Preparation, Characterization, and Optimization of Amnion Membrane Scaffold for Improved Wound Healing

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A thesis submitted to the National University of Sciences and Technology, Islamabad,

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Master of Science in

Biomedical Sciences

Supervisor: Dr. Muhammad Asim Waris

Co-Supervisor: Dr. Aftab Ahmed

School of Mechanical and Manufacturing Engineering

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Islamabad, Pakistan

(2024)

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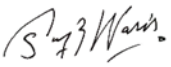
Name of HOD: Dr. Muhammad Asim Waris Signature: 

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To my parents, my unwavering source of love and support, I dedicate this thesis. Your belief in me and your sacrifices have been the driving force behind my journey. With heartfelt gratitude, I honor your unwavering dedication and love.

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LIST OF SYMBOLS, ABBREVIATIONS AND ACRONYMS

EDTA	Ethylenediaminetetraacetic acid
FTIR	Fourier transform infrared spectroscopy
H&E	Hematoxylin-Eosin staining
PBS	Phosphate-Buffered Saline
SDS	Sodium Dodecyl Sulfate
SEM	scanning electron microscopy

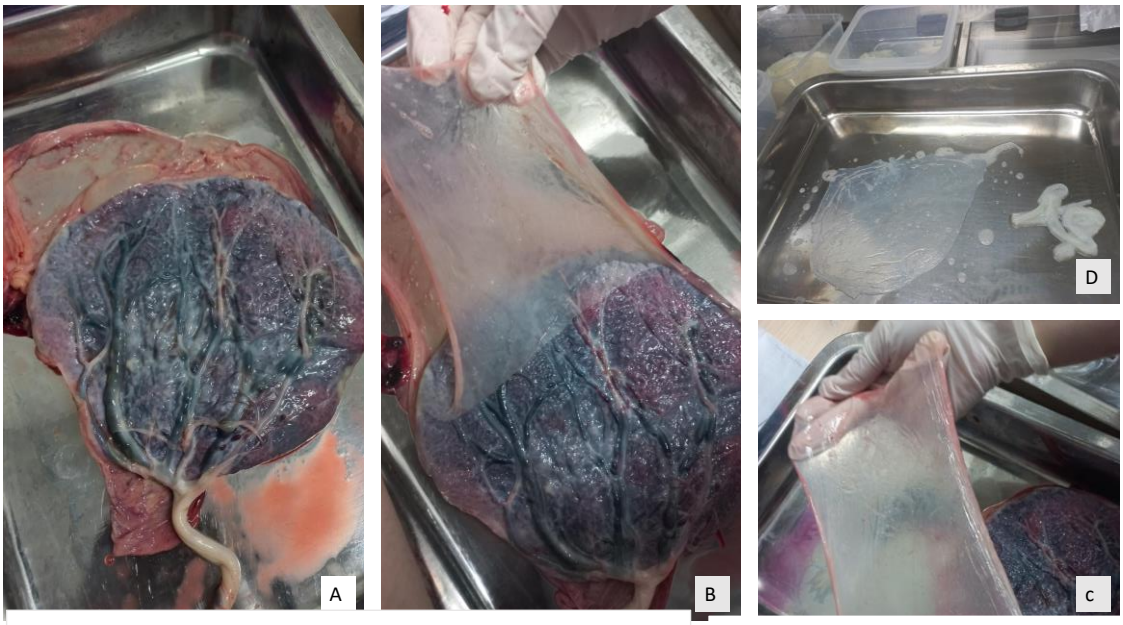
ABSTRACT

The human amniotic membrane is a transparent, thin, and resilient tissue composing the innermost layer of the placenta during pregnancy, enriched in bioactive components, stem cells, and growth factors. Globally recognized, it finds extensive application in regenerative medicine, tissue repair, and wound healing, highlighting versatile therapeutic potential. This study devised a cost-effective method to decellularize the human amniotic membrane, highlighting its potential for effective wound healing in diverse therapeutic applications. The human amniotic membrane was decellularized using 0.5% of Sodium Dodecyl Sulfate (SDS) solution and characterized using Hematoxylin-Eosin (H&E) assay, Masson's trichrome staining, scanning electron microscopy (SEM), and Fourier transform infrared spectroscopy (FTIR-ATR). In the in-vivo experiment, a skin-wounded rat model was used to evaluate the healing activity of the amniotic membrane. The H&E staining results indicated the membrane is well-preserved and has intact structural integrity, particularly the basement membrane. The FTIR graphs revealed the presence of collagen which is effective in wound healing during treatment. This processed membrane showed antimicrobial activity against three bacterial strains, including E. coli, S. aureus, and B.subtilus. Rats treated with the decellularized amniotic membrane exhibited accelerated wound healing, the original wound size was 8 mm completely healed in our treated rats in 14 days, resulting in complete recovery leaving no scar behind in comparison to the control groups. Along with the healing of wounds, the fur of treated rats was also restored within 2 weeks. Based on our findings, the amniotic membrane decellularized with the proposed method has the potential to establish a favorable environment for cell growth and have a positive influence on the overall process of wound healing

Keywords: Amniotic membrane, decellularization, wound healing, SDS, Characterization, wound analysis.

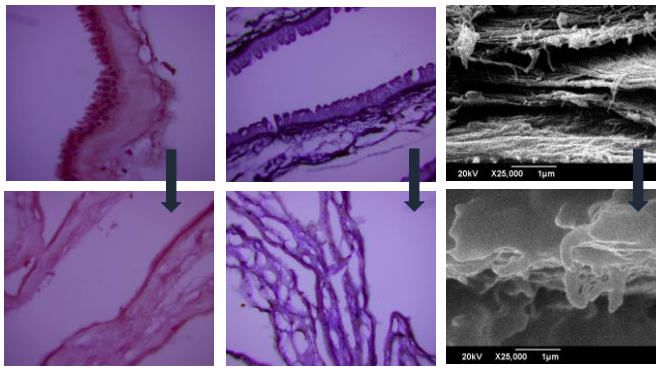
GRAPHICAL ABSTRACT

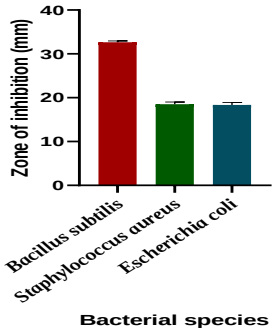
Decellularized Amniotic membrane preparation



Characterization of amniotic membrane

Antimicrobial activity





In-vivo wound healing analysis

CHAPTER 1 : INTRODUCTION

Skin plays a crucial role in defending against external harm (Bi & Jin, 2013). It serves as the primary safeguard, offering functions such as sensation and maintaining water balance (Harder, Schröder, & Gläser, 2013) (Hu & Wang, 2012). Skin injuries, whether from burns, chemical exposure, or tearing, can lead to infections, ulcers, and eventual scarring during the wound-healing process (Sheikh, Sheikh, & Fetterolf, 2014).

Skin healing after an injury requires an appropriate wound-healing mechanism to occur in an organized and timely manner. Hemostasis, inflammation, proliferation, and remodeling are the four steps of this process (Castellanos, Bernabe-Garcia, Moraleda, & Nicolas, 2017; C. G. Insausti, Moraleda, Castellanos, & Nicolás, 2016; Singer & Clark, 1999). Chronic wounds are difficult to heal completely. There are several reasons why this happens, such as poor cytokine levels, fibrotic and dehydrated tissues, and poor vascularization and oxygenation. These elements typically result in cell death, an altered proliferative phase, and a persistent inflammatory process (Castellanos et al., 2017).

Effective treatment involves promptly identifying a suitable wound dressing to facilitate healing and restore skin functions (Dhivya, Padma, & Santhini, 2015). Traditional approaches include autologous skin grafts, allogeneic skin transplantation, xenogeneic grafts, and artificial synthetic skin substitutes (Climov et al., 2016). However, these methods face limitations such as donor scarcity, immunological rejection, and the likelihood of transmitting the disease, restricting their widespread application. Therefore, finding an optimal skin substitute is a critical priority for skin regeneration in clinical settings (Yu et al., 2015).

The amniotic membrane constitutes the innermost layer of placental membranes. Typically, this thin and semitransparent membrane tissue has a thickness that varies between 0.02 and 0.5 millimeters. Structurally, the amniotic membrane comprises three primary layers: a single layer of epithelial cells, a basement membrane, and a stromal layer (Parthasarathy, Sasikala, Gunasekaran, & Raja, 2014). The stromal layer, in turn, is

characterized by three contiguous yet distinct layers—namely, the inner compact layer, the middle fibroblast layer, and the outermost spongy layer (Song et al., 2017).

The amniotic membrane represents a plentiful and easily accessible tissue with compelling attributes that render it a desirable material in regenerative medicine (Díaz-Prado et al., 2011). The advantages of amniotic membrane for human therapies are well documented; these include its application as a full amniotic membrane dressing as well as the use of its cell components or amniotic membrane extracts along with another matrix vehicle (Murphy et al., 2017; Parolini & Caruso, 2011). Notably, it exhibits limited immunogenic response, possesses anti-inflammatory properties, and allows for the isolation of cells without the need to sacrifice human embryos. Given that it is discarded post-partum, the amniotic membrane holds potential utility in the realms of regenerative medicine and cell therapy (Díaz-Prado et al., 2011).

From 1910 onward, one of the most commonly used and medically acceptable biomaterials for the healing of burn wounds is the human amniotic membrane (Bujang-Safawi, Halim, Khoo, & Dorai, 2010; Davis, 1910). Because of the existence of substances including fibronectin, elastin, collagen types I, III, IV, V, VI, and hyaluronic acid, it serves as a foundation for the proliferation and differentiation of new epithelial cells (Higa, Shimmura, Shimazaki, & Tsubota, 2005; Riau, Beuerman, Lim, & Mehta, 2010; Tehrani, Ahmadiani, & Niknejad, 2013).

The amniotic membrane has acquired significance due to its reported capacity to diminish scarring and inflammation (Solomon et al., 2001) (Tseng, Li, & Ma, 1999), promote wound healing and epithelialization (Tsubota et al., 1999), and exhibit antimicrobial and antiviral properties (Fernandes, Sridhar, Sangwan, & Rao, 2005) (Inge, Talmi, Sigler, Finkelstein, & Zohar, 1991). Also, research has shown that it doesn't cause the body's immune system to react strongly, and there haven't been cases of rejection when transplanted (CA Akle, Welsh, Adinolfi, Leibowitz, & McColl, 1981) (Adinolfi et al., 1982).

Over a century ago, the initial utilization of amniotic membrane was documented, reporting its application as a biological wound dressing to expedite the healing process in

skin ulcers (Leila et al., 2017). Amniotic membrane has since been recognized for its immunomodulatory properties, facilitation of epithelization, anti-inflammatory effects, inhibition of angiogenesis, and anti-fibroblastic features, all of which contribute significantly to the healing of various types of wounds. Tissue engineering for wound healing using amniotic membranes has been extensively reviewed by various authors, highlighting its prominent role in this field (Tettelbach et al., 2019). Amniotic membrane has been used in multiple studies to treat burns, ocular surface reconstruction, chronic leg ulcers, diabetic foot ulcers, and skin wounds. It has also been used to prevent tissue adhesion during surgical procedures (C. L. Insausti, Alcaraz, et al., 2010; Parolini, Soncini, Evangelista, & Schmidt, 2009; M. Valiente et al., 2018).

To prevent de-epithelialization, the amniotic membrane can be applied denuded, deprived of amniotic epithelium, or intact (C Akle et al., 1985; Wilshaw, Kearney, Fisher, & Ingham, 2006). Amniotic membrane is utilized fresh in certain situations, preserved, and decellularized in other situations using different techniques. It's widely accepted these days that using fresh amniotic membranes for clinical purposes is impractical (Zelen, Snyder, Serena, & Li, 2015).

In the pursuit of effective decellularization methods for the amniotic membrane, various techniques have been explored in existing literature. These include enzymatic approaches such as the diaspase (Espana et al., 2003) and trypsin–EDTA methods (Sangwan, Vemuganti, Singh, & Balasubramanian, 2003) (Madhira et al., 2008), chelation using ethylene diaminetetraacetic acid (EDTA), denaturation with urea, and ethanol incubation. Additionally, the hypotonic buffer, SDS, and nuclease method have been investigated, involving a sequential process of hypotonic buffer incubation, SDS treatment, and enzymatic digestion (Germain, Guignard, Rouabhia, & Auger, 1995). Laleh et al., also used SDS and nucleases method for decellularization (Laleh et al., 2023).

Amidst these approaches, my research focuses on streamlining the decellularization process by exclusively utilizing 0.5% SDS for 3 days, followed by thorough washing with distilled water to ensure the removal of any residual detergent. This optimized procedure

is essential for maintaining the purity and biocompatibility of the decellularized amniotic membrane, laying the groundwork for its application in diverse biomedical contexts.

1.1 Objectives

The objectives for the project "Preparation, Characterization, and Optimization of Amnion Membrane Scaffold for Improved Wound Healing" are as follows:

1. Create and implement the simplest and most cost-effective technique to effectively decellularize the amniotic membrane while preserving its structural integrity.
2. Employ a rat model to assess the wound healing properties of the amniotic membrane, observing its effectiveness in promoting tissue regeneration and reducing the healing time.

These objectives collectively aim to enhance our understanding of the amniotic membrane's characteristics and wound healing potential, ultimately contributing to the development of an optimized scaffold for improved wound healing applications.

CHAPTER 2 : LITERATURE REVIEW

2.1 Skin

The skin, being the body's largest organ, covers approximately 1.7 square meters and constitutes about 15% of the total body weight. Its primary role is to shield us from the external environment. Across different body areas, the thickness, pigmentation, and arrangement of the skin's appendages differ, adapting to each region's specific functions and requirements (Lanigan, Zaidi, Zaidi, & Lanigan, 2010).

2.2 Wound

The skin functions as a protective barrier, safeguarding the body by preventing the entry of harmful agents like bacteria and viruses. It also plays a crucial role in maintaining the internal environment by regulating body hydration and temperature (Percival, 2002).

When the skin, mucosal surfaces, or organ tissue is compromised, it develops a wound. Wounds can arise from various causes, including disease processes, accidental injuries, or intentional actions (Velnar, Bailey, & Smrkolj, 2009).

