FEASIBILITY OF NANOPOLYMER AND 3D PRINTED SKIN IN LOWER LIMB WOUNDS: PROSPECTIVE PRECLINICAL AND CLINICAL ASSESSMENT

By

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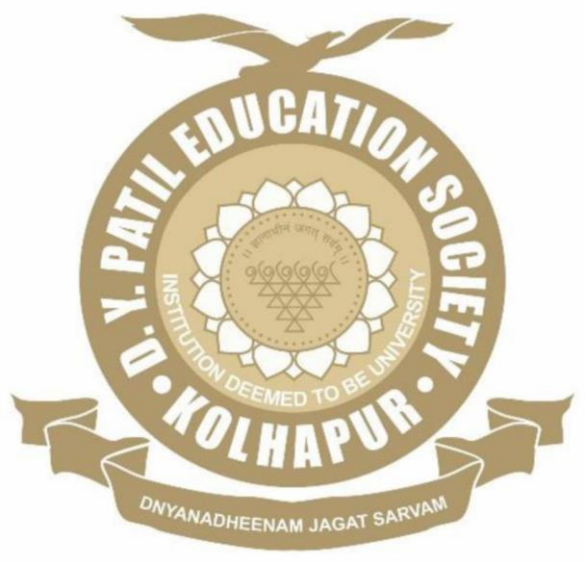

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BY

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DECLARATION

I hereby declare that the work presented in this thesis entitled “Feasibility of Nanopolymer and 3D Printed Skin in Lower Limb Wounds: Prospective Preclinical and Clinical Assessment” is entirely original and was carried out by me independently in the D. Y. Patil Education Society (Deemed to be University), Kolhapur under the guidance of Dr. Meghnad G Joshi, Professor and Head, Department of Stem Cells and Regenerative Medicine, D.Y. Patil University (Deemed to be University), Kolhapur (M. S.) India and co-guidance of Dr. Vaishali V Gaikwad, Professor and MS, Department of Surgery, Dr. D. Y. Patil Medical College, Hospital, and Research Centre, Kadamwadi, Kolhapur (M.S.), India. I further declare that present work has not form the basis for the award of any degree, diploma, fellowship or similar title of any University or institutions. The extend information derived from the existing literature has been indicated in the body of the thesis at appropriate places giving the references.

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Certificate

This is to certify that the work incorporated in the thesis "Feasibility of Nanopolymer and 3D Printed Skin in Lower Limb Wounds: Prospective Preclinical and Clinical Assessment" submitted herewith for the degree of Doctor of Philosophy in Stem Cell and Regenerative Medicine of D. Y. Patil Education Society (Deemed to be University), Kolhapur by Mrunal N Damle was carried out under my supervision. This thesis or any part of it was part of any not submitted for any degree or diploma or any academic elsewhere.

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SUMMARY OF RESEARCH WORK

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1. Formulation and synthesis process of mucosal connective tissue loaded gel-based topical agent for diabetic wound healing applications: Application Number: 202521006571
2. Formulation and synthesis process of human placental extract loaded topical gel for diabetic and non-healing wound ulcers; Application Number: 202521006565

B) Papers Published at International Journals:

1. **Damle, Mrunal N., Leena Chaudhari, Kishor Tardalkar, Nilesh Bhamare, Swapnali Jagdale, Vaishali Gaikwad, Dharvi Chhabra, Balvinder Kumar, Anju Manuja, and Meghnad G. Joshi. "A biologically functional bioink based on extracellular matrix derived collagen for 3D printing of skin." *International Journal of Biological Macromolecules* 258 (2024): 128851.**
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2. Kshersagar, Jeevitaa, Lavanya Pulgam, **Mrunal N. Damle**, Kishore Tardalkar, Rakesh Sharma, and Meghnad G. Joshi. "Transplantation of human placenta derived mitochondria promotes cell communication in endometrium in a murine model of disturbed endometrium." *Stem Cell Reviews and Reports* 19, no. 5 (2023): 1384-1401.
3. Kshersagar, Jeevitaa, **Mrunal N. Damle**, Poonam Bedge, Rakhi Jagdale, Kishor Tardalkar, Dhanaji Jadhav, Swapnali Jagadale, Yashwant Toro, Rakesh Sharma, and Meghnad G. Joshi. "Downregulation of MICA/B tumor surface expressions and augmented soluble MICA serum levels correlate with disease stage in breast cancer." *Breast Disease* 41, no. 1 (2023): 471-480.
4. Tardalkar, Kishor R., Leena R. Chaudhari, **Mrunal N. Damle**, Akshay A. Kawale,

Nilesh C. Bhamare, Jeevitaa R. Kshersagar, Tanvee S. Kulkarni, and Meghnad G. Joshi. "Extracellular-matrix Composite Bioink for 3D bioprinting and molding of small diameter vascular grafts." *Bioprinting* 34 (2023): e00300.

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7. Joshi, Meghnad G., **Mrunal N. Damle**, and Rakesh Kumar Sharma. "Application route of mitochondrial transplantation." In *Mitochondrial Transplantation and Transfer*, pp. 231-280. Academic Press, 2024.
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C) National/International Conferences

1. International conference on biomedical and clinical research 2022., organized by Shri Dharmasthala Manjunatheshwara University at Ishavaasyam, Dharwad on November 21 and November 22, 2022
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D) Paper Presentation (Oral)

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E) Poster Presentation

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F) Webinars attended:

2. Attended online National Conference on Present Day Biology: Recent Advancements in Biological Sciences 10 – 11 December 2021 organized by St. Xaviers College, Ahmadabad
3. Attended online National Conference on Bioinnovation & Entrepreneurship (NCBE-2022) organised by Department of Biotechnology KL foundation – 12th Feb 2022
4. Attended series webinar arranged by Springer Nature Experiments Training Webinar Sept 202
5. Attended online webinar on Merck Spectroscopy and Microscopy: Principles and Applications – 29th June 2022
6. Attended online webinar on 2D Electrophoresis (2DE): Principles & Applications organized by MERCK Innovation Lab, Banglore, chandigarh, - 22nd August 2022
7. Biosafety Levels in Research Laboratory organized by organised by MERCK Innovation Lab, Banglore, chandigarh - 5th sept 2022
8. Attended online webinar on Attended online webinar on ' Mass spectroscopy: concept, instrumentation and application' organised by MERCK Innovation Lab, Banglore, chandigarh, 26th sept. 2022.
9. Onine webinar attended on ' cell based funtional assay' organised by MERCK Innovation Lab, Banglore, chandigarh, 26th sept. 2022.
10. Participated in 'Maharashtra Startup Yatra 2022' and presented idea in bootcamp organized at RIT college of engineering, Sangli, 14th oct. 2022.
11. Novel methods for the culture and development of stem cell-derived 2D and 3D models, 12 december 2022, Cell microsystems

12. Novel Immuno-regulatory methods using designer extracellular vesicles, 25 jan 2023, Biocompare
13. Developing a spatio-temporal single-cell type map of adult human tissues, Feb 21 2023, Akoya Biosciences
14. Advanced Therapies: Scalable and Sustainable Cell and Gene Therapies, tissue engineering, may 16 2023
15. Bioprinting strategies for vascularized bone tissue engineering, 23 may 2023, Cellink
16. 3D Bioprinting for Biomedical Applications, 19th Dec 2023, Celllink and ALTEM technologies
17. See what machine learning can do for nano and biomaterial characterizations, 30 Nov 2023, Select Sceience
18. Dr. Joshua Morgan, Assistant Professor at the University of California to explore the culture of Vascularized Human Skin Equivalents (VHSEs), Nov 16, 2023

CONTENT

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS

%	Percentage
^{0}C	Degree Celsius
3D	Three-dimensional
AB	Alcian Blue
Ang	Angiopoietin
AS	Artificial 3D printed Skin
BFGF	Basic Fibroblast Growth Factor
BSA	Bovine Albumin Serum
CAD	Computer-aided Design
CAM	Chorioallontoic Membrane Assay
cDNA	Complementary Deoxy ribonucleic acid
CK	Cytokeratin
cm	Centimetres
Col 1	Collagen type 1
COMP	Cartilage Oligomettric Matric Protein
CTGF	Connective Tissue Growth Factor
CTRI	Clinical Trial Registry of India
D/W	Distilled water
DAPI	4',6-diamidino-2-phenylindole
dECM	Decellularized Extracellular Matrix
DFU	Diabetic Foot Ulcers
DMSO	Dimethyl sulfoxide
DNA	Deoxyribo Nucleic Acid
DPBS	Delbucco's Phosphate Buffer Saline
E-cad	E cadherin
ECM	Extra cellular matrix
EDS	Energy Dispersive X-ray Spectroscopy
EDTA	Ethylenediaminetetra acetic Acid
EGF	epidermal growth factor
EGFR	Epidermal Growth Factor Receptor
EM	Elastic Modulus
EpCAM	Epithelial Cell Adhesion Molecule
EPCs	Endothelial Progenitor Cells
FDA	Food and Drug Administration
FGF	Fibroblast Growth Factor
FOX 1	Forkhead Box M1
F-T	Freeze- Thaw cycles
FTIR	Fourier Transform Infrared Spectroscopy
g	Gram
g	Gravitational Force
GAGs	Glycosaminoglycan
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GF	Growth Factors
h	Hour/s
HA	Hyaluronic Acid
HbA1c	Hemoglobin A1C
HE	Hematoxylin and Eosine
HPLC	High Performance Liquid Chromatography

IEAC	Institutional Ethical Committee
IGF	Insulin- like Growth Factor
IL	Interleukin
IPD	Inpatient Department
kg	kilogram
KGF	Keratinocyte Growth Factor
mg	milligrams
Min	minutes
ml	millilitres
Mm/s	millimetre per second
mm	millimetres
MMMLV	Murine Leukaemia Virus
MMP	Matrix Metalloproteinase
MNCs	mononuclear cells
mRNA	Messenger Ribonucleic Acid
MT	Massion's Trichrome
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
N	Newton
NaCl	Sodium Chloride
NaOH	Sodium Hydroxide
NCCS	National Centre of Cell Science
NCIM	National Collection of Industrial Microorganisms
nm	Nano molar
NS	Normal saline
OD	Optical Density
Oligo dT	oligo deoxythymidines
OPD	Outpatient Department
Pa	Pascal's
PBS	Phosphate Buffer Saline
PCL	Polycaprolactone
PCR	Polymerase Chain Reaction
PDGF	Platelet-derived growth factor
PDGF-BB	Platelet-Derived Growth Factor Subunit B
PEG	Poly- ethylene Glycol
PLA	Polylactic Acid
PLGA	Polylactic-co-Glycolic Acid
PMSCs	Placental-derived Mesenchymal cells
PRP	Platelet Rich Plasma
MSCs	Mesenchymal Stem Cells
PU	Polyurethane
PVA	Poly-vinyl Acid
PVD	Peripheral vascular disease
qPCR	Quantitative Polymerase Chain Reaction
RBC	Red Blood Cells
RNA	Ribo Nucleic Acid
RPM	Rotations per minute
RS	Rabbit Skin
SD	Standard Deviation

SEM	Scanning Electron Microscopy	UV	Ultraviolet
STL	Stereolithographical	VCAM	Vascular Cell Adhesion Molecule
STZ	Streptozotocin	VEGF	Vascular endothelial growth factor
TGA	Thermo-Gravimetric Analysis	Wt	Weight
TGF	Transforming Growth Factor	XRD	X-Ray Diffraction Analysis
UCB MSCs	Umbilical cord blood mesenchymal stem cells	αSMA	Smooth Ac
UTS	Ultimate Tesile Strength		

CHAPTER 1: GENERAL INTRODUCTION AND LITERATURE SURVEY

1.1. Introduction

Skin being the vast organ, serves as the primary defence mechanism against external stimuli while also playing a significant role in haemostasis, sensation and immune responses. The skin is composed of three layers namely; epidermis, dermis and hypodermis, each function for the protection and complex physiological processes. However, different forms of injuries can disturb the usual structure of skin leading in disruption of tissue architecture and its function (Kumar, MA, 2024).

The restoration of injured tissue is an active and complex route of cascade including four phases of hemostasis, inflammation, proliferation, and remodelling. There are number of growth factors and cellular components responsible for these cascades to work properly. These growth factors are transforming growth factor beta (TGF-β), vascular endothelial growth factor (VEGF), and platelet-derived growth factor (PDGF) and many more. Despite the capability of the body to heal the injured tissue, certain wounds, such as those associated with diabetes, burns, and surgery, stand significant challenges due to their complex etiology and pathophysiology (Pakyari M, et al., 2013). The patients with diabetic wounds have major complications like prolonged inflammation, impaired angiogenesis. Similarly, burns and surgical wounds can result in significant tissue damage, infection, and delayed healing. Advanced therapeutic strategies are required for the management of these chronic wounds and acute wounds that extend beyond conventional wound care (Davis FM, et al., 2018).

Recent developments in tissue engineering and regenerative medicine have opened up some advanced treatments methods of including use of hydrogels, synthetic and biological polymers as well as the usage of stem cells. The goal of these technologies is to refine the wound healing process by providing scaffolds which supports cell propagation, proliferation, delivery and tissue regeneration. The clinical translation of these treatments is often hampered by cost effectiveness, mechanical properties, biocompatibility despite their potential.

There are several products currently available in the market for the treatment of diabetic wounds. However, they have limitations and risk of incomplete healing, high recurrence rate and unwanted side effects. The ongoing clinical trials focus their outcomes of using the new therapeutic applications and overcoming the limitations and patient care.

The purpose of this review is to provide a comprehensive overview of the skin structure, the wound healing process, and the etiology and pathophysiology of different wound types. It also discusses the role of growth factors in wound healing and evaluates the effectiveness of new treatments, including tissue engineering and regenerative medicine. Additionally, the current

picture of diabetic wound products and ongoing clinical trials are examined and the advances and challenges in the area of wound healing administration have been discussed.

