Original Article

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Protease-Mediated Recovery of Amnion from Placenta and Its Application as a Bioprocessed Therapeutic Biomaterial for Wound Healing

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Abstract:

Objective: Wound healing presents significant challenges to healthcare systems, particularly in the management of chronic wounds. Amnion, a collagen-rich membrane traditionally discarded after childbirth, has shown promise in skin regenerative medicine due to its anti-inflammatory, antimicrobial, and biocompatible properties. However, conventional methods for separating amnion from the placenta often result in tissue damage and inconsistent material quality, limiting their therapeutic potential. This study introduces an enzymatic process using *Bacillus sp. S2 MTCC 13117* protease to separate the amnion from the placenta while preserving its collagen structure and mechanical properties.

Material and Methods: The enzymatic treatment effectively removes non-collagenous proteins and epithelial layers, creating high-quality amnion suitable for wound-healing applications. Biophysical characterization of the biomaterials demonstrated suitable tensile strength, permeability to oxygen and water vapor, and high surface area, all critical for effective wound coverage and healing.

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Results: In vitro studies showed that amnion scaffolds promoted fibroblast proliferation with low toxicity and high biocompatibility, while *in vivo* rat models indicated significantly faster wound healing, with complete closure observed in 7 days compared to 12–14 days for controls. Histological analysis confirmed enhanced collagen organization and skin structure regeneration in treated wounds.

Conclusion: These results demonstrate that enzymatically processed amnion scaffolds are an effective and cost-efficient alternative for wound care, with superior healing outcomes compared to conventional therapies. This study supports the use of amnion-derived biomaterials in treating chronic wounds, including diabetic and venous leg ulcers.

Keywords: amnion, chronic wounds, collagen scaffolds, enzymatic recovery, wound healing

Introduction

Wound healing involves blood cells, the extracellular matrix, growth factors, and cytokines. It includes granulation tissue formation and connective tissue synthesis. Coordinated interactions are crucial, as imbalances can cause chronic wounds, increasing infection risk and severe complications like sepsis and amputations[1,2]. Acute wounds heal within a standard timeframe, while chronic wounds, often lasting months or years, result from prolonged healing marked by persistent inflammation and tissue degradation. Common in conditions like diabetes, leprosy, and venous insufficiency, they significantly affect health and quality of life. Diabetic foot ulcers occur in 1–4.1% annually, with amputation rates up to 1.37%[3]. A history of foot ulceration in diabetic patients correlates with a 47% higher 10-year mortality rate compared to those with no prior ulcers[4].

Treating chronic wounds is costly for both patients and healthcare providers[5], highlighting the need for faster, more efficient healing methods and strategies to prevent recurrence, particularly in diabetic patients[6]. Chronic wounds are characterized by excessive exudates, increased protease activity, dysfunctional extracellular matrix (ECM), and impaired epithelialization and vascularization, which hinder healing. These local factors contribute to systemic effects, such as elevated production of chemokines and inflammatory cells, further delaying wound healing[7]. Underlying conditions like neuropathy, diabetes, peripheral vascular disease, infections, and overall health status exacerbate delayed healing, increasing the risk of recurrence, amputation, and mortality[8]. Effective ulcer management requires addressing both local and systemic factors to reduce infection risks, prevent ECM degradation, and promote healthy granulation tissue growth[9].

Biomaterials offer a means to induce gradual changes at the wound site, making them effective as wound dressings for drug delivery, growth factors, or cell therapy[10,11]. They also promote systemic effects that support healing, addressing challenges that impede healing. For effective treatment, it is crucial to address both local and systemic factors. Various biomaterials, such as collagen or amnion-based dressings, keratin compositions, phytochemicals, and natural polysaccharides, provide options for treating acute and chronic wounds. Their efficacy varies based on factors like adherence, angiogenesis, hydration, and the delivery of healing substances[12–16]. Though chitosan, collagen, amnion, and silicon-based dressings are commonly used, their effectiveness in treating chronic ulcers remains suboptimal.

Amniotic membrane (AM), a collagen-rich, translucent membrane, is a valuable source of stem and progenitor cells. It offers anti-adhesive, antimicrobial, anti-inflammatory, and biocompatible properties with minimal immunogenicity, making it useful in skin regenerative

medicine and clinical applications[17]. Amnion and amnion-based scaffolds are increasingly used in regenerative medicine for treating burns, wounds, diabetic ulcers, leprosy ulcers, corneal ulcers, venous leg ulcers, ocular surface disorders, and other conditions[16,18].

Rich in collagen proteins, amnion is a promising resource for developing affordable biomaterials with therapeutic potential[16]. Amnion-derived biomaterials provide progenitor cells, growth factors, and collagen, modulating the wound environment to enhance healing in chronic wounds[15]. This study develops a protease-based process for safer, more efficient amnion separation, overcoming the limitations of manual dissection and chemical treatments. Using *Bacillus sp. S2 MTCC 13117* protease, the placental membranes are separated after cleansing and enzyme immersion. Recovered membranes undergo physico-chemical analysis, scanning electron microscopy (SEM), histochemical examination, and animal histology for skin regeneration assessment.

Material and Methods

Standard care and ethical permits

Human placenta was collected post-delivery from mothers who tested negative for infectious agents, including human immunodeficiency virus (HIV), hepatitis viruses, and syphilis, confirmed by the Department of Microbiology, Dr. ALM Post Graduate Institute of Basic Medical Sciences (ALM PGIBMS), University of Madras, Chennai. The experiments involving placenta collection, processing, amnion recovery, and physical and chemical sterilization were conducted at the Tissue Bank Facility at the King Institute of Preventive Medicine and Research (KIPMR), Chennai. All procedures were approved by the institutional ethical committee (KIPMR/IEC/2019/003/TB/Amn, dt 24/01/2018). Dulbecco's Modified Eagle Medium (DMEM) and Fetal Bovine Serum (FBS) were purchased from Invitrogen, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) from Hi-Media Laboratories, India. Antimicrobials, including amphotericin B, gentamicin, chlorhexidine, and metronidazole, were also sourced from Hi-Media. Animal procedures adhered to national guidelines, approved by the ethical committee at the Department of Microbiology, ALM PGIBMS, University of Madras, Chennai, with oversight by an in-house veterinarian[19,20]. Skin samples were carefully dissected from the perfused mice and placed in 4% Paraformaldehyde (PFA) in Phosphate Buffered Saline (PBS), where they were kept overnight at 4 °C for fixation. All unused biological materials were disposed of as biowaste, adhering strictly to standard guidelines for biowaste handling.

Enzymatic and conventional processing of human placenta and preparation of amnion membrane

A total of 12 fresh human placenta samples were obtained from the Tissue Bank Facility of the King Institute of Preventive Medicine and Research, Chennai, which collects placentas from healthy deliveries—after obtaining informed consent from mothers in the maternity wards of the tertiary care hospitals in Chennai. Blood clots and debris were removed with saline and 0.1 molar (M) PBS. Placentas were immersed in PBS with bacterial protease (500 casein digestion unit (CDU)/kg, Bacillus sp.), free of collagenase and elastase. Protease activity, measured via casein substrate[21], was maintained at 30 °C for 30 minutes. A 45-minute enzymatic treatment (1,000 CDU/g amnion) removed the epithelial layer. Membranes were washed with PBS containing antimicrobials, ultra violette (UV) sterilized (30 minutes), and radiosterilized (25 kilogray (kGy), ^{60}Co gamma)[22].

Biophysical characterization of biomaterials and validation studies

Biophysical characterization included tensile and tear strength, water uptake, porosity, oxygen and water vapor

permeability, degradation, and swelling analysis for Human Amniotic Membrane (HAM) and Human Amniotic Membrane Scaffold (HAMS) recovered conventionally and enzymatically[14]. Thickness was measured using a magnetic induction probe on dried 1×3 square centimeter (cm^2) membranes (n=6). Tear and tensile strength tests used 2×3 centimeters (cm) films on a Mocon Permatran at 36±1 °C per ASTM F 1249-90 standards, yielding stress, strain, and WVTR data. A 5 psi Pressure Transducer (Sensotec, Model GM-A) measured pressure. Water uptake was assessed by weighing films before and after immersion. Swelling analysis incubated 200 micrometer (µm) thick, 1 cm^2 blocks in PBS (pH 7.4) for one week. Tests compared manual and enzymatic processing for therapeutic suitability.

Preparation of collagen scaffolds from amnion

The prepared membranes underwent enzymatic treatment in a 100% weight/volume (w/v) Amn bath with 1,000 CDU/kg protease for 2 hours to obtain epithelium-free amniotic membranes for collagen extraction. Ten grams of epithelium-free HAM was treated with 100 milliliters (mL) of 0.5 M acetic acid for 24 hours, homogenized at 15,000 revolutions per minute (rpm) (4 °C) for 20-30 minutes. A 5% Chitosan-Gelatin stock was prepared. To 20% HAM extract, 3% Chitosan (90% deacylated) was added, homogenized at 14,500 rpm for 5 minutes, then mixed with 5% Gelatin and Triton X-100. The foam solution was frozen at -80 °C overnight and lyophilized for 5-6 hours to obtain HAMS.

Fourier transform infrared spectroscopy (FT-IR)

FT-IR was used to obtain the infrared spectra (IR) of collagen scaffold biomaterials prepared as described previously. The measurements were conducted at a range of 4,000 to 650 cm^{-1} with a phase resolution of 128 and an averaging rate of 25 scans per minute using a Bruker IFS 28 Equinox infrared spectrophotometer.

SEM

SEM was employed for the analysis of collagen scaffolds, as described earlier, prepared from amnion separated by conventional and enzymatic methods. For SEM analyses of scaffolds, the dried scaffold samples were coated with gold (20 nanometer (nm)) by direct current sputtering under vacuum. The cross sections of coated samples were viewed using a JEOL 8401 scanning electron microscope operated at an accelerating voltage of 12 kilovolts (kV).

Cell culture and cell viability studies

To assess the effect of amnion-derived collagen scaffolds on cell viability, circular patches were prepared from enzymatically isolated amnion and placed in 96-well plates. Human foreskin fibroblasts ($4x10^3$ cells/well) were incubated overnight in DMEM with 10% FBS and Pen-Strep. Pre-soaked scaffolds were placed on cells for 2 days, followed by MTT assay[23]. Cells ($3x10^4$ cells/well) were treated with material concentrations (250–1,500 micrograms (µg)/mL) for 120 hours, including solvent (0.2% DMSO) and cell controls. After treatment, 20 microliter (µL) of MTT solution (1 mg/mL) was added, and plates were incubated at 37 °C for 3 hours. Absorbance was measured at 620 nm to determine cytotoxic concentration (IC50). Growth induction and scaffold toxicity were further assessed via 2′,7′-dichlorofluorescein (DCF) and 4′,6 diamidino-2-phenylindole (DAPI) staining, examining fibroblast cell morphology microscopically.

Histology studies

For pre-processed, post-enzymatic (protease-treated), and conventionally processed placenta or recovered amnion, tissue samples were embedded in paraffin following standard procedures, and 5 µm sections were mounted onto glass slides. These sections were prepared for microscopic examination to assess any pathological changes. The slides

containing amnion were subjected to a series of staining techniques, including Haematoxylin and Eosin (HE) staining to examine overall tissue architecture and cell morphology, Masson's Trichrome (MT) staining to assess collagen integrity, Alcian Blue-Periodic acid-Schiff (AB-PAS) staining to detect polysaccharides and glycoproteins, Picrosirius Red (PR) staining to analyze collagen content and Verhoeff's staining for determining elastin content, allowing for a comprehensive analysis of tissue composition and structure.

Wound healing studies using the Wistar albino rat model

Healthy Wistar albino rats (250 grams, male) were used. Excisional wounds (0.5 mm^2) removed epidermal, dermal, hypodermal, and panniculus carnosus layers[24]. Rats were grouped into control (no biomaterial), AMN-treated, and AMNS-treated. Biomaterials were fitted to wounds and secured. Infected rats were replaced. Healing was tracked via daily observations, photos, and wound diameter reduction. On day 14, 0.5 square millimeter (cm^2) of healed skin was excised, fixed in paraformaldehyde, paraffin-embedded, and sectioned (5 micrometers (µm)). Staining assessed pathological changes in the healed tissue.

Results

Enzymatic recovery of amnion membrane from placenta

The bacterial protease used to separate placental membranes, such as amnion and chorion, from the placenta achieved complete and gentle recovery of amnion within 30 minutes at 30 °C, without causing damage. The enzyme concentration used for separation was 500 CDU. Further, treatment of the recovered amnion with 1,000 CDU enzyme concentration for 45 minutes resulted in an amnion membrane devoid of the epithelial layer. The recovered translucent amnion had a high surface area and was free from tissue debris, blood cells, and blood clots. The harvested amnion was treated with 0.1 M sodium acetate and cross-linked with glutaraldehyde in order to enhance membrane stability.

Table 1 Physical features of amnion membranes recovered by manual and enzymatic methods

Physical and mechanical features	Conventional method		Enzymatic method	
	Amnion with epithelial layer	Amnion[#] devoid of epithelial layer	Amnion with epithelial layer	Amnion[##] devoid of epithelial layer
Thickness (µm)	55.11±3.9	47.23±4.6	52.50±4.7	42.87±2.9
Water content (%)	88.91±1.9	89.12±1.7	89.35±2.1	90.44±1.0
Strain value (E_f) by Bulge test	43.30±1.7	39.67±2.8	41.54±3.1	39.32±2.4
Porosity	87.68±2.5	90.22±2.3	89.51±1.4	92.74±1.7
Water uptake				
Tensile strength (MPa)	36.22±2.1	37.13±1.6	34.50±2.4	33.29±1.5
Elongation at break (%)	93.34±1.5	92.65±2.4	91.11±2.4	90.79±2.9
Oxygen permeability for Amnion ($cc/m^2/24$ h)	1891±57	1937±65	2001±71	2137±69
Water vapour permeability for Amnion ($g/m^2/24$ h)	27.31±1.43	28.99±2.01	29.01±1.37	29.49±1.94

[#] Amnion recovered by the conventional method is subjected to enzymatic treatment to obtain amnion devoid of epithelial membrane

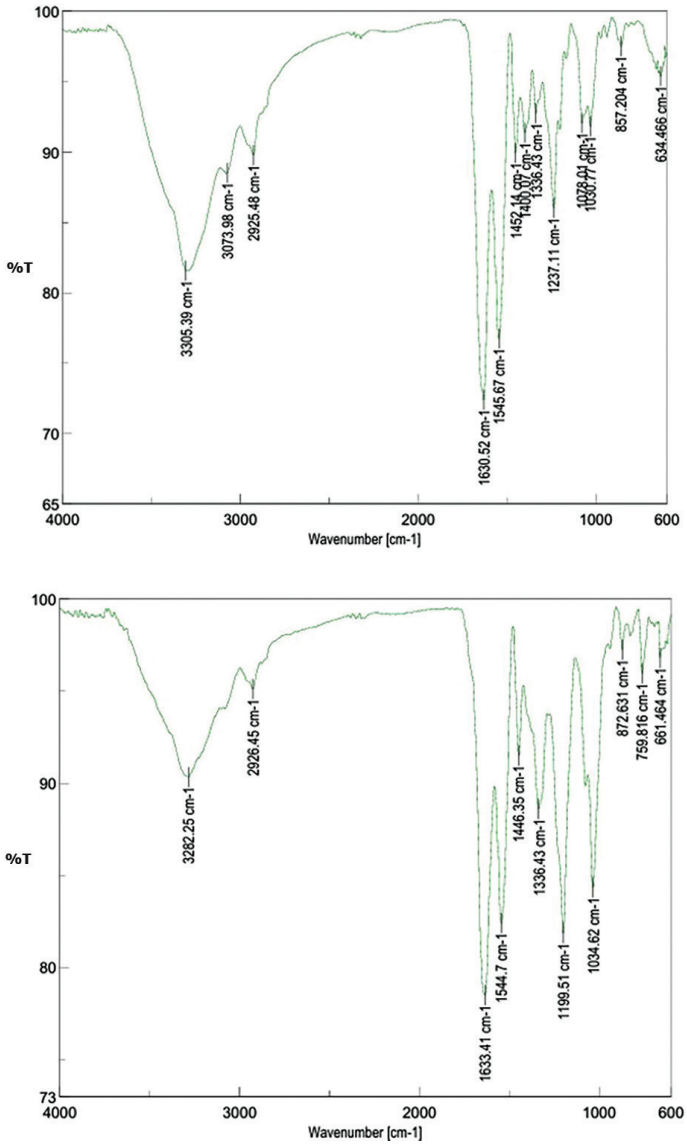
[##]Amnion recovered by the enzymatic method is subjected to further enzymatic treatment to obtain amnion devoid of epithelial membrane