When the skin gets damaged, it forms an opening that allows bacteria to enter, resulting in local inflammation, infection, or potential spread throughout the body. Equally important, any breach in the epidermis can result in the loss of tissue fluid. As a result, the primary objective in treating any wound is to promote rapid healing through the simplest means possible, while the secondary objective is to minimize the formation of scars that may result from the healing process (Percival, 2002).

When an injury occurs, various cellular and extracellular pathways are triggered in a highly regulated and coordinated manner to initiate the restoration of tissue integrity. The process of wound healing is conventionally classified into four distinct phases: hemostasis, inflammation, proliferation, and tissue remodeling. It is noteworthy that despite the complexity of this healing cascade, it often progresses smoothly without complications.

However, several factors can disrupt this process, leading to delayed wound healing, heightened patient morbidity and mortality, and unfavorable cosmetic results (Ueno, Hunt, & Hopf, 2006).

Wounds greatly impact society and the economy because they are very common, especially among older people. Besides the usual wounds that heal relatively quickly, many long-lasting wounds are associated with health conditions like diabetes and ulcers caused by poor blood circulation. These chronic wounds are hard to heal and add to the overall problem. Moreover, acute wounds can develop into chronic wounds when they don't heal properly and on time. Chronic wounds are more challenging to manage because they come with complications that make the healing process harder to control and regulate (Natarajan, Williamson, Stiltz, & Harding, 2000).

Diabetic foot ulcers are chronic wounds and are difficult to heal **Figure 2. 1** (Moura, Dias, Carvalho, & de Sousa, 2013). About 15 percent of the 200 million people with diabetes worldwide suffer from diabetic foot disease, a serious health issue. Diabetes's worst side effect is major amputation, either above or below the knee (Dalla Paola & Faglia, 2006). If not identified and treated immediately, diabetic foot ulcers are linked to substantial morbidity and mortality, which may result in hospitalization and lower limb amputation. (Reardon et al., 2020).

The primary challenges to diabetic wound healing include diabetic neuropathy, peripheral vascular dysfunction, and abnormal cellular activity. A significant and ongoing issue in the development of innovative and effective wound dressings is diabetic foot ulcers. Ideally, the best wound dressing should eliminate wound exudate, offer protection against secondary infections, keep the site moist, and encourage tissue regeneration (Moura et al., 2013).

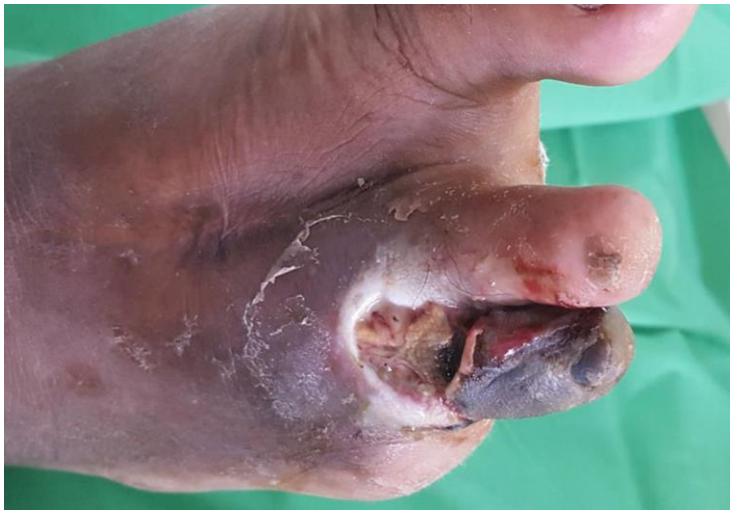


Figure 2. 1: Diabetic foot ulcer (Mishra, Chhatbar, Kashikar, & Mehndiratta, 2017)

2.2.1 Wound healing process

The process of wound healing encompasses four primary phases: coagulation and hemostasis, inflammation, proliferation, and wound remodeling **Figure 2. 2**. (Hunt, Hopf, & Hussain, 2000).

2.2.1.1 Hemostasis

Immediately after an injury, the wound undergoes coagulation and hemostasis, where blood clotting occurs to stop bleeding and form a protective barrier (George Broughton, Janis, & Attinger, 2006). The primary objective of these mechanisms is to stop excessive bleeding and safeguard the vascular system after an injury, ensuring the well-being of vital organs. Additionally, they aim to establish a framework that allows essential cells to infiltrate the site as healing progresses. This framework provides a supportive environment for the subsequent phases of the healing process to proceed smoothly (Lawrence, 1998). The process of haemostasis is controlled by a delicate interplay among endothelial cells, thrombocytes (platelets), coagulation factors, and fibrinolysis. This intricate balance governs the amount of fibrin (a protein involved in clot formation) deposited at the wound site, subsequently influencing the progression of the reparative processes during wound healing (Pool, 1977). When blood vessels are injured, a neuronal reflex mechanism triggers rapid constriction of the vessels due to the contraction of

vascular smooth muscle cells in the circular muscle layer. This strong contraction is effective in stopping bleeding from smaller arterioles (about 0.5 cm in diameter) when the vessel is interrupted horizontally.

However, this reflex vasoconstriction is not as effective in longitudinally severed arterioles and may widen the gap (Pool, 1977). While reflex vasoconstriction can temporarily reduce or halt bleeding, it is not a long-term solution. After a short duration, hypoxia and acidosis within the wound area cause the smooth muscle cells to relax passively, and bleeding resumes.

To address this issue, the body forms an insoluble fibrin plug at the wound site. This fibrin plug, along with other haemostatic mechanisms, is essential for effective long-term control of bleeding, ensuring the wound healing process can progress efficiently (Strecker-McGraw, Jones, & Baer, 2007).

In addition to the haemostatic events, the coagulation cascade is set into motion through both the extrinsic and intrinsic pathways. This activation leads to platelet aggregation and the formation of a clot, which is crucial for limiting blood loss at the site of injury (Jespersen, 1988).

When blood gets into the wound area, platelets come in contact with the exposed collagen and other extracellular matrix components. This contact stimulates the release of clotting factors from the platelets, initiating the formation of a blood clot. The clot is composed of various proteins, including fibronectin, fibrin, vitronectin, and thrombospondin. Together, these components work to form a stable clot that helps seal the wound and prevent further bleeding (Lawrence, 1998).

The blood clot formed with trapped platelets serves not only to stop bleeding but also acts as a temporary matrix for cell migration during the subsequent haemostatic and inflammatory phases. Within the platelets, α-granules contain growth factors and cytokines like platelet-derived growth factor, transforming growth factor-β, epidermal growth factor, and insulin-like growth factors, which play essential roles in the healing process (Lawrence, 1998).

These molecules play a key role in promoting the process of wound healing. They activate and attract other cells to enhance wound healing. Platelets also contain vasoactive amines, which dilate the blood to increase permeability. This causes the fluid to leak into the extracellular matrix which causes tissue swelling, which further amplifies during the subsequent inflammatory phase (Strecker-McGraw et al., 2007).

2.2.1.2 Inflammation

After the initial inflammation, the wound healing process enters the humoral and cellular inflammatory phase, to stop and kill the coming pathogens. This phase consists of two stages: early and late inflammatory phases, working together to enhance defense mechanisms, promote healing, and prevent infections. During the late coagulation phase, the early inflammatory response activates the complement cascade, attracting neutrophils to the wound site. Neutrophils play a critical role in protecting against infection by phagocytosing invading pathogens and damaged cells and tissues, important for effective wound healing. Keeping the wound free from infection is essential for successful healing (Hart, 2002).

Within 24 - 36 hours of injury, various chemo-attractive agents attract neutrophils to the wound site. Due to changes in surface adhesion molecule regulation, neutrophils become sticky and adhere to the endothelial cells in the post-capillary venules around the wound through a process called margination. They then roll along the endothelial surface, supported by selectin-dependent interactions. Endothelial cell-secreted chemokines trigger a stronger adhesion system mediated by integrins. Neutrophils stop rolling and migrate out of the venules by diapedesis. Their subsequent movement relies on chemokines and other chemotactic agents. Once in the wound, neutrophils phagocytose foreign material and bacteria, destroying them with proteolytic enzymes and oxygen-derived free radicals. As days pass and bacterial removal is complete, neutrophil activity changes. Redundant neutrophils are eliminated from the wound through extrusion as slough and apoptosis. This process allows their disposal without causing tissue damage or prolonged inflammation. The dead cells are then removed and phagocytized by macrophages (Flanagan, 2000).

During the late inflammatory phase after 48-72 hours of injury, macrophages appear and continue phagocytosis. They activate crucial growth factors for healing and play a key role in tissue repair. Lymphocytes enter the wound site later, attracted by specific factors, contributing to collagen remodeling and proper wound healing (Witte & Barbul, 1997).

2.2.1.3 Proliferation

Once the ongoing injury has stopped, and hemostasis and the immune response have been established, the wound enters the tissue repair phase. Around the third day of healing the proliferative phase begins and continues for approximately two weeks. During this process the fibroblasts migrate to the wound site and deposit a newly synthesized extracellular matrix, which replaces the old network of fibrin and fibronectin. At a macroscopic level, the proliferative phase is evident as an abundant formation of granulation tissue. This tissue serves as a foundation for further healing and plays a critical role in the formation of new blood vessels and the production of collagen, which provides structural support to the wound. The proliferative phase is a dynamic process, paving the way for subsequent stages of wound healing (Diegelmann & Evans, 2004).

At the site of injury, the migration of epithelial cells begins promptly at the edges, usually within a few hours. At first, a single layer of cells forms to wound defect. There is a noticeable increase in the mitotic activity of epithelial cells around the wound edges, indicating their active proliferation to facilitate healing. As the epithelial cells migrate across the wound, they attach to the provisional matrix present below. As the growing epithelial cells meet, migration stops and the process of basement membrane formation commences. The basement membrane is a critical structure that provides support and a foundation for the new epithelial tissue, facilitating proper wound closure and healing (Hunt et al., 2000).

2.2.1.4 Wound remodeling

Remodeling is the last phase of wound healing in this phase new epithelium develops, and scar tissue forms. The synthesis of the extracellular matrix begins along with

granulation tissue development during the proliferative and remodeling phases. This stage may extend for a duration of 1 or 2 years, or possibly even more (Ramasastry, 2005).

The remodeling process is carefully regulated to maintain a delicate balance between collagen degradation and synthesis which is important for normal healing. Collagen bundles grow larger, and hyaluronic acid and fibronectin undergo breakdown. (Baum & Arpey, 2005). While collagen fibers may recover approximately 80% of their initial strength, the tissue can never completely regain its original strength. (Clark, 1993).

The ongoing production and breakdown of collagen and extracellular matrix eventually reach a stable condition approximately three weeks after the injury. Matrix metalloproteinase enzymes, generated by neutrophils, macrophages, and fibroblasts, break down collagen. Their function is controlled by inhibitory factors, and tissue inhibitors of metalloproteinases encourage the accumulation of new matrix (Clark, 1993; Toy, 2005).

Over time, the new collagen matrix becomes more structured and organized. Wound contraction, initiated during the proliferative phase, further contributes to the organization. The underlying connective tissue contracts, bringing the wound edges closer together through fibroblast interactions with the extracellular matrix. Factors like PDGF, TGF-β, and FGF play key roles in this process (Pierce, Berg, Rudolph, Tarpley, & Mustoe, 1991).

As the healing process advances, the number of fibroblasts and macrophages decreases because of apoptosis. Capillary growth ceases, blood flow reduces, and metabolic activity decreases at the wound site (Baum & Arpey, 2005). The outcome is a fully matured scar with fewer cells and blood vessels but with a high tensile strength (Falanga, 1998).

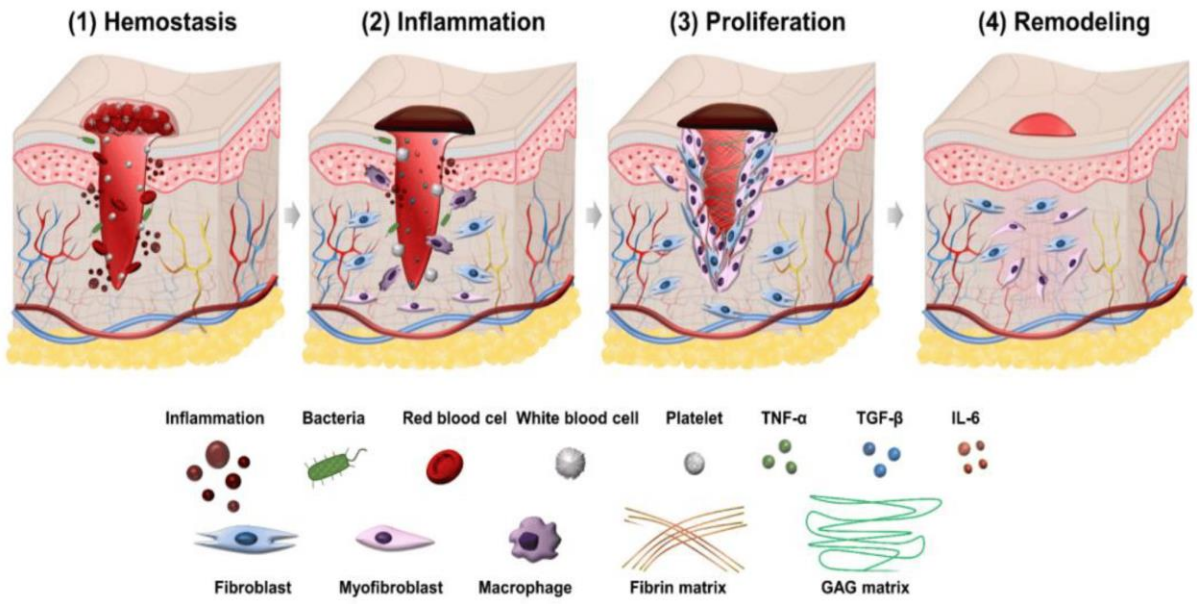


Figure 2. 2: Four Phases of wound healing process (Trinh et al., 2022)

2.3 Amniotic membrane structure

Fetal development relies on an intricate system involving the umbilical cord, amniotic fluid, and placenta **Figure 2. 3**. The fetal membranes consist of two layers: chorion which is the outer layer and encounters maternal cells, and the amniotic membrane which is the inner layer. Also referred to as the amnion, it is a thin membrane that forms the amniotic cavity, which is filled with amniotic fluid, and entirely encloses the fetus on the inside of the placenta (Pollard, Aye, & Symonds, 1976). Nerves, muscles, and lymph vessels are absent from the translucent biological tissue, the amniotic membrane. It gets oxygen and nutrition from sources such as chorionic fluid, amniotic fluid, and fetal surface vessels. Nutrients are transported to the tissue through diffusion (Toda, Okabe, Yoshida, & Nikaido, 2007). The amniotic membrane's thickness varies from 0.02 to 0.5 mm and is composed of three primary histological layers: the epithelial layer, the substantial basement membrane, and the non-vascular mesenchymal tissue (Bourne, 1960). The uniform layer of cuboidal epithelial cells that are firmly linked to the basement membrane makes up the innermost layer, which is in direct contact with the amniotic fluid. Collagen types I, II, and V make up the compact acellular layer that is attached to this basement membrane. (Sadler, 2022). Amniotic epithelial cells possess numerous microvilli on their upper surface,

suggesting their likely involvement in active secretion and intracellular as well as intercellular transport processes (Pollard et al., 1976).