1.2. The skin

The skin is the most extensive organ in the human body. It keeps body temperature stable and stops water loss. In addition, it acts as primary barrier against many infections, environmental germs and trauma. It contributes to metabolism through the synthesis of vitamin D and the formation of hormones. Derived from the ectoderm, skin is composed of multiple layers of cells and connective tissue that allow it to heal itself after an injury. Depending on the location, the skin is thickest on the soles of the feet and palms and thinnest on the eyelids. It consists of sensory receptors, sweat and sebaceous glands, hair follicles and blood vessels. The structure varies depending on the body part, with the face, soles of the feet and scalp having particular characteristics. The basement membrane, dermis and epidermis are the three primary skin layers that cover the subcutaneous tissue and subcutaneous fat (Walters KA and Roberts MS 2002; Yadav N. et al., 2019).

The epidermis consists largely of keratinocyte layers, but also contains non-epithelial cells such as melanocytes, Merkel cells and antigen-presenting dendritic Langerhans cells. Layers are classdied into four different categories based on the arrangement of their cells: stratum corneum, s. granulosum, s. lucidum, s. spinosum and s. basale (McGrath JA. et al., 2004). Melanocytes, Langerhans cells and Merkel cells are also found in the epidermis, and keratinocytes undergo mitosis to regenerate the skin. Neural crest cells give rise to melanocytes, which generate UV-blocking melanin. Originating from bone marrow stem cells, Langerhans cells serve as immune system cells that present antigens. Merkel cells are light touch mechanoreceptors in the stratum basale and are connected to nearby keratinocytes (Koster MI., 2009; Yousef, et al., 2017).

The basement membrane is composed of ECM and other proteins like collagen, laminin, fibronectin and glycosaminoglycans; and has receptors like integrins and dystroglycan to support the mechanical strength of epithelial cells. It consists of different layers, including the basal lamina, the lamina lucida, the lamina densa and the lamina reticularis. The proteins it contains include heparan sulfate proteoglycans, perlecan, laminin and collagen type IV. This membrane plays a crucial role in angiogenesis by acting as a selective barrier, promoting cell division and controlling cell growth during tissue development and repair. The basement membrane consists of ECM proteins such as collagen, laminin, fibronectin and

glycosaminoglycans and supports and anchors epithelial cells through receptors like integrins and dystroglycans. It has a vital part in angiogenesis and promotes cell division and tissue development.

The dermis serves as the skin's innermost layer, is composed of two layers viz the reticular and papillary layers. Blood vessels, hair follicles, lymphatic vessels, sweat glands, sebum, and fibrous and elastic tissue makes up the dermis structure. In addition to having mast cells, blood vessels, collagen fibers, fibroblasts, nerve endings, and lymphatic vessels, the dermis offers flexibility and protection. It also contains connective tissue and mucopolysaccharides. The dermis layer provides mechanical and nutritional support and regulates body temperature. The hypodermis is located near the dermis and houses fat lobules and other skin appendages. The skin, the outermost layer, is vulnerable to trauma and injury that can result in wounds. Recovery from skin wounds depends on cellular function, but management practices limit healing rather than restoring tissue integrity.

1.3. Wound and Classification of Wounds

"A wound is defined as a break in tissues' epithelial integrity, which can occur due to physical trauma such as tearing, cutting, or puncturing the skin."

Wounds are being classified into two categories such as acute and chronic. This classification is based on their healing time and underlying pathophysiological mechanisms (Whitney JD, 2005).

An acute wound is defined as a quick injury to the skin or tissue that heals normally within an expected period which can be usually a few days to weeks. As discussed above these wounds heal through the four phases of wound healing; hemostasis, inflammation, proliferation, and remodelling (Fig. 1). Common examples of acute wounds include:

- Surgical wounds: Cuts during surgical procedures.
- Traumatic wounds: Cuts or abrasions leading from accidents or injuries.
- Burns: Damage caused by heat, chemicals, or electricity to the skin

Acute wounds are treated by following standard protocol such as cleaning and dressing changes, resulting to complete wound closure without any complications.

Whereas, a chronic wounds are complicated characterized by a prolonged healing process that lasts longer than 30 days and has impaired wound healing (Chaby G et al., 2007).

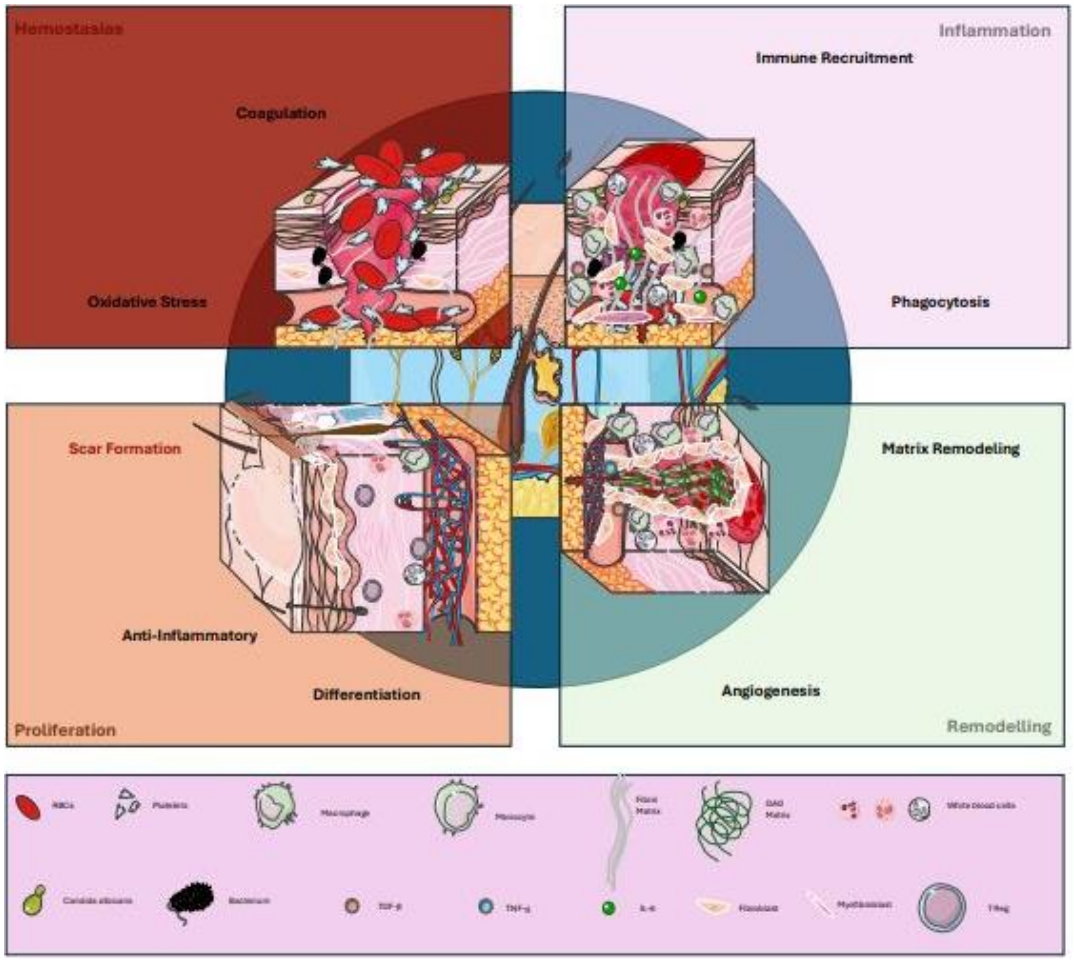

Figure 1.1 Phases of wound healing

These wounds may persist in the inflammatory phase for longer periods due to various factors, including health conditions. Examples of chronic wounds are as follows.

The ulcers are caused by underlying disease or an internal reason. The ulcer can be formed by different reasons like;

- Diabetic ulcers: Commonly found on the feet of individuals with diabetes due to poor circulation and nerve damage.
- Venous ulcers: Resulting from improper blood flow in the veins.
- Pressure ulcers (bedsores): Caused by prolonged pressure on the skin, often in individuals with limited mobility

Chronic wounds are associated with stubborn inflammation and may require dedicated treatment approaches that deal with the problems associated with circulatory issues or diabetes management.

Wounds are caused by damage or disruption of normal structure that results in injury. They can result from any disease or accidental causes. The wound can cause bleeding, vasoconstriction,

complement activation and an inflammatory reaction. The non-treatable wounds like diabetic wounds are classified as acute or chronic (Man E and Hoskins C., 2020; Cheng B, et al., 2021). Acute wounds caused by surgery or trauma normally heal within 5-10 days (Raziyeva K, et al., 2021). Chronic wounds caused by factors such as infections, necrosis, tissue hypoxia and inflammatory cytokines can lead to prolonged tissue healing (Falanga V, et al., 2022). The infection which can arise from trauma, post-infection, or large tissue removal is classified into complex wounds; a combination of acute and chronic wounds (Markiewicz-Gospodarek A, et al., 2022).

Diabetes is a metabolic syndrome characterized by hyperglycemia and results in various complications such as diabetic foot ulcers. These ulcers occur in 85% of cases and can lead to amputations of the lower limbs. The risk of foot ulcers in diabetics is between 1.9% and 2.2%. Risk factors include peripheral neuropathy, peripheral vascular disease, foot abnormalities, arterial insufficiency, trauma, and reduced resistance to infection. Motor neuropathy caused by diabetes impairs the body's coordination of movements and causes osteomyelitis, resulting in atrophy of the foot muscles and changes in the architecture of the foot. Additionally, Neuropathy raises a question about infection in diabetic foot. Peripheral vascular disease (PVD) in the lower limbs is an atherosclerotic occlusive condition (Kim J., 2023).

Every year millions of people suffer from burns, causing various serious complications. The pathophysiology of burns involves tissue damage from heat, chemicals, or other sources that results in inflammation and vascular changes (Arturson, 2007; Singer and Clark, 1999). This reaction hinders tissue perfusion and slows healing by causing oedema, fluid leakage, and cell damage. Surgical wounds emerged from medical operations should be treated with high care to achieve full recovery. Surgical wounds heal through the phases of inflammation, tissue growth, and remodelling; risks include infections brought on by skin barrier disruption and microbial exposure (Alexander et al., 2018; Allegranzi et al., 2016). Good surgical practices and sterilization help reduce these risks.

1.4. Cellular Basis of Wound Healing

Healing takes place in different phases, each of which is essential for repair. The hemostasis phase initiates (Fig. 1) the process and stops bleeding through rapid constriction of blood vessels and the formation of platelet plugs. This phase also forms a fibrin scaffold that releases cytokines and growth factors required for subsequent healing (Coller, 2015; Davi and Patrono, 2007). The next phase is the inflammatory phase (Fig. 1), in which immune cells remove deposits and fight infections. Neutrophils and macrophages release cytokines and growth

factors to coordinate repair and attract new cells to the site (Gallucci and Sloan, 2015; Koh and DiPietro, 2011). During the proliferation phase (Fig. 1), fibroblasts, endothelial cells and keratinocytes actively rebuild tissue. Fibroblasts form a collagen-rich matrix, while endothelial cells form new vessels and keratinocytes cover the wound surface, reducing the risk of infection (Gurtner et al., 2008; Gallucci and Sloan, 2015). Finally, the remodelling phase (Fig. 1) strengthens and organizes the tissue and gradually transforms it into a functional scar. ECM remodelling and collagen alignment improve tissue strength and functionality (Koh and DiPietro, 2011; Gurtner et al., 2008). In diseases such as diabetes, wound healing is impaired and often stops at one stage, causing problems like amputation.

1.5. Impaired wound healing:

Major clinical concern, impaired wound healing is illustrated by persistent wounds that do not resolve throughout the usual phases of wound healing. The delayed wound closure causes risks of secondary infections. Understanding the mechanisms that responsible to impaired wound healing is essential to created focused treatments strategies to expand clinical consequences and decrease the burden of chronic wounds.

The dysregulation of growth factors and cytokines involved in the wound healing process is one of the key factors for impaired wound healing. Growth factors like platelet-derived growth factor (PDGF), transforming growth factor beta (TGF-β), and vascular endothelial growth factor (VEGF) contributes significantly in controlling diverse phases of wound healing, including inflammation, proliferation, and remodelling (Eming et al., 2014; Bielefeld et al., 2019). Dysregulated expression or activity of these growth factors can impair cellular responses critical for tissue repair, resulting in delayed wound closure and impaired angiogenesis.

Reduced expression of PDGF receptors on fibroblasts and impaired responsiveness to PDGF stimulation contribute to reduced fibroblast proliferation and impaired collagen synthesis, which impairs granulation tissue development and delays wound closure (Brem et al., 2017; Ennis et al., 2019). Similarly, defective TGF-β signalling been noted in chronic wounds, where excessive TGF-β production promotes fibrosis and ECM deposition, causes the development non-healing wounds with excessive scar tissue (Ennis et al., 2019; Han et al., 2018). Furthermore, inadequate VEGF expression and impaired angiogenesis contribute to poor wound bed vascularization, further compromising tissue perfusion and nutrient supply required for effective wound healing (Bielefeld et al., 2019; Han et al., 2018).

Cellular dysfunction also plays an important role in impairing wound healing, with changes in the function and phenotype of immune cells, fibroblasts, and endothelial cells contributing to impaired repair processes. In chronic wounds, persistent inflammation, characterized by persistent infiltration of inflammatory cells like neutrophils and macrophages, contributes to tissue damage and delays in the transition to the proliferative phase (Eming et al., 2014; Rodero and Khosrotehrani, 2010). Dysfunctional macrophages in chronic wounds exhibit an impaired can turn from a proinflammatory to a proreparative phenotype, resulting in sustained cytokine release and impaired ECM remodelling (Ennis et al., 2019; Rodero and Khosrotehrani, 2010). Similarly, fibroblasts in chronic wounds exhibit senescence and reduced proliferation capacity, compromising their ability to produce ECM components necessary for tissue repair (Ennis et al., 2019; Lerman et al., 2012). Endothelial dysfunction and impaired angiogenesis further exacerbate tissue hypoxia and ischemia, perpetuating a cycle of impaired wound healing and tissue damage (Han et al., 2018; Bielefeld et al., 2019).

