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Research Article

DEVELOPMENT OF PLACENTAL MEMBRANE BASED THERAPEUTIC MATERIALS FOR WOUND HEALING

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ABSTRACT

Wound healing is a fundamental physiological response to tissue injury that results in restoration of tissue integrity with optimal functions. It is characterized by the formation of granulation tissue and synthesis of major connective tissue components through the coordinated interplay of many extracellular matrix (ECM) proteins, growth factors and cells. Several wound healing biomaterials are available for various types of wounds with varied outcomes and efficacy. Amnion based scaffolds find a range of applications in regenerative medicine and there is a growing demand for quality amnion material for various therapeutic purposes. In this study, a new novel enzymatic process is developed for separation as well as processing of the membrane to improve its properties to cure both acute and chronic wounds. As the amnion is processed enzymatically using non collagenolytic protease, the non-collagenous antigenic components are removed whereas collagen proteins are preserved in the placental membrane. The processed amnion is envisaged to have high compatibility upon application for healing wounds as it contains collagen protein contents which are non-antigenic in nature. The enzymatically separated amnion membrane is characterized for its biophysical and cell viability or toxicity properties along with the amnion separated by conventional method and the characteristic studies suggest that the processed amnion materials are having desirable features for acting as a wound healing scaffolds.

KEYWORDS: Wound healing, Amnion, Biomaterials, Collagen, Protease, Skin regeneration

INTRODUCTION

Wound healing is a multifaceted dynamic skin regenerative process comprising the regulatory events of suppression of inflammatory reactions and opportunistic infections, induction of proliferation of connective tissue cells such as fibroblasts, collagen deposition and remodeling of extracellular matrix[1]. The management of chronic wounds of diabetic and leprosy, venous and pressure ulcers, burns injury represents a significant burden, not only in terms of direct costs to healthcare services but also in terms of lack of effective therapeutic strategies, economic loss and impaired quality of life experienced by patients[2,3].

The cost of treating wounds is rising sharply, and the international market for the wound therapeutic products is expected to reach $20.4 billion by 2021[4]. High costs of treatment can deter patients from seeking wound care, potentially leading to the development of chronic wounds[5]. Use of several wound healing biomaterials or substances are available for various types of wounds and they include collagen or amnion based dressing materials, compositions based on keratin, hyaluronic acid, chitosan, phytochemicals, natural polysaccharides etc[6,7]. The efficiency of these biomaterials widely varies in terms of adherence, angiogenesis, hydration effects, delivery of wound heal substances, collagen turn over, techno-economic viability, safety as well as physical, chemical and biological properties. The outcomes of management of chronic ulcers are frequently dissatisfactory especially in the case of neuropathic wounds of leprosy and diabetes origins as patients harbor wounds for years that result in high economic and social costs due to disability, decreased productivity, and loss of independence.

The ultimate goal of wound management is to minimize the risk of (a) opportunistic infection and (b) damage to the ECM of skin connective tissue *via* proteolytic degradation while promoting the development of healthy granulation tissue and complete skin regeneration. An ideal wound dressing material should achieve rapid healing and skin regeneration at reasonable cost with minimal inconvenience to the patient.

Amnion, the most internal placental membrane, is considered as a collagen rich translucent biotherapeutic material composed of a single layer of epithelial cells that lie on a basement membrane, and of a nonvascular collagenous stroma. The membrane, containing a variety of collagen proteins, is an affordable source to develop biomaterials for various therapeutic purposes as the non immunogenic membrane represents an advantageous source of progenitor cells, tissue regenerative growth factors and substances, and various types of collagen[8]. These components give amnion the beneficial properties such as anti-adhesive, antimicrobial, anti-inflammatory, low toxicity, wound protection, pain reduction, epithelialization effects and biocompatibility with low risk of immunogenicity[9]. Amnion and amnion based scaffolds find a range of applications in the field of regenerative medicine and there is a growing demand for quality amnion material for various therapeutic purposes such as treating burns, wounds, difficult to cure diabetic, leprosy, corneal and venous leg ulcers, ocular surface disorders etc[10].