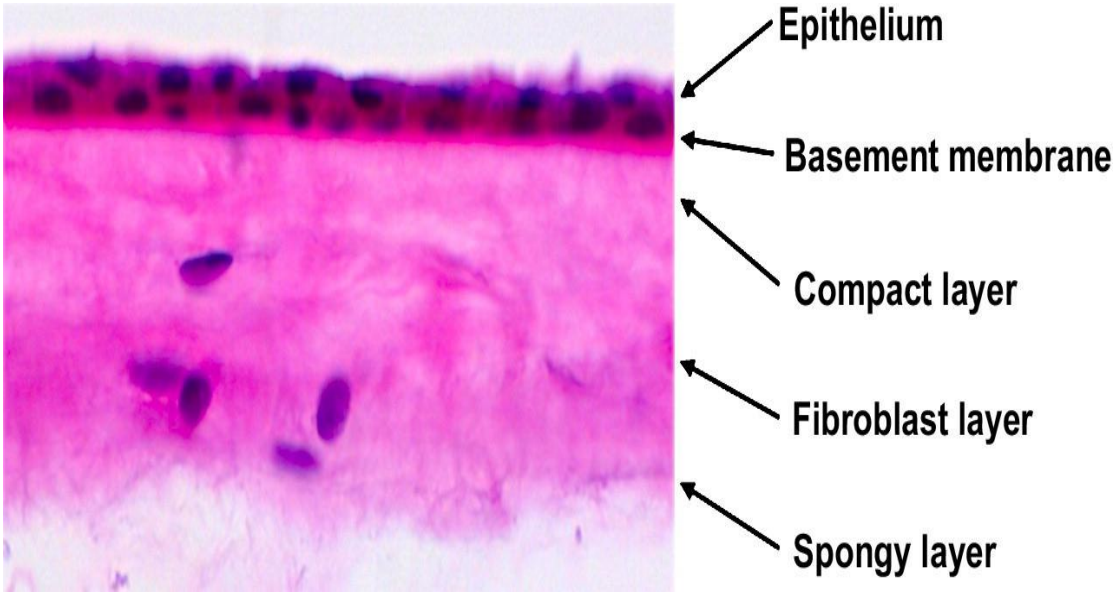


Figure 2. 3: The histology section of the human amniotic membrane.

Figure 2. 3 reveals the five distinct layers: epithelium, basement membrane, compact layer, fibroblast layer, and spongy layer, as demonstrated by hematoxylin-eosin staining (Riau, Beuerman, Lim, & Mehta, 2010).

The basement membrane harbors significant quantities of proteoglycans, abundant in heparan sulfate, which act as a permeable barricade for amniotic macromolecules. It also supports various molecules with structural roles that contribute to upholding membrane integrity. Actin, α-actinin, spectrin, ezrin, many cytokeratins, vimentin, desmoplakin, and laminin are a few of these molecules (King, 1985). Laminin expression has garnered significant attention in research due to its substantial role in promoting cell survival, facilitating differentiation, influencing cellular morphology and motility, and playing a crucial part in preserving tissue characteristics.(Takashima et al., 2008).

The mesenchymal fibroblast-like cells that make up the exterior layer of the amniotic membrane are scattered across a full-term membrane and are most likely derived from the mesodermal embryonic plate. The mesenchymal layer, rich in collagen, contributes to heightened tensile strength. Some researchers refer to the outermost amnion

layer as the "zona spongiosa" due to its high concentration of proteoglycans and glycoproteins, which makes histological preparations appear spongy. This layer is almost cellular and is located next to the chorion laeve. Its non-fibrillar meshwork is mostly made of type III collagen (Toda et al., 2007).

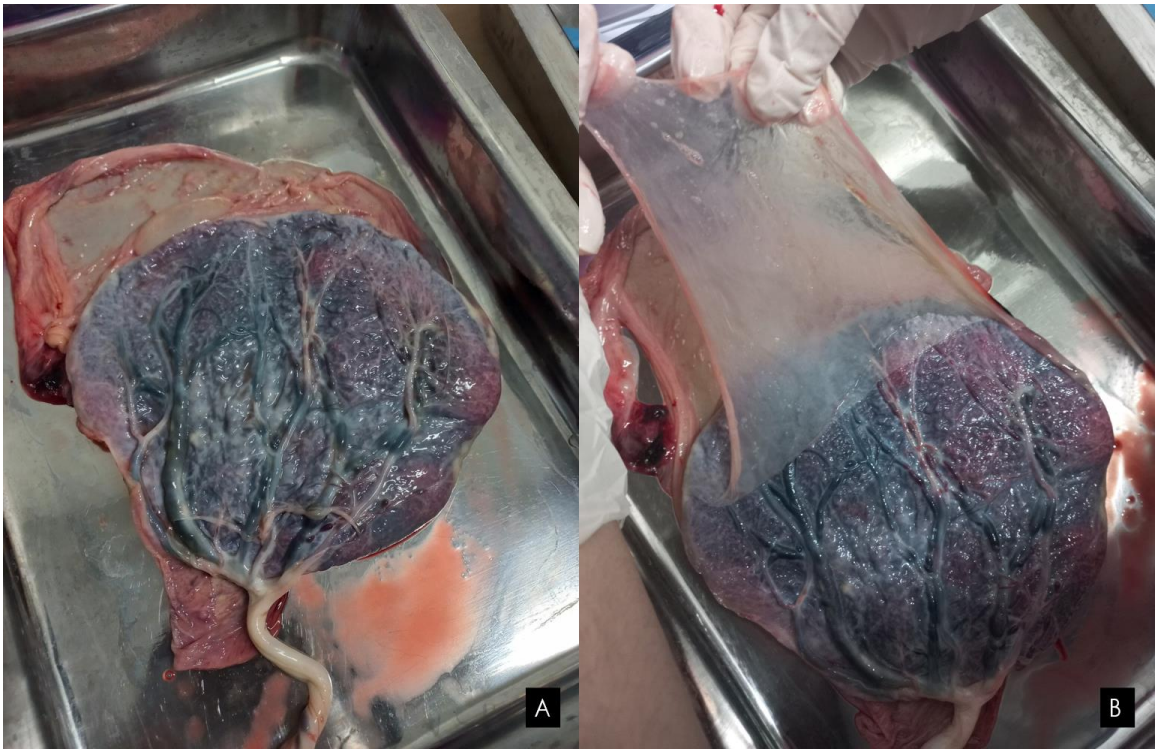


Figure 2. 4: Human placenta (A) and amniotic membrane separation (B).

2.3.1 Amniotic membrane function

The amniotic membrane goes beyond being a mere avascular structure; it encompasses a range of metabolic functions. These include the facilitation of water and soluble material transport, as well as the synthesis of bioactive substances like vasoactive peptides, growth factors, and cytokines (Cunningham et al., 2014). A fundamental role of the amniotic membrane is to shield the developing embryo from dehydration and offer a buoyant setting that permits uninterrupted growth, free from external pressures. This protective function stems from the robust network of interstitial collagen, including types I, II, and elastin, which primarily contributes to the amniotic membrane's resistance against tractional forces. In contrast, the elasticity of the amnion is primarily ascribed to the presence of collagen type III (Sadler, 2022). The presence of interstitial collagens imparts

a significant characteristic to the amniotic membrane, its notable resilience against proteolytic agents (Fukuda, Chikama, Nakamura, & Nishida, 1999).

2.3.2 Amniotic membrane application for wound healing

For nearly a century, amniotic membranes have been utilized in wound treatment, starting with the initial use of natural amniotic membranes sourced from labor and delivery for diverse burns and wounds. Abundant in collagen and various growth factors, the amniotic membrane facilitates the healing process, enhancing wound closure and mitigating scar formation (Fetterolf & Snyder, 2012). Comparing amniotic membrane application to skin transplantation reveals significant benefits. Its application does not result in rejection or uncontrolled growth (C. L. Insausti, Blanquer Blanquer, et al., 2010). The human amniotic membrane exhibits many qualities of optimal skin replacement. It serves as a proficient barrier, adhering well to wounds without causing immune reactions. This membrane also has bacteriostatic properties and offers advantages like diminishing water loss, protein reduction, and the dissipation of heat from the wound area. Moreover, it hinders additional bacterial contamination (Rodríguez-Ares et al., 2009). The membrane can sustain a naturally moist microenvironment that facilitates the process of healing (Dua, Gomes, King, & Maharajan, 2004). In 1910, Davis utilized an amniotic membrane for skin transplantation, and subsequently, in 1913, Stern and Sabella used it for the treatment of skin burns and superficial wounds (Davis, 1910; SABKLLA, 1913; Stern, 1913).

Amniotic membranes are readily accessible and more cost-effective compared to other bioengineered skin substitutes (Meller, Pauklin, Thomasen, Westekemper, & Steuhl, 2011). The amniotic membrane is an extracellular matrix rich in collagen, displaying exceptional biocompatibility. In the realm of tissue engineering, this naturally occurring substance offers numerous advantages for wound healing. It exhibits various positive properties such as anti-inflammatory, anti-bacterial, anti-viral, anti-fibrotic, and analgesic effects. Specifically for skin repair, the human amniotic membrane stands out for its ability to facilitate oxygen and water vapor transmission, providing essential moisture to the wound bed. This physiologically humid micro-environment significantly contributes to the

wound healing process, aiding re-epithelialization and minimizing scarring (Arrizabalaga & Nollert, 2018).

The generation of anti-inflammatory proteins and the inhibition of transforming growth factor B expression, which results in a reduction in pro-inflammatory cytokines like interleukin 10, appear to be connected to the anti-inflammatory qualities of the amniotic membrane. Furthermore, the anti-inflammatory properties of the amniotic membrane and the accelerated process of epithelialization are responsible for the observed decrease in scarring after wound treatment (Hao, Ma, Hwang, Kim, & Zhang, 2000; Tseng et al., 1999). Rats' split-thickness skin grafts showed a faster epithelialization when Maral et al. covered them with the amniotic membrane (Maral et al., 1999). According to Loeffelbein et al. the release of growth factors may have accelerated the creation of the basement membrane in wounds treated with amniotic membrane. One important feature of the amniotic membrane as a skin substitute is its pain-relieving properties, which are probably due to decreased inflammation, improved hydration of the wound bed, and protection of exposed nerve endings (Loeffelbein et al., 2014).

Studies have showcased the efficacy of amniotic membrane grafts in wound healing. A study was conducted by Robson and associates to investigate the effectiveness of the human amniotic membrane in treating open wounds, in which 150 patients were involved (Robson, 1973) (Robson & Krizek, 1974). Additionally, Cobcho and colleagues (Colocho, Graham, Greene, Matheson, & Lynch, 1974) examined the effectiveness of the amnion layer alone as a physiological wound dressing in 107 patients who had split-thickness graft donor sites and partial-thickness burns. Mermet et al. applied amniotic membrane grafts to 15 chronic leg ulcers, resulting in complete healing for all patients (Mermet et al., 2007). Similarly, Pesteil et al. treated eight patients with resistant vascular ulcers using cryopreserved amniotic membrane, demonstrating positive outcomes (Pesteil et al., 2007). Sheikh et al. used dehydrated amnion to promote four patients' chronic wounds to heal, and the treated wounds showed sustained recovery without recurrence during long-term follow-up (Sheikh et al., 2014). A comparable investigation by Zelen et al. involved the use of a dehydrated amniotic membrane in diabetic foot ulcers, resulting in complete healing for 37 out of 40 ulcers (Zelen, Serena, & Snyder, 2014). Cryopreserved

amniotic membrane grafts were utilized by M. R. Valiente et al. to treat diabetic foot ulcers in patients with diabetes. Fourteen individuals with diabetic foot ulcers received treatment. Every patient in the research had their wound fully epithelialized. There were no adverse incidents noted (M. R. Valiente et al., 2018).