Additionally, genetic factors might have a role in poor wound healing; discrete susceptibility to chronic wounds is influenced by variation in the genes linked to inflammation, ECM remodelling, angiogenesis, and cell proliferation (Han et al., 2018; Ennis et al., 2019). The expression or activity of cytokines, growth factors, and their receptors can be impacted by gene polymorphisms, which can lead to abnormal extracellular matrix deposition, dysregulated inflammatory responses, and impaired angiogenesis (Bielefeld et al., 2019; Han et al., 2018). As an illustration of the significance of genetic predisposition to wound healing outcomes, differences in genes encoding elements of the TGF-β signaling pathway have been linked to an increased risk of chronic wounds (Ennis et al., 2019; Han et al., 2018).

A complex mechanism, including impaired growth factor signalling, cellular dysfunction, and genetic predisposition, underlies impaired wound healing. Patients with impaired wound healing may benefit from targeted therapy techniques that alter growth factor activity, improve cellular function, and address genetic predispositions to restore normal wound healing processes. It is crucial to comprehend the fundamental processes that result in insufficient wound healing in order to increse tissue repair and lessen the burden of chronic wounds. (Wilkinson HN and Hardman MJ., 2023; Kolimi P, 2022).

1.6. Growth Factor Dynamics in Wound Healing

1.6.1 Angiogenic Growth Factors:

1.6.1.1 Vascular Endothelial Growth Factor (VEGF):

VEGF is a crucial signalling protein (Fig. 2) that enhances angiogenesis by inducing the growth of fresh capillaries from the established vasculature (Firmansyah Y et al., 2024). In the area of wound healing, angiogenesis has established role in supplying oxygen and nutrients to the proliferating cells in the wound bed and for facilitating the removal of metabolic waste products. VEGF stimulates the proliferation, migration and capillary tube formation of endothelial cells, thereby aiding in the creation of functional blood vessels within the granulation tissue. In addition, VEGF increases vascular permeability, which makes it easier for growth factors and inflammatory cells to enter the wound site, thereby promoting tissue repair. Dysregulation of VEGF expression or signaling has been linked to impaired tissue repair conditions like chronic wounds and diabetic ulcers (Liu F, et al., 2024).

1.6.1.2. Angiopoietins:

Angiopoietins are another class of crucial angiogenic factors (Fig. 2) that play a substantial role in neovascularization during wound healing (Raina N et al., 2021). In particular, angiopoietin-1 (Ang-1) is known to facilitate the maturation of endothelial cells and the stabilization of newly formed blood vessels. Ang-1 increases the bone marrow ability to mobilize endothelial progenitor cells (EPCs) to damaged site, enhancing neovascularization and supporting tissue regeneration (Balaji S et al., 2015). According to recent data, diabetic wounds with overexpressed Ang-1 have better EPC recruitment and higher capillary density, which considerably increases wound closure rates then the controls (Balaji S et al. (2015). This indicates that targeting angiopoietin signalling may be a promising strategy to improve healing because diabetic patients often have impaired vascularization.

1.6.1.3. Platelet-Derived Growth Factor (PDGF)

PDGF is a critical regulator of wound healing and is best known for its role in promoting the proliferation and migration of fibroblasts, which are critical for the formation of granulation tissue (Fig. 2). PDGF is present in the alpha granules of platelets and released at the site of injury upon activation. It acts through paracrine and autocrine mechanisms and stimulates not only fibroblasts but also monocytes and vascular smooth muscle cells. Current studies highlight the importance of PDGF in improving wound healing outcomes. Developed PDGF-BB has shown increased re-epithelialization and granulation tissue development in diabetic wound models, indicating its potential for the management of chronic wounds (Jian K et al., 2022; Peña OA and Martin P. 2024). Additionally, combining PDGF with other growth factors has been shown to have synergistic effects on healing. The combining therapies like mixing of PDGF with insulin-like growth factor 1 (IGF-1) has shown improved wound healing compared

to either factor alone, highlighting the importance of timing and combination therapies in optimizing healing responses (Ganapathy N et al., 2012; Peña OA , and Martin P. 2024)

1.6.2 Epithelial Growth Factors:

1.6.2.1. Epidermal Growth Factor (EGF)

EGF plays a key role in the wound healing process and primarily influences the proliferation and migration of keratinocytes, which are essential for re-epithelialization (Firmansyah Y et al., 2024). EGF engages with epidermal growth factor receptor (EGFR) on the exterior of target cells, triggering a series of molecular events that facilitate cell proliferation and enhance survival (Fig. 2). Current studies have showed the effectiveness of EGF in improving wound healing outcomes. EGF has been shown to accelerate healing of partial burns and surgical wounds by promoting migration of keratinocytes and increasing collagen deposition in the ECM (Dogan S et al., 2009). Furthermore, EGF treatment has demonstrated improved tensile strength in skin incisions in various animal models, indicating its potential for therapeutic applications to treat wound. Furthermore, the presence of proteases in chronic wounds can degrade EGF, limiting its availability and effectiveness. This has led to investigations into direct administration of EGF to wound sites to improve its therapeutic potential. Such strategies aim to overcome the challenges presented by the wound microenvironment, specifically patients with diabetic patients where wound healing is often compromised (Alavi SE et al., 2024).

1.6.2.2. Fibroblast Growth Factor (FGF):

FGFs are belong to the family of growth factors that play diverse roles in wound healing, including stimulating fibroblast proliferation, promoting angiogenesis, and improving re-epithelialization (Firmansyah Y et al., 2024) (Fig. 2). FGFs work by attaching themselves and activating specific tyrosine kinase receptors (FGFRs) on the surface of target cells, leading to downstream signalling cascades that regulate cell behaviour. In the beginning of the stages of wound healing, FGFs stimulate the migration and proliferation of fibroblasts, which are responsible for producing the collagen-rich ECM that forms the scaffold for tissue repair. FGFs also stimulate angiogenesis by inducing endothelial cell proliferation and migration and accelerating the expression of VEGF. In addition, FGFs play a role in re-epithelialization by stimulating the proliferation and migration of keratinocytes at the wound edges, leading to the formation of a new epithelial layer over the wound surface (Liu Y, et al., 2021).

1.6.2.3. Insulin-Like Growth Factor-1 (IGF-1)

IGF-1 is another essential growth factor involved in wound healing, particularly known for its effects on fibroblast activity and keratinocyte migration (Fig. 2). It plays a multifaceted role in promoting tissue repair. Recent research demonstrated that persistent release of IGF-1 from silk fibroin films accelerated wound healing in diabetic mice models. The IGF-1 treatment resulted in enhanced granulation tissue formation and improved re-epithelialization compared to controls (Lin MJ et al., 2021). IGF-1 has been shown to stimulate keratinocyte migration, which is crucial for closing wounds. It also promotes the synthesis of hyaluronan, a component that aids in maintaining tissue hydration and elasticity during healing (Garoufalia Z et al., 2021). Additionally, topical application of IGF-1 creams has been associated with increased expression of myofibroblasts, which are vital for contracture of wound and ECM remodelling (Rajalekshmy GP et al., 2024).

1.6.3 Fibrotic and Remodelling Factors:

1.6.3.1. Transforming Growth Factor-alpha (TGF-α)

TGF-α belongs to the TGF superfamily and serves as a central function in cell proliferation, differentiation and survival. It is mainly produced by macrophages, keratinocytes and fibroblasts at the wound site. TGF-α acts through the epidermal growth factor receptor (EGFR) and stimulates various cellular processes important for wound healing. Recent studies have showed the role of TGF-α in promoting granulation tissue formation and re-epithelialization (Berlanga-Acosta J et al., 2024). TGF-α has been shown to accelerate epithelial cell proliferation, allowing for faster wound closure (Fig. 2). It modulates angiogenesis by promoting mobilization and multiplication of endothelial cells, thereby Assisting in the generation of new blood vessels necessary for nutrient supply during healing (Bilalov BE et al., 2024). However, excessive TGF-α signalling can lead to pathological fibrosis. In fibrotic diseases, elevated TGF-α levels are associated with increased collagen deposition and extracellular matrix (ECM) remodelling, which can impair organ function (Effendi WI and Nagano T, 2022). This dual role of TGF-α, beneficial for normal healing but detrimental in chronic fibrosis, shows its importance as a therapeutic product in fibrotic conditions.

1.6.3.2. Transforming Growth Factor-Beta (TGF-β):

The multifunctional cytokine TGF-β is important for regulating inflammation, proliferation, and tissue remodelling, among other phases of wound healing (Firmansyah Y et al., 2024). TGF-β controls the activation and proliferation of immune cells, with neutrophils and macrophages, during the inflammatory phase (Fig. 2), which are necessary to clear the

wound site of debris and pathogens. TGF-β promotes fibroblast migration and proliferation during the proliferative phase, which results in the release of extracellular matrix (ECM) elements like collagen and fibronectin. TGF-β also promotes angiogenesis by stimulating the formation of vascular endothelial growth factor (VEGF) and other proangiogenic factors, thereby facilitating the development of fresh blood vessels within the wound site. In addition, TGF-β is involved in the regulation of myofibroblast differentiation, which is important for wound closure. (Frangogiannis NG. 2020).

1.6.3.3. Connective Tissue Growth Factor (CTGF)

CTGF, also known as Cellular Communication Network Factor 2 (CCN2), is a multifunctional protein that plays a critical role in fibrogenesis. It is often upregulated in response to TGF-β signalling and is considered a key mediator of TGF-β's profibrotic effects (Nikolaos G. 2020; Lin YM et al., 2024). CTGF is essential for tissue repair and remodelling through proliferation of fibroblast and ECM production (Fig. 2). Recent research suggests that CTGF not only increases collagen synthesis, but also influences the differentiation of fibroblasts into myofibroblasts, which are important effector cells in fibrosis. Several fibrotic disorders, such as intestinal and liver fibrosis, have been linked to elevated CTGF levels. (Liu S, 2024; Lin YM et al., 2024). Furthermore, CTGF has emerged as a promising biomarker for fibrotic activity due to its significant correlation with disease severity. Its status as a "fibrogenic master regulator" emphasizes its critical involvement in fibrosis, making it a compelling target for the treatment of fibrotic disorders (Gressner OA et al., 2008).

1.6.3.4. Matrix Metalloproteinases (MMPs) and their regulation:

MMPs belong to the family of zinc-dependent endopeptidases responsible for degradation and remodelling of the extracellular matrix (ECM) throughout wound healing. In the initial stages of wound healing, MMPs are upregulated and serve to break down the provisional ECM, allowing cell migration and tissue reorganization. MMPs are also involved in angiogenesis by promoting the release of bioactive ECM fragments that activate endothelial cell migration and tube formation (Fig. 2). However, as with chronic wounds and non-healing ulcers, impaired MMP activity has been responsible for increasing ECM degradation and poor wound healing. MMP-9 is a key regulator in the transportation of endothelial progenitor cells (EPCs) from the bone marrow, which is critical for neovascularization during wound healing (Balaji S et al., 2015). Recent research, overexpressing angiogenic factors like angiopoietin-1 can improve wound healing outcomes in diabetic models by accelerating the recruitment of EPCs through mechanisms involving MMP-9. Therefore, tight regulation of MMP activity is essential for

proper wound healing, and dysregulation of MMP expression or activity can be detrimental to the repair process (Kandhwal M, et al., 2022).

1.6.3.5. Role of cytokines:

Interleukins are a cluster of cytokines that play diverse roles in immune regulation, inflammation, and tissue repair. In the context of wound healing, interleukins such as IL-1 and IL-6 are involved in orchestrating the inflammatory response and modulating the behavior of different cells at the wound area (Fig. 2). IL-1 promotes inflammation by activating endothelial cells and recruiting leukocytes into the region of damage, in turn activating cellular repair. IL-6 is involved in stimulating fibroblast and keratinocyte proliferation, promoting collagen synthesis, and facilitating tissue repair (Kuridze N et al., 2024). The anti-inflammatory cytokine IL-10 is critical for resolution of inflammation and transition to the reparative phase of the healing process, as extreme or chronic inflammation can impede healing (Jayanthi S et al., 2017). IL-12, produced by dendritic cells and macrophages, promotes Th1 cell differentiation, which is crucial for fighting infections during the healing process (Peignier A et al., 2024). In addition, interleukins play a role in controlling the shift between pro-inflammatory and anti-inflammatory signals during wound repair, thereby ensuring a structured and effective repair response (Wilson SE., 2021). During the proliferative phase, interleukins such as IL-4 and IL-13 promote fibroblast activation and collagen synthesis. These Th2 cytokines facilitate tissue remodelling and accelerate the development of vascular endothelial growth factor (VEGF), which supports angiogenesis (Heidari Z, 2024). In the final stages of wound healing, IL-22 was identified as a crucial regulator. The induction of genes responsible for cell proliferation and its survival promotes epithelial regeneration and tissue repair (Sonnenberg et al., 2010). A recent study has highlighted the role of IL-17A in wound healing. IL-17A, produced by Th17 cells, has been shown to recruit neutrophil in the early stages of inflammation but also contributes to tissue damage when overexpressed. This balance highlights the complex interaction of various interleukins in achieving optimal wound healing results (Ni Q et al., 2024).

Interleukins play a key role in the complex molecular network that controls the process of wound healing. Dysregulation of their expression or signalling pathways can lead to impaired healing and the development of chronic wounds. Understanding the role of these interleukins in wound healing may provide insights into potential therapeutic targets to increase tissue rejuvenation and improve clinical outcomes in wound treatment.