µm-micro molar

Ef=Strain value measured during the bulge test, MPa=Megapascal, $cc/m^2/24$ h=cubic centimeters per square meter per 24 hours, $g/m^2/24$ h=grams per square meter per 24 hours

Biophysical characterization of amnion materials

Characterization studies of enzymatically separated amnion (experimental) and conventionally recovered amnion (control) analyzed their properties. Samples were conditioned for 1 day at 25 °C and 65±2% humidity before physical testing. Optimized enzymatic conditions were used. No significant biomechanical differences were found (Table 1). Chorio-amnion thickness (361±52) exceeded the sum of separated layers, indicating basement membrane digestion. Enzymatic digestion slightly reduced thickness, strain, and tensile strength, but other mechanical properties remained comparable. Enzymatically separated membranes showed



FT-IR=fourier transform infrared spectroscopy

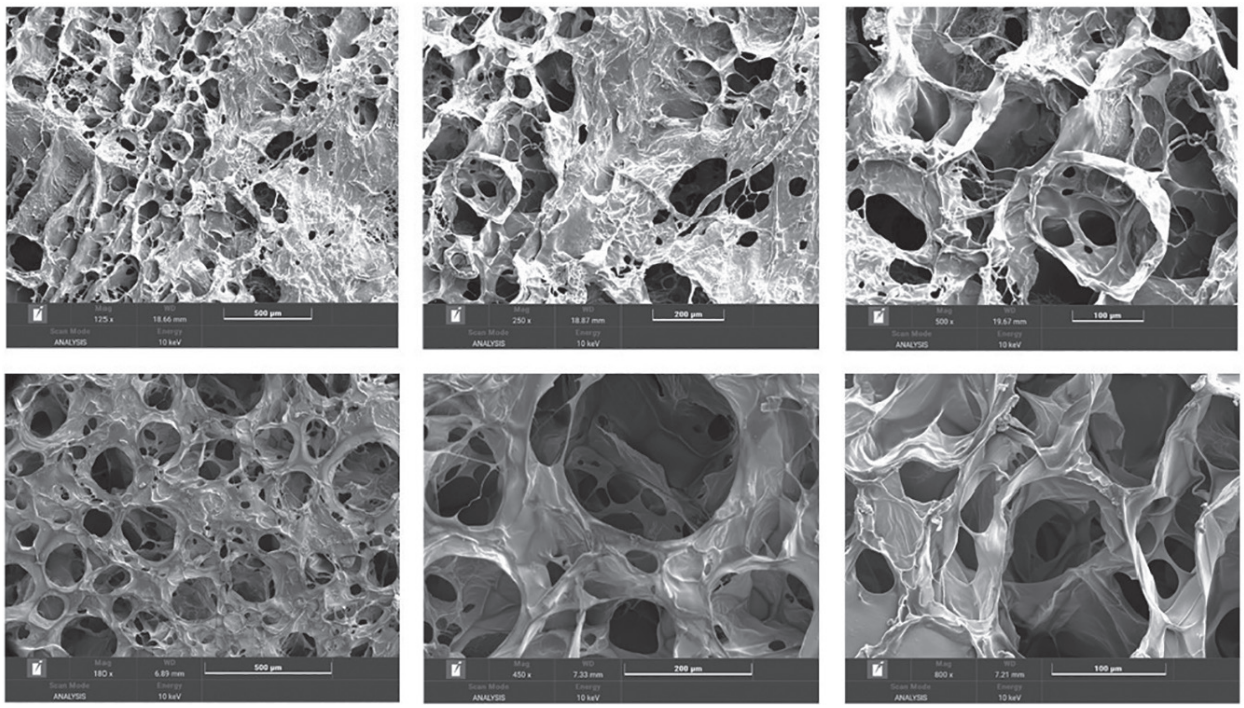
Figure 1 FT-IR spectrum of collagen scaffolds prepared from amnion separated by Conventional and Enzymatic methods

high oxygen/water vapor permeability, enhanced collagen, and reduced immune rejection, benefiting wound healing applications.

FT-IR studies

The FT-IR spectra of collagen scaffolds from amnion, prepared via conventional and enzymatic methods, showed distinct IR bands for collagen and chitosan. The spectrum (Figure 1) displayed characteristic collagen bands at 1545 cm–1 (amide II) and 1236 cm–1 (amide III). The amide I band arises from C=O stretching in protein amides, while amide II results from N–H bending and C–N stretching. Amide III involves C–N stretching, N–H bending, and CH2 wagging from glycine and proline. Additional bands at 1450 cm–1 (pyrrolidine ring stretching) and 1205 cm–1 (N–H stretching) were observed.

Chitosan absorption bands at 3280-3305 cm–1 and 2923-2926 cm–1 corresponded to –OH and –CH3 groups, respectively. Bands at 1545-1630 cm–1 and 1400 cm–1 were linked to N–H and –OH bending, while 1337 and 1030 cm–1 corresponded to C–O–N and C–O stretching. Bands at 1034 and 872 cm–1 indicated glycosidic bond vibrations, while 1631 cm–1 represented C=O stretching and the amide I band. The collagen triple helix's integrity, assessed by the 1235/1450 cm–1 absorbance ratio, was 0.96 in scaffold (a) samples, confirming that chitosan did not destabilize the collagen's structure, which is crucial for its biological and mechanical properties.



SEM=scanning electron microscopy

Figure 2 SEM analyses of Collagen scaffolds prepared from amnion separated by Conventional (A-C:125x; 250x; 500x) and Enzymatic methods (D-E:180x; 450x; 800x)