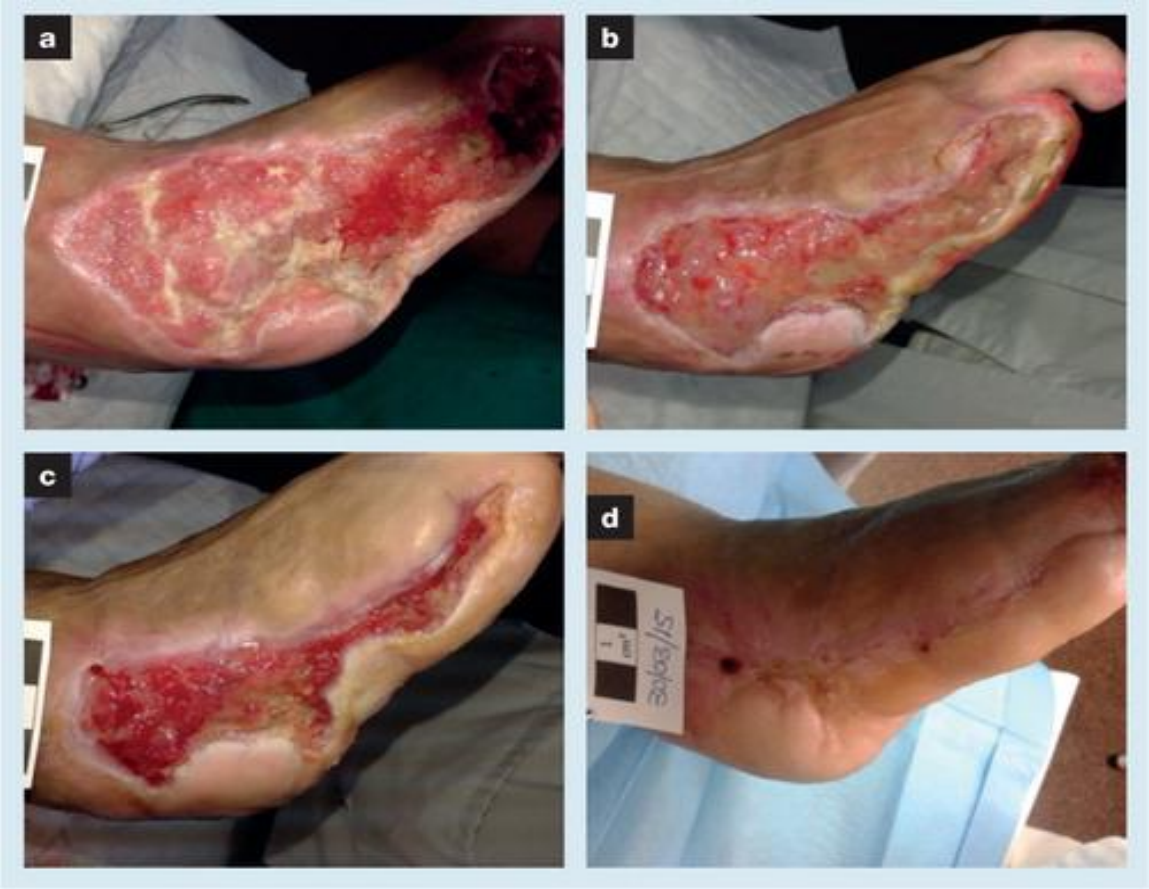


Figure 2. 5: Diabetic foot ulcer healing by using amniotic membrane (M. R. Valiente et al., 2018)

2.3.3 Processes by which amniotic membrane promotes healing

Most of the processes behind amniotic membrane-induced skin re-epithelialization remained unclear. Research has demonstrated that the amniotic membrane may influence wound healing by accelerating the migration of keratinocytes from the wound edge and stimulating their differentiation, which helps to produce intact epithelium (Lee & Tseng, 1997). Growth factors and progenitor cells produced by the amniotic membrane mediate the stimulatory effect on epithelialization from the wound bed and/or the wound edge (Niknejad et al., 2008). It has also been noted that preservation of the stromal matrix and

basement membrane integrity enhances the amniotic membrane's healing capacity and is essential for accelerating re-epithelialization (Kubo, Sonoda, Muramatsu, & Usui, 2001).

Various amniotic membrane cell types determine the amniotic membrane's capacity for wound regeneration. The specific amniotic membrane cell type that controls the amniotic membrane's advantageous effects on chronic wound epithelialization is not evident from the available data. A recent paper by Zhao et al. asserted that certain elements of amniotic membrane on keratinocytes may be replicated using a conditioned medium derived from human amniotic epithelial cells (Zhao et al., 2016) (C. L. Insausti, Alcaraz, et al., 2010). Similar to the conditioned medium from the amniotic membrane, the conditioned medium from these cells was able to trigger the activation of ERK, JNK, and AKT, albeit in a transitory manner (C. L. Insausti, Alcaraz, et al., 2010).

However, it appears that the amniotic basal membrane extracellular matrix has a particular function in either preserving the capacities and characteristics of amniotic cells or perhaps influencing their differentiation (Kopaczka, Skowron, Kolanko, & Czekaj, 2016). The makeup of the basement membrane is like that of the amniotic membrane. Even though a denuded (without cells) amniotic basement membrane can help expanded limbal cells grow, stratify, and differentiate, it doesn't maintain the stem cell characteristics of these expanded limbal cells, for example (Dietrich-Ntoukas, Hofmann-Rummelt, Kruse, & Schlötzer-Schrehardt, 2012).

2.3.4 Decellularization techniques

Decellularization is a commonly used technique in the creation of scaffolds that imitate the properties of the extracellular matrix seen in tissues or organs. With this method, a tissue's cellular constituents are removed, leaving only the extracellular matrix. Numerous methods have been explored for decellularization, broadly categorized into three main groups: physical, chemical, and biological techniques (Crapo, Gilbert, & Badylak, 2011). This process significantly reduces the risk of immunogenic rejection, enhancing the tissue's compatibility for various applications (Taghiabadi, Nasri, Shafieyan, Firoozinezhad, & Aghdami, 2015). Hence, the primary objective of the decellularization

process is the complete removal of epithelial cells while preserving a smooth, uniform basement membrane with adequate mechanical strength and biological activity. Various experimental protocols have been developed and employed for the decellularization of the human amniotic membrane, each yielding different degrees of success in achieving these goals (Hashim et al., 2016).

Physical methods encompass techniques like freeze-thawing cycles (Proulx et al., 2009) (Utomo et al., 2015) (Ide, Tohyama, Yokota, Nitatori, & Onodera, 1983) (Rahman, Griffin, Naik, Szarko, & Butler, 2018). Chemical agents include ionic detergents like sodium dodecyl sulphate (Zilic, Wilshaw, & Haycock, 2016), and non-ionic detergents such as Triton X-100 (San Choi et al., 2010). Biological methods for decellularization involve enzymes such as trypsin, and dispase. These diverse approaches offer various means to achieve the process of decellularization (Chen, Ho, Tsai, & Sheu, 2004; Wu et al., 2009).

2.3.4.1 Diaspase method

Dispase has been used to decellularize amniotic membranes (Espana et al., 2003). The literature reports clinical utilization of denuded amniotic membrane achieved with 1.2 U/ml Dispase II enzyme, exposing the membrane to treatment durations between 5 minutes and 2 hours at room temperature (Ti, Anderson, Touhami, Kim, & Tseng, 2002) (Yam et al., 2002). After this, gentle scraping is performed. Dispase II treatment leads to disruption of the basement membrane, particularly the lamina densa (Lim et al., 2009), causing irreversible physical and biological damage. Hopkinson et al. observed the absence of all basement membrane components after only 10 minutes of incubation, suggesting either complete removal or significant enzymatic degradation of the proteins (Hopkinson et al., 2008).

2.3.4.2 EDTA method

Another technique uses ethylene diaminetetraacetic acid (EDTA), a chelating chemical that affects calcium ion-regulated cell-cell interactions. (Sedar & Forte, 1964). Studies have utilized EDTA concentrations ranging from 0.02% to 0.25% for durations of 10 minutes to 2 hours at 37 °C (Koizumi et al., 2007; Nakamura et al., 2003; Nakamura,

Inatomi, Sotozono, Koizumi, & Kinoshita, 2004; Sun et al., 2005), thereafter mechanically scraping any remaining amniotic epithelial cells.

2.3.4.3 Trypsin–EDTA method

The Trypsin–EDTA method, commonly employed for detaching cells from culture flasks, has been reported for decellularization of amniotic membrane (Madhira et al., 2008; Sangwan, Vemuganti, Iftekhar, Bansal, & Rao, 2003; Sangwan, Vemuganti, Singh, et al., 2003). However, the harsh nature of trypsin–EDTA treatment may lead to the degradation of extracellular matrix components and growth factors.

2.3.4.4 Urea

Another technique involves the use of urea, a protein denaturant that can solubilize proteins (Bennion & Daggett, 2003). The amniotic membrane is decellularized by treating it with 5 minutes of ice-cold 5 M urea treatment, which is followed by gentle scraping (Mehta, Beuerman, Thein, & Tan, 2007).

2.3.4.5 Ethanol method

The ethanol method for decellularization involves incubating the amniotic membrane in 20% ethanol for 30 seconds, followed by mechanical scraping. Similar to the urea technique, the advantages include quick ethanol availability and a short incubation period. However, because of the ethanol's weaker denaturation, this technique requires more forceful scraping. The decellularized amniotic membrane has a smooth membrane surface, with a few epithelial cell fragments still visible, according to scanning electron microscopy (SEM) images (Bennion & Daggett, 2003).

2.3.4.6 SDS, and nuclease method

The amniotic membrane is incubated in a hypotonic 10 mM tris buffer with 0.1% EDTA and 10 KIU/ml aprotinin at 4 °C for 16 hours in the hypotonic buffer, SDS, and nuclease technique. This causes the cells to swell and absorb water, which causes cell lysis (Germain et al., 1995). The amniotic membrane is subsequently treated for a whole day at room temperature with an ionic detergent called sodium dodecyl sulphate. By splitting into

the lipid bilayer and dissolving it, the detergent attaches itself to cell membranes. (Germain et al., 1995). After that, the amniotic membrane is incubated for three hours at 37 °C with 50 U/ml DNase and 1 U/ml RNase to make sure that all remaining DNA and ribonucleic acid are removed from the matrix. It is possible to isolate amniotic epithelial cells without the need for mechanical scraping. The amniotic membrane was found to include no loss of collagen type IV, glycosaminoglycans, or elastin content. Additionally, there was no noticeable distinction between the denuded and intact amniotic membranes in terms of the total amount of denatured collagen (Wilshaw et al., 2006). Laleh et al., also used SDS and nucleases method for decellularization (Laleh et al., 2023).

In the extensive body of literature, diverse methodologies for decellularizing the amniotic membrane have been explored. In the context of my research, I have opted to employ the SDS method. In streamlining the procedure, my approach involves the exclusive use of 0.5% SDS over a period of 3 days. Following this, the membrane will undergo thorough washing with distilled water, a critical step aimed at effectively removing any residual detergent. This meticulous process is integral to ensuring the purity and biocompatibility of the decellularized amniotic membrane for subsequent applications in various biomedical contexts.

CHAPTER 3 : MATERIALS AND METHODS

3.1 Placenta collection

Five placentae were collected from a consenting donor after their cesarean section deliveries from Dr. Naveeda Wasif, road, Westridge III Allahabad, Rawalpindi, Punjab 46000. All donors were screened serologically for HIV, Hepatitis B, and C. The human placenta was collected and stored at 4°C until further use. For the removal of the amniotic membrane, the placenta was washed thoroughly several times using distilled water **Figure 3. 1**. All the blood content was removed. The amniotic membrane was dissected from the chorion manually using scissors and surgical blades. The translucent amniotic membrane was washed again with distilled water to remove any blood clots. Then the amniotic membranes were washed with 100ml sterile saline. The amniotic membranes were stored at -20°C for further use.

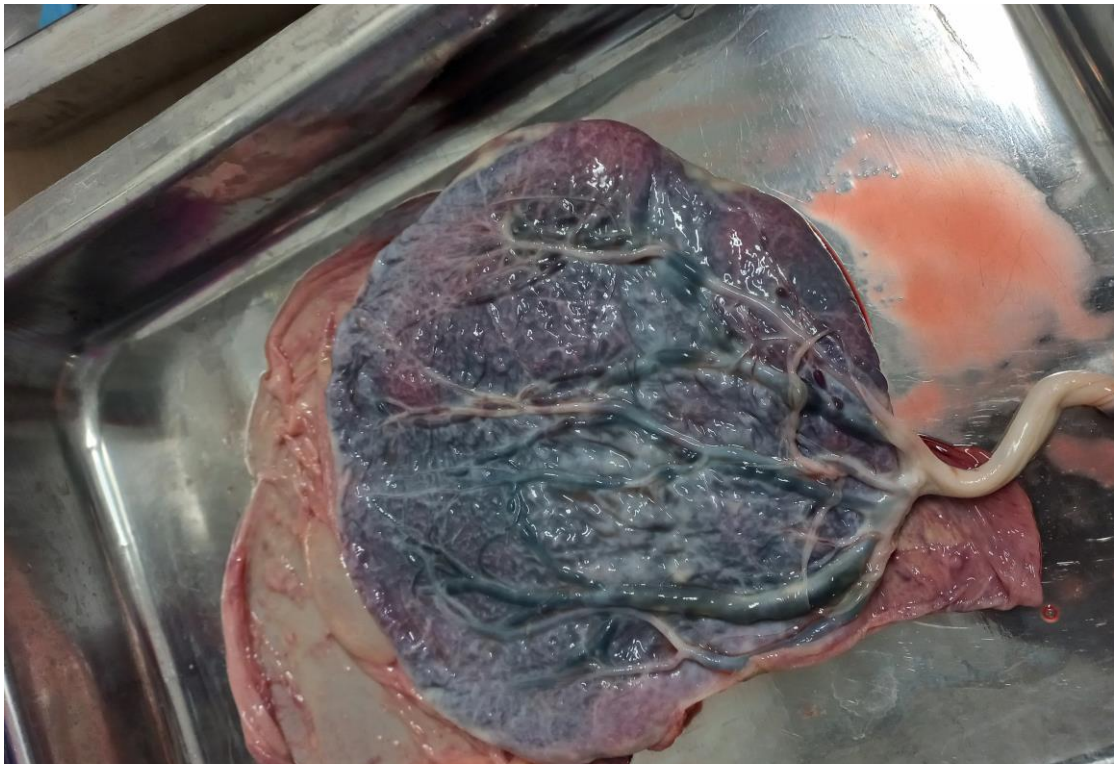


Figure 3. 1: Human Placenta.

3.2 Amniotic membrane decellularization

The membranes were thawed at room temperature. Distilled water was added, and the membranes were kept in a shaking incubator for 3-4 hours for thawing purposes. **Figure 3. 3** shows the complete methodology.

3.2.1 SDS solution

SDS solution was used to decellularize the amniotic membranes. A 0.5% SDS solution was prepared for this purpose. In the preparation of the 0.5% Sodium Dodecyl Sulfate (SDS) solution, the first step involved precise weighing of the SDS powder using a laboratory scale, adhering to the ratio of 0.5 grams of SDS per 100 mL of solution for a 0.5% concentration. Subsequently, the weighed SDS powder was placed in a beaker, and distilled water was added to initiate the dissolution process. Vigorous stirring with a stirring rod ensued until the SDS powder was completely dissolved. Post-dissolution, the volume of the solution was carefully assessed, and adjustments were made, if necessary, with distilled water to achieve the intended final volume. Following the preparation, the solution was transferred to a reagent bottle, ensuring meticulous adherence to safety protocols and the utilization of personal protective equipment due to the potential irritative nature of SDS to the skin and eyes. The entire process was conducted in alignment with established laboratory safety guidelines.