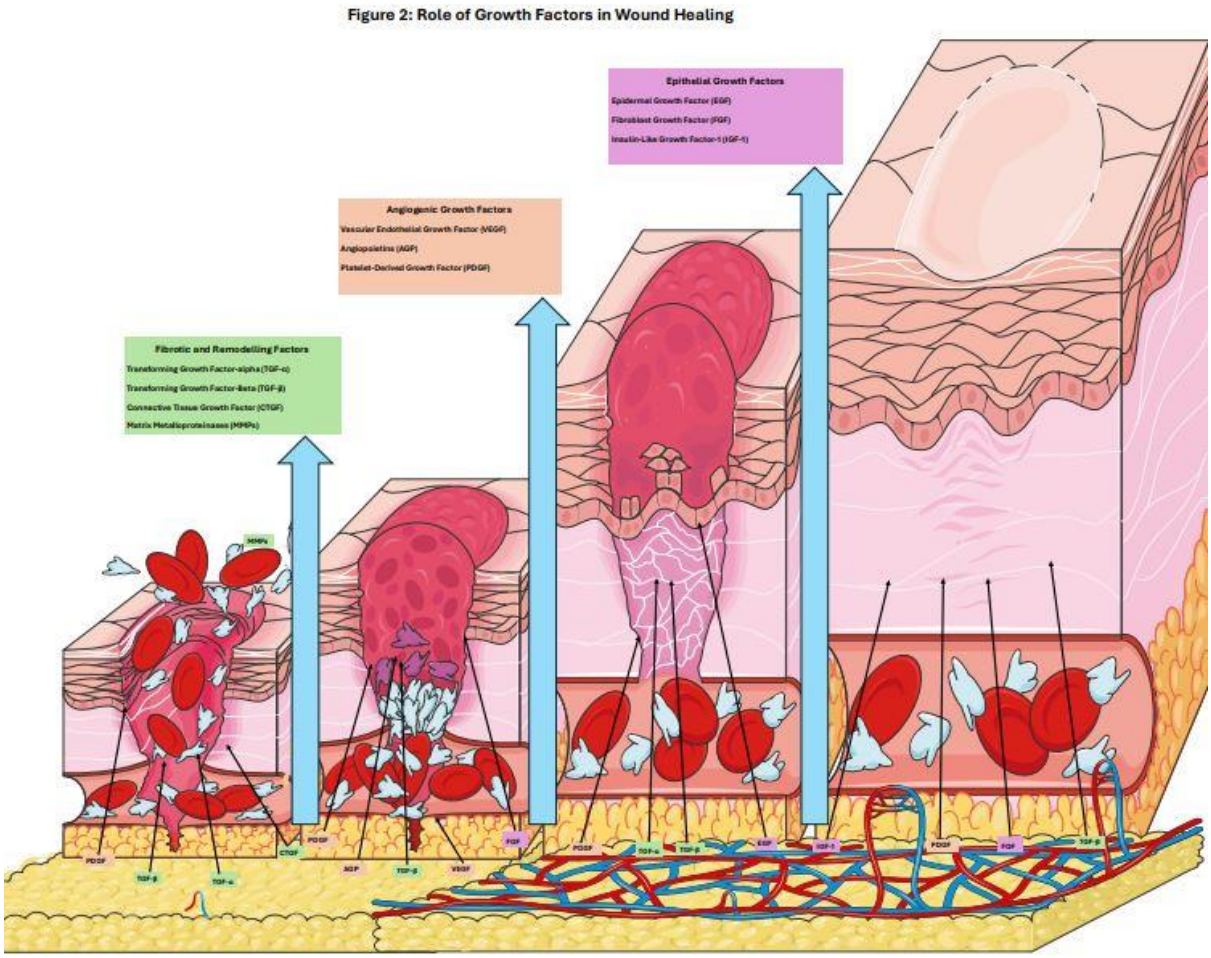

Figure 1.2: Role of growth factors in wound healing

1.7. Innovative Therapeutic Approaches

1.7.1 Tissue engineering and 3D bioprinting:

Tissue repair is a vital process ensuring the re-establishment of skin integrity after injury and functionality after injury. The healing process is inherently complex and efficient, but can be significantly hampered in cases of chronic illness, infection, or severe tissue loss, resulting in the formation of non-healing wounds. These diseases represent a major clinical challenge because traditional wound management strategies – including dressings, debridement and topical agents – are often insufficient to promote complete or timely healing in severe cases (Rodrigues et al., 2019). Such limitations highlight the major need for novel approaches that address the underlying biological challenges of complex wound healing.

Tissue engineering has emerged as a revolutionary approach in the field of regenerative medicine, offering innovative solutions for wound healing. By incorporating mechanisms from biology, engineering, and material science, tissue engineering aims to develop biological

replacements that aid in restoring, preserving, or optimizing tissue function (Eming et al., 2014). This multidisciplinary field seeks to harness the body's inherent healing capabilities by creating engineered constructs that facilitate and enhance the natural repair processes (Deng et al., 2020).

In current years, the advent of 3D printing technology has significantly propelled the field of tissue engineering forward. 3D printing, also known as 'additive manufacturing', facilitates precise generation of intricate, three-dimensional structures layer by layer (Choudhury et al., 2018). This technology has opened fresh opportunities to create customized, patient-specific tissue constructs, offering unprecedented control over the architecture and composition of engineered tissues (Das et al., 2023).

This review focuses on the synergy between tissue engineering and 3D bioprinting as a pioneer in wound healing research and application. In particular, we explore the integration of scaffolds, cells and growth factors into technical constructs and their application in the production of skin substitutes. Furthermore, we are investigating how 3D bioprinting might be used to create customized treatments, specifically for the regulation of diabetic foot ulcers (DFUs), a prevalent and crippling side effect of diabetes. By assessing recent advances, clinical trials, and emerging technologies, this review aims to highlight the transformative ability of tissue engineering and 3D bioprinting in revolutionizing wound care and improving patient outcomes (Wu et al., 2021).

1.7.2 Polymers and hydrogels:

The development of wound scaffolds is crucial for improving tissue repair and regeneration. Both natural and synthetic polymers are being extensively studied for their important applications in wound care, each providing exclusive benefits and challenges. Polymers of natural origin extracted from plants, animals, and microorganisms are gaining popularity because of their superior biocompatibility, bioactivity, and ECM mimicking capabilities (Chen M et al., 2022) (Fig. 3).

Common natural polymers used in wound scaffolds include:

- **Collagen:** Collagen-based scaffolds are found mostly in ECM and support cell adhesion and growth while promoting wound healing.
- **Chitosan:** Chitosan is derived from chitin and has antimicrobial properties and biocompatibility, appropriate choice for wound dressings.
- **Hyaluronic Acid:** This polysaccharide plays a critical role in tissue hydration and cell migration, enabling faster healing processes.

- **Silk fibroin**: Silk fibroin scaffolds are known for their mechanical power and biocompatibility and have shown promise in various tissue engineering applications.

Wound healing linked with diabetes is often hampered by things like decreased blood flow, persistent inflammation, and weakened tissue repair abilities. Advanced therapeutic techniques such as hydrogels, synthetic materials and biological cells are increasingly being used to solve these problems. Hydrogels deliver medication or growth factors to the wound site, promoting healing in a moist environment. Man-made materials provide structural support and can be used to deliver drugs. They are often manufactured to resemble natural composition of the extracellular matrix. To address deficiencies in diabetic wound healing, biological cells – including stem cells and genetically modified cells – are used to improve tissue regeneration and regulate the immune response. Combined, these cutting-edge methods provide a comprehensive plan to improve outcomes for diabetic wounds.

Despite their advantages, natural polymers often face challenges such as variability in source materials, limited mechanical properties, and difficulties in processing. Recent advances in processing techniques have improved the fabrication of these materials into functional scaffolds suitable for clinical applications (Ansari M and Darvishi A, 2024).

Synthetic materials have core function in wound recovery in diabetic patients, delivering versatility and controllability in addressing the complicated challenges related to impaired wound healing processes (Anderson, JM. and Shive, MS. 2012). These materials include a broad spectrum of polymers, nanofibers, and smart materials designed to provide structural support, regulated discharge of bioactive molecules, and tailored therapeutic interventions (Boateng, J. and Catanzano, O. 2015). Polymers are vastly used in diabetic wound dressings because of their biocompatibility, mechanical strength and ease of production (Lee, KY. and Mooney, DJ. 2012). Polymeric dressings like polyethylene glycol (PEG), polyvinyl alcohol (PVA) and polyurethane (PU) provide benefits like sustained release of medicinal substances and preserving a moist wound environment that promotes healing (Boateng, J. and Catanzano, O. 2015). Nanofiber-based dressings represent a promising advance in synthetic materials for diabetic wound healing. Ultrafine fibers with diameter in the nanoscale to micrometer range can be produced via electrospinning, providing enhanced mechanical qualities and a high area to volume ratio (Li, D. and Xia, Y. 2004). Nanofiber dressings can be loaded with bioactive agents to promote wound healing and prevent infections (Jayakumar, R., et al., 2011).

Smart materials that can react to particular stimuli have attracted interest for wound healing applications in diabetics. These materials can release bioactive substance triggered by changes

in the wound microenvironment, thereby stimulating cellular regeneration and accelerating wound sealing (Cao, Z. and Chen, X. 2007). In addition, smart materials can integrate sensors and actuators for tracking the healing process of wounds in real time (Kim, SH., et al., 2015). Synthetic polymers typically offer better structural stability compared to natural counterparts. However, they may lack the bioactive cues required for optimal cellular behavior and tissue integration. Recent studies have focused on blending natural and synthetic polymers for developing composite scaffolds that harness the powers of both species while mitigating their weaknesses (Amini S et al., 2021). Although promising, synthetic materials face concerns regarding biocompatibility and scalability. Ensuring sustained durability and biodegradability is essential for the successful clinical translation of these materials. (Veiseh O and Tang BC, 2015). However, ongoing research aims to address these challenges and further optimize synthetic materials for diabetic wound healing applications.

1.7.3 Biological Materials:

Biologically derived materials demonstrate significant efficacy in diabetic wound care, advancing tissue healing by leveraging the intrinsic repair mechanisms of natural tissues. (Veiseh O., and Tang B. C. 2015). These materials encompass extracellular matrix (ECM) scaffolds, growth factors, cytokines, and stem cells, offering biocompatibility and bioactivity (Kim S. H., et al., 2015) (Fig. 3).

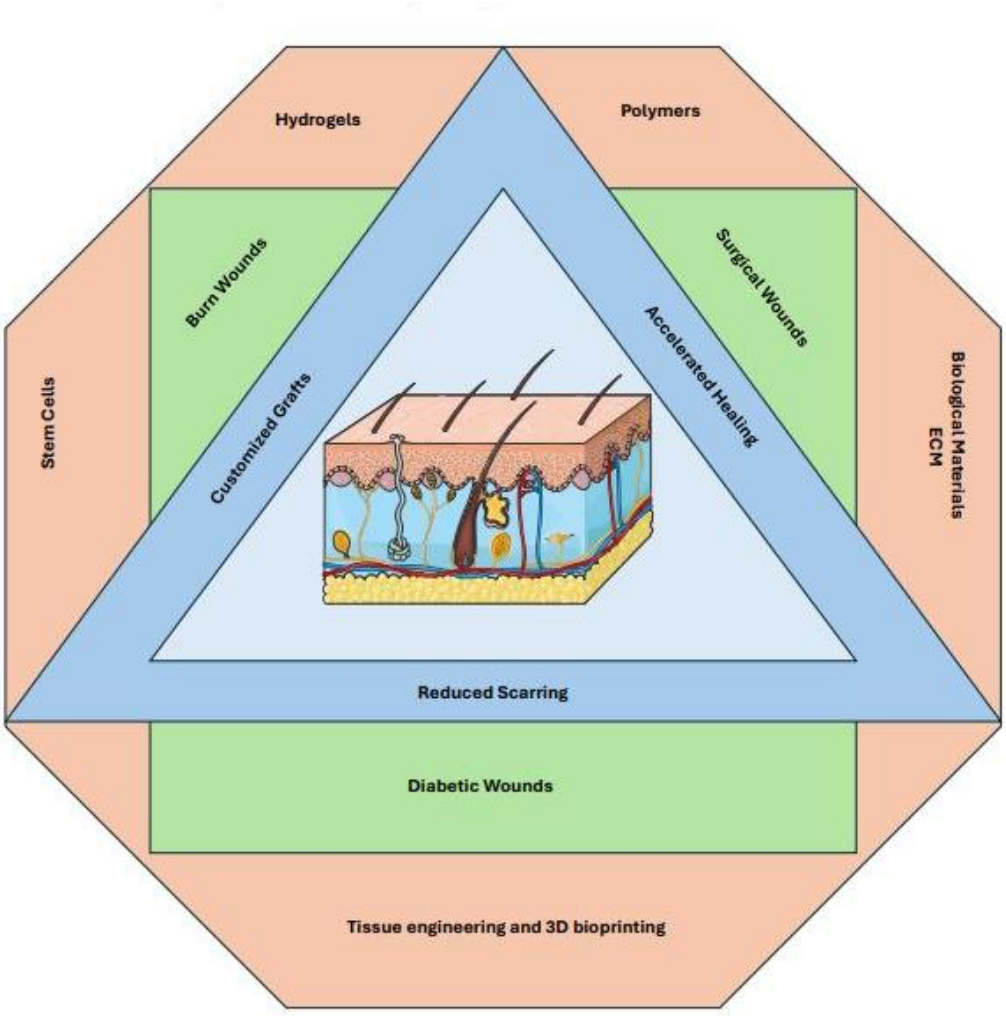

Figure 1.3: Innovative Therapeutic Approaches for skin substitute

A new bioreactor system was created by Ksheersagar J. et al., 2018, that uses a modified detergent-based technique (SDS, DMSO, and SD) to decellularize many human amnion samples at once and efficiently remove cellular components. Numerous histological and mechanical investigations verified that the resultant scaffolds were non-immunogenic and maintained their structural integrity. The decellularized amnion enhanced wound healing in mice by encouraging the creation of epidermis, keratinocyte proliferation, and decreased scarring within seven days when triggered with platelet rich plasma (PRP) and calcium chloride. The characteristics of scaffold were maintained by cryopreservation using DMEM, which makes it a viable off-the-shelf skin substitute that may be used right away for wound treatment.