SEM studies

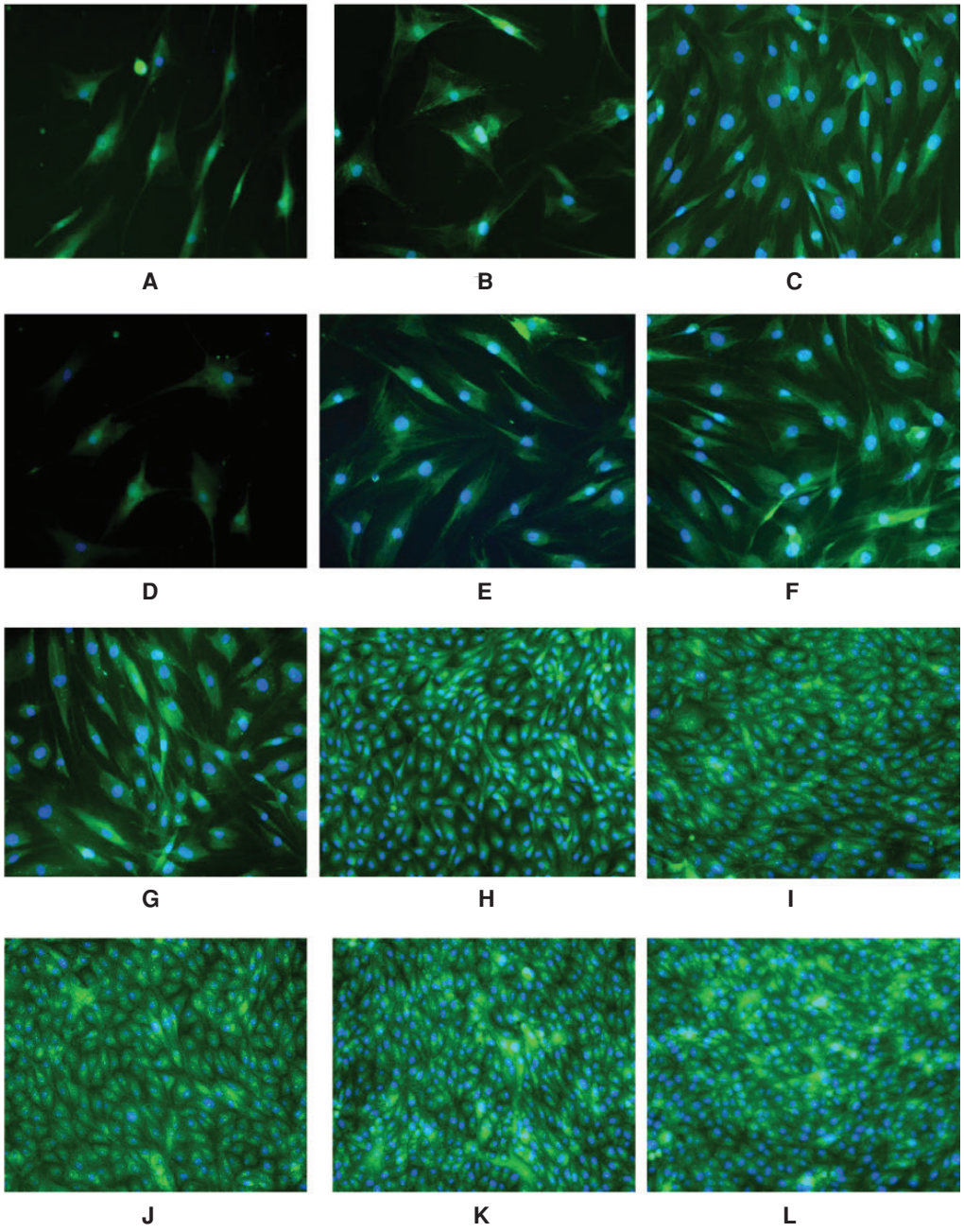
SEM analysis of collagen scaffolds from amnion, isolated via the conventional and enzymatic methods, showed distinct structural differences (Figure 2). Conventional scaffolds had rough collagen distribution, uneven porosity, and compact debris, limiting effectiveness. Enzymatic scaffolds exhibited smoothness, uniform porosity, and no non-collagenous content, indicating effective interfibrillar substance removal. Higher magnification SEM revealed well-opened, undamaged collagen fibers, a networked collagen mesh, and evenly spaced, hollow fiber bundles. These properties enhance oxygen exchange, drug delivery, and collagen integrity. Enzymatically processed amnion offers superior physical and biological traits, promoting oxygen permeability, preventing hypoxia and desiccation, and supporting improved wound healing.

Cell viability studies

The *in vitro* effects of collagen scaffolds prepared from enzymatically isolated amnion were assessed by treating human foreskin fibroblast cells with scaffold concentrations ranging from 50 to 1,000 µg/mL, against untreated cells (Figure 3). Cells cultured with scaffolds made from enzymatically isolated amnion (Figure 3D-L) exhibited increased growth and viability, even after serum withdrawal from the medium, in contrast to the untreated control cells, which displayed reduced cell distribution throughout the culture period (Figure 3A-C). Scaffold-treated cells reached confluence at 120 hours, while untreated cells took 160 hours, indicating enhanced cell growth. DCF-DAPI staining confirmed that the scaffold promoted fibroblast proliferation and viability, with intact cell morphology at 1 mg/mL, supporting the scaffold's nontoxicity. The scaffold derived through the enzymatic method demonstrated an IC_{50} concentration of approximately 1.5 mg/mL for the fibroblast cell lines, indicating that the material is non-toxic even at the highest concentration tested. Figure 3H-L further demonstrates the intact morphology of cells exposed to scaffold concentrations ranging from 50 µg/mL to 1 mg/mL, a range found to be non-toxic.