3.2.2 Antibiotic

Novidet injection is also used to inhibit the growth of bacteria.

3.2.3 Decellularization

A 0.5% SDS solution, comprising 50 ml, was introduced into a container containing amniotic membranes. The container was then placed in a shaking incubator for a duration of 24 hours. In order to facilitate the decellularization process, the SDS solution was changed twice daily. To enhance the effectiveness of the decellularization, 1 ml of novidet was added to the SDS solution. The SDS solution, which thickened as a result of the

amniotic membrane decellularization, was subsequently discarded. This procedural sequence was iteratively conducted over a span of three days, ensuring thorough and repetitive treatment for the optimal removal of cellular components from the amniotic membranes. **Figure 3. 2** shows the intact and decellularized amniotic membrane.

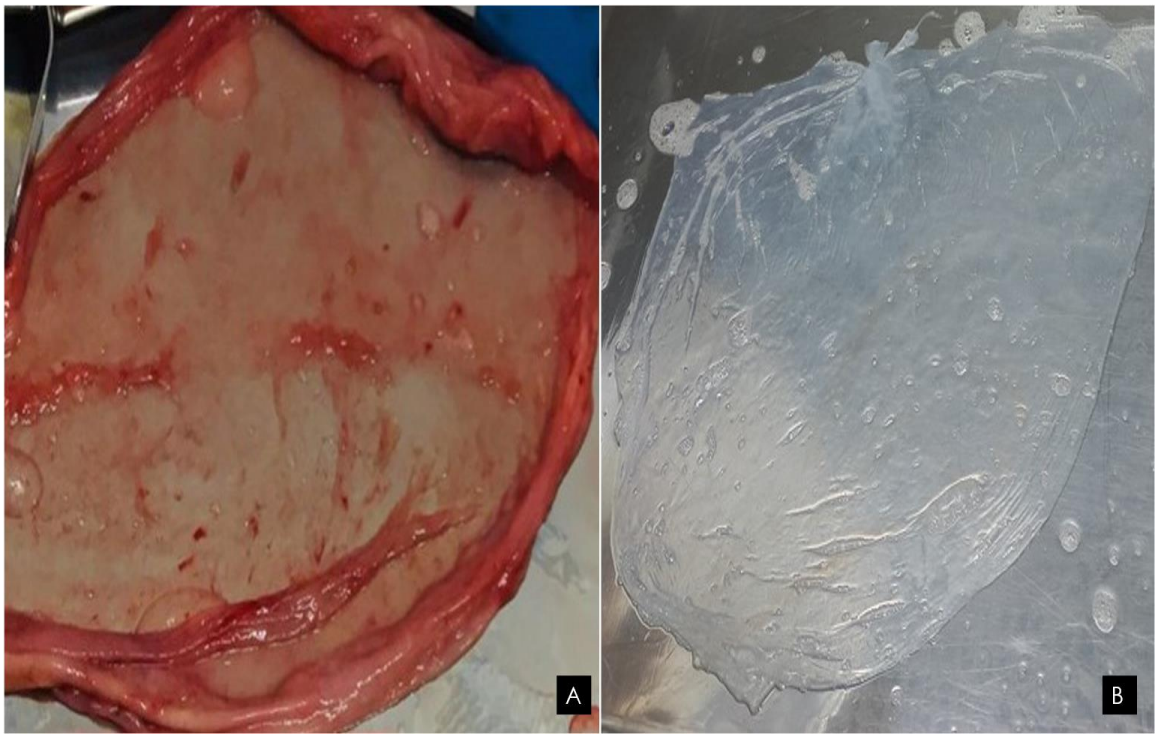


Figure 3. 2: Intact (A) and Decellularized amniotic membrane (B).

3.2.4 Extensive Washing

The decellularized membrane was washed thoroughly with distilled water to remove any residual SDS solution. Finally, the membranes were washed with 0.9% saline.

3.2.5 Membranes Cutting

Aseptic forceps and scissors were utilized to cut the amniotic membranes into 5x5 cm pieces. Following this, the cut pieces of amniotic membranes were submerged in 70% ethanol for sterilization purposes.

3.2.6 Membranes spreading

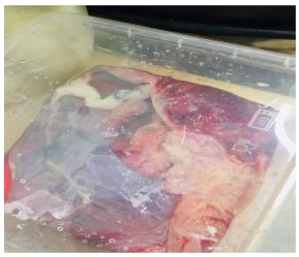
The individual pieces of amniotic membranes were carefully laid out on aluminum foil, creating an even spread. Subsequently, this arrangement was placed within a controlled environment, typically under a hood, to undergo air drying. This process is crucial for the removal of any residual moisture from the amniotic membranes, ensuring that they are thoroughly dried and ready for subsequent stages in the experimental protocol. The use of aluminum foil provides a stable surface for the membranes during the drying process.

3.2.7 Membranes drying

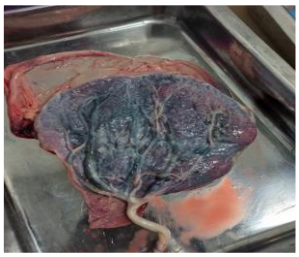
The drying process of the membranes took approximately 6-7 hours, during which they were left undisturbed in the designated environment. This duration allowed for the effective removal of moisture, ensuring that the amniotic membranes became thoroughly dry.

3.2.8 UV light

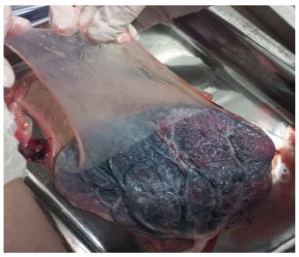
The membranes were sterilized by exposure to UV light within a sterile hood for 20 minutes. Following the sterilization process, the membranes were carefully stored in a sterile container.



i. Human Placenta



ii. Washing



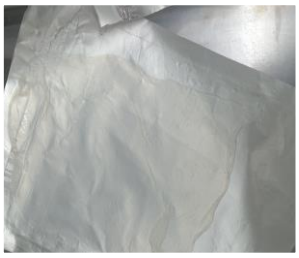
iii. Amniotic membrane Separation

iv. Amniotic membrane washing

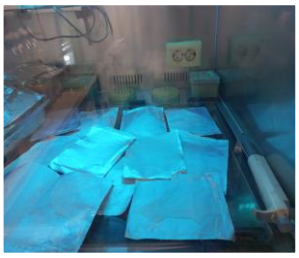
v. Decellularizatin By 0.5% SDS

vi. Washing with Distilled water

vii. Ethanol wash



viii. Membrane spreading



ix. UV Light

x. Dry Membrane

Figure 3. 3: Amniotic membrane processing and decellularization.

3.3 Anti-microbial activity

The anti-microbial activity of the amniotic membrane is assessed by the disc diffusion method.

3.3.1 Media preparation

Nutrient agar was used for bacterial culture. To prepare 500 ml of agar, 18.5 grams of nutrient agar powder were meticulously measured and mixed with distilled water. The resulting mixture was autoclaved to ensure sterility, and the pH level was carefully adjusted to 7.0, creating an optimal environment for bacterial cultivation.

3.3.2 Sterilization

Every Petri plate, flask, and media bottle utilized in the experiments underwent a thorough sterilization process. The items were subjected to autoclaving at a temperature of 121°C for 30 minutes. This sterilization protocol ensured the elimination of any potential contaminants, creating a sterile environment for the experimental procedures, thereby maintaining the integrity of the culture media and facilitating reliable and uncontaminated results.

3.3.3 Pouring into sterilized plates

After the autoclaving process, allow the agar medium to cool down to approximately 45°C. Once the agar medium has cooled to the desired temperature, add 25 ml of the agar into each plate, ensuring that the plates are laid on a clean, level surface. Swirl the plate gently to ensure even distribution of the agar, and then let it solidify.

3.3.4 Bacterial strains

Bacillus subtilius, *Staphylococcus aureus* and *Escherichia coli* were used in the assay in which the *Bacillus subtilius*, *Staphylococcus aureus* were gram-positive and the *Escherichia coli* was gram-negative.

3.3.5 Inoculation of Petri plates

A volume of 100 µl from the bacterial cultures was carefully dispensed onto a Petri plate containing 25 ml of solidified nutrient agar. The even distribution of the bacterial suspension was achieved by spreading it across the agar surface using a glass rod. This method ensures uniform inoculation of the bacterial cultures onto the agar medium, facilitating the subsequent growth and observation of bacterial colonies.

3.3.6 Disc preparation

Amniotic discs were prepared by cutting a 6/6 mm square from the amniotic membrane, utilizing sterile scissors to maintain aseptic conditions. Subsequently, employing sterile forceps, the dried and impregnated discs were delicately placed on the surface of the inoculated agar plate.

3.3.7 Incubation

The agar plates were incubated in an inverted position (with the lid on the bottom) at the specified temperature for a duration of 16 to 18 hours. Following the incubation period, the zone of inhibition was measured and recorded as the mean value along with the standard deviation, providing a quantitative assessment of the inhibitory effects observed in the bacterial cultures on the agar plates.

3.4 Characterization of Amniotic membrane

3.4.1 Histological Analysis

Intact and decellularized Human Amniotic Membranes were fixed in 10% natural-buffered formalin at room temperature for 24 hours. Subsequently, the samples underwent dehydration through a graded alcohol series and were embedded in paraffin wax. Sections,S approximately 4 mm in thickness, were obtained using a Manual Rotary Microtome. Following this, the sections were subjected to staining with Hematoxylin and Eosin (H&E) and Masson's Trichrome (MT) from Sigma-Aldrich, USA. The stained

samples were then examined under a light microscope (LOBMED, Lobo America, Inc.) for detailed analysis and observation.

In the examination of intact and decellularized Human Amniotic Membranes, Hematoxylin and Eosin (H&E) staining serves as a foundational tool for revealing cellular morphology and overall tissue architecture. H&E staining allows for the identification of cell nuclei and differentiation of cytoplasmic components, aiding in the assessment of cellular integrity. Masson's Trichrome staining is employed to specifically highlight collagen fibers, providing crucial information about the extracellular matrix. In the context of intact amniotic membrane, H&E staining can unveil the native cellular composition, while Masson's Trichrome staining helps assess the distribution and preservation of collagen. In decellularized amniotic membrane, these staining techniques play a pivotal role in evaluating the success of the decellularization process, confirming the absence of cells, and ensuring the preservation of the extracellular matrix, particularly collagen fibers.

3.4.2 SEM

The amniotic membrane was observed using a Scanning electron microscope (SEM) (JEOL JSM-64900). The membranes were coated with gold using an ion spectrum device. The SEM was equipped with a secondary electron beam detector and a 20 kV accelerating voltage for the analysis. Both the decellularized and pure amniotic membranes were examined for their structure using SEM. For structural analysis surface and cross-sectional images were taken.

3.4.3 FTIR

For the characterization of the amniotic membrane, FTIR was performed. FTIR (Fourier Transform Infrared) spectroscopy is a common analytical technique used to identify and characterize the molecular composition of a wide range of materials. It measures the absorption of infrared light by the sample, which can provide valuable information about the functional groups and chemical bonds present in the material.

ATR-FTIR analysis was conducted to assess alterations in functional groups and surface chemistry of intact and decellularized amniotic membranes. FTIR spectra were

collected using an Agilent Cary 630 FTIR spectrometer (U.S.A.) employing the Attenuated Total Reflection (ATR) technique. This analysis aimed to investigate specific chemical group changes in the modified membranes.

3.5 In-vivo wound healing experiment

In this study involving in-vivo experimentation, I procured six-month-old Wistar outbred albino rats from the Atta ur Rahman School of Biological Sciences, following ethical approval by the NUST Animal Ethics Committee.

For the experiment, the rats were divided into three groups. Rats in Group 1 received no treatment at all and no intervention. Polyfex, a common ointment, was used to treat Group 2. Rats in Group 3 received an amniotic membrane treatment. There were five rats in each group, for a total of fifteen rats in the experiment. This arrangement made it possible to compare the impact on the observed results of regular ointment treatment, amniotic membrane treatment, and no treatment at all.

To maintain a sterile environment, the surgical site was cleansed with 70% ethanol, and all instruments were properly sterilized. Anesthesia was administered using Ketamine hydrochloride (75 mg/kg) and xylazine (10 mg/kg), both sourced from Sigma, USA. Subsequently, sterile scissors were employed to remove fur from the designated wound area, surgical blade was used to shave the back of the rats **Figure 3. 4**. Punch biopsy was used to create an 8mm full-thickness wound. To eliminate any residual blood, the wounds were thoroughly cleansed with 0.9% saline solution.

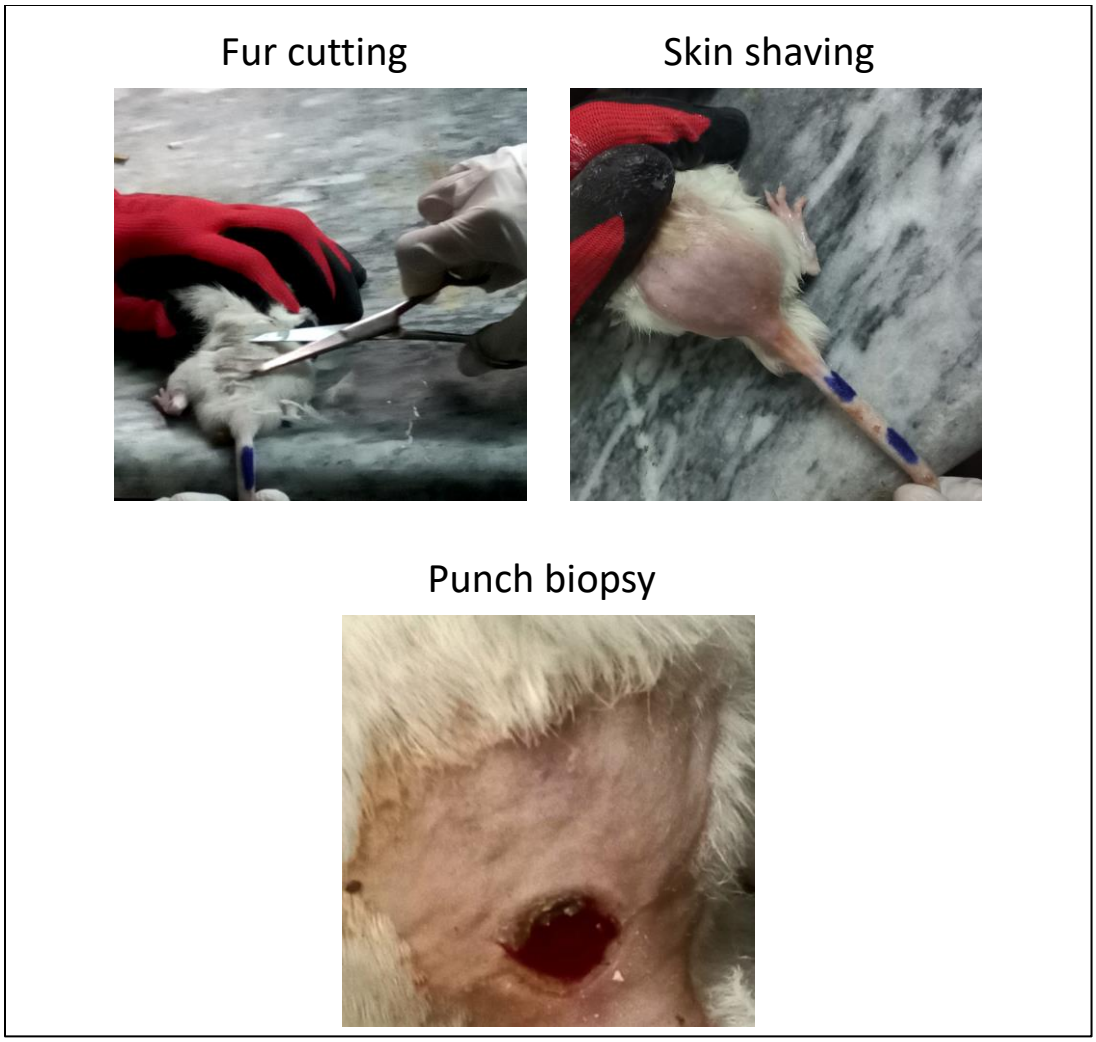


Figure 3. 4: Rat Wound Model

CHAPTER 4 : RESULTS

4.1 Phenotypic Analysis:

In this study, the intact amniotic membrane is described as glistening, translucent, and pliable with a smooth surface and inherent moisture due to amniotic fluid. It exhibits strong adhesion to the chorion and is recognized for its translucency, pliability, and adhesion, making it valuable in biological and clinical contexts. After meticulous decellularization, the membrane maintains translucency and pliability but becomes thinner and acquires a matte finish. The decellularized membrane's porous structure, resulting from effective cell removal, enhances cellular integration, making it ideal for regenerative applications. Despite increased fragility, it retains structural integrity, proving invaluable in regenerative medicine for tissue repair and cell engraftment **Figure 4. 1**.

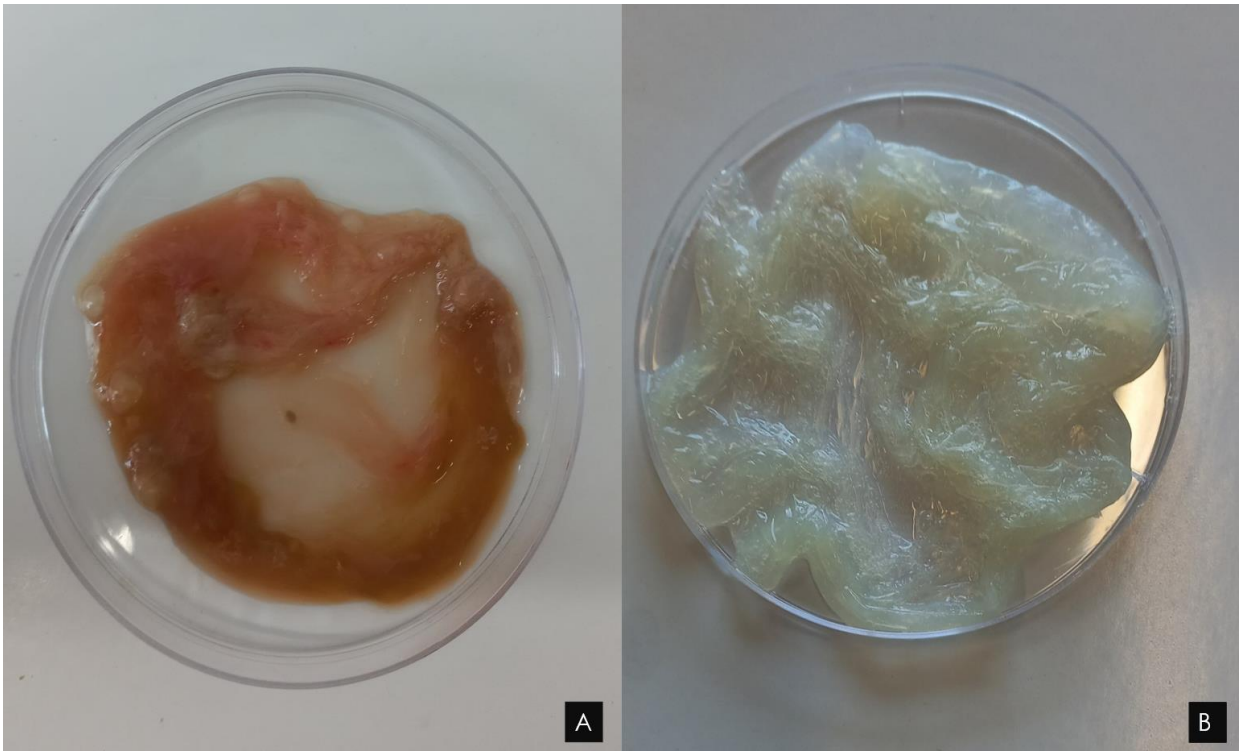


Figure 4. 1: Difference between Intact (A) and decellularized amniotic membrane (B).

4.2 Histological Analysis:

Microscopic analysis was performed with a light microscope (LOBMED, Lobo America, Inc.) which confirmed the absence of cellular components, including nuclei, cytoplasm, and cellular membranes, through meticulous examination. The intact amniotic membrane contains the amniotic epithelium, whereas the decellularized amniotic membrane lacks this cellular component.

The efficacy of the decellularization process was affirmed by the absence of cells post-procedure. To create a natural, ECM-rich scaffold suitable for long-term storage, we implemented a cost-effective method using 0.5% SDS to remove cells from the amniotic membranes. The resultant decellularized amniotic membrane scaffolds retained their elasticity and mechanical integrity, albeit with discernible differences when compared to intact amniotic membrane structures.

H&E stain **Figure 4. 2** results showed the elimination of cellular material while preserving the integrity of the extracellular matrix including the basement membrane. The decellularized amniotic (b) membrane exhibited a lack of epithelial layer, cellular components, and nuclear material in contrast to the native amniotic membrane (a). Masson's trichrome staining revealed the preservation of the complete extracellular matrix within the decellularized amniotic membrane, highlighting the presence of intact collagen fibers. This observation is depicted in the associated **Figure 4. 2** (d), where both the absence of cells and the maintained structural integrity are discernible.

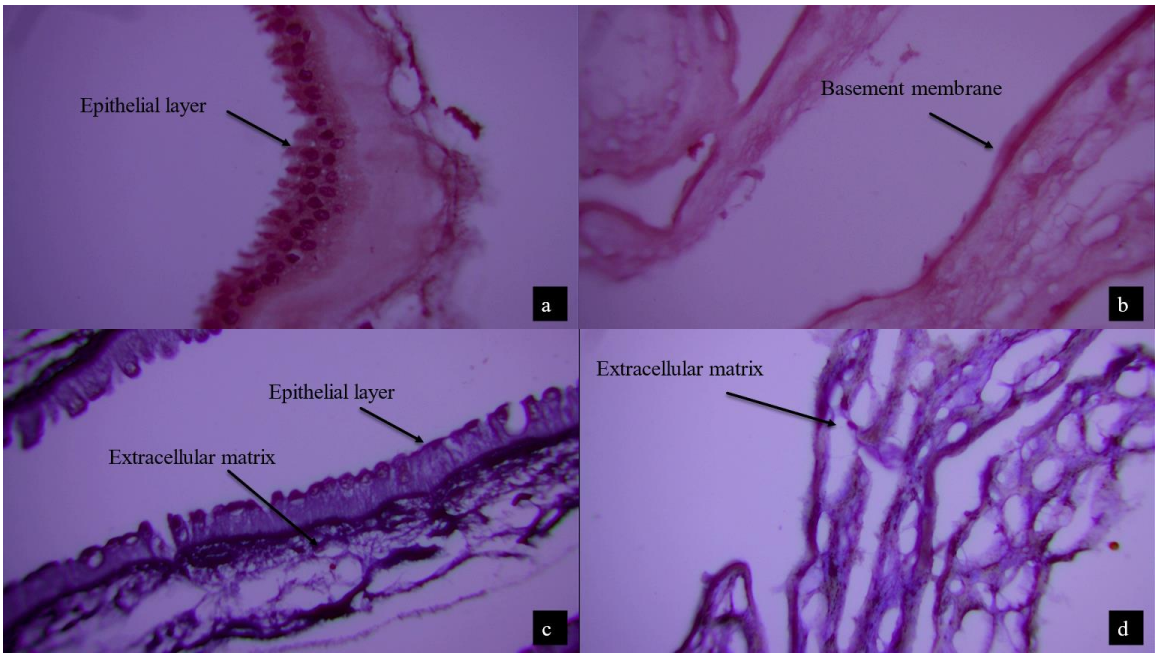


Figure 4. 2: Microscopic observation of Amniotic Membranes through H&E staining, a. intact b. decellularized and Masson's Trichrome staining, c. intact d. decellularized.

4.3 Antimicrobial activity:

The assessment of antimicrobial activity has demonstrated that the amniotic membrane can impede the growth of microorganisms. Specifically, the amniotic membrane exhibited antimicrobial effects against three types of bacteria: *Bacillus subtilis*, *Staphylococcus aureus*, and *Escherichia coli.*

The findings are presented in **Figure 4. 3** which outlines the results of these antimicrobial tests. The antimicrobial activity of the amniotic membrane against each bacterium was assessed three times, and the results were reported as the mean ± standard deviation **Figure 4. 4**. Notably, the Decellularized Amniotic membrane displayed a significant inhibitory effect against the growth of *Bacillus subtilis*. The term "zone of inhibition" refers to the area around a substance where microbial growth is prevented. In the context of the table, a high zone of inhibition suggests that the decellularized amniotic membrane was particularly effective in restraining the growth of Bacillus subtilis bacteria. The zones of inhibition for *Bacillus subtilis*, *Escherichia coli (E. coli)*, and *Staphylococcus aureus* were measured at 32 mm ± 0.28, 18 mm ± 0.5, and 19 mm ± 0.57 units, respectively.

This data is noteworthy as it indicates the potential of the amniotic membrane, especially in its decellularized form, to serve as an antimicrobial agent against specific types of bacteria. Such information holds promise for various applications where controlling microbial growth is essential, such as wound healing or medical device development.

Table 4. 1: Antimicrobial activity of Amniotic membrane.

Sample	Antimicrobial activity (zones of inhibition mm)		
	Bacillus subtilis	*Staphylococcus aureus*	*Escherichia coli*
Decellularized Amniotic membrane	32 mm ± 0.28	18.5mm ± 0.5	18 mm ± 0.57

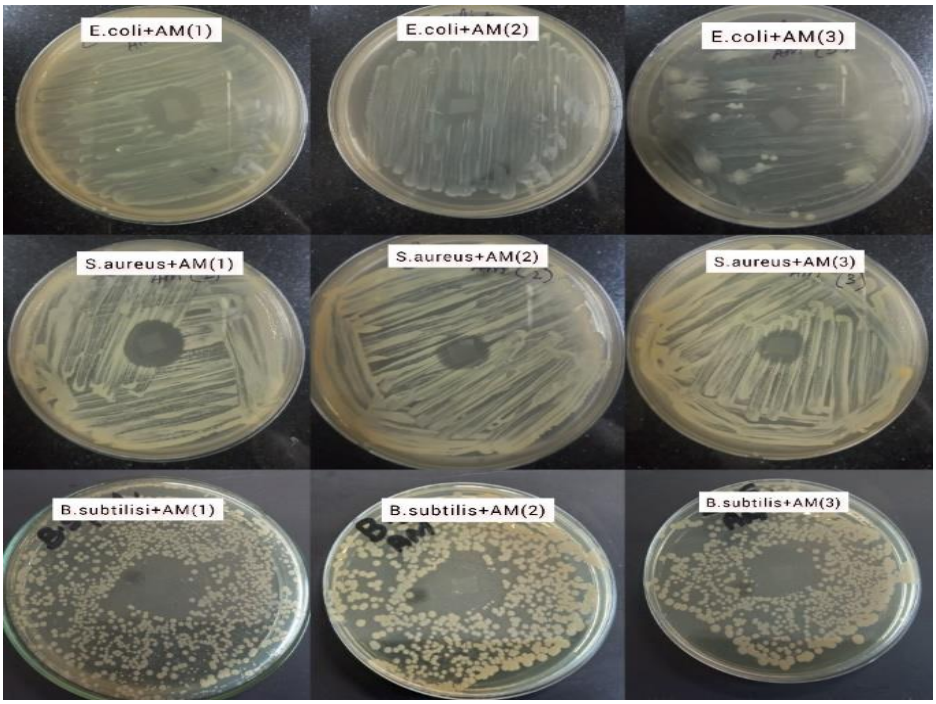


Figure 4. 3: Antimicrobial activity of decellularized amniotic membrane.

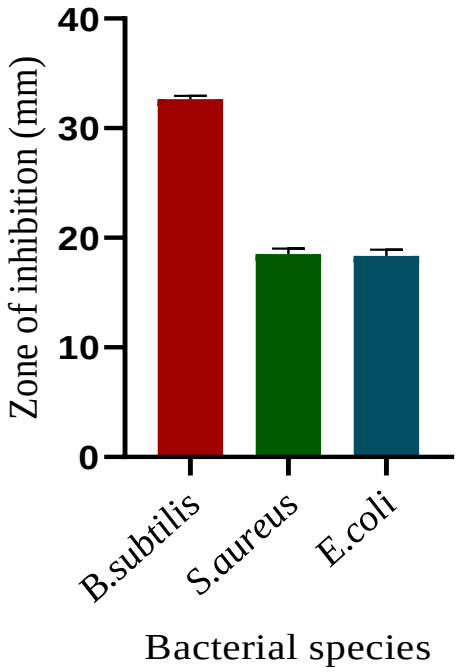


Figure 4. 4: Amniotic membrane antimicrobial activity.

4.4 SEM:

SEM analysis was conducted at various magnification levels, including X10,000, and X25,000, using micrometer sizes of 1um. The purpose was to compare the natural amniotic membrane with a decellularized one, and the distinctions between them. The SEM images of intact and decellularized amniotic membranes are labeled as **Figure 4. 5** (a, b, c) and **Figure 4. 5** (d, e, f) respectively.

Figure 4. 5 (a) highlights the surface structure of the intact amniotic membrane, while (d) demonstrates the surface structure of the amniotic membrane after decellularization. **Figure 4. 5**(b, c), a cross-sectional perspective of the intact amniotic membrane in contrast **Figure 4. 5** (e, f) exhibits the cross-sectional view of the amniotic membrane after the decellularization process. In **Figure 4. 5** (a, b, c) of the SEM images, the area appeared densely populated, indicating the presence of cells on the surface of the intact amniotic membrane. These dense regions suggested that the original membrane retains its cellular composition. Contrastingly, in **Figure 4. 5** (d, e, f) the corresponding images display

areas with less density and noticeable empty spaces. These characteristics indicate the decellularization of the amniotic membrane. The empty spaces in these images suggest that the cellular components have been effectively removed from the amniotic membrane. This observation aligns with the expected outcome of the decellularization procedure, where the goal is to eliminate cellular elements while preserving the structural integrity and composition of the extracellular matrix. The SEM results provide a comprehensive visual comparison between the intact and decellularized amniotic membranes, showing their structural differences across various magnification levels and micrometer sizes.

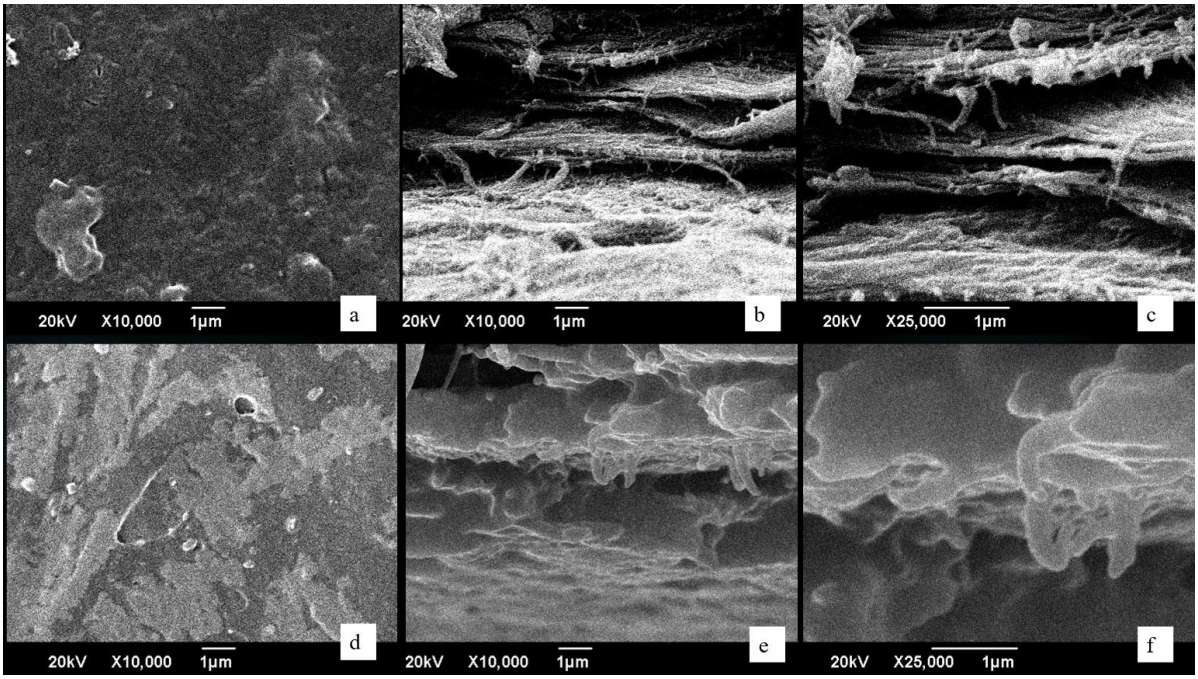


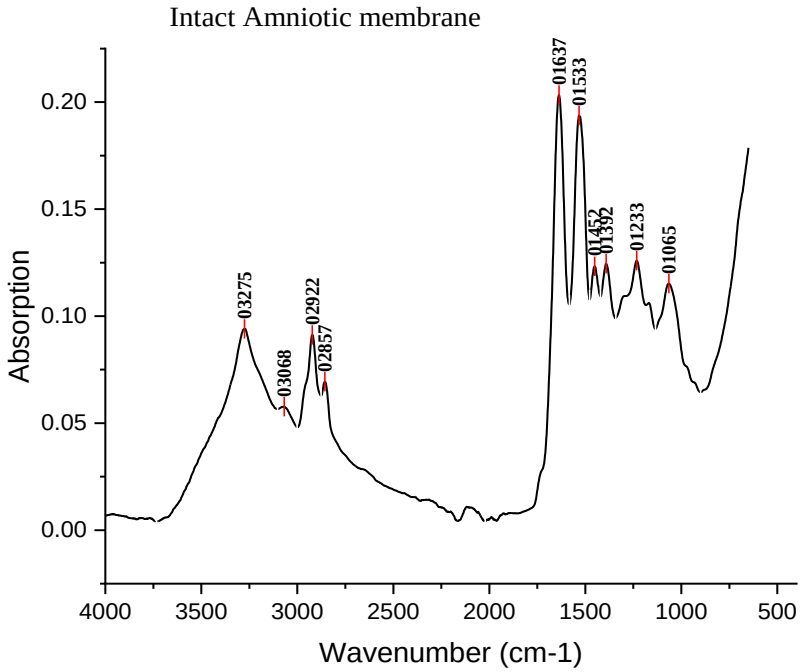
Figure 4. 5: Scanning Electron Micrographs of intact (a, b, c) and decellularized Human Amniotic Membranes (d, e, f). (a,d) Amniotic membranes external surface SEM images. (b, c, e, f) Amniotic membranes cross-sectional SEM images.

4.5 FTIR:

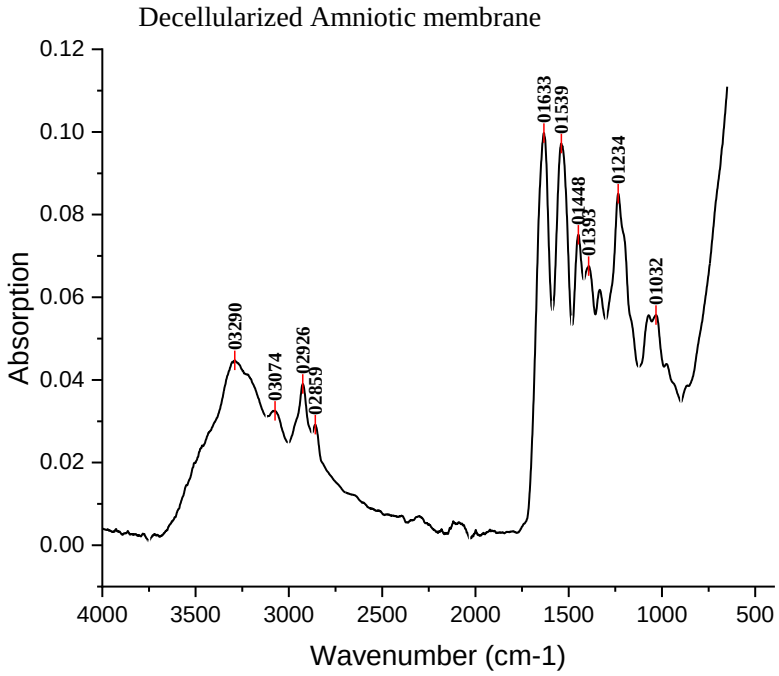
The **Graph 4. 1** and **Graph 4. 2** display FTIR spectra recorded within the 400–4000 cm^{-1} range. The Amide I band, typically found in the range of 1600-1700 cm^{-1}, arises from the coupling of C=O stretching vibrations with N–H bending vibrations. The Amide II band arises from N-H bending in the range of 1500-1600. On the other hand, the Amide III bands observed between 1300–1200 cm^{-1}, are a result of the interaction between N–H bending and C–N stretching vibrations. The amide I band serves as a sensitive indicator of

protein secondary structure and conformational alterations (Doyle, Bendit, & Blout, 1975). The positions of the amide I and amide II bands are highly responsive to protein secondary structure due to the involvement of both C=O and N-H bonds in the hydrogen bonding interactions occurring within various secondary structural elements. In the intact amniotic membrane, the Amide I band was observed at 1637 cm^{-1}, the Amide II was observed at 1533 cm-1 and the Amide III band was detected at 1233 cm^{-1}. Conversely, in the decellularized amniotic membrane, the Amide I band was noted at 1634 cm^{-1}, the amide II band was observed at 1539 cm^{-1} and the Amide III band was identified at 1231 cm^{-1}, demonstrating the presence of a collagen structure and the existence of functional groups (such as amide groups) suggests enhanced cell attachment to the scaffold. The band located near 1450 cm^{-1} likely corresponds to C–H bending vibrations. For the decellularized amniotic membrane, slight shifts were observed in the absorption bands.

The distinct bands associated with collagen are still observable after the decellularization process, indicating that collagen remains present in the membrane. However, noticeable variations in the intensity of these spectral bands are apparent. These differences can be attributed to the interaction between the membranes and the SDS (Sodium Dodecyl Sulfate) solution. The SDS solution might have influenced the membrane properties, leading to changes in the intensity of the collagen bands. This observation suggests that the decellularization procedure, while preserving collagen, also induces alterations in the membrane structure likely due to its interaction with the chemical solution.



Graph 4. 1: FTIR Spectra of Intact Amniotic membrane



Graph 4. 2: FTIR Spectra of Decellularized amniotic membrane.

4.6 Phenotypic analysis of wound healing:

In the assessment of decellularized amniotic membranes' impact on wound healing, a rat model was utilized, involving three distinct groups **Figure 4. 6**. The findings revealed a significantly accelerated rate of wound closure in both Group 3 and Group 2. These groups, treated with decellularized amniotic membrane and standard ointment (Polfex), respectively, demonstrated remarkable improvements in wound healing compared to they control group (untreated). Particularly noteworthy was the outstanding progress observed in Group 3, where not only did the wounds exhibit advanced closure, but they also healed completely without any noticeable scarring within 14 days. This compelling result underscores the efficacy of decellularized amniotic membranes in promoting rapid wound closure and minimizing scarring, suggesting their promising potential as a therapeutic intervention for optimizing the wound healing process.

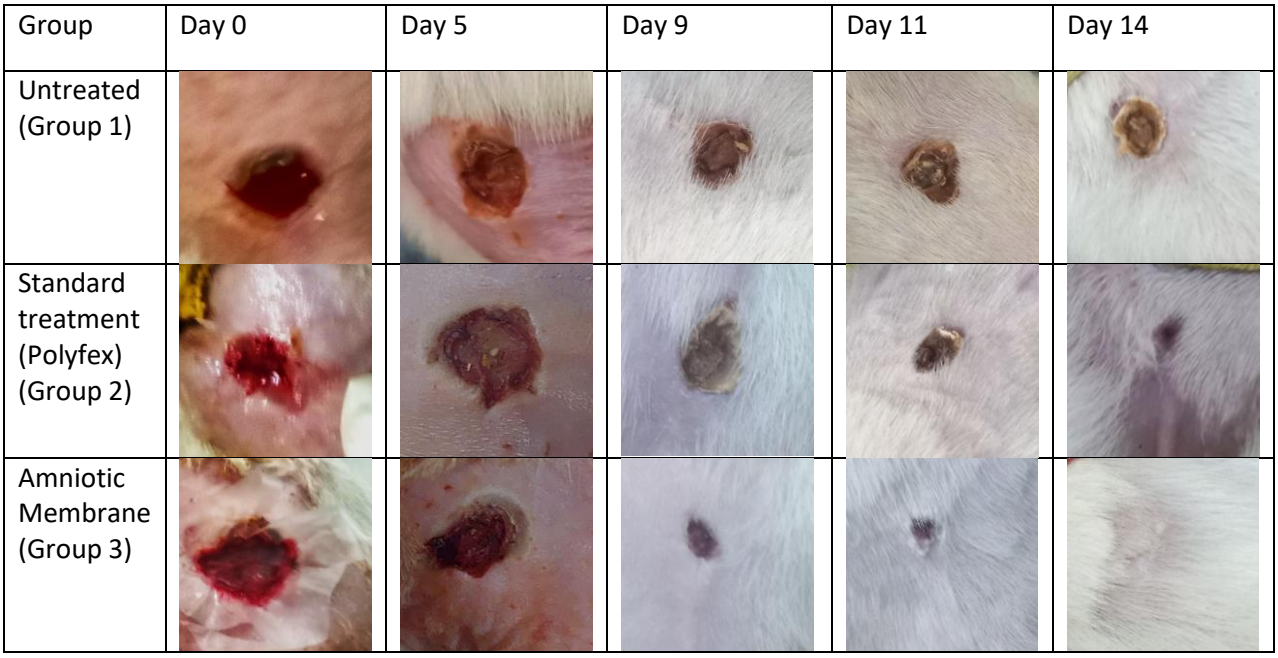


Figure 4. 6: Wounds healing in Rats model.

4.6.1 Histological Analysis:

In the histological examination conducted after 14 days, rat wound tissues were analyzed using H&E staining. The study aimed to compare the effectiveness of

decellularized amniotic membrane with two other groups: the control group (Group 1) receiving no treatment, and Group 2 treated with a standard ointment (polyfex). The histological analysis of treated wounds is shown in **Figure 4. 7**.