Damle M, et al., 2023, created artificial skin that resembles human skin. A potential remedy could be artificial skin made with 3D printing technology that contains collagen and other

epidermal and dermal components. PVA (polyvinyl alcohol) and gelatin were combined with the skin-specific bioink, which was made from digested chicken skin.

ECM scaffolds derived from decellularized tissues provide a biomimetic microenvironment that promotes angiogenesis, collagen deposition, and tissue remodelling (Suggs, LJ., and Mikos, AG. 2003). Growth factors like platelet-derived growth factor (PDGF) and vascular endothelial growth factor (VEGF) stimulate tissue repair processes and accelerate wound closure (Langer R., and Vacanti, JP. 1993).

Stem cells provide an innovative approach to accelerating wound recovery in diabetes as they possess immunomodulatory and proangiogenic properties (Viseh O. and Tang BC. 2015). Mesenchymal stem cells (MSCs) can be delivered directly to the wound bed or integrated into tissue-engineered frameworks to support tissue renewal (Veiseh O. and Tang BC. 2015). MSCs are multipotent cells found in various tissues, including bone marrow, adipose tissue, and umbilical cord. These cells have the power to specialize into various functional cells such as osteoblasts, chondrocytes and adipocytes, making them valuable for regenerative therapies. Recent studies have highlighted their immunomodulatory properties, which can reduce inflammation and promote healing of injured tissue. Current research has shown that MSCs can improve wound healing by secreting different growth factors and cytokines that facilitate angiogenesis and cellular restoration. A study by Kalhori D et al., (2022) confirmed that human MSCs embedded in 3D bioprinted hydrogels could support myocardial engagement in cardiac tissue engineering, indicating their potential for cardiac repair applications. Furthermore, MSCs derived from umbilical cord tissue have been displayed to have improved proliferation and differentiation capabilities compared to MSCs from adult sources, available for clinical applications (Zhidu S et al., 2024). Placental-derived cells, including trophoblasts and placental mesenchymal stem-like cells (PMSCs), represent a hopeful source of stem cells due to their unique properties. These cells are immunologically privileged, which reduces the risk of rejection in allogeneic transplantation. Recent studies have shown that PMSCs can promote wound healing through their paracrine effects. Placenta-derived MSCs have been reported to release several growth factors that promote fibroblast migration and proliferation, thereby accelerating wound closure (Xiao J et al., 2024). In addition, PMSCs have anti-inflammatory characteristics that can favorably influence the wound healing environment. iPSCs are created by reprogramming transforming somatic cells into pluripotent cells so they can undergo differentiation into any cell type. This technology offers significant advantages for personalized medicine and disease modeling. iPSCs can be derived from various tissues,

including skin fibroblasts, making them amenable to patient-specific therapies. Recent advances have focused on optimizing differentiation protocols for iPSCs to generate specific cell types for regenerative applications. For example, studies have successfully caused iPSCs to differentiate into endothelial cells and cardiomyocytes for cardiovascular applications. In addition, iPSCs have been investigated for their potential in treating chronic wounds by improving angiogenesis and promoting epithelialization through their secretome (Takahashi, K. and Yamanaka S. 2006).

Although promising, biological materials face challenges such as variability and immunogenicity. Standardization and optimization of administration methods are essential for their clinical translation (Viseh O. and Tang BC., 2015). However, research is still going on to address these issues and further refine biological materials for diabetic wound healing applications.

1.7.4 Hydrogel-Based Innovations

Hydrogel materials have been recognized as a potential platform for diabetic wound healing, providing a versatile and customizable platform for delivering bioactive molecules and stimulating cellular regeneration (Hoffman AS., 2012). These materials include injectable and 3D bioprinted hydrogels with enhanced bioactivity and mechanical properties (Murphy SV. and Atala A., 2014).

Hydrogel dressings maintain optimal moisture levels that promotes tissue repair and absorb excess exudate from the wound bed (Boateng J. and Catanzano O., 2015). Additionally, hydrogels can be tailored to manage growth factors, cytokines, and antimicrobial agents to stimulate wound healing and avoid infections (Jayakumar R., et al., 2011). Current advances in 3D bioprinting technology have allowed the fabrication of patient-specific hydrogel scaffolds with fine-tuned regulation of architecture and composition (Murphy SV. and Atala A., 2014). These personalized scaffolds provide tailored treatment strategies for diabetic wounds and promote tissue regeneration and wound closure (Ng WL. and Yeong WY., 2016). Despite their promise, hydrogel materials face difficulties like deterioration and mechanical charcateristics kinetics. The optimization of these parameters is essential for their clinical implementation (Hoffman AS., 2012). However, the research is going on to conquered these challenges and further refine hydrogel materials for diabetic wound healing applications. In summary, synthetic, biological and hydrogel materials offer significant benefits for wound healing in diabetic patients, ranging from the controlled release of bioactive agents to the promotion of tissue regeneration. Ongoing research aims to address challenges and further

optimize these materials for clinical implementation, ultimately improving wound healing outcomes for diabetic patients.

1.7.5 Regenerative Medicine in Wound Care

The field of regenerative medicine has made remarkable suceess with the development of 3D-printed skin substitutes. These innovative constructs aim to copy the complex structure and activity of natural skin, and provid solutions for a different types of wounds, including chronic ulcers, burns, and other skin injuries. The content, material composition, and specific applications of these 3D-printed skin substitutes vary significantly, reflecting the different technological approaches and therapeutic needs in this area.

1.8. Advancements in Wound Care Technologies

A popular product sold in clinics is Apligraf®, a bi-layered skin substitute composed of human keratinocytes and fibroblasts. The upper layer of Apligraf® contains keratinocytes that form the epidermal layer, while the lower layer contains fibroblasts that form the dermal layer. This biologically based product is incorporated with human cells to create a skin-like structure. Apligraf® is used to treat and heal chronic wounds like diabetic foot and venous leg ulcers. The aim is to support the healing process by providing a functional skin barrier and promoting tissue regeneration (Tavakoli and Klar, 2021).

Dermagraft ® is another known skin substitute, a cryopreserved human fibroblast-derived dermis skin replacement. This product creates a scaffold for new tissue to grow and simulates the skin's dermal layer. Dermagraft®, like Apligraf ®, is biological and is composed of the human cells that can withstand in cryopreservation to allow their functional capacity. According to Cahn B, and Lev-Tov H. (2020), Dermagraft® is effective in treating diabetic foot ulcers as it enhances wound closure and hastens repair processes.

In the realm of individualized medicine, SkinTE™ is unique, being a bi-layer skin substitute consisting of autologous keratinocytes and fibroblasts. Being patient's own cells, SkinTE™ minimizes risk of immune reaction and increases compatibility. This product, which helps in treating chronic wounds and burns, can greatly improve the outcome in skin reconstruction by providing skin mimetic treatment to skin extremities (Bade Y,et al., 2021).

The Integra® Dermal Regeneration Template is a system that promotes skin regeneration. It is composed of two layers: deep dermal layers made of glycosaminoglycan and collagen, specifically harvested from cows, and a temporary silicone layer which forms the skin. This device is effective for burn wounds, chronic ulcers, and many more (Chang DK, 2019).

An innovative application of 3D bioprinting of tattooed skin models, which combines the bio-printing of the skin with specific patient cells on the skin with patterns that are predefined. This model is primarily for research and cosmetic applications, but it also serves to understand the possibilities in both skin regeneration and cosmetology, showcasing potential in developing custom-made and appealing-looking skin substitutes (Nizam et al., 2024).

1.9. Ongoing clinical trials

DFUs represent a major challenge in medical care, and researchers are continually exploring innovative treatments to improve healing outcomes. Current clinical trials associated to wound healing technologies are summarized in Table 1. Some of the exciting clinical trials that have been completed and are currently underway are highlighted in the following section.

NexagonTM for Skin Wounds: A product of OcuNexus Therapeutics, Nexagon^{TM} uses synthetic substances to interrupt cellular signals at the site of the wound. This approach helps to heal the wound faster, by reducing inflammation and pain, which is a new therapy. It was evaluated in a Phase 1 study (ClinicalTrials.gov ID: NCT00736593).

StrataGraft® for Partial-Thickness Burns: StrataGraft®, a living skin substitute, was studied for deep partial-thickness burns, replacing autograft, with encouraging wound healing (ClinicalTrials.gov ID: NCT01437852).

Silicon-Based Medicines for Skin Lesion: This was a study looking at the silicone-based medical equipment to cure laser-induced microscopic skin lesions. This novel formulation has already been approved as a Class IIa device in Europe and as a Class I device by the FDA for the treatment of wound care (ClinicalTrials.gov ID: NCT05614557).

Modified Polyurethane Film Dressing: The modified polyurethane film dressing was evaluated for its effectiveness in controlling leakage at skin graft donor sites. When used in conjunction with secondary absorbent dressings, it demonstrated strong wound management capabilities, making it a promising advancement for graft site care (ClinicalTrials.gov Identifier: NCT00600457).

Microporous Annealed Particle (MAP) Wound Matrix: The MAP wound matrix is a specialized microporous scaffold designed for clean surgical wounds. Its safety and effectiveness are currently being studied, presenting a promising option for advanced wound healing technologies (ClinicalTrials.gov Identifier: NCT06600152).

Hyalo4 Skin Gel for Bed Wounds: Hyalo4 Skin Gel from Fidia Farmaceutici is being tested to see if it's safe and effective for treating chronic bed sores. So far, we know that it improves wound appearance after 14 days (ClinicalTrials.gov NCT06103812).

PEP to Treat Donor Graft Wounds: This is a prospective, prospective investigation into leukocyte-poor blood products from collective human platelets as a new approach to treating donor graft wounds. This preparation is called PEP, and it could significantly boost wound healing (ClinicalTrials.gov ID: NCT04664738).

1.9.1 Exploring Cellular Therapies

Platelet-Rich Plasma and Keratinocyte Suspensions: Human keratinocytes and PRP were compared to see if they could facilitate faster healing of wounds. This Phase 1 trial showed better tissue repair than conventional therapy (ClinicalTrials.gov ID: NCT00856934).

Expanded Bone Marrow Stem Cells for Chronic Wound Healing: A Phase 2 trial used $CD90^{+}$enriched bone marrow-derived mesenchymal stem cells in the treatment of diabetic patients with ischemia-related chronic wounds that were successful in repairing the wounds (ClinicalTrials.gov NCT01065337).

Epidermal Grafting for Wound Healing: Epidermal grafting was explored as an alternative to split-thickness skin grafting, providing finer, minimally invasive options for treating chronic wounds (ClinicalTrials.gov Identifier: NCT02535481).

ABCB5+ Stem Cells for Chronic Venous Ulcers: This study investigated allogeneic ABCB5+ MSCs to treat chronic venous ulcers resistant to standard therapy. It demonstrated improved outcomes in wound healing during Phases 1 and 2 (ClinicalTrials.gov Identifier: NCT03257098).

SyntrFuge™ Processed Adipose Tissue: This technology utilizes autologous processed adipose tissue for the treatment of DFUs. The SyntrFuge™ system was adapted to utilize regenerative capacity and bioactive properties of adipocytes (ClinicalTrials.gov Identifier: NCT05519501).

Umbilical Cord TTAX01 for DFUs: Cryopreserved umbilical cord-derived material (TTAX01) was tested for non-healing DFUs, showing enhanced healing rates in late-stage cases. This finding is particularly significant because it suggests potential new avenues for treatment (ClinicalTrials.gov Identifier: NCT04450693).

Platelet-Rich Plasma with Fibroblasts: An autologous dermal fibroblast with PRP that improved tissue regeneration which led to enhanced wound healing (ClinicalTrials.gov Identifier: NCT04483934).

1.9.2 Innovative Drug Delivery Systems

Gene Therapy for Diabetic Wounds: In this trial, gene-activated matrix technology was used to deliver PDGF-B genes into wound sites, aiming to activate effective tissue repair in diabetic ulcers (ClinicalTrials.gov Identifier: NCT00065663).

However, the implications of this approach are significant, because it could potentially transform the treatment landscape for such conditions. The preliminary results of the study are encouraging, but there are still challenges related to long-term consequences of gene editing techniques.

1.9.3 Novel Biological Dressings and Biomaterials

Tissue-Engineered Skin Substitutes: Researchers evaluated safety and efficacy of tissue-engineered skin substitutes incorporated with mesenchymal stem cells (MSCs). The Phase 1 trial shed light on their ability to regenerate DFUs (ClinicalTrials.gov Identifier: NCT02668042).

Amnion Wound Covering: This study evaluated human amnion membrane powder to cover skin graft donor sites and the trial affirmed its safety and effectiveness in ameliorating wound conditions (ClinicalTrials.gov Identifier: NCT03754218).

Topical Collagen Powder: The efficacy of Nuvagen™, a collagen-based powder, was tested on acute full-thickness wounds. Application of Nuvagen™ resulted in improved healing rates (ClinicalTrials.gov Identifier: NCT03481907).

Rising Tide—Amniotic Allograft Treatments: Amniotic allografts have demonstrated the ability to speed wound healing and lower complications when used with standard care for DFUs. (ClinicalTrials.gov Identifier: NCT06681428).

Acellular Skin Substitute for Burns: Acellular skin substitutes have been tested as an alternative to autografts for partial-thickness burns, offering a less invasive treatment with favourable healing outcomes (ClinicalTrials.gov Identifier: NCT01454310).

Bioengineered Skin Substitute: A bioengineered, two-layer skin substitute consisting bovine collagen and donor cells has been tested for its efficacy in chronic wound management. The results of the trial were encouraging (ClinicalTrials.gov Identifier: NCT00007280).

Silk Sericin Dressing for Graft Donor Sites: In this clinical study, silk sericin was evaluated in combination with collagen as a biological dressing for cleft graft donor sites, which

demonstrated improved wound healing and reduced scarring (ClinicalTrials.gov Identifier: NCT04743375).

Fish Skin ECM for Chronic Wounds: Fish skin-derived extracellular matrix (ECM) was tested for its safety, no immunogenicity, and effectiveness in healing chronic wounds. The results of the clinical trial were encouraging (ClinicalTrials.gov Identifier: NCT01348581).