Reports disclose various methods for the separation of amnion from placenta utilizing non-enzymatic methods such as manual, mechanical and chemical methods[11, 12]. However, these methods suffer drawbacks such as tissue damage, loss in consistency of the material, difficulty in scale-up and demands manual handling of the material. Hence, there exists a need to develop an enzymatic process for the separation of placental membranes from placenta, which is devoid of above said drawbacks. Hence, in this study, we developed a bioscaffold from placental membrane involving a novel enzymatic method to separate the amniotic membrane which is further subjected to characterization studies to identify its potential as wound heal dressing material.

MATERIALS AND METHODS

Chemicals and Reagents

The experiments pertaining to collection of placenta, processing, amnion recovery, physical and chemical sterilization were carried out at Tissue Bank facility at King Institute of Preventive Medicine and Research (KIPMR), Chennai. All procedures have been approved by institutional ethical committee (IEC NO KIPMR Lett- 002/DIR/RESEARCH/KIPMR dt 05/09/2013). Dulbecco's Modified Eagles Medium (DMEM) and fetal bovine serum (FBS) were purchased from Invitrogen. MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide] was purchased from Hi-Media Laboratories, India. Amphotericin B, gentamicin, chlorhexidine, and metronidazole were from Hi-Media, India. A bacterium *Bacillus subtilis* was used for protease production. Medium components used for the production of protease as well as salting out were of commercial grade. Hammersten casein (assay grade) for determining protease activity was obtained from SRL, India. All other reagents and chemicals were of the highest purity grade and are mentioned in the respective description of the protocols.

Human placenta processing and preparation of amnion membrane

Human fresh placentas, weighing about 700–800 g, were obtained by normal healthy delivery without a history of premature rupture of membranes and were moved to processing unit at Tissue Bank of King Institute of Preventive Medicine and Research, Chennai. Blood clots and debris of the umbilical cord were completely removed through rinse with saline and extensive wash with 0.1 M phosphate-buffered saline (PBS), followed by dipping them in 100% w/v of PBS bath containing 1000 casein digestion units of a bacterial protease (for 1 kg of placenta) lacking collagenase and elastase activity from *Bacillus subtilis* at 30°C for 30 min. The bath was drained off, and the detached amniotic membranes from chorion and decidua of placenta were recovered. Post-enzymatic separation, Amnion materials were washed extensively with 0.1 M PBS-containing antimicrobial chemicals such as 25–50 µM each of amphotericin B, gentamicin, chlorhexidine, and metronidazole and immersed in the said antimicrobial solution for 30 min. In the case of conventional mode of amnion separation, tweezers were used. Postchemical disinfection, the membrane was spread onto the filter paper and kept in the Biosafety Level II laminar hood and subjected to ultraviolet (UV) sterilization for 30 min. Post-UV sterilization, the material was radiosterilized using 25 kGy ^{60}Co gamma radiation. All unused biological materials were discarded as biowaste, and standard guidelines of biowaste handling were followed.

Protease Activity Determination

The protease activity is expressed in terms of tyrosine equivalents using Hammarsten casein. To 1.9 mL of 1% casein solution prepared in 0.1 M carbonate buffer of pH 9.0, 0.1 mL of suitably diluted enzyme solution is added and the reaction mixture is kept at 30 °C for 10 min. The reaction is terminated by the addition of 3 mL of 5% trichloroacetic acid (TCA) solution (Qualigens, India). The absorbance of the trichloroacetic acid soluble filtrate at 280 nm is measured and one unit of enzyme activity is defined as the liberation of 1 µM tyrosine equivalent of substrate per ml or per gram of enzyme/min (casein digesting unit [CDU] = release of 1 µM tyrosine from casein substrate/mL or g/min)[13].

Cell Culture

Human skin fibroblasts were maintained and cultured in Dulbecco's Modified Eagles Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1X penicillin-streptomycin (Pen-Strep) (Invitrogen). Confluent cells were split 1:3 to 1:5 using TrypLE Express (Invitrogen). The cells were maintained at 37 °C with 5% CO_2 in a humidified CO_2 incubator for 48 h. Cells were dissociated with trypsinization (Tryple E™ Express 1x, Gibco).

Biophysical characterization and material validation studies

Biophysical characterization studies such as tensile and tear strength, water uptake capacity, biomaterial swelling analysis (mechanical properties) etc were carried out as per the methods reported earlier[1]. Briefly, for thickness measurements, 1 x 3 cm^2 measuring membranes were cut (n=6), dried using filter paper and these strips were placed on the center of the cover glass and a second glass was placed without squeezing the membrane. Likewise six sandwiched glass plates were prepared and placed under magnetic induction probe. Similarly, in another set of sandwich plates were prepared without membranes. The thickness of differences between the two sets of sandwich plates was provided for 6 pieces of membranes. For determining tear and tensile strengths, the prepared films (2 cm × 3 cm) were first dried and the water vapour transmission rate (WVTR) of the membrane films was calculated by Mocon Permatran, according to the standard of ASTM F 1249-90. The experimental temperature was close to normal human body temperature (36.5 ± testing machine, with a loading speed of 5 mm/min. To maintain humidity in the specimens, a liquid spray was continuously administered. Upon experimental completion, the following indices were automatically generated from the automatic control electronic universal testing machine: maximum load, maximum displacement, maximum stress, maximum strain, elastic limit load, elastic limit stress, and stress-strain curve: 1.0°C. Using the equipment 5 psi Pressure Transducer with Pressure signal indicator (Sensotec, Model GM-A), the measurements were carried out. For measurements, 9 x 9 cm^2 dried strips of membranes were used. The above tests were performed to compare the membranes obtained manually and enzymatic process and finding out the suitability of the materials for therapeutic applications. For measuring water uptake capacity, dried films were weighed and placed in watch glass filled with deionized water and retained for 20 min. Difference in the weight was measured to find out the actual water uptake capacity. Biomaterial of 200 µm thickness were cut into 1 cm^2 blocks and placed in petri-dish with 10 mL PBS (pH 7.4) and incubated at room temperature for 1 week. After 7 days, biomaterials were photographed and the increase in thickness was measured.

Fourier Transform Infrared Spectroscopy (FT-IR)

Infrared spectra (IR) of membrane biomaterials was obtained using phase resolution 128, and averaging 25 scans/min, using a Bruker IFS 28 Equinox infrared spectrophotometer.

Scanning Electron Microscopy (SEM)

The amnion scaffolds were fixed with 1.5% glutaraldehyde in PBS for 20 min at room temperature, washed three times with PBS and dehydrated stepwise with ethanol. For SEM analysis, after critical point drying, the samples were gold coated under vacuum and viewed with a JEOL 8401 scanning electron microscope.

Cell viability assay

To assess the effect of amnion materials on cell viability, the sheets were cut into circular patches to fit into a well of a 96 well plate. Human foreskin derived fibroblast cells grown to 80% confluency were taken, trypsinized and plated into a 96 well plate at a density of 3000 cells per well. The plate was incubated overnight with DMEM, 10% FBS, Pen-Strep media, following incubated, the media was replaced with DMEM, 1X Pen-Strep with or without 10% FBS. Cut circular amnion membranes were presoaked in DMEM for 20 min and were added on top on the cells in 96 well plate. The plate was incubated for 2 days and cell toxicity was assessed using MTT assay (Mosmann, 1983). Briefly, monolayer of cells (at a density of $2x10^4$ cells/well) seeded in 96-well microplates was treated with different weights of materials (500, 1000 and 1500 µg/mL) for 72 h. Both solvent (0.2% DMSO) and cell controls were included in the study. Then 20 µL of MTT solution (1 mg/mL) was added to each well. The plates were incubated at 37 ^{0}C for 3 h followed by the addition of 0.2% DMSO to each well. The absorbance of each well was measured at 620 nm using 96-well microplate reader (Thermo Multikan EX, USA). The cytotoxic concentration (IC_{50}) was determined for the materials.

RESULTS

The bacterial protease, which is used to separate placental membranes such as amnion and chorion from placenta, achieves complete and easy recovery of amnion from placenta within 30 min at 30 °C without causing any damage to amnion. The concentration of enzyme used for achieving separation was 1000 CDU. The recovered translucent amnion is having high surface area and is devoid of any tissue debris, blood cells, blood clot etc. The harvested amnion was treated with 0.1 M sodium acetate and further cross linked with gluteraldehyde to provide stability to the membrane.

Biophysical characterization of amnion materials

All the characterization studies for enzymatically separated amnion (experimental group) were performed along with amnion recovered by conventional method (control group) and the properties were compared. Before testing the samples for physical characteristics, the samples were conditioned at 1 day at 25 °C and 65 ± 2 relative humidity. For, experimental groups involving enzymatic method, the optimized conditions were employed. The physical characteristics studies employing amnion showed that there were no significant differences between the control (conventional) and the experimental (enzymatic) groups **(Table 1)**. This suggested that the enzymatically separated membranes were having biomechanical properties comparable to that of conventional methods of separation. The thickness of chorio-amnion was higher than the sum of amnion and chorion and this implied the presence of basement membrane between amnion and chorion that could be extensively digested by protease enzymes. There was a slight reduction in the physical parameters for experimental group was observed when compared to control group; this could be due to the enzymatic digestion of certain components such as non-collagenous proteins, proteoglycans, stromal matrix components etc. However, the mechanical properties such as strain value and tensile strength were comparable between control and experimental groups indicating that the amnion membrane could be exploited for therapeutic purposes including the wound healing purposes since the collagen contents were probably preserved in these membranes. Loss in the content of collagen due to enzymatic digestion potentially affects the strength properties. This indicated that the enzyme preparations used in the study is non collagenase in nature but affects other proteins. The enzymes act on non-collagenous protein components and proteoglycans of basement membrane (BM) intermediate layer that cements amnion and chorion together. Due to digestion of these components of BM layer by enzyme treatment, the amnion and chorion were separated. High oxygen and water vapor permeability values of enzymatic method validated higher porosity and well opened up fiber structure for the experimental amnion membranes. Hence, the developed enzymatic method of placental membrane separation is advantageous in terms of bio-mechanical properties and could be conveniently used for therapeutic applications.

The chemical compatibility of prepared samples were analysed by FT-IR spectroscopy (Fig. 1 and 2; Table 2 and 3) in the wavelength range of 500-4000 cm^{-1}. Amnion membranes derived by both methods show all necessary peaks of collagen in IR bands at the region of 1646 cm^{-1} (C=O stretching) for amide I; 1550 cm^{-1} (N–H bending) for amide II; 1247 cm^{-1} (C–N stretching) for amide III and 3428 cm^{-1} for O–H stretching[15,16]. However, the amnion membrane obtained by enzymatic treatment showed various distinct peaks in the region of very broad band spreading 3100–3500 cm^{-1} due to N-H and polyhydroxy OH group; 2955 cm^{-1} due to C-H stretch; 1240 cm^{-1} due to S=O of sulfate esters; 1076 cm^{-1} due to C=O stretch of cyclic ethers; 626 cm^{-1} due to C–O–S of axial sulphate on C-4 of galactose. The FT-IR spectra of prepared Amnion derived by both methods shows all characterise peaks of amnion collagen indicating the chemical integrity of the materials.

Scanning Electron Microscopy (SEM) studies

Scanning electron microscopy analyses (SEM) of amniotic membrane derived by conventional method pointed out a topography of rough collagen rich amnion layer with approximately 6 µm thick columns packed together (Fig. 3). Moreover, the conventional separation method showed that the collagen in the amnion was not significantly fibrous when compared to enzymatic method. Transverse sections of the Amnion material (conventional) showed the columns are not be regularly spaced long and hollow whereas amnion derived by enzymatic treatment showed uniformly well spaced or even-porous nature with devoid of non-collagenous content indicating that the material could facilitate exchange of oxygen, transportation of drugs if it gets loaded with and undamaged internal collagen architecture.

Further, the SEM images of enzymatically treated and separated amnion show the following features: (a) collagen fibers are well opened up without any damage; (b) Well networked fibers (collagen mesh); and (c) spacing of collagen fibers and fiber bundles evenly; long & hollow. These properties indicated that

the amnion processed by enzymatic method is having better physical properties than amnion obtained by conventional mode. Permeability to oxygen allows the wound to prevent hypoxia and desiccation due to porosity and well opened fibrous architecture of the materials.

Cell Viability Assay

It was observed that homogenized amnion derived from enzymatic method exhibited IC_{50} concentration of about 1.5 mg/mL against fibroblast cell lines (Fig. 4C) indicating that the material is non toxic in nature even at its highest concentration when compared to control untreated cells (Fig. 4A). Fig. 4B shows the intact nature of cells treated with non toxic concentration (1 mg/mL) of homogenized amnion.

The cell viability studies further indicate that the high content of collagen present in amnion scaffold might have facilitated the cell growth up to a threshold of 1.5 mg/ml. The collagen is basically non toxic nature and it is safe to use because of high compatibility and non antigenicity. The amnion devoid of non-collagenous content (as non collagenous proteins are removed by enzymes) is also safe to use for therapeutic purposes.

Table 1. Physical properties of membranes obtained by conventional (manual) and enzymatic methods

	Conventional method	Enzymatic method
Amnion thickness (µm)	64 ± 4.7	61 ± 5.4
Chorion thickness (µm)	295 ± 46	283 ± 36
Chorioamnion thickness (µm)	391 ± 58	373 ± 48
Water content of Amnion (%)	86.8 ± 3.1	87.4 ± 2.2
Water content of Chorion (%)	89.1 ± 2.8	88.4 ± 2.4
Water content of Chorioamnion (%)	87.3 ± 3.2	88 ± 3.5
Amnion/Chorion wet mass ratio (%)	10.4 ± 3.2	9.8 ± 2.7
Amnion/Chorion dry mass ratio (%)	14.1 ± 1.7	13.2 ± 2.3
Strain value (E_f) by Bulge test	46.2 ± 2.1	44.9 ± 2.3
Tensile strength (N mm^{-2}) for Amnion	218 ± 36	222 ± 43
Tensile strength (N mm^{-2}) for Amnion	28 ± 3.7	29.2 ± 4.2
Elongation (%) for Amnion	9535±62	10910±49
Oxygen permeability for Amnion (cc/m^2/24 h)	1931±36	2109±28
Water vapour permeability for Amnion (g/m^2/24 h)	26.14±1.68	28.36±1.11

Experiments were performed in triplicates and expressed as mean + SD

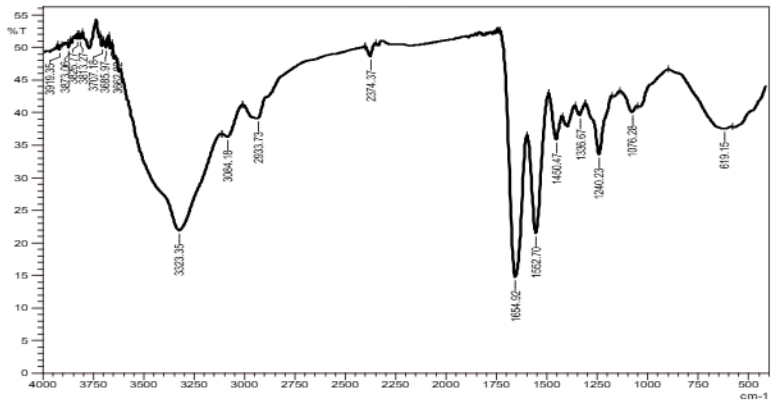


Fig. 1. FT-IR studies on amnion obtained by conventional separation

Table 2: FT-IR studies on amnion obtained by conventional separation (Control)

Functional Group	Value of the peaks	Characteristic absorptions (cm^{-1})	Type of vibration	Intensity
O-H	3323.35	3200 – 3600	Stretch, H-bonded	Strong, broad
N-H	3084.18	2500-3300	Stretch	Strong, Very broad
C=O	1654.92	1620-1680	Stretch	Variable
C=C	1552.70	1400-1600	Stretch	Medium-weak, multiple bands
C-N	1240.23	1080-1360	Stretch	Medium-weak
C-0	1076.28	1000-1300	Stretch	Two bands or more

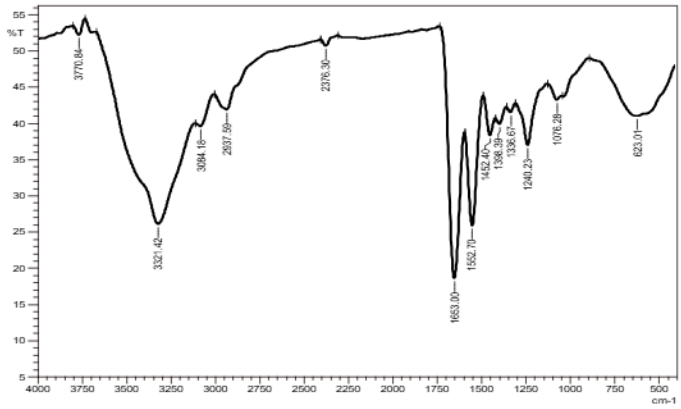


Fig. 2: FT-IR studies on amnion obtained by enzymatic treatment and isolation

Table 3: FT-IR studies on amnion obtained by enzymatic treatment (Experimental)

Functional Group	Value of the peaks	Characteristic absorptions (cm^{-1})	Type of vibration	Intensity
N-H	3321.42	3100-3500	Stretch	Unsubstituted, has two bands
C=O (amide)	1653.00	1640-1690	Stretch	Strong
N-H (amide)	1552.70	1550-1640	Bending	-
C=C	1452.40	1400-1600	Stretch	Medium-weak, multiple bands
-C-H	1398.39	1350-1480	Bending	Variable

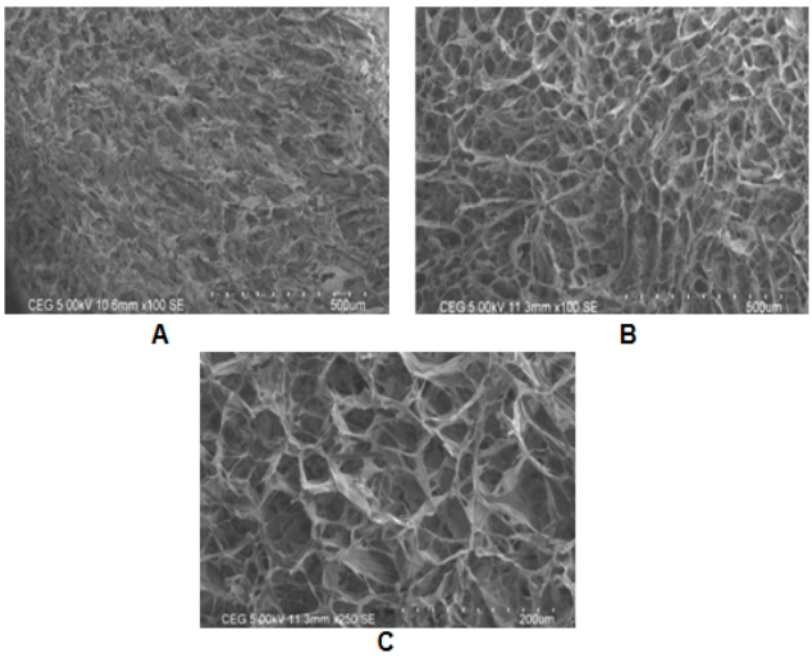
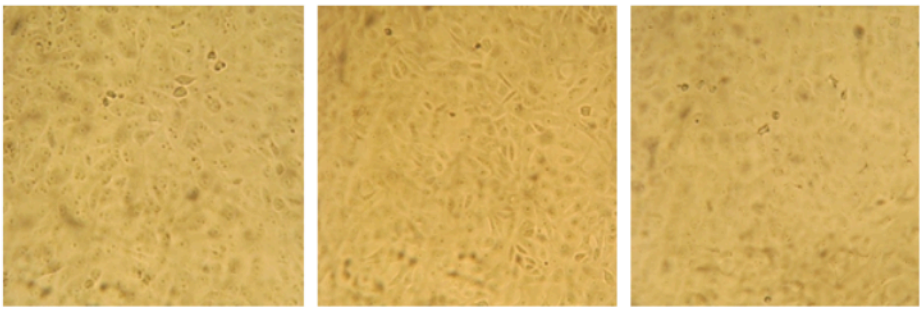