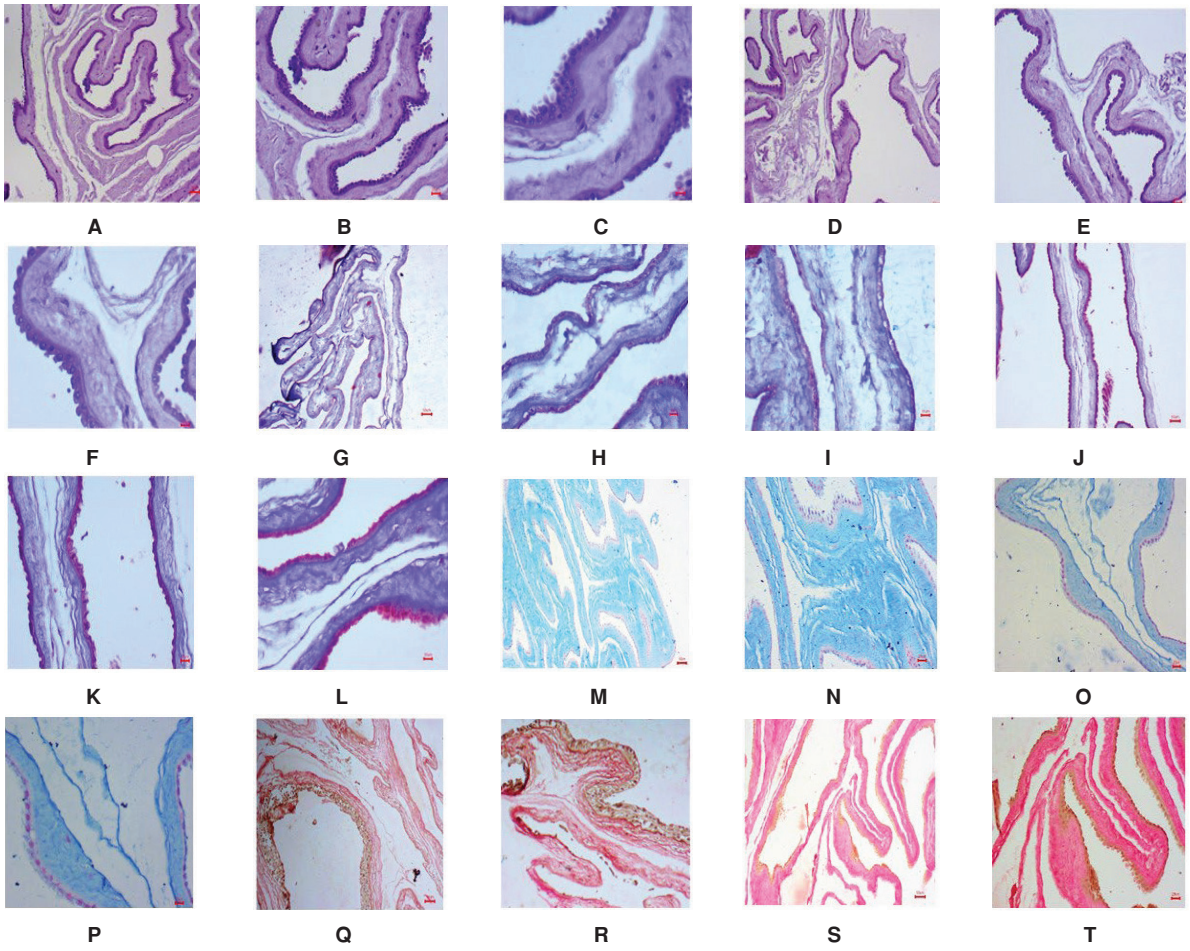
Histochemical studies

Histochemical examination of amnion separated by both conventional and enzymatic methods revealed that amnion recovered by either method is of good quality, exhibiting comparable collagen content and a uniform epithelial layer firmly attached to the amniotic mesenchymal or fibroblast layer through the basement membrane (Figure 4). HE and MT staining revealed a distinct epithelial layer with amniocytes on the surface of the amniotic membrane, with collagen stained light purple (HE) or blue (MT). AB-PAS and PR staining confirmed connective tissue matrix components. The enzymatic method resulted in a slightly loosened collagen structure, suitable for wound healing. Histochemical studies also demonstrated that the epithelial layer was completely removed when enzymatically recovered amnion was subjected to an additional 45-minute enzyme treatment (Figure 5). This is particularly important for applications where amnion without an epithelium is needed, as epithelial cells (amniocytes) are slightly differentiated and may trigger immunogenic responses when the amnion material is applied to wounds or damaged tissues for regenerative purposes[26]. HE, MT and AB-PAS staining of post processed amnion by additional enzyme treatment showed the presence of only the collagen layer, with a complete absence of the epithelium, epithelial cells, fibroblast cells, and other components in the amnion suggesting its biocompatible feature as collagen, in general, is non immunogenic in nature (Figure 5B, D, F).



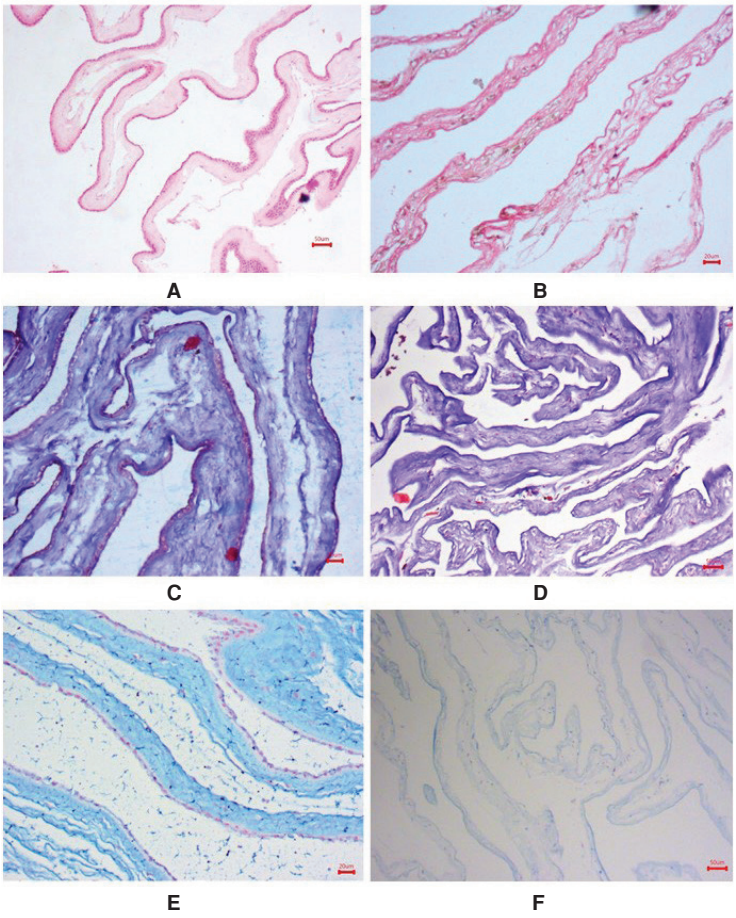
h=hour, µg=microgram, mL=milliliter

Figure 3 Cell viability studies. Untreated control cells (A–C): (A) 24 h; (B); 72 h; and (C) 120 h. Cells treated with amnion derived collagen scaffold (50 µg/mL) (D–H): (D) 24 h; (E); 48 h; (F); 72 h; (G); 96 h; and (H); 120 h. Scaffolds showing their nontoxic nature when the cells were treated in the concentration ranging from 50 to 1,000 µg/mL for 120 h (H–L): 50 µg/mL (H); 100 µg/mL (I); 250 µg/mL (J); 500 µg/mL (K) and 1000 µg/mL (L).



HE=Hematoxylin and Eosin, MT=Masson's Trichrome, AB-PAS=Alcian Blue-Periodic Acid Schiff, PR=Picrosirius Red

Figure 4 Histological examination of amnion separated by conventional and enzymatic methods. HE staining of amnion separated by the conventional method (A) 50x; (B) 100x; and (C) 200x and the enzymatic method (D) 50x; (E) 100x; and (F) 200x. MT staining of amnion separated by the conventional method (G) 50x; (H) 100x; and (I) 200x and the enzymatic method (J) 50x; (K) 100x; and (L) 200x. AB-PAS staining of amnion separated by the conventional method (M) 50x and (N) 100x and the enzymatic method (O) 50x and (P) 100x. PR staining of amnion separated by the conventional method (Q) 50x and (R) 100x and the enzymatic method (S) 50x and (T) 100x.



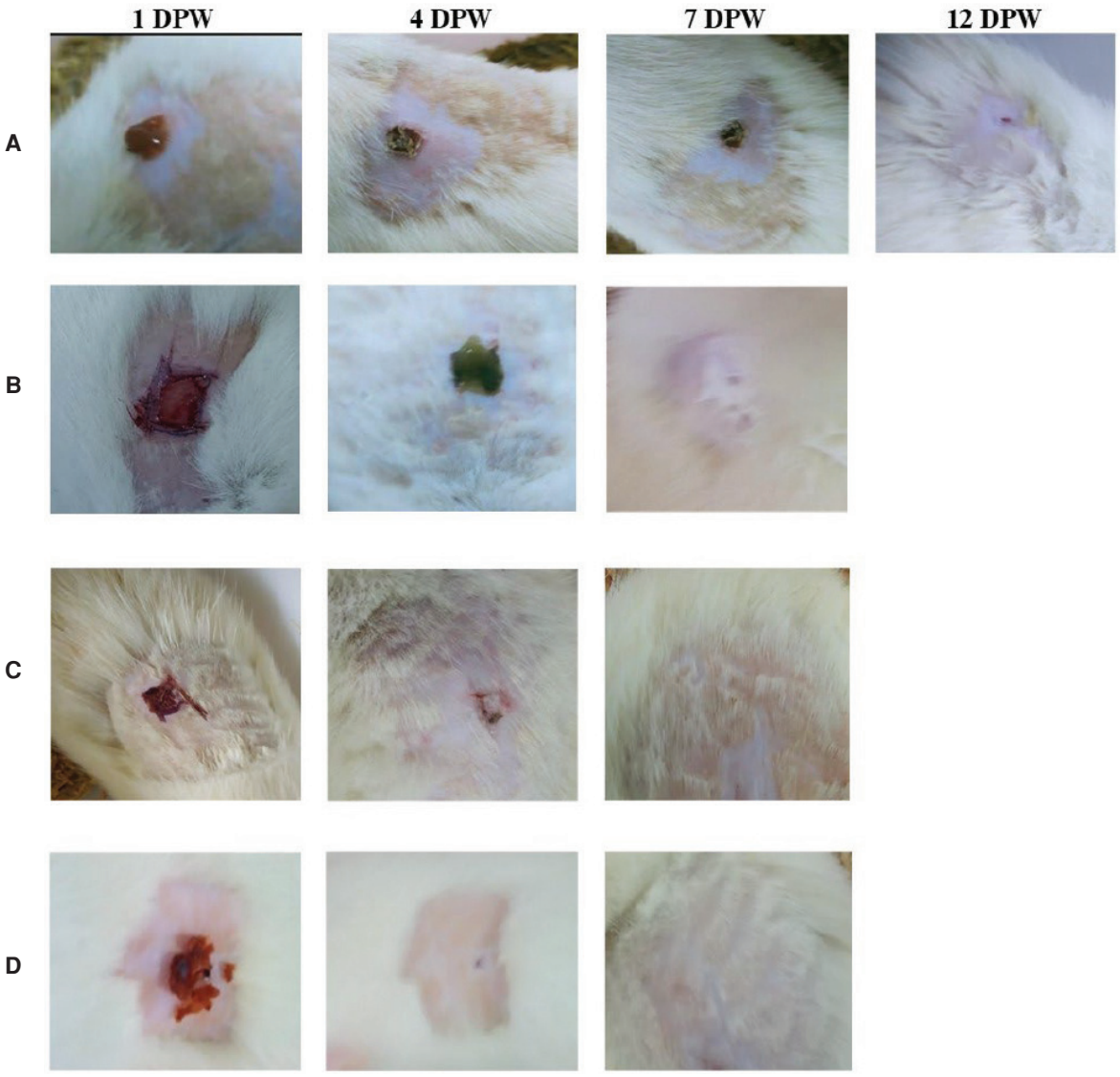
HE=Hematoxylin and Eosin, MT=Masson's Trichrome, AB-PAS=Alcian Blue-Periodic Acid Schiff

Figure 5 Histological studies on enzyme-treated amnion devoid of the epithelial layer against conventionally obtained amnion. HE staining of enzyme-treated amnion (B) against the conventional method (A); MT staining of enzyme-treated amnion (D) against the conventional method (C); AB-PAS staining of enzyme-treated amnion (F) against the conventional method (E).

In vivo effects of the biomaterials

Biophysical, microscopic, and in vitro analyses showed that enzymatically separated amnion and its derived scaffolds promote healing. To assess their therapeutic potential, we conducted in vivo evaluations using a rat model. One day post-wounding (DPW), rats were divided into 4 groups: (a) untreated control, (b) amnion recovered conventionally, (c) enzymatically recovered amnion, and (d) collagen scaffold from enzymatically recovered amnion (Figure 6). Fresh biomaterial was applied every other day. Compared to controls, treated rats had no exudates and showed healthy pink skin by 2 DPW. Wound closure occurred by 7 DPW with epidermis and keratin formation, whereas controls required 12–14 days. Controls had exudates on 1

DPW, pink skin by 4 DPW, and complete healing by 15 DPW. While all biomaterials improved healing, the collagen scaffold from enzymatically recovered amnion showed the best results, suggesting it is a superior therapeutic material compared to unprocessed amnion.

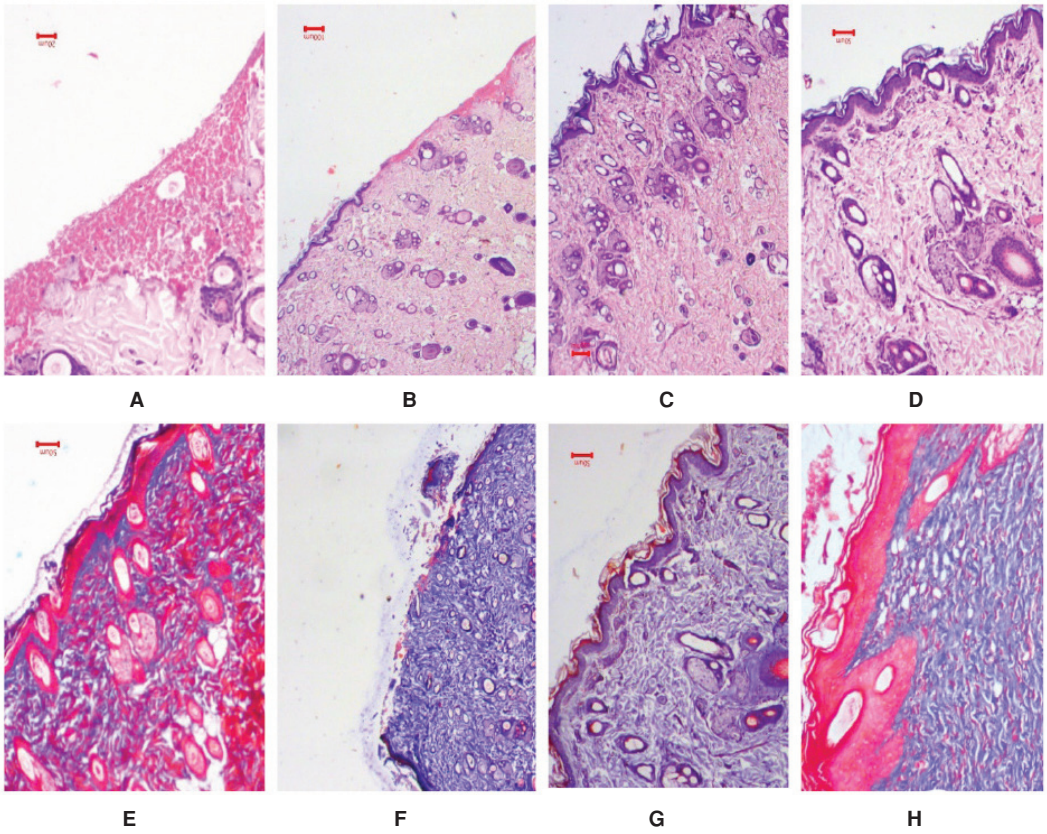


DPW=days-post wounding

Figure 6 Rat model based wound healing evaluation of the efficacy of Amnion biomaterials against untreated control through images of DPW. (A) Untreated control rat; rat treated with (B) amnion separated by the conventional method; (C) amnion separated by the enzymatic method; and (D) scaffold prepared from enzymatically recovered amnion.

Animal histological studies

Histological studies using various staining methods (HE, MT, AB-PAS, Verhoeff's) revealed a healthy epidermis with intact epithelial layers, collagen, elastin, proteoglycans, hyaluronic acid, hair follicles, and glandular structures (Figure 7 and Supplementary Figure 1). Amnion-treated skin showed faster, more complete regeneration than untreated skin. Enzymatically recovered amnion and its scaffolds enabled full epidermal regeneration, while conventionally recovered amnion showed partial regeneration. Enzyme-treated skin exhibited improved collagen distribution and regenerated hair follicles, shafts, and glands. Amnion biomaterials stabilized wounds, prevented desiccation, and promoted fibroblast growth, collagen production, and tissue regeneration, accelerating healing and closure.



DPW=days-post wounding, HE=Hematoxylin and Eosin, MT=Masson's Trichrome

Figure 7 Histopathological studies on wound healing in rats on 7 DPW. HE staining of untreated control (A); rat treated with (B) amnion separated by conventional method; (C) amnion separated by enzymatic method; and (D) scaffold prepared from enzymatically recovered amnion. MT staining of untreated control (E); rat treated with (F) amnion separated by conventional method; (G) amnion separated by enzymatic method; and (H) scaffold prepared from enzymatically recovered amnion.

Discussion

Wound management poses a significant burden on healthcare systems, requiring control of both the local wound environment and systemic effects[7]. Wound healing is a complex regenerative process involving the suppression of inflammation and infections, stimulation of fibroblast proliferation, collagen deposition, and ECM remodelling[27]. Effective therapeutic biomaterials should activate cells at the wound site from a senescent to a healthy state, facilitating these processes for complete skin regeneration. Collagen-rich amnion has proven to be effective for wound healing[28,29]. Widely recognized as a safe and multifunctional material, amnion can also serve as a substrate for excess proteases from the wound site, helping to reduce protease-mediated obstacles to healing[14,15,30,31]. The placenta has 2 layers: decidua and chorio-amniotic. For therapeutic use, the amnion must be separated, typically using blunt dissection, manual, mechanical, or chemical methods, which often cause tissue damage and quality issues. These methods also fail to remove non-collagenous ECM components that hinder wound healing. This study developed a method using *Bacillus* sp. S2 MTCC 13117 protease, which selectively removes non-collagenous proteins while preserving collagen and elastin, yielding high-quality amnion with favorable mechanical properties for wound healing.