1.9.4 Combination Therapies

AC5® Advanced Wound System: A combination of biological and synthetic materials, the AC5® Advanced Wound System has been tested for its ability to improve healing in DFUs (ClinicalTrials.gov Identifier: NCT06028386).

Porcine Placental ECM and Standard Care: Porcine placental placenta extracellular matrix (PPECM) was combined with standard wound care to improve outcomes in venous leg ulcers (ClinicalTrials.gov Identifier: NCT06616844).

Amniotic tissue and SOC for DFUs: Amniotic tissue allografts were used combined with the standard treatment for treating DFUs, which accelerated healing and improved outcomes (ClinicalTrials.gov Identifier: NCT06681428).

As clinical trials continue to advance, each study paves the way for more effective treatments for diabetic foot ulcers (DFUs) in the future. These findings aim to ease the difficulties associated with these chronic wounds and enhance quality of life. They present promising options for achieving better outcomes in managing these complex medical issues.

1.10. Discussion:

Wound management specifically of prolonged and complicated types such as burns, diabetic, and surgical wounds is still an important aspect of clinical practice. The healing of wounds is a highly orchestrated process that depends on the coordinated actions of various cellular and molecular pathways at different stages. While the body is naturally endowed with the ability to heal and regenerate, some conditions such as underlying diseases like diabetes will impede this process giving rise to non-healing chronic wounds.

Diabetic wounds, especially DFUs, present a major health-care challenge in the world because of their high incidence, associated morbidity and life-threatening complications such as amputation. The pathophysiology of diabetic wounds is multifactorial and characterized by ischemia, neuropathy, and altered immune response. This leads to the factors combining and creating a slower healing process along with an increased chance of infection. Likewise,

surgical and burn wounds are also classified as acute but they often require special care to prevent infection, minimize scarring and support the healing process.

The classical therapeutic modalities for such type of wounds include debridement and wound dressings, while it includes further advanced techniques like tissue engineering and regeneration medicine. Revolution in the field including hydrogels, synthetic and biological polymers and stem cell therapies have also increased expectations for improved healing outcomes for instance, hydrogels can provide a moist wound healing environment which will lead to faster recovery times and may be loaded with bioactive compounds to improve therapeutic efficacy. In contrast, synthetic and biological polymers have been used to develop scaffolds with enhanced biocompatibility-mechanical properties that favor tissue regeneration. However, the advancement of these therapies has some challenges for clinical translation. Importantly, effective uptake of these technologies is limited by treatment cost effectiveness, industrial scalability of the technology and biocompatibility to the material. In addition, although stem cell treatment has promising preclinical data in the current research era, the application of stem cell therapy for wounds remains in its infancy, with several unresolved issues around optimal source / delivery of these cells and their long-term safety.

The wound-healing market has many different products for diabetic wound treatment, including growth factor therapy, bandages, and bioengineered skin substitutes. Such products are not very effective though and come with many disadvantages like poor efficacy, high recurrence rates, and potential adverse effects. It highlights the need for ongoing innovation and research into even more reliable and effective treatment options. In this regard, clinical trials will remain a constant necessity due to the data they provide towards novelty of administration tools and their safety. As an example, fabrication of 3D bio-printing technology may be developed to formulate personalized wound healing treatment fitting the needs of each patient. But, based on the complexity of 3D bio-printing processes and the challenges in mimicking the architecture and functional properties of human skin, it seems that some more studies need to be carried out before these technologies can be applied regularly in a clinical setting.

In summary, despite the all the research in our knowing of wound healing and development of advanced treatment modalities, management of chronic wounds particularly diabetic wounds is still an evolving specialty. Innovative biomaterials, regenerative medicine, and tissue engineering offer new promise for future directions, but additional research, testing, and ingenuity will easily be needed to solve current challenges and improve patient outcomes.

Wound care will likely evolve into additional integrative strategies requiring the collaboration of biologists, engineering and the clinical physician to formulate more tailored and cost-effective approaches.

1.11. Conclusion:

The repair of an injury is a multifaceted, and dynamic process during which a coordinated series of events occur that are classified into healing and regeneration remodelling through inflammation to repair. However, in certain circumstances such as diabetes, the evidence of rapid recovery is significantly impaired, resulting in a chronic, non-healing wound that is very difficult to treat. Although the conventional modalities have been proven to be effective, they are often insufficient in ensuring that the root factors of defective healing. These have propelled the emergence of next-generation treatment strategies such as: hydrogels, synthetic materials and biological product usage that are bringing new approaches to promote wound healing.

The efficacy of these novel therapeutic alternatives has been confirmed by preliminary clinical trials, which have demonstrated enhanced tissue restoration and superior wound healing. In this regard, tissue engineering and regenerative medicine in particular will impact on medical research with the help of stem cells, growth factors, and biomaterials that support the regeneration of damaged tissue.

These novel therapies will not only improve living-standard of patients having chronic wounds but in due course they have the potential to also transform wound care by offering more efficient and target specific therapy as research makes progress. The hurdles of aberrant wound healing and its implications need to be addressed through developmentally controlled regenerative modalities, the clinical translation of which will require further exploration with the promise to shift towards other solutions in clinical settings.

1.12. Hypothesis

The study hypothesized that nanopolymer and 3D printed skin would be an ideal dressing material for wound healing applications.

1.13. Aim and Objectives

Aim:

To study the efficacy of nano polymer and 3D printed skin in diabetic wound healing in animal models and human subjects.

Objectives:

- Efficacy of nano-polymer on animal models to study wound healing.
- Efficacy of 3D printed skin on animal models to study wound healing.
- Study of nanopolymer in diabetic wounds in human subjects (Phase I study)
- Study of 3D printed skin in diabetic wounds in human subjects (Phase I study)

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Table 1.1 summarizing the current clinical trials related to wound healing technologies, including the name of the trial, its focus, and relevant details (Dated:21-11-2024).

Sr.	Name of the Trial	Content of the Trial	Biological or Synthetic Material	Phase	Sponsor	Present Status	ClinicalTrials.gov Identifier:
1	A Study Evaluating Nexagon™ in the Treatment of Skin Wounds	Promotes wound healing by temporarily disrupting cellular communication at the wound site, thereby promoting accelerated healing, reducing inflammation and pain.	Synthetic Material	1	OcuNexus Therapeutics, Inc.	Completed	NCT00736593
2	Impact of Silicon-based Formulations on Wound Healing of Laser-induced Microscopic Skin Lesions (SPASM11)	Silicone-based formulation registered as a European class IIa, TGA (Therapeutic Goods Administration) and FDA listed Class I medical device (wound dressing).	Synthetic Material	NA	Stratpharma AG	Completed	NCT05614557
3	The Activity of Tissue Engineering Skin Substitutes (MSCs)	Living Tissue Engineering Skin Substitutes Safety and Efficacy Studies for the Treatment of Difficult to Heal Wounds	Biological	1	Chinese PLA General Hospital	Completed	NCT02668042
4	Modified Polyurethane Film Dressing For Skin Graft Donor Sites	To determine whether the combination of modified polyurethane film and secondary absorbent dressing for skin graft donor sites avoids the regular uncontrolled leakage	Biological	1	Klinik Bogenhausen	Completed	NCT00600457

4	Modified Polyurethane Film Dressing For Skin Graft Donor Sites	To determine whether the combination of modified polyurethane film and secondary absorbent dressing for skin graft donor sites avoids the regular uncontrolled leakage	Biological	1	Klinik Bogenhausen	Completed	NCT00600457
5	Amnion Wound Covering for Enhanced Wound Healing	human amnion membrane powder can be safely used as a covering for wounds and can improve the condition of skin graft donor sites.	Biological	1	Wake Forest University Health Sciences	Completed	NCT03754218
6	Autologous Regeneration of Tissue (ART) for Wound Healing	This device will harvest hundreds of full-thickness columns of skin tissue (500 micrometer diameter) using single-needle, fluid-assisted harvesting technology.	Biological	NA	University of Miami	Active, not recruiting	NCT03796988
7	Topical Collagen Powder for Healing of Acute Full-thickness Wounds	investigate the effect and potential utility of topical NuvagenTM (collagen powder) on the rate and quality of wound healing in healthy volunteers using the punch biopsy method.	Biological	NA	Adam Friedman, George Washington University	Completed	NCT03481907
8	An Acellular Epithelial Skin Substitute in Deep Partial-thickness Burns.	To compare an acellular epithelial skin substitute with autologous split-thickness skin grafts (STSGs) in deep partial-thickness burns.	Biological	4	Medical University of Vienna	Completed	NCT01454310

9	Study With an Autologous Dermo-epidermal Skin Substitute for the Treatment of Full-Thickness Skin Defects in Adults and Children.	To evaluate the Safety and Efficacy of an Autologous Bio-engineered Dermo-epidermal Skin Substitute (EHSG-KF) for the Treatment of Full-Thickness Defects in Adults and Children in Comparison to Autologous Split-thickness Skin Grafts (STSG)	Biological	2	CUTISS AG	Active, not recruiting	NCT03394612
10	Evaluate Performance and Safety of Hyalo4 Skin in Acute and Chronic Wounds	The evaluation of performance and safety of Hyalo4 Skin Gel in the amelioration of bed wound appearance after 14 days of treatment.	Biological	NA	Fidia Farmaceutici s.p.a.	Recruiting	NCT06103812
11	Treatment of Patients With Non-healing Wounds and Trophic Ulcers Using Autologous Dermal Fibroblasts	Treatment of patients with non-healing wounds and trophic ulcers using local LED phototherapy with local transplantation of autologous dermal fibroblasts	Biological	1 and 2	Institute of Biophysics and Cell Engineering of National Academy of Sciences of Belarus	Completed	NCT04483934
12	Epidermal Grafting in Wound Healing (EPIGRAAFT)	Epidermal grafting (EG) is an alternative method of autologous skin grafting that 'harvests' a finer layer of skin than traditional Split thickness skin grafting (SSG).	Biological	NA	University College, London	Completed	NCT02535481

13	Safety of Microporous Annealed Particle (MAP) Wound Matrix in Patients with Clean Surgical Wounds. (MOSAIC)	Investigational Safety Evaluation of the Microporous Annealed Particle (MAP) Wound Matrix (TT101) Device As a Volumetric Biomaterial Scaffold	Biological	NA	Tempo Therapeutics	Recruiting	NCT06600152
14	Effect of Platelet Rich Plasma and Keratinocyte Suspensions on Wound Healing	Autologous keratinocytes isolated from skin biopsy suspended in platelet rich plasma before spraying.	Biological	1	Centre Hospitalier Universitaire Vaudois	Completed	NCT00856934
15	ExpressGraft-C9T1 Skin Tissue as a Treatment of Diabetic Foot Ulcers.	ExpressGraft-C9T1 skin tissue is provided as a suturable, biologically-active, circular skin tissue with a fully-stratified epithelial compartment comprised of human keratinocytes (NIKSC9T1) and a dermal compartment containing fibroblasts.	Biological	1	Stratatech, a Mallinckrodt Company	Completed	NCT02657876
16	StrataGraft® Skin Tissue as an Alternative to Autografting Deep Partial-Thickness Burns.	Study Evaluating the Safety and Efficacy of StrataGraft® Skin Tissue in Promoting the Healing of the Deep Partial-Thickness Component of Complex Skin Defects	Biological	1	Stratatech, a Mallinckrodt Company	Completed	NCT01437852
17	Examining the Effectiveness of DermGEN™ in the Treatment of	Examine the effectiveness of a decellularized dermal matrix (DermGEN™) in improving	Biological	NA	DeCell Technologies Inc.	Not yet recruiting	NCT05251480

	Diabetic Foot Ulcers in First Nations People	wound healing, quality of life and associated costs of treatment of DFUs					
18	Bioengineered Skin and Wound Healing	bioengineered skin (BSC), is a two-layered sheet made from purified beef tendon collagen, donor skin cells, and a substance that maintains the cells until they are grafted	Biological	1	Roger Williams Medical Center	Completed	NCT00007280
19	PEP on a Skin Graft Donor Site Wound	Study of PEP (a leukocyte depleted blood preparation derived from human U.S. sourced pooled apheresed platelets) in patients with at least two donor split-thickness skin graft wounds.	Biological	1	Rion Inc.	Active, not recruiting	NCT04664738
20	Use of AC5® Advanced Wound System in the Treatment of Diabetic Foot Ulcers	AC5® Advanced Wound System as an active synthetic cellular tissue product	Biological + Synthetic	NA	Arch Therapeutics	Recruiting	NCT06028386
21	Healing Chronic Venous Stasis Wounds With Autologous Cell Therapy	The treatment group will undergo a small liposuction procedure and receive placement of autologous cell therapy (stromal vascular fraction or SVF) injected around the rim of venous stasis wound	Biological	NA	InGeneron, Inc.	Unknown	NCT02961699
22	Efficacy of Porcine Placental Extracellular Matrix Augmented Plus Standard of Care (SOC)	Evaluating the Efficacy of Porcine Placental Extracellular	Biological	NA	ConvaTec Inc.	Not yet recruiting	NCT06616844

	Versus SOC Alone for the Management of Diabetic Foot Ulcers (IDEAL)	Matrix Augmented Wound Care					
23	Use of the Epidermal Micrografts for Wound Healing After Mohs or Excisional Surgery for Skin Cancer	The Epidermal Expansion System (designed by MoMelan Technologies) will generate an array of small microblisters and transfer the micrografts to a sterile wound dressing for application to the subject's surgical area.	Biological	NA	Momelan Technologies	Unknown	NCT01536444
24	Cryopreserved Human Umbilical Cord (TTAX01) for Late Stage, Complex Non-healing Diabetic Foot Ulcers (AMBULATE DFU II).	TTAX01 is a cryopreserved human umbilical cord product derived from donated human placental tissue	Biological	3	BioTissue Holdings, Inc	Recruiting	NCT04450693
25	Observational Study to Investigate the Use of Sterilized Porcine Placental Tissue in the Treatment of Chronic VLU	To evaluate the use of InnovaMatrix AC sterilized porcine placental ECM to treat chronic VLUs .	Biological	NA	ConvaTec Inc.	Completed	NCT06400875
26	INNOVEN: Efficacy of Porcine Placental Extracellular Matrix Plus Standard of Care (SOC) Versus SOC Alone	the efficacy of porcine placental extracellular matrix (PPECM) and standard of care (SOC) versus SOC alone in the closure of non-healing venous leg ulcers (VLUs).	Biological	NA	ConvaTec Inc.	Recruiting	NCT06606210
27	Clinical Efficacy of Silk Sericin Dressing With Collagen for Split-thickness Skin Graft Donor Site Treatment	Sericin dressing with collagen or Bactigras will be used as a primary dressing for treating the STSG donor site.	Biological	NA	Chulalongkorn University	Unknown	NCT04743375

28	Rapid Construction of Tissue-engineered Skin for Repairing W ounds	This method is composite of skin grafting over human acellular dermal matrix scaffold the investigators used before with skin basal cell as seed cells	Biological	NA	First Affiliated Hospital, Sun Yat-Sen University	Recruiting	NCT02070809
29	Application of Cultured Autologous Keratinocytes for Burn Wound Healing (KC)	In this study the treatment of full thickness burn wounds with cultured autologous keratinocytes in combination with meshed split skin autograft versus meshed split skin graft alone will be compared.	Biological	3	Association of Dutch Burn Centres	Completed	NCT00832156
30	Long Term Status of Free Dermal Fat Autografts for Complex Craniofacial Wounds (FTFDT2)	This study will evaluate the use of free autologous dermal fat grafting (also called free dermal fat autografting) to treat complex craniofacial wounds that have failed standard treatment	Biological	NA	Dufresne, Craig, MD, PC	Unknown	NCT03880188
31	Use of Fish Skin Extracellular Matrix (ECM) to Facilitate Chronic Wound Healing	The clinical study is designed to assess the effectiveness, safety and non-immunogenicity of fish skin wound dressing extracellular matrix (ECM) in treating chronic wounds.	Biological	NA	Kerecis Ltd.	Completed	NCT01348581
32	Acellular Dermal Allograft for Chronic Diabetic Wounds	To determine the efficacy of a novel decellularized dermal matrix (DDM) DermGEN™ for	Biological	NA	University Health Network, Toronto	Not yet recruiting	NCT06227520

		the treatment of diabetic foot ulcers (DFU).					
33	SDRM® vs. Collagen for Diabetic Foot Ulcers	SDRM as an active synthetic wound closure matrix, FIBRACOL™ Plus Collagen Wound Dressing with Alginate dressing	Biological + synthetic	NA	Polymedics Innovations Inc.	Completed	NCT05883098
34	Hematopoietic Stem Cell Therapy in Chronic Wounds Using a Pressure Sore Model	Injection of autologous stem cells in suspension (50'000 CD 34+ cells in 100 microliter saline per cm2 of wound surface) on one half of the total wound surface.	Biological	1 and 2	University Hospital, Basel, Switzerland	Unknown	NCT00535548
35	Allogeneic ABCB5-positive Stem Cells for Treatment of CVU	the efficacy and safety of allogeneic ABCB5-positive mesenchymal stem cells (MSCs) on wound healing in patients with chronic venous ulcer (CVU).	Biological	1 and 2	RHEACELL GmbH and Co. KG	Completed	NCT03257098
36	Evaluating The Efficacy Of A Keratin Graft In Treating Non-Healing Diabetic Foot Ulcers	To collect patient outcome data on a commercially available, keratin-based skin substitute matrix: ProgenaMatrix®.	Biological	NA	ProgenaCare Global, LLC	Completed	NCT05797285
37	ChitoCare Medical Wound Healing Gel PMCF Study on Healing of Chronic Wounds (CHITOCHRONIC)	The purpose of this PMCF study is to evaluate the safety and efficacy of ChitoCare medical Wound Healing Gel (chitosan) for the healing of chronic wounds.	Biological	NA	Primex ehf	Completed	NCT05570877

38	Gene Therapy to Improve Wound Healing in Patients With Diabetes	Gene activated matrix (GAM) technology offers the opportunity to place a therapeutic gene (platelet-derived growth factor-B-GAM501) contained within a structural matrix into a wound site.	Biological	1	Tissue Repair Company	Completed	NCT00065663
39	The Study of the Effectiveness of Tissue Equivalent on the Basis of Cultured Cells to Heal Skin Blemishes (SETES)	Study of the efficacy of skin equivalent comprising living cells and skin biodegradable substrate for the treatment of skin lesions	Biological	2	Ural State Medical University	Unknown	NCT01884831
40	A Study to Evaluate the Efficacy of an Acellular Dermal Template for the Treatment of Full Thickness Skin Defects.	the efficacy of an acellular dermal template (Novomaix), combined with split thickness skin grafts	Biological	2	Association of Dutch Burn Centres	Completed	NCT02373566
41	Comparative Assessment of Adjuvant Effect of Cultured Epidermal Autografts Versus Skin Allografts on Wound Healing of Burns Treated With Widely Expanded Skin Autograft Using Meek Micrografting Technique MEEKADEAU (MEEKADEAU)	To compare the results on wound healing of 2 adjuvant treatments to Meek micrografting technique : Cultured Epidermal Autografts and cryopreserved skin allografts.	Biological	3	Assistance Publique Hopitaux De Marseille	Unknown	NCT01330407
42	Dermacyte® Amniotic Wound Care Liquid for	To evaluate the efficacy and safety of Dermacyte® Amniotic	Biological	2	Merakris Therapeutics	Recruiting	NCT04647240

	the Treatment of Non-Healing Venous Stasis Ulcers	Wound Care Liquid (Dermacyte® Liquid). Subjects will receive localized subcutaneous injection of Dermacyte® Liquid					
43	Evaluation of Safety and Activity of Celaderm in Healing Venous Leg Ulcers	This pilot study was designed to test the safety of Celaderm (Frozen Cultured Epidermal Allograft) in treating venous leg ulcers.	Biological	NA	Shire	Completed	NCT00399308
44	Allogeneic ABCB5-positive Dermal Mesenchymal Stromal Cells for Treatment of Therapy-resistant CVU (Phase III)	to investigate the efficacy and safety of Allogeneic dermal ABCB5-positive Mesenchymal Stromal Cells (ABCB5+ MSCs) administered topically on therapy-resistant non-healing CVUs	Biological	3	RHEACELL GmbH and Co. KG	New, Not yet recruiting	NCT06489028
45	Tissue-engineered Skin Graft Repair of Autologous Scar Dermal Scaffolds	Tissue-engineered Skin Grafts With Autologous Scar Dermal Scaffolds for the Repair of Hypertrophic Scars	Biological	NA	First Affiliated Hospital, Sun Yat-Sen University	Recruiting	NCT04389164
46	Induced Wound Healing by Application of Expanded Bone Marrow Stem Cells in Diabetic Patients With Critical Limb Ischemia	Application of Expanded Autologous Bone Marrow Stem Cells enriched in CD90+ mesenchymal stem cells administered intramuscular in Diabetic Patients With Ischemia-induced Chronic	Biological	2	Ruhr University of Bochum	Completed	NCT01065337

		Tissue Ulcers Affecting the Lower Limbs					
47	Safety and Efficacy of realSKIN® to Provide Complete Wound Closure of Burn Wounds as an Alternative to Autografting	to Evaluate the Safety and Efficacy of realSKIN® to Provide Complete Wound Closure. is a live biotherapeutic, bi-layered, split-thickness, membranous, skin xenotransplant wound dressing manufactured from living porcine skin sourced from genetically engineered, alpha-1,3-galactosyltransferase knock-out (GalT-KO) porcine (Sus scrofa) donors	Biological	3	XenoTherapeutics, Inc.	Recruiting	NCT06223269
48	Study to Assess the Safety of DERMASEAL for Diabetic Foot Ulcers	To evaluate the safety of the DERMASEAL advanced wound care dressing in the treatment of DFU	Biological	1	Vitruvian Medical Devices, Inc.	Recruiting	NCT05586542
49	PHASE 1, open label safety study of umbilical cord lining mesenchymal stem cells (CORLICYTE®) to heal chronic diabetic foot ulcers.	to establish the safety and tolerability of Corlicyte mesenchymal stem cells (MSCs) in the treatment of patients with DFUs	Biological	1	University of Colorado, Denver	Completed	NCT04104451
50	Randomized Controlled Clinical Investigation Evaluating the Effect of Adipose Tissue Processed With the SyntrFuge™ System in the Healing of Diabetic Foot Ulcers	To evaluat the efficacy of the adipose tissue processed with the SyntrFuge™ system in DFUs	Biological	NA	Syntr Health Technologies, Inc.	Completed	NCT05519501

51	A Comparative Efficacy Study of DermaPure™ to Treat Diabetic Foot Ulcers	Evaluate safty and efficacy of DermaPure™ (a decellularized dermal skin substitute) treating hard-to-heal DFUs.	Biological	4	TRx Wound Care Limited	Unknown	NCT02081352
53	Feasibility Study of DermGEN for Diabetic Foot Ulcer Treatment	To determine the safety and feasibility of DermGEN (decellularizes and sterilizes donated human tissue) treatment of DFUs	Biological	1	Dr. Paul F. Gratzer, DeCell Technologies Inc.	Completed	NCT02184455
52	PriMatrix for the Management of Diabetic Foot Ulcers	To evaluate the efficacy of PriMatrix Dermal Repair Scaffold in the management of DFUs	Biological	NA	Integra LifeSciences Corporation	Completed	NCT03010319
	Rising Tide - Amniotic Tissue(s) Treatments for Chronic Diabetic Foot Ulcers	The safety and efficacy of Amnion/Chorion/Amnion allograft , Amnion/Chorion allograft, and/or Amnion/Amnion allograft, plus Standard of Care (SOC) each versus SOC alone in the treatment of DFUs	Biological	NA	Tides Medical	New, Recruiting	NCT06681428

CHAPTER 2: EFFICACY OF NANO-POLYMER ON ANIMAL MODELS TO STUDY WOUND HEALING

2.1. Introduction

A wound occurs when the usual structure of the skin and function are disrupted due to an injury, whether caused by physical trauma, burns, or other damage (Diegelmann Rf and Evans MC, 2004). These disruptions not only impact the physical barrier of the skin but also compromise its ability to perform vital protective functions. Wounds can be classified into two main types: acute wounds, which heal quickly and follow a predictable recovery process, and chronic wounds, which do not heal in the anticipated amount of time and commonly stem from underlying diseases like diabetes or poor circulation. The restoration of tissue is a highly intricate biological mechanism where the body repairs the damage, restores the structure of the skin, and regains its functional integrity (Meyers M. et al., 2020; Tavakoli S. and Klar AS. 2020).

Traditionally, wound care relied on basic materials such as cotton, gauze, and bandages to cover and protect wounds. However, modern wound care emphasizes creating an environment conducive to faster and more effective healing (Sikka MP. et al, 2019). Research has shown that maintaining a moist environment around the wound significantly improves healing outcomes (Park YG et al., 2017). Smart dressings, like hydrogels, are designed to keep the wound moist, protect it from infections, and provide structural support for tissue regeneration (Naskar YG et al., 2020). Despite these advancements, there is no single type of dressing that works perfectly for every wound. Effective wound care requires a tailored approach based on factors like the wound type, the occurrence of infection, and the overall health condition of patient (Moura L, et al., 2013).

A variety of different things must be studied in order to manage wounds effectively (Tavakoli S. & Klar AS. 2020) such as the type of wound, the healing process, conditions of the wound in terms of health and infection (e.g. diabetes), societal and environmental conditions as well as the physical and chemical characteristics of the available dressing material. (Mehrabi T et al., 2020; Stan D. et al., 2021). Wound dressing is important to keep the wound environment optimal to prevent infections and exudates. Usage of the biomaterials like PVA, PEG, gelatine, have gained its importance due to its favourable qualities in wound healing (Boateng JS et al., 2008). PVA is used in medicine because of its biocompatibility, high water contents, elastic nature, absorb toxic products, decomposing necrotic masses, and reduce blood loss (Schmedlen RH. et al., 2002). PVA was the first material approved by the FDA for intravascular use (Vaidya S et al., 2008). PEG is a versatile polyether utilized in various medicine due to its non-immunogenic and biocompatible nature (Qiu M. et al., 2016). Both the polymers are widely deployed in scaffolding and wound healing applications ((Schmedlen RH. et al., 2002). PRP

gain significant attention in wound healing applications as ability to promote and quicken the healing process of wounds (Lee M., et al., 2013). Platelet rich plasma is composed of growth factors like platelet-derived growth factor (PDGF), epidermal growth factor (EGF), fibroblast growth factor (FGF), insulin-like growth factor (IGF_1, IGF_2), vascular endothelial growth factor (VEGF), transforming growth factor (TGF-β), and keratinocyte growth factor (KGF) which are recongnized to play a role in healing of wounds.

These factors are pivotal in accelerating tissue regeneration, supporting cell division, and fostering vascular development. However, PRP alone has some limitations, such as its short-lived activity and difficulty in maintaining a sustained release of growth factors at the wound site. To overcome these challenges, this study focuses on combining PRP with PVA and PEG to create a unique nanopolymer scaffold. This combination is designed to enhance the wound healing process by supporting tissue regeneration, providing a controlled release of PRP's growth factors, and offering antibacterial properties to reduce the risk of infections. This innovative scaffold demonstrates significant potential in optimizing clinical wound management and expediting the healing process.

2.2. Materials and Methods:

2.2.1. Preparation of nanopolymer

The nanopolymer was prepared using a patented protocol (Indian Patent No. 379395). In short, 15% polyvinyl alcohol (PVA) is mized with the solution and stirred until completely dissolved, followed by the addition of 5% polyethylene glycol (PEG). Subsequently, 20 % of platelet-rich plasma (PRP) is incorporated into the PEG and PVA solution as shown in the figure 2.1. The resulting PRP-infused PEG/PVA scaffold is then subjected to three freeze-thaw (F-T) cycles to enhance polymer cross-linking. Before preparing the scaffold, antibiotics (120 mg gentamicin and 120 mg streptomycin) are added to the mixture to ensure sterility. The entire mixture is then placed on a magnetic stirrer and stirred at 85°C for 3 h. Subsequently, the prepared mixture is placed at –20°C to freeze for 12 hours and then allowed to thaw at room temperature over the next 12 hours. This freeze-thaw cycle is repeated three times to achieve optimal cross-linking of the polymer network. After the completion of the third cycle, the prepared scaffold was stored at +4°C. The process is shown in figure 2.1.

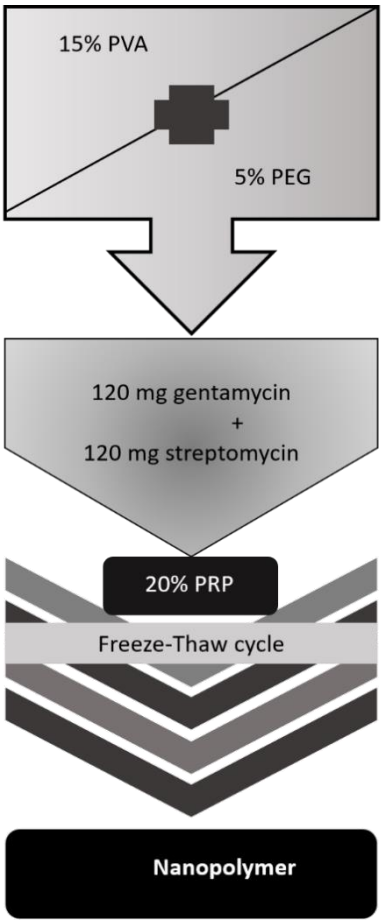


Figure 2.1: Preparation of Nanopolymer

2.2.2. Scanning Electron microscopy (SEM)

The JEOL JSM-6360 Scanning Electron Model was used for SEM examination at Shivaji University Department of Physics in Kolhapur, India. Glutaraldehyde was used to fix and dry the nanopolymer. To enhance the quality of the image, the specimen was sputtered with gold during processing. A Scanning electron microscope was performed on the developed Nano-polymer bio-ink working at a voltage of 18kV. The SEM pictures were analyzed using the open-source imaging program Image-J.

2.2.3. Concentration of platelet and other growth factors

An automated hematology analyzer was used to determine the total concentration of platelets in the blood that was drawn. The concentration of growth factors was measured at the time of manufacture and at the end of each month for four months in order to assess the effectiveness of the created nanopolymer. Growth factors such insulin-like growth factor (IGF), vascular endothelial growth factor (VEGF), and basic fibroblast growth factor (BFGF) were measured in relation to the nanopolymer. The amounts of VEGF (SVE00, rndsystems), bFGF

(MBSS90001, mybiosource), and human insulin-like growth factor (IGF1) (ab108873, abcam) in nanopolymer were measured using commercial sandwich ELISA kits. These processes were conducted in accordance with the rules provided by the manufacturer. Each growth factor data was shown as concentration (ng/ml) versus time in months.

2.2.4. High performance liquid chromatography (HPLC)

The Nanopolymer was mixed in ratio of 1:1 with HPLC water and subjected for sonication for 10min. The mixture was then centrifuged to remove any suspended particles. The compounds to be detected were of Collagen and Hyaluronic Acid (HA). Standards for both Collagen and HA were prepared in different concentrations. Collagen standard was prepared in different concentration such as 12.5 µg/ml, 25 µg/ml and 50 µg/ml and HA as 12.5 µg/ml, 25 µg/ml and 50 µg/ml, respectively. The solvents used for HPLC was Acetonitrile and Methanol. Solvents were prepared by measuring 25 ml of Acetonitrile and Methanol, respectively, and sonicating it for 10min. The flow rate was kept 2 ml/min and injection rate was 2 µl.

HPLC Conditions

- **Instrument**: High-Performance Liquid Chromatography (HPLC) system.
- **Column**: C18 column (150 mm × 4.6 mm, 5 µm)
- **Mobile Phase**: 50% acetonitrile and 50% Methanol
- **Flow Rate**: 2 mL/min
- **Injection Volume**: 20 µl
- **Detection Wavelength**: 254 nm
- **Retention Time**: 6 min

2.2.5. Fourier Transform Infrared Spectroscopy

The nanopolymer scaffold was analyzed using Fourier Transform Infrared Spectroscopy (FTIR) spectroscopy to identify its functional groups and assess chemical interactions. The nanopolymer sample was analysed using an FTIR spectrometer in the range of 4000–400 cm^{-1}. The sample was prepared in solid form and placed on the FTIR sample holder for measurement. The spectrum was recorded, and the corresponding peaks were analysed to identify the characteristic functional groups.

2.2.6. X-Ray diffraction analysis

Cu Kα radiation was used in an XRD analyser to evaluate the nanopolymer diffraction patterns. For efficient polymer analysis, the sample was prepared with the following characteristics: it

was homogenized, finely powdered, and had an average bulk composition. Following the study, the intensity vs. 2-theta graph was displayed.

2.2.7. Energy Dispersive X-ray Spectroscopy

The elemental composition of the nanopolymer scaffold was assessed using Energy Dispersive X-ray Spectroscopy (EDS). The beam was focused on the selected area of the nanopolymer surface, and the X-ray spectrum was collected over a range of 0–10 keV. The elemental peaks were identified, and their relative intensities were analyzed for quantitative assessment.

2.2.8. Spreadability assay

The nanopolymer scaffold was assessed for its spreading capabilities using spreadability assay. Initially, a 2 cm diameter circle is marked on a glass plate and 0.5 ml of nanopolymer is dispersed within the marked 2 cm circle. Following this, a second plate (10 × 10 cm^2) facing the flat surface is placed along with five-hundred-gram weight on the nanopolymer, which is examined every five minutes. As seen in figure 2.2, the diameter of the circle following the nanopolymer spreading is measured (n = 5). We have computed the % spread and the rate of spread (cm/min) in relation to time. The formula that used to calculate the rate of speed

Spread rate (A1) = A2/time …….…… (1)

Where, A1: the pre-marked circle diameter (in centimetres) on a glass plate.
A2: last region (cm) after spreading.
time: the duration of the measurement (min).

The percent spread (%) was calculated using equation:

% spread by area = A1 × 100 …… (2)

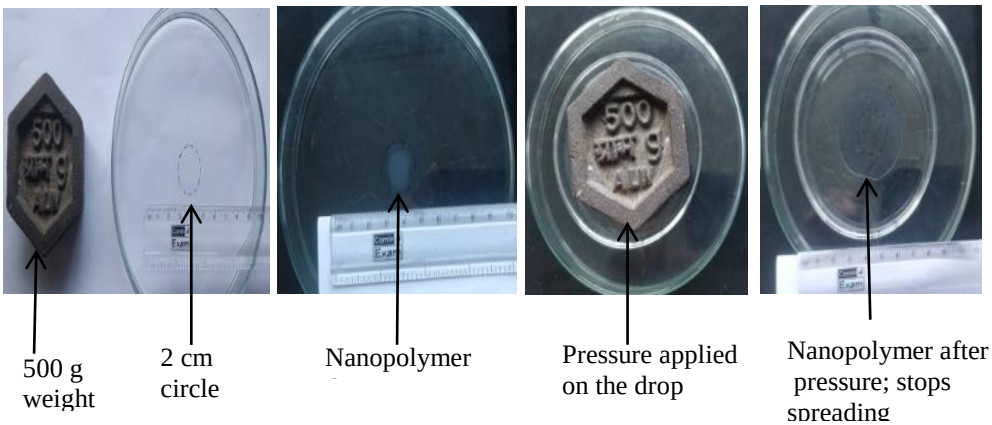


Figure 2.2: Spreadability assay of nanopolymer

2.2.9. Tensile strength

Analyses was performed on Naval Tech Fabrication (Model no. NTF CTS 3201), with a capacity of 500 Kg and variable speed of 50 to 500 mm/min. The nanopolymer was turned into scaffold by layering it onto a glass sheet. The measurement of the scaffold was recorded with a digital Vernier Caliper. Measurements included the width, thickness and length of scaffold and were recorded as 21.2 mm, 0.46 mm, and 64.9 mm, respectively.

2.2.10. Antibacterial Assay

E. Coli (NCIM 2832), *Salmonella typhi* (NCIM 5255), *Staphylococcus aureus* (NCIM 2802), and *Pseudomonas aeruginosa* (NCIM 2036) were the four bacterial isolates utilized in the investigation. The cultures were acquired from the National Collection of Industrial Microorganisms (NCIM), located in Pune, Maharashtra. The agar plate surfaces were inoculated by evenly spreading microbial inoculum by cell spreader over entire agar surface. Then three wells were created by using a punch cork borer of 6 to 8 mm. A drop of nanopolymer was added in one of the well. The D/W was maintained as a negative control and a drop of silver sulfadazine as a positive control. Further the zones of inhibition were counted and compared for the results.

2.2.11. Hemolysis assay

The hemocompatibility of the nanopolymer was conducted using the human erythrocytes (Guler C et al., 2023). With informed consent, ten milliliters (ml) of blood were extracted from a healthy human subject in a vacutainer containing ethylenediaminetetraacetic acid (EDTA). In order to obtain the RBC pellet, the collected blood was carefully transferred to the centrifuge tube and subjected to centrifugation at 3000 rpm for 15 minutes. Phosphate buffered saline (PBS) was used to wash the RBC pellet. Heparin-coated nanopolymer thin films were introduced to 200 µl of RBC that had been diluted with PBS. 200 µl of diluted fresh whole blood was added to the positive control and negative control, where 10 ml of distilled water (D/W) and 10 ml of an aqueous solution containing 0.9% NaCl (Normal Saline NS) was used as the positive and negative control respectively. Incubation at 37°C for two hours preceded a 15-minute centrifugation at 3000 rpm for each sample. Following centrifugation, spectroscopic examination was performed at 540 nm using the supernatant. The following formula was used to determine the percentage of hemolysis:

OD_{540} (sample) - OD_{540} (0% lysis)/OD_{540} (100% lysis) - OD_{540} (0% lysis) ×100%..... (3)

2.2.12. MTT assay

An increasing volume of nanopolymer (5–25 µl/well) was applied to the wells of a 96-well plate (n=3) in order to monitor the proliferation of cells in the presence of nanopolymer. On the scaffolds, cord blood MNCs were sown at a density of 1.2 x 10^4 cells. The control group consisted of cells that were planted in wells without scaffolds. After that, the cells were left to multiply for twenty-four hours at 37°C in a CO_2 incubator. MTT was added to each well after a 24-hour period, bringing the total MTT concentration in the media to 0.5 mg/ml. After five hours of additional incubation in a CO_2 incubator, the plate was carefully aspirated to remove the solution and nanopolymer. After that, 150 µl DMSO was used to dissolve the formazan crystals. The absorbance was measured at 540nm after half an hour.

2.2.13. Animal study

The animal experiments were conducted as per the guidelines of Institutional Ethical Committee, D.Y. Patil Education Society. Sixteen wistar rats (*Rattus norvegicus*) were utilized to investigate the ability of nanopolymer to heal wounds. Two groups containing 8 rats each were divided to create two groups; diabetic wound model and burn wound model.

2.2.13.1 Diabetic wound model:

For creating diabetic wound model, first the induction of diabetes using streptozotocin was performed. All the rats were injected with STZ (2.0 ml/kg) intravenously to induce diabetes. For 21 days in a row, the routine blood and urine glucose level checks were performed. The rats were anesthetized by ketamine (50 mg/kg) to create deep wounds on the dorsal region. The wounds were created using a punch biopsy of size about 9-10mm. Each rat received three wounds; one was treated with nanopolymer, other with silver sulfadiazine and one left without any treatment. The experiment was carried out for 15 days. The 2 mm biopsy sample was obtained by punch biopsy for histological and immunohistochemical analysis.

2.2.13.2. Burn wound model:

For creating burn wound model, animals were anesthetized by ketamine (50 mg/kg). The burn wound was created of size 9-10 mm by using heated metal rod. The heated rod held on the surface of skin for 20 sec. The process was repeated for three times to achieve deep burn wound. Four wounds on the dorsal side of each rat were created. The one wound was treated with silver sulfadiazine. The other two wounds were treated with nanopolymer gel. The one was maintain without any treatment. The application of nanopolymer to rats was done twice a day. The experiment was carried out for 15 days. On day 15, a little (2 mm) sample of skin was removed for immunohistochemistry and histology.

2.2.14. Histochemistry

After the obtaining biopsy samples from rats on day 15, the skin sample was treated right away with 10% formalin buffer to fix the tissue for analysis using the HE (hematoxyline and eosine), AB (alcian blue), and MT (masson's trichrome) staining methods. For typical staining methods, the tissue samples were first dehydrated and then rehydrated. Skin biopsy samples were promptly fixed for histological analysis in a 10% formalin solution. A microtome (YORCO sales Pvt. Ltd., India) was used to cut the paraffin wax embedded specimens into 3-5 μm slices on a glass slide after they had been isolated, processed, and dehydrated with alcohol grades, enlightened in xylene, and paraffin wax embedded. The rehydrated sections on the coated glass slides were stained with MT stain, AB stain, and the HE technique in accordance with normal protocol for nuclear organization and cytoplasm.

2.2.15. Immunohistochemistry

Tissue sections that were three μm thick and embedded in paraffin were used for the immunofluorescence examination. The portions were graded for rehydration using xylene and alcohol. Heat-induced epitope retrieval methods were performed using Tris citrate buffers (pH 6). Goat and rabbit serum was utilized for blocking. Following that, primary and fluorescent secondary antibodies were added to the samples. Cell adhesion markers E-cadherin (G-10) (CAT #sc-8426, 1/100 dilution, Santa Cruz Biