on a redox reaction that occurs in the mitochondria of the cells; the colored product transported out of the cell, and optical density can be measured^[13]. The cell-scaffold constructs were removed from the culture plates at 2, 4, 8, 16, 32, 64 and 128 hours, washed with PBS and placed into 6-well culture plates. For each construct, 2 ml of phenol red removed medium containing 10% Alamar blue (R7017, Sigma) was added directly, followed by incubation for 24 hours at 37°C at 5% CO₂ incubator. As a negative control, AB added to the medium without cells.

For measure absorbance, 100 μl solution were transferred to 96 well plates, and absorbance measured with the plate reader at 540 nm and 630 nm. The number of viable cells corresponded to the magnitude of dye reduction and showed as a percentage of AB reduction^[14]. In order to remove differences due to medium colour, the culture media filtered with sterile activated charcoal to remove phenol red from culture media. Briefly, in the culture medium, 17.5 mg activated charcoal added per 1

ml medium, the bottle was thoroughly shaken for 40 minutes, centrifuged at 210 x G (Thermo Fisher Scientific, USA) for 10 minutes and the supernatant was filtered to yield a colourless medium.

Cell viability and Distribution over the scaffold

After distribution of the mesenchymal stem cells in the amniotic membrane, the cell viability of each allograft was characterized using fluorescence staining with Live/Dead assay kit (K501-100, Biovision). Briefly, the cell-scaffold constructs (5×10^4 cells/scaffold) were removed from the culture plates at different days of incubation, washed with Hank's balanced salt solution (HBSS; 14025092, Gibco). Then, the allografts were incubated with fluorescein diacetate (FDA) and propidium iodide (PI) at 37°C for 60 minutes, washed with HBSS, and examined under a fluorescence microscope using a band-pass filter (Olympus, Japan). The healthy live cells were visualized through green fluorescein, whereas dead cells appeared as red.

Cell Attachment and Morphology Scanning Electron Microscopy (SEM)

The microstructure of the fresh and dehydrated AM was examined with Resolution 2.0nm at 30kV by versatile analytical scanning electron microscope (SEM, ZEISS EVO 50). Before SEM analysis, samples were dehydrated with graded ethanol from 10% to 100%, dried overnight, coated with gold, and then observed under SEM. The diameter of individual pores in the amniotic membrane was measured directly from the SEM images with 100X magnification.

Statistical analysis

All statistical analyses were performed using GraphPad Prism Software (La Jolla, CA, USA). Results were expressed as the mean \pm standard deviation (SD) for illustration. The statistical significance of metabolic activity and proliferation potential among harvested cells was determined using two-way ANOVA. $P < 0.0001$ was considered statistically significant.

Results

Appearance and Morphologic features

The allograft used in our study was wafer-like, very light and thin, easy to handle, and suture without ripping (Figure 1A). It became charming and elastic on hydration, similar to cryo-preserved amniotic membrane (Figure 1B). The results of the sterility tests performed were all negative. The basal lamina was present and intact, forming a continuous flat and generally smooth layer above the fibrous collagen stroma. The dHAM chorion and epithelial cells had been successfully removed from the amnion layer. The basal lamina was also clearly visible. Figure 3C shows that the stroma of the dHAM also appeared normal and the collagen fibers making up the AM stroma are well preserved by the freeze-drying technique.

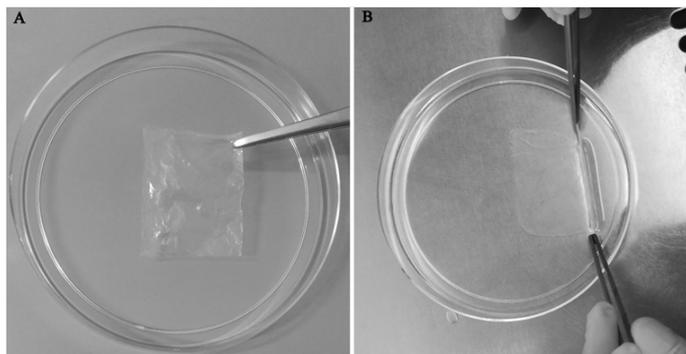


Figure 1: (A) The Sterilized, freeze-dried amniotic membrane was light and thin. (B) It became smooth and elastic on hydration, similar to the cryopreserved amniotic layer.

Cytotoxicity evaluation of dHAM

The cytotoxicity of amniotic membrane scaffold seeded with WJ-MSCs was confirmed by quantitative analysis using the MTT assay. The absorbance values of test samples (TS1 and TS2) after exposure to the medium for 64 hours were corresponding to the number of metabolically active cells. The absorbance values of TS1 and TS2 showed good viability and continuous metabolic activity of the MSCs on the dHAM than the positive control (PC) and known samples (cells) control (KS1-KS4).

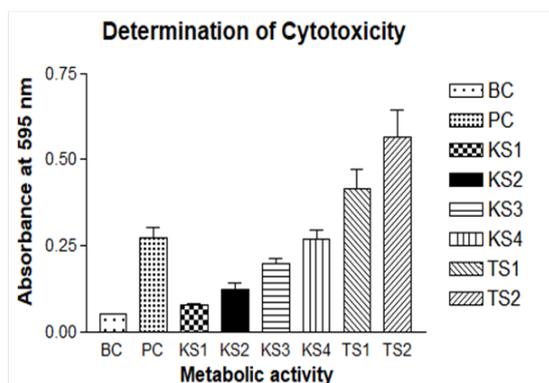


Figure 2: Determination of cytotoxicity of allograft by MTT assay. (BC): Blank Control - Culture medium without scaffold and Cells, (PC): Positive Control -dHAMwithout cells, (KS1 to KS4): Known Sample - 25000, 50000, 75000 and 100000 MSCs seeded without scaffold respectively, (TS1 and TS2): Test sample – 50000 and 100000 cells seeded on dHAMrespectively. (Absorbance at 595 nm). Data represent mean \pm SD of five independent experiments.

Thus the results Figure 2 showed that the metabolic activity of WJ-MSCs on the scaffold was not suppressed in the medium and from the computable scores, it was determined that the extracts of the dHAM proved no cytotoxic reactivity in this experiment.

Cell attachment and Cell proliferation

Figure 3 shows the percentage reduction of AB with different incubation hours and at different initial cell densities and including the standard curve of percentage (%) AB reduction versus the logarithm of cell growth. Higher AB reduction was observed in the test culture TS2 compared with TS1 over the whole culture period. Similarly, AB reduction percentage of TS2 was similar to the percentage of KS1 were showed that the constant cells

growth rate and quantified proliferation of MSCs on the scaffold. In the culture, cell proliferation increased with culture time over the first three days. The metabolic activity of the cells growing in the medium seems to slow-down by day 5, suggesting that the surfaces were advancing into maximum confluence.

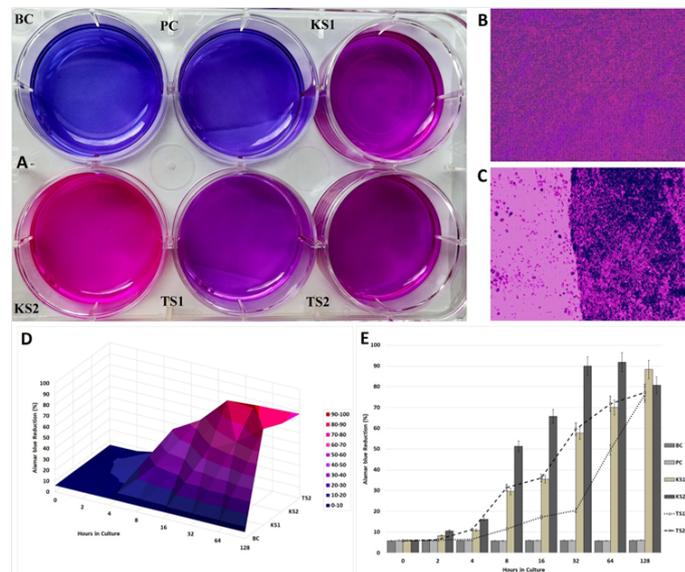


Figure 3: Cell proliferation evaluation by Alamar blue assay. (A) Changes of medium colour associated with AB reduction from blue (oxidized) to pink (reduced) quantified by measuring absorbance at wavelengths of 540 nm and 630 nm. (B) Microscopic images of AB assay for viability/ proliferation of WJ-MSCs (KS2) and, (C) Cells were cultured on dHAM (TS2) up to 5 days (Magnification 10X). (D) Graph showing % of AB reduction with different incubation hours and at different initial cell densities. (E) Standard curve of % AB reduction versus the logarithm of cell growth. Test sample (TS1 and TS2): 50000 and 100000 cells seeded on dHAM, and known sample control (KS1 and KS2): 50000 and 100000 MSCs seeded without dHAM respectively. Whereas Blank Control (BC): Culture medium without scaffold and Cells, and Positive Control (PC): dHAM without cells accordingly.

Cell viability and distribution over the allograft

The viability and spreading of WJ-MSCs on the fresh amniotic membrane (fHAM) and dHAM visualized after day 7 of cells seeding, which showed a significant proportion of living cells on the dehydrated allograft. As shown in Figure 4, more than 80% of cells on fresh AM and considerable cells on dHAM were live and randomly spread over the membrane and formed a matrix across the surface of the layer. The mesenchymal stem cells were proliferated, and their distribution increased from day 1 to day 7. Up to 14 days of the cultivation period, it observed that no profound change in the viability and morphology.

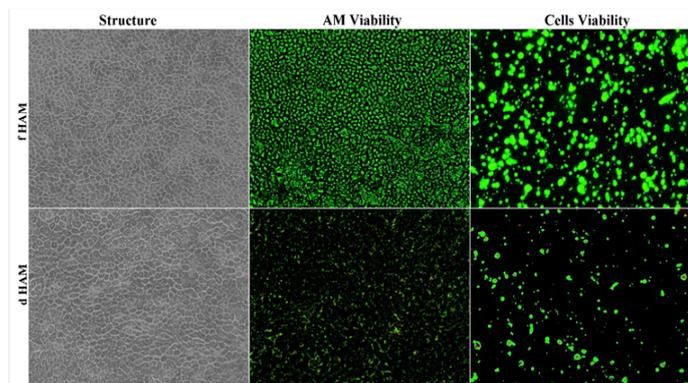


Figure 4: Structure of fresh and dehydrated amniotic membrane: (Left; Top to Bottom). Fresh and dehydrated AM analysed by Live /Dead staining (Centre; Top to Bottom). Live /Dead staining of seeded cells in both fresh and dehydrated AM demonstrates the majority of the cells on fresh and dehydrated AM remain viable (green fluorescence) with only a few dead (red fluorescence) after seven days (Right; Top to Bottom) (Magnification 10X).

SEM Analysis

Through electron microscopy, the fresh amniotic membrane exhibited a fractal-like structure, having a pore surface area appearance (Figure 5A); dehydrated amniotic membrane exhibited a basilar membrane-like structure (Figure 5B). Investigation of the dHAM by scanning electron microscopy revealed a continuous flat, porous layer of smooth basement membrane (Figure 5A and

nearly similar morphology of a fresh amniotic membrane (Figure 5B). Besides, morphology of MSCs cultured for 5 days on the fresh amniotic membrane and complete confluence growth of MSCs (Figure 5C) and dehydrated amniotic membrane exhibited MSCs adhered onto the scaffold (Figure 5D) revealed the mesenchymal stem cell attachment and growth on the scaffolds.

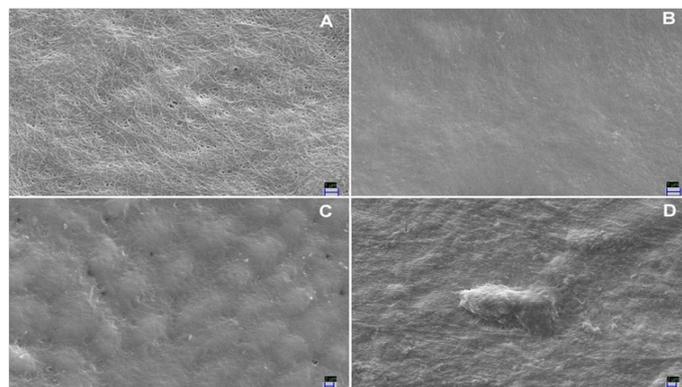


Figure 5: Investigation by scanning electron microscopy, Morphology of fresh amniotic membrane (Figure 5A); and dehydrated amniotic membrane (Figure 5B) (Magnification 15KX respectively), Morphology of MSCs cultured for 5 days on the fresh amniotic membrane (Figure 5C); and dehydrated amniotic membrane (Figure 5D) (Magnification 5KX respectively).

Table 1: Concentration of growth factor, chemokine, and cytokine in the homogenized dehydrated amniotic membrane. The content of cytokines and growth factors analyzed by ELISA and the software used for data interpretation is Magellan

Cytokines / Growth factors	Systematic Name	Function/ Category	Values reported in literature	fresh Amniotic Membrane (fAM)	dehydrated Amniotic Membrane (dAM)
EGF	Epidermal growth factor	Growth factors	20-308pg/ml; 70-85 pg/mg	119.2 pg/ml	116.4 pg/ml
FGF2	Fibroblast growth factors	Growth factors	NA	<5ng/L	<5ng/L
IL10	Interleukin 10	Proliferative cytokines	33-651.4pg/ml; 0-2.18 pg/mg	47.9 pg/ml	15 pg/ml
IL4	Interleukin 4	Anti-inflammatory cytokines	33-210.4pg/ml; 0.7-0.86 pg/mg	135.25 pg/ml	134.50 pg/ml
IL6	Interleukin 6	Proliferative and Pro-inflammatory cytokines	22.3 pg/mg; 4.11-4905.90pg/g tissue	634.7 pg/ml	213 pg/ml
IL8	Interleukin 8	chemotactic cytokines (Chemokines)	474.4 pg/mg; 36.99-9259.04pg/g tissue	34 pg/ml	132.4 pg/ml
NGF	Nerve growth factor	Growth factors	NA	<7 pg/ml	<7 pg/ml
PDGF-BB	Platelet-derived growth factor	Growth factors	22-142 pg/mg	120.6 pg/ml	117.9 pg/ml
PGE2	Prostaglandin E2	Anti-inflammatory cytokines	24 to 390 ng/ml (Prostanoids quantification)	63.6 pg/ml	27.4 pg/ml
SDF1 α	Stromal-derived factor 1 Alpha	chemotactic cytokines (Chemokines)	20-26 pg/mg	9.2 pg/ml	10.4 pg/ml
TGF β	Transforming growth factor	Growth factors	29-658.5pg/ml; 1.3-431.6 pg/mg	45 pg/ml	30 pg/ml
TIMP1	Tissue inhibitor of metalloproteinase 1	Metalloproteinase	87.3-6356.8 pg/mg,	110.9 pg/ml	89.3 pg/ml
TIMP4	Tissue inhibitor of metalloproteinase 4	Metalloproteinase	36.2 -2692.9 pg/mg	113 ng/ml	11.8 ng/ml
VEGF	Vascular endothelial growth factor	Growth factors	0-94 pg/mg	36.3 pg/ml	34.9 pg/ml

Cytokine quantification by ELISA

The cytokines and growth factors are known to be present within the amniotic membrane, in which evaluated for dosage in the fresh and dehydrated human amniotic layer. ELISA assays were performed on samples of dHAM and showed quantifiable levels of the following factors: vascular endothelial growth factor (VEGF), transforming growth factor-beta (TGF- β), tissue inhibitors of metalloproteinases 1 (TIMP-1), TIMP-3, stromal-derived factor 1 Alpha (SDF1 α), prostaglandin E2 (PGE-2), platelet-derived growth factors (PDGF-BB), nerve growth factor (NGF), interleukin 4 (IL-4), IL-6, IL-8, IL-10, fibroblast growth factor 2 (FGF-2) and epidermal growth factor (EGF) has determined to be present are provided in Table 1 and the scientific evidence of the cytokines and growth factors primary function in wound healing were highlighted in Table 2.

Table 2: Scientific evidence of the cytokines and growth factors primary function in wound healing

Cytokines / Growth factors	The primerole in wound healing
EGF ^[15,16]	Re-epithelialization of skin wounds and promotion of wound closure
FGF2 ^[17,18]	Exert a cytoprotective function in would repair, supporting cell survival under stress conditions. Promotes mitogenic activity for keratinocytes and fibroblasts at the wound site. FGF1 and FGF2 stimulate angiogenesis. Basic fibroblast growth factor (bFGF) enhances the proliferation of endothelial cells and smooth muscle cells.
IL10 ^[19,20]	Regulates differentiation and/or growth of keratinocytes, endothelial and various immune cells, Regulates the infiltration of macrophage-derived neutrophils into the wound site, promotes the expression of pro-inflammatory cytokines and reduces matrix deposition and thereby inhibiting scar formation
IL4 ^[21,22]	They play a primary role in limitation and termination of inflammatory responses
IL6 ^[23,24]	Possess both pro-inflammatory and anti-inflammatory activities under different conditions of the wound-healing process, Promotes angiogenesis formation, Promotes epithelial cell migration, Plays an axial role in wound healing by regulating cellular responses
IL8 ^[25,26]	Increases keratinocyte proliferation and stimulate re-epithelialization in human skin grafts, both in-vitro and in-vivo. IL-8 and its receptor act as a chemoattractant for neutrophils and Enhances the migration of epithelial cells in-vitro
NGF ^[20,27]	Involved in fibroblast migration, increasing expression of actin by smooth muscle and collagen gel contraction by these cells. Stimulates proliferation of keratinocytes and inhibits apoptosis in-vitro, and supports the proliferation of human dermal in wound healing
PDGF-BB ^[15,28]	Potent chemoattractant and activator of neutrophils and monocytes. Promote angiogenesis; also proliferative, migration stimulatory effects and Stimulates DNA synthesis, attracts fibroblasts to wound sites, enhances their production of collagenase, collagen, and glycosaminoglycan
PGE2 ^[22,29]	They play a primary role in limitation and termination of inflammatory response
SDF1 α ^[16,30]	Promotes wound closure and Induces cell migration
TGF β ^[20,31]	Activin B supports wound repair and regeneration of hair follicles, promotes wound closure, Activins which are members of TGF- β family act as enhancers for granulation tissue fibroblasts and the induction of extracellular matrix deposition and Enhances proliferation of epithelial cells, expression of antimicrobial peptides and release of chemotactic cytokines
TIMP1 ^[32,33]	TIMPs (TIMP-1, -2, -3, and -4) considered as "classical" endogenous inhibitors of MMPs. The expression of TIMP-1 and -3 mRNAs detected in proliferating keratinocytes between the third and fifth day after injury. In the case of chronic wounds, the expression pattern of TIMPs altered. The lack of TIMP-2 in the zone with migrating keratinocytes of the wound edges may lead to uncontrolled activation of MMP-2 in chronic ulcers.
TIMP4 ^[32,33]	Matrix metalloproteinases (MMPs) play important roles in wound healing, and their dysregulation leads to prolonged inflammation and delayed wound healing. MMPs are multi-domain proteins, and their activities regulated by tissue inhibitors of metalloproteinases (TIMPs)
VEGF ^[15,16]	VEGF stimulates endothelial cell migration, proliferation, and survival; potent stimulator of angiogenesis and Promotes wound closure.

Discussion

Critical steps in tissue engineering are the selection of material and technology to fabricate scaffold. The main objective of all scaffold fabrication technique is to fabricate scaffold with materials which can mimic the extracellular matrix of targeted tissue as close as possible. We have reviewed the properties and therapeutic potential of dehydrated, as well as biological scaffolds. A wide range of materials including natural, synthetic, ceramic, metals, biological and their composites can be fabricated as scaffold in tissue engineering and regenerative medicines and there is continuous research going on to enumerate their full potential. Still, state-of-the-art synthetic scaffolds have to undergo clinical trials, and there is a long way to go from bench to bedside. However, the advancements in processing and preservation technology have enhanced the popularity of dHAM as allograft in various tissue engineering applications. The dehydrated amniotic membrane provides many advantages over synthetic scaffolds firstly it is available in ample amount at low cost and processing is also very simple. The preservation procedures allow it to store for a longer time and use it when required. These preservation procedures also remove the risk of any infection transmission. Furthermore, amniotic membrane being

natural material gets easy acceptance from host and there are no reports of graft rejection with amniotic membrane. In addition to biocompatibility, it is permeable, stable, flexible and restorable with time.

In developing countries such as in India and in cost-sensitive segments of developed countries, the available skin graft market limited by expensive products. There is a need for a clinical approach for wound management that can reduce the burden of society. The progress made in stem cell science has opened new perspectives for their usage in clinical therapeutic applications. Amniotic membrane is non-immunogenic and non-vascular tissue and has anti-inflammatory, anti-microbial and anti-scarring properties^[6] make it a potential scaffold to be used for cell adhesion and proliferation^[34]. The allograft derived from extracellular matrix (ECM) and amniotic membrane tissue. There are various sources of ECM such as blood vessels, skin, nerves, tendons, small intestinal submucosa, and placental amniotic membrane. ECM has been successfully used in multiple preclinical and clinical applications in tissue engineering. Individual components of the ECM such as collagen, laminin, fibronectin, and hyaluronic acid can be isolated and used both *in-vitro* and *in-vivo* to facilitate cell growth and differentiation. Various forms of intact ECM have been used as biologic scaffolds to promote the constructive remodeling of tissues and organs. ECM scaffold comprises a complex mixture of molecules that has structural and biological properties. ECM is obtained by decellularisation of tissue, through a number of techniques utilizing chemical, enzymatic, or mechanical disruption which removes xenogenic and allogeneic cellular composition from tissue without disturbing the structure and component of ECM.

Amniotic membrane has unique properties of easy proliferation of epithelial cells which helps healing process to complete with minimal inflammation, angiogenesis, and scarring. It also possesses several inherited biological properties which makes it potential for wound healing. For many years amniotic membrane transplantation has been widely used for various clinical applications. For evidence, we investigated the cytotoxicity, stemcell attachment, proliferation, viability and distribution over the allograft; and the growth factors, cytokines, proteases, and inhibitors secreted by dHAM, compared with fHAM were evaluated. The result is a durable, bioactive allograft with natural barrier properties which may store at ambient conditions for up to five years. Each batch of the allograft was subjected to stringent quality control checks following national and international quality standards and only qualifying grafts tests are released for further use. Such as, the sterility tests as per USP (United States Pharmacopeia) 71: The allograft is assessed to be sterile, the all burst test as per USP: The tensile strength of tissue is evaluated and proven to be not less than 0.25 N/m², Protein Assay by BCA method: Amount of protein in tissue is evaluated and shall not be less than 0.05 mg/cm², Moisture content test as per USP: Moisture shall not exceed the prescribed limits of 15% respectively.

In brief, the Cytotoxicity evaluation of by MTT assay showed that the amniotic membrane allografts seeded with mesenchymal stem cells prepared in this study were nontoxic. The MSCs seeded, and the method of freeze-dried dHAM proved the overall good viability of the cells, demonstrating that these allografts were free of toxic substances and appropriate

for *in-vitro* and *in-vivo* studies. Besides, when MSCs are alive on the scaffold retain a reducing environment within the cytosol of the cell. Resazurin is a blue dye and dynamic element of AB reagent, is negligibly toxic to alive cells, cell penetrable compound and itself weakly fluorescent. Upon entering cells, resazurin is reduced into further resorufin, a compound that is red in color and highly fluorescent^[14,35]. Therefore, viable MSCs constantly convert from resazurin to resorufin, increase the complete fluorescence and color of the culture media surrounding the cells. Hence, this test demonstrated that the reducing environment of MSCs on AM and the attraction of AB incorporated in the reagent were determined the continuous growth of stem cells proliferation on the allograft.

The study of mesenchymal stem cells viability and distribution on the allografts proved that proliferation of the WJ-MSCs on the amniotic membrane with precise distribution and proper growth. Live dead staining revealed that the WJ-MSCs were live and spread throughout the biological and dehydrated scaffold. It proves that the cells formed as a cell network across the surface of the membrane and the high number of cells spread over the broad area of the layer. Scanning electron microscopy images showed that the large surface area of the porous structure offHAM and dHAM allowed MSCs to adhere spread and grow on the allografts. The flat morphology and excellent spreading in and around the interconnected porous structure indicated strong cellular adhesion and growth of cells. Instead, the fresh and dehydrated allografts contain multiple growth factors, including EGF, FGF2, NGF, PDGE-BB, TGF β . ELISA assays measured the presence of other biological regulatory proteins, IL- 4, 6, 8 and 10, and TIMPs 1 and 4. This list of growth factors and regulatory proteins does not encompass the entire array of physiologically important and biologically active regulatory molecules present in fHAM and dHAM. However, these particular growth factors are likely to be responsible for some of the clinical benefits of the allograft, specifically for wound healing or soft tissue repair. Hence the overall possible mechanism of this study demonstrated that these allografts would provide not only a bridging role but also a microenvironment suitable for regeneration. Moreover, human amniotic membrane and mesenchymal stem cells can be easily obtained and there is no need to perform the second surgery after transplantation. Therefore, amniotic membrane loaded with WJ-MSCs for wound management brings to bear the promising potential for clinical application and especially for wound care management. In further to evidence, we stretching the study to prove the *in-vitro* and *in-vivo* wound healing effect in animal model and cytokines profiling towards different composition of allografts to target as close as possible.

Conclusion

Amniotic membrane provides many advantages over synthetic scaffolds firstly it is available in ample amount at low cost and processing is also very simple. The dHAM preservation procedures allow it to store for longer time and use it when required. These preservation procedures also remove the risk of any infection transmission. Furthermore, amniotic membrane being natural material gets easy acceptance from host and there are no reports of graft rejection with amniotic membrane. In addition to biocompatibility, it is permeable, stable, flexible and resorbable

with time. Published literature exhibits the extensive usage of amniotic membrane in ophthalmology and wound healing and continued to be explored in periodontics, cartilage, tendon, etc. Further studies are needed to have optimized scaffolds and its powder that imitate biological tissues in terms of both structure and function. We initiated a various study project to further investigate the applications and its optimization of bioactive allograft tissue matrix dehydrated (ReadyHeal).

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Authors' Contributions: Dr. Jaianand Kannaiyan performed experiments, analysed the data, and wrote the manuscript. S.K. performed experiments. S.N. and F.M. interpreted the data. All read and finalized the manuscript.

Disclosure of Potential Conflict of Interest

The authors have no conflicting interest.

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