The ease of preparing these materials provides a cost-effective alternative to existing therapies. These materials should possess well-defined therapeutic components and desirable physical, chemical, and biological properties to support optimal delivery of therapeutics at the wound site. These biomaterials must have a large surface area with porosity and mass transfer features to enable effective wound coverage, aeration, exudate removal, and therapeutic delivery[32]. Biophysical characterization indicates that these materials effectively modulate the local wound environment and influence systemic healing responses. Their tensile strength ensures suitable mechanical and elastic properties for clinical applications, while permeability to oxygen and water vapour prevents hypoxia and desiccation, aiding the diffusion of essential healing substances[14,33]. An ideal wound dressing adheres quickly, conforms to the wound's contours, and prevents air or fluid pocket formation. High absorption capacity is vital to manage exudate, protecting surrounding healthy tissue from maceration[34]. SEM analysis of the amnion-derived scaffold showed a collagen-rich, porous, free of non-collagenous content, which can support oxygen exchange, drug delivery, and retain its internal architecture. Enzymatic processing preserved the amnion's structure, allowing it to absorb exudates and be loaded with drugs, while completely removing the chorion.

This study demonstrated that extensive enzymatic treatment of conventionally or enzymatically derived amnion resulted in amnion devoid of the surface epithelial layer containing amniocytes. Removing cellular content from amnion promotes healing, integration with host tissues, and minimizes foreign body or immunogenic reactions[26]. Decellularization of amnion, though less reported, involves chemical, enzymatic, physical, or combined methods to remove cellular components while preserving the ECM[35]. The choice of the decellularization method significantly impacts the ECM structure, tissue integrity, and biomechanical properties, necessitating a balance between effective cellular removal and ECM preservation. While various approaches have been applied to decellularize placental tissues, some methods extensively damage amnion constituents. Notably, the enzyme used in this study preserved both the structural and functional integrity of the membrane. Leonel et al. (2017) reported Trypsin, a protease, as an effective agent for decellularizing placental membranes[36].

Amnion biomaterials of this study stimulate fibroblast proliferation while demonstrating low toxicity and high biocompatibility. The high collagen content in the amnion scaffold may have promoted cell growth up to a threshold of

1 mg/mL[37]. Collagen's non-toxic nature, along with its high compatibility and non-antigenicity, makes it safe for use, especially after the enzymatic removal of non-collagenous proteins in the amnion.

Histochemical analysis confirmed superior amnion recovery via enzymatic over conventional methods, enhancing biocompatibility by removing non-collagenous components. In vivo rat studies showed faster healing with enzymatically processed amnion and collagen scaffolds, achieving wound closure in 7 vs. 12–14 days. Various animal models showed ~60% wound closure within 2 weeks, proving their efficacy in wound care and tissue regeneration[38].

Over the past decade, randomized controlled trials have highlighted the effectiveness of amnion-derived biomaterials, including dehydrated human amnion, amnion powder, dried amnion, hypothermically stored amnion, etc, in treating hard-to-heal ulcers like diabetic foot ulcers, venous leg ulcers, and leprosy ulcers[15,36]. These studies consistently show that placental-derived biomaterials significantly improve healing outcomes, including higher healing rates, faster healing times, and reduced ulcer size, compared to standard wound care.

Conclusion

The enzymatic process for separating amnion from placenta offers several advantages, including its scalability. It ensures consistent product quality, reduces manpower and energy needs, and facilitates cleansing by removing unwanted substances. The process minimizes infection transmission, shortens separation time, and efficiently isolates both amnion and chorion membranes. Extending enzyme treatment enhances processing, enabling the extraction of valuable components like amniocytes, progenitor cells, collagen proteins, growth factors, etc. It also improves membrane adherence to damaged tissues. The physico-chemical properties of the developed materials, along with their *in vitro* and *in vivo* effects and pre-clinical evaluation, suggest that biomaterial-based gradual alteration of the wound site is a promising and cost-effective approach for wound healing.

Ethical considerations

All animal studies were conducted in accordance with national guidelines and were approved by the Institutional Animal Ethics Committee (IAEC) of Dr. ALM Post Graduate Institute of Basic Medical Sciences, University of Madras, Taramani Campus, Chennai (IAEC Approval No. 205/GO/ReBi-S/Re-L/2000/CPCSEA). The study protocols were reviewed and approved by the committee. Additionally, human placenta samples were collected and used in the study through approval from the Institutional Human Ethics Committee (IEC Approval No. KIPMR Lett- KIPMR/IEC/2020/003/TB/Amn, dt 24/01/2018).

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Conflict of interest

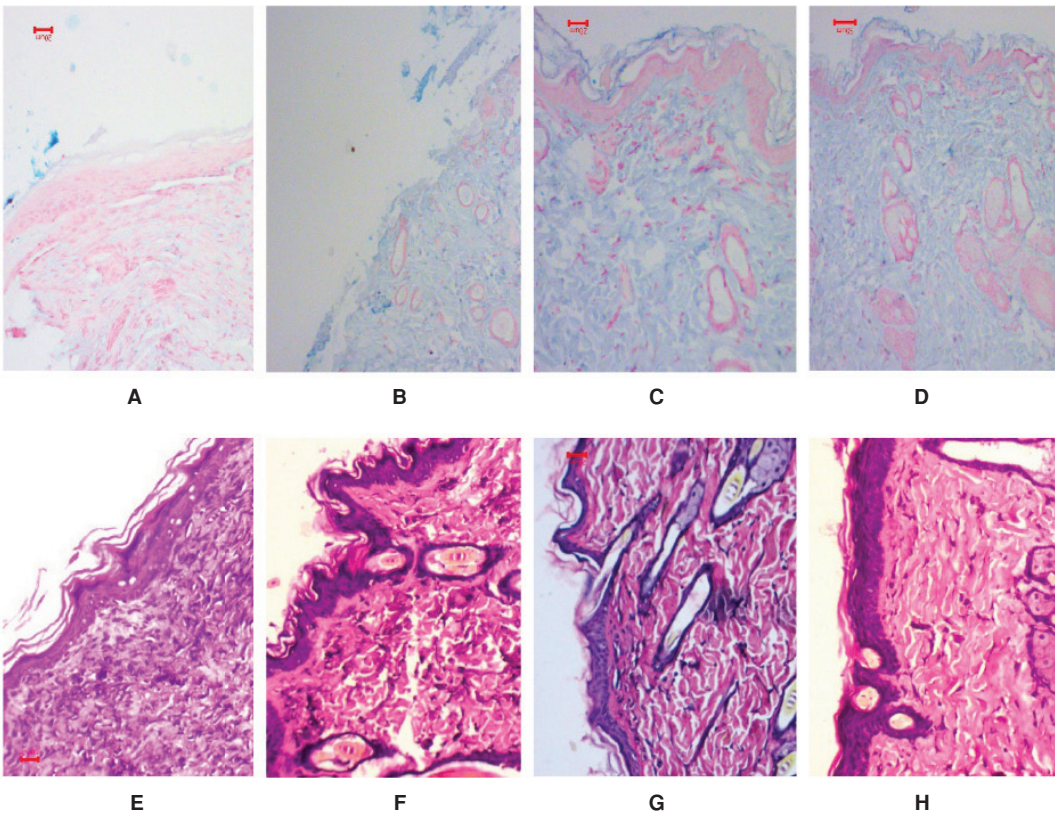
The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

References

1. Ertl J, Pichlsberger M, Tuca AC, Wurzer P, Fuchs J, Geyer SH, et al. Comparative study of regenerative effects of mesenchymal stem cells derived from placental amnion, chorion and umbilical cord on dermal wounds. Placenta 2018;65:37-46.
2. Zahorec P, Koller J, Danisovic L, Bohac M. Mesenchymal stem cells for chronic wounds therapy. Cell Tissue Bank 2015; 16:19-26.

3. Bartus CL, Margolis DJ. Reducing the incidence of foot ulceration and amputation in diabetes. Curr Diab Rep 2004;6: 413–8.
4. Mrugala A, Sui A, Plummer M, Altman I, Papineau E, Frandsen D, et al. Amniotic membrane is a potential regenerative option for chronic non healing wounds: a report of five cases receiving dehydrated human amnion/chorion membrane allograft. Int Wound J 2016;4:485–92.
5. Iversen MM, Tell GS, Riise T, Hanestad BR, Østbye T, Graue M, et al. History of foot ulcer increases mortality among individuals with diabetes: ten-year follow-up of the Nord-Trøndelag Health Study, Norway. Diabetes Care 2009;32:2193–9.
6. Cavanagh P, Attinger C, Abbas Z, Bal A, Rojas N, Xu ZR. Cost of treating diabetic foot ulcers in five different countries. Diabetes/metabolism research and reviews 2012;28:107–11.
7. Harding KG, Morris HL, Patel G. Healing chronic wounds. BMJ 2002;324:160–3.
8. Liu ZJ, Velazquez OC. Hyperoxia, endothelial progenitor cell mobilization, and diabetic wound healing. Antioxid Redox Signal 2008;10:1869–82.
9. Reinar LM, Forsetlund L, Bjørndal A, Lockwood DN. Interventions for skin changes caused by nerve damage in leprosy. Cochrane Database Syst Rev 2008;3. doi: 10.1002/14651858. CD004833.pub4.
10. Das S, Baker AB. Biomaterials and nanotherapeutics for enhancing skin wound healing. Front Bioeng Biotechnol 2016; 4:82.
11. Rahimnejad M, Derakhshanfar S, Zhong W. Biomaterials and tissue engineering for scar management in wound care. Burns Trauma 2017;5. doi: 10.1186/s41038-017-0069-9.
12. Dreifke MB, Jayasuriya AA, Jayasuriya AC. Current wound healing procedures and potential care. Mater Sci Eng 2015;48: 651–62.
13. Penny H, Rifkah M, Weaver A, Zaki P, Young A, Meloy G, et al. Dehydrated human amnion/chorion tissue in difficult-to-heal DFUs: a case series. J Wound Care 2015;24:104–11.
14. Sivasubramanian S, Chandrasekar G, Svensson Akusjärvi S, Thangam R, Sathuvan M, Kumar RBS, et al. Phenotypic screening identifies synergistically acting natural product enhancing the performance of biomaterial based wound healing. Front Pharmacol 2017;8:433.
15. Sivasubramanian S, Mohana S, Maheswari P, Victoria V, Thangam R, Mahalingam J, et al. Leprosy-associated chronic wound management using biomaterials. J Glob Infect Dis 2018; 10:99–107.
16. Nejad AR, Hamidieh AA, Amirkhani MA. Update review on five top clinical applications of human amniotic membrane in regenerative medicine. Placenta 2021;103:104–19.
17. Niknejad H, Peirovi H, Jorjani M. Properties of the amniotic membrane for potential use in tissue engineering. Eur Cells Mater 2008;15:88–99.
18. Toda A, Okabe M, Yoshida T, Nikaido T. The potential of amniotic membrane/amnion-derived cells for regeneration of various tissues. J Pharmacol Sci 2007;105:215–28.
19. Deferrari G, Ravera M, Berruti V. Treatment of diabetic nephropathy in its early stages. Diabetes Metab Res Rev 2003;19: 101–14.
20. Phiel CJ, Wilson CA, Lee VM, Klein PS. GSK-3alpha regulates production of Alzheimer's disease amyloid-beta peptides. Nature 2003;423:435–9.
21. Kunitz M. Crystalline soyabean trypsin inhibitor II. General properties. J Gen Physiol 1947:30:291–310.
22. Zahari NK, Sheikh Ab Hamid S, Yusof N. Effects of different doses of gamma irradiation on oxygen and water vapour transmission rate of preserved human amniotic membrane. Cell Tissue Bank 2015;16:55–63.
23. Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods 1983;65:55–63.
24. Dovi JV, He LK, DiPietro LA. Accelerated wound closure in neutrophil-depleted mice. J Leukoc Biol 2003;73:448–55.
25. Fernandes LL, Resende CX, Tavares DS, Soares GA, Castro LO, Granjeiro JM. Cytocompatibility of chitosan and collagen-chitosan scaffolds for tissue engineering. Polímeros 2011;21:1–6.
26. Aamodt JM, Grainger DW. Extracellular matrix-based biomaterial scaffolds and the host response. Biomater 2016;86:68–82.
27. Martin P. Wound healing--aiming for perfect skin regeneration. Science 1997;276:75–81.
28. Guo X, Kaplunovsky A, Zaka R, Wang C, Rana H, Turner J, et al. Modulation of cell attachment, proliferation, and angiogenesis by decellularized, dehydrated human amniotic membrane in in vitro models. Wounds 2017;29:28–38.
29. Sabol TJ, Tran GS, Matuszewski J, Weston WW. Standardized reporting of amnion and amnion/chorion allograft data for wound care. Health Sci Rep 2022;5:e794.
30. Litwiniuk M, Bikowska B, Niderla-Bielinska J, Jozwiak J,

Kaminski A, Skopinski P, et al. Potential role of metalloproteinase inhibitors from radiationsterilized amnion dressings in the healing of venous leg ulcers. Mol Med Rep 2012;6:723-8.
31. Castellanos G, Bernabe-Garcia A, Moraleda JM, Nicolas FJ. Amniotic membrane application for the healing of chronic wounds and ulcers. Placenta 2017;59:146-53.
32. Varley M, Neelakantan S, Clyne T. Cell structure, stiffness and permeability of freeze-dried collagen scaffolds in dry and hydrated states. Acta Biomaterialia 2016;33:166-75.
33. Zaman HU, Islam JM, Khan MA, Khan RA. Physico-mechanical properties of wound dressing material and its biomedical application. J Mech Behav Biomed Mater 2011;4:1369-75.
34. Chattopadhyay S, Raines RT. Collagen-based biomaterials for wound healing. Biopolymers 2014;101:821-33.
35. Protzman NM, Mao Y, Long D, Sivalenka R, Gosiewska A, Hariri RJ, et al. Placental-derived biomaterials and their application to wound healing: a review. Bioengineering 2023;10:829.
36. Leonel LC, Miranda CM, Coelho TM, Ferreira GAS, Caãada RR, Miglino MA, et al. Decellularization of placentas: establishing a protocol. Braz J Med Biol Res 2017;17:51.
37. Hadjipanayi E, Mudera V, Brown RA. Close dependence of fibroblast proliferation on collagen scaffold matrix stiffness. J Tissue Eng Regen Med 2009;3:77-84.
38. Dunn L, Prosser HC, Tan JT, Vanags LZ, Ng MK, Bursill CA. Murine model of wound healing. J Vis Exp 2013. doi: 10.3791/50265.



DPW=days-post wounding, AB-PAS=Alcian Blue-Periodic Acid Schiff

Supplementary Figure 1 Histopathological studies on wound healing in rats on 7 DPW. AB-PAS staining of untreated control (A); rat treated with (B) amnion separated by conventional method; (C) amnion separated by enzymatic method; and (D) scaffold prepared from enzymatically recovered amnion. Verhoeff's staining of untreated control (E); rat treated with (F) amnion separated by conventional method; (G) amnion separated by enzymatic method; and (H) scaffold prepared from enzymatically recovered amnion.