4.6.2 Control Group (Group 1):

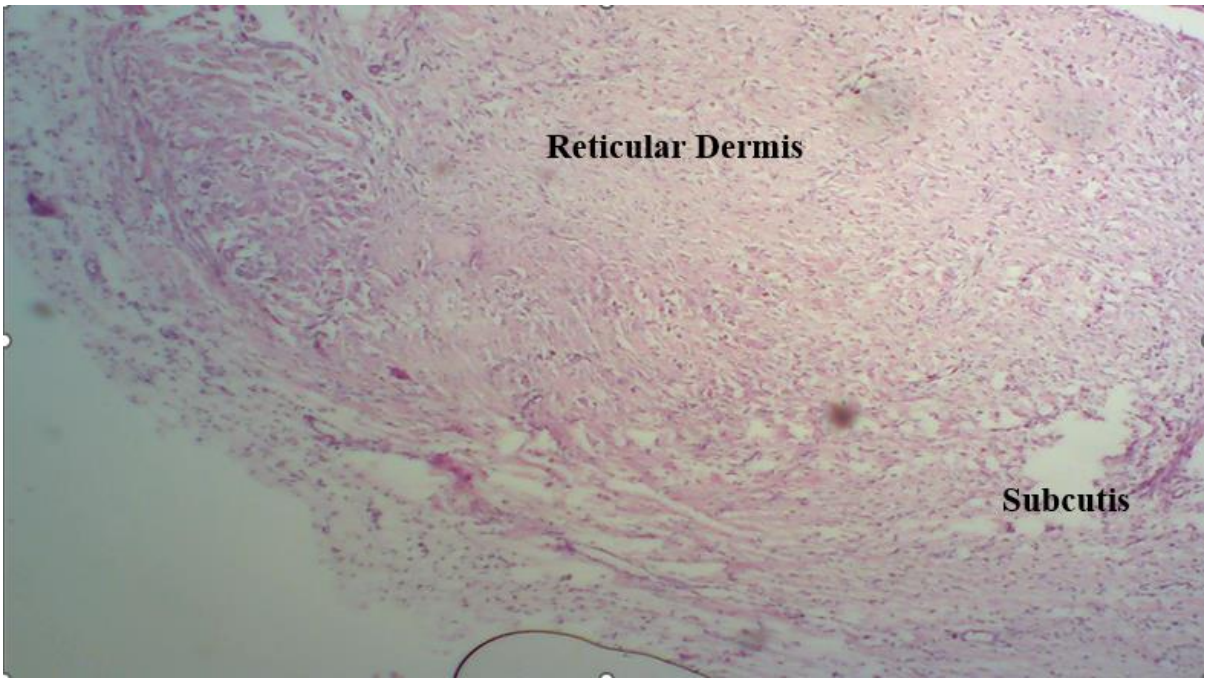
In image A, **Figure 4. 7** representing untreated rats, the histological slide illustrates incomplete wound healing. Dermal and epidermal layers were not formed, indicating persistent inflammation, and limited natural healing response.

4.6.3 Standard Ointment Group (Group 2):

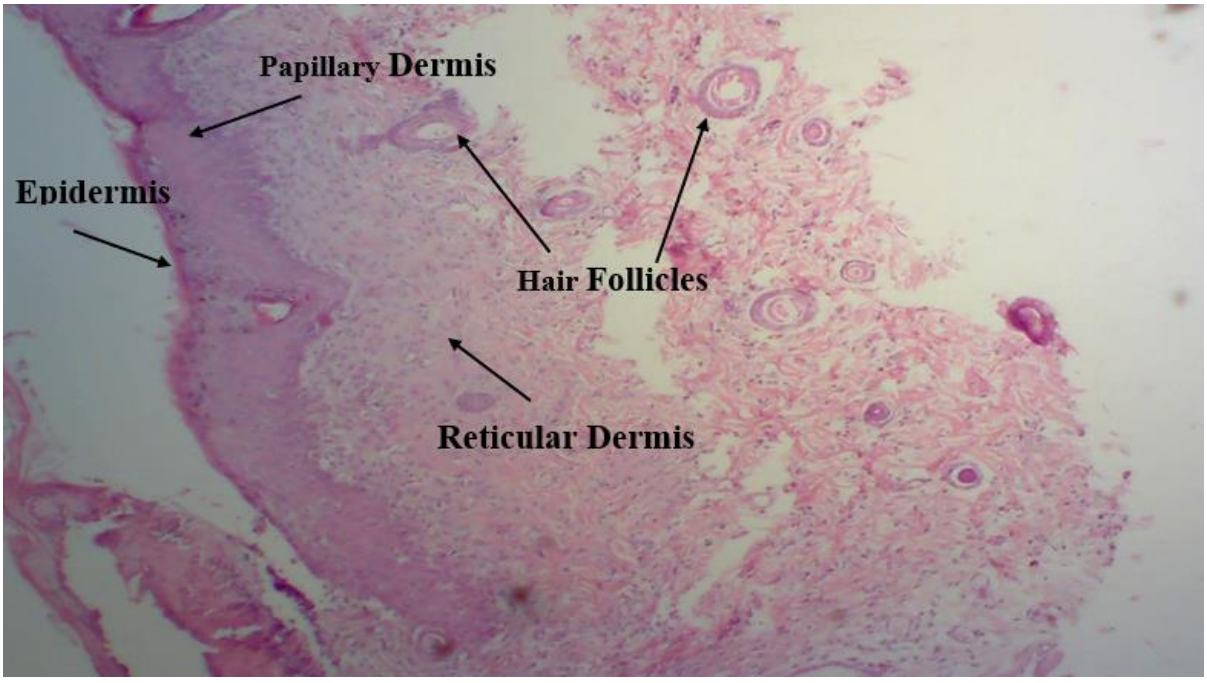
Image B, **Figure 4. 7** displays the histological slide of Group 2 rats treated with standard ointment (polyfex). Here, the slide demonstrates wound healing progress with the development of hair follicles, epidermis, and dermis, indicating a partial recovery due to the ointment treatment.

4.6.4 Decellularized Amniotic Membrane Group (Group 3):

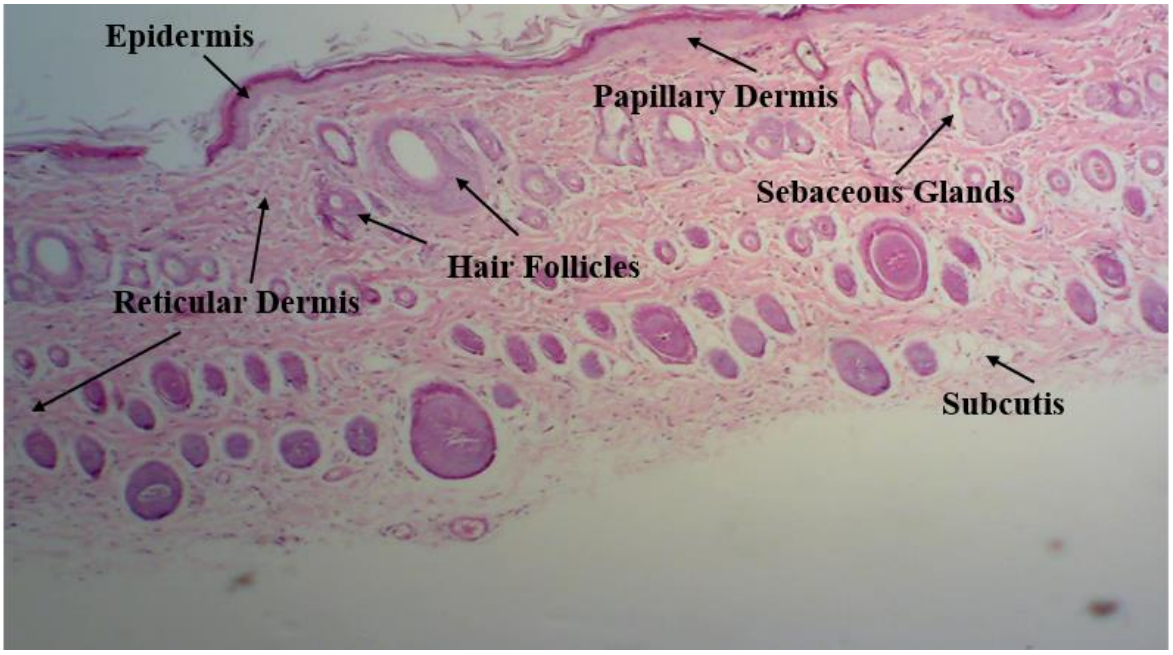
Image C, **Figure 4. 7** shows the histological slide of Group 3 rats treated with decellularized amniotic membrane. Remarkably, the slide reveals a fully developed epidermis, hair follicles, and sebaceous glands. The presence of a well-formed epidermis and stratum corneum indicates efficient wound healing and scar removal. This observation highlights the superior efficacy of the amniotic membrane in promoting wound closure and tissue regeneration compared to the control and standard ointment-treated groups.



A. Untreated wound



B. Wound treated with standard Ointment.



C. Wound treated with amniotic membrane.

Figure 4. 7: Histological analysis of rat wounds.

CHAPTER 5 : DISCUSSION

Numerous studies have validated that the amniotic membrane is effective in promoting wound healing. Recent investigations have highlighted the enhanced healing capabilities, along with reduced infection and pain, attributed to the use of dressings made from the human amniotic membrane.

Decellularized human amniotic membranes have been studied in the past for their potential as a scaffold without cells to heal corneal and skin abnormalities or as a transporter for other cell types (Liu et al., 2020) (Leal-Marin et al., 2021). In parallel to previous studies on the amniotic membrane, our approach was focused on developing a cost-effective method based on 0.5% SDS only, to decellularize the amniotic membrane and then characterize it to see if the integrity, functionality, and oridinality of the membrane remain intact to the reported studies.

Different studies reported histological analysis of amniotic membrane (Khosravimelal, Momeni, Gholipur, Kundu, & Gholipourmalekabadi, 2020), our membrane histological analysis (**Figure 4. 2**) correlates with other reported studies (Milan et al., 2020). Histological slides that were stained with H&E showed the intact amniotic membrane that exhibited a single layer of epithelium, whereas this layer was absent in the decellularized amniotic membrane. It is noteworthy to highlight that the findings of the current study align with those reported by Villamil Ballesteros et al., who previously employed 0.1% SDS, 0.1 M NaOH, PAA, + ascorbic acid to decellularize the amniotic membrane (Villamil Ballesteros et al., 2020). Milan et al. have used 2% SDS for the decellularization of the amniotic membrane and it was observed that the extracellular matrix and basement membrane have not been preserved (Milan et al., 2020). While in this study the trichrome stain revealed that the collagen fibers are preserved. In the H&E slides the basement membrane can be seen in the decellularized amniotic membrane shown in **Figure 4. 2**. The basement membrane of the amnion plays a key role in effectively regenerating damaged skin tissue, modulating the development and differentiation of

fibroblasts and keratinocytes, and facilitating the construction of epithelial tissue (Niknejad et al., 2008).

Amniotic membrane grafts not only act as biological barrier but also show anti-bacterial and anti-viral properties. The risk of infection is decreased by preventing bacterial penetration and growth by covering the wound surface with an amniotic membrane (BARADARAN, Aghayan, Arjmand, & Javadi, 2007). A variety of bacterial strains exhibited varied degrees of growth inhibition when exposed to amniotic membranes, including *E. coli, Bacillus cereus, Klebsiella pneumonia, Streptococcus pyogenes, Pseudomonas aeruginosa, Staphylococcus aureus, Shigella flexneri,* and *Lactobacillus plantarum* (Zare-Bidaki, Sadrinia, Erfani, Afkar, & Ghanbarzade, 2017). This study investigated the antimicrobial efficacy of decellularized amniotic membranes against *E. coli, Staphylococcus aureus, and Bacillus subtilus*, revealing notable zones of inhibition.

Earlier investigations using SEM analysis demonstrated that the native amniotic membrane displayed collagen fibers interwoven with tissue cells on an uneven surface. In contrast, the decellularization process in those studies effectively eliminated cellular contents while maintaining the integrity of the native extracellular matrix (Villamil Ballesteros et al., 2020). In the current study, SEM micrographs of the native amniotic membrane revealed a dense composition, indicating the presence of cells. Conversely, the decellularized amniotic membrane micrographs exhibited lower density containing only the extracellular matrix.

In this study, the amide I, II, and III were observed at 1637 cm^{-1}, 1533 cm-1 1233 cm^{-1} respectively which correlate with the reported studies. Previous studies showed that the Amide I band is generally located in the 1600–1700 cm^{-1} range. Between 1500 and 1600, N-H bending gives birth to the Amide II band. However, the combination of N–H bending and C–N stretching vibrations results in the Amide III bands that are seen in the 1300–1200 cm^{-1} range. The amide I band serves as a sensitive indicator of protein secondary structure and conformational alterations (Doyle et al., 1975).

Rats subjected to amniotic membrane graft treatment demonstrated accelerated healing in comparison to the control groups, indicating the potential therapeutic efficacy

of this intervention, which was also reported previously (Milan et al., 2020). In our study, along with the wound contraction and healing the fur at the site of wound of treated rats were also restored within 2 weeks.

CONCLUSION

In conclusion, our study has achieved significant milestones in the development of an efficient and cost-effective method for the decellularization and preservation of Human Amniotic Membrane, thereby amplifying its therapeutic potential for wound healing applications. The outcomes emphasize the robustness of the proposed decellularization protocol, showcasing its ability to preserve human amniotic membranes at room temperature and positioning it as a promising biological scaffold for wound healing. The successful validation of decellularization through H&E staining and the confirmation of collagen presence via Trichrome staining further affirm the method's efficacy.

Moreover, our in-vivo wound healing experiments provide compelling evidence of the amniotic membrane's effectiveness in facilitating accelerated and efficient wound healing. The amalgamation of cost-effectiveness, simplicity, rapid processing, and safety aspects solidify this method as a noteworthy strategy for advancing decellularized human amniotic membranes in the realms of wound healing and tissue engineering. These findings collectively contribute to the growing body of knowledge in regenerative medicine, offering a practical and impactful approach that holds promise for future clinical applications.

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