Fig. 3: SEM analysis of amnion scaffold obtained by (A) Conventional and (B) Enzymatic methods; (C) Magnified image of enzymatically separated amnion



A B C

Fig. 4: MTT assay shows non toxic nature of contents of amnion bioscaffold. (A) Conventional and (B) Enzymatic methods; (C) Magnified image of enzymatically separated amnion

DISCUSSION

Effective wound therapeutic biomaterials should induce conversion of cells at the wound site from senescence to active state so as to achieve complete regeneration of healthy skin. Besides, the wound dressing biomaterials should necessarily contain well-defined therapeutic components and desirable physical, chemical and biological properties to support optimal delivery of therapeutics at the site of wound. The therapeutic biomaterials are expected to have large surface area with porosity and mass transfer features for enabling effective wound cover, aeration, exudate removal and delivery of therapeutics, to facilitate proliferation of cells and controlled biodegradation for replacement with neo-tissues besides exhibiting low toxicity and high biocompatibility[17].

Collagen-based biomaterials remain as a treatment of choice for wound therapy as they possess the chemotactic ability of activating fibroblasts, and thereby encourage the deposition and organization of newly formed collagen so as to create an environment that fosters healing. Also, they have other preferable properties such as smooth adherence to the wound bed, absorbance of wound exudates, preserving moist environment, shielding against mechanical harm and preventing secondary bacterial infections, facilitating fibroblast growth and minimizing matrix metalloproteinase (MMP) activities, biocompatibility, and safety[18,19]. Collagen based dressing materials suffer the limitation of the requirement of high collagen content with right collagen types as well as other ECM substances for effecting wound healing.

Biotherapy with amnion has more advantages than other bioengineered skin substitutes or collagen materials in terms of wide availability, easy processing and low-cost production[20]. Though natural placental membranes and placental membrane based dressing materials provide high collagen with right collagen types for wound healing, they suffer the limitation of the presence of non collagenous ECM substances or changes in the structure of collagen arrangements due to processing. These unwanted non-collagenous ECM substances have to be eliminated from amnion for its use as biotherapeutic purposes. Hence, there exists a need to develop a process using protease for separation of placental membranes from placenta, which is devoid of above said drawbacks. To address this need, a one step enzymatic process to separate amnion from placenta was developed using protease. The protease acts on non-collagenous protein components and proteoglycans of Basement Membrane (BM) intermediate layer that cements amnion and chorion together. Due to digestion of these components of BM layer by enzyme treatment, the amnion and chorion were separated. Proteases, having the ability to act on proteins of stromal matrix separating amnion and chorion membranes as well as proteins of chorion and desidua of the placenta, can be used in the process to produce quality amnion rich in collagen without any damage as caused by mechanical and chemical methods. Besides, the proteolytic action yields quality biomaterial product having the desired mechanical properties. The protease lacked the collagenolytic and elastolytic activities otherwise they would have reduced the quality of amnion material by damaging collagen and elastin components.

The physical and SEM validation of amnion materials recovered by enzymatic method was carried out while comparing the properties with conventionally recovered amnion. Their biophysical characterization indicates that the material permits modulating the local wound environment and by its systemic effects. Tensile strength of the materials developed here reinforces the desirable mechanical properties suitable for handling them for clinical applications, while permeability to oxygen and water vapor allow the wound to prevent hypoxia and desiccation as well as to facilitate diffusion of wound heal substances, the important factors in wound healing[21,22,23,24,25]. High oxygen and water vapor permeability values of enzymatic method validated higher porosity and well opened up fiber structure for the experimental amnion membranes. The FT-IR spectra of prepared amnion derived by both methods shows all characteristic peaks of amnion and collagen indicating the chemical integrity of the materials.

SEM studies with the recovered amnion material (conventional) showed collagen rich scaffold characterized with uniformly well spaced or even-porous nature with devoid of non-collagenous content indicating that the material could facilitate exchange of oxygen, transportation of drugs if it gets loaded with and undamaged internal collagen architecture. These analyses indicated that enzymatic processing retained the original architecture and amnion scaffold therefore could be molded into various configurations and its hollow columns acting as an absorbent to imbibe exudates from the wound site, or be loaded with drugs. SEM analyses indicated that enzymatic processing completely removed chorion while retaining the desirable architecture of the amnion layer.

The enzymatically recovered amnion exhibited IC_{50} concentration of about 1.25 mg/mL against fibroblast cell lines suggesting that the material is non toxic in nature even at its highest concentration. The high content of collagen present in amnion scaffold might have facilitated the cell growth up to a threshold of 1 mg/ml. The collagen is basically non toxic nature

and it is safe to use because of high compatibility and non antigenicity. As non collagenous proteins in amnion are removed by enzymes, the amnion is also safe to use for therapeutic purposes.

Further, the process and the materials have the following salient features and advantages over the other conventional methods disclosed in the literature: (a) consistency and uniformity in the process, leading to better product quality, avoid tearing, rupturing, abrasion, scratching etc. and associated damages to the membranes; (b) reduction or elimination of manpower and energy; (c) cleansing of membranes is facilitated since unwanted substances such as blood, protein etc. are removed; (d) Transmittance of infection, if any, can be reduced with respect to handling of the placental materials; (e) Duration for the separation is reduced, especially, dealing with bulk quantity; (f) single step process enables to isolate both amnion and chorion membranes from placenta; (g) upon extension of enzyme treatment duration, the contents of the amnion and chorion membranes are loosened further, which facilitates easier processing of the membranes to isolate progenitor cells, collagen proteins, hyaluronic acid, glycosaminoglycans, growth factors etc; and (h) improves the adherence of the amniotic membrane on to the damaged surface in the human body. Since the process can be executed in controlled environment, scalability and development of process validation abiding to Good Manufacturing Practice (GMP) norms is possible.

CONCLUSION

Amnion based tissue regenerative materials are advantageous than collagen biomaterials due to their affordability and efficiency. To add more therapeutic value to the amnion based wound dressing materials in terms of retaining high collagen content as well as tissue regenerative substances devoid of antigenicity, we used protease enzyme to treat placenta and further isolate amnion from placental deciduas. The amnion recovered from this method has enormous advantages in terms of scalability, desirable mechanical and biophysical features, high cell viability but no toxicity, biocompatibility etc. Besides, the porous material is rich in collagen contents and can be suitably loaded with drugs for the optimal delivery of skin regenerative substances. This is the first study on use of enzymes to recover therapeutically valuable placental membranes. Further *in vivo* studies using animal model would explore the potential of this material for clinical applications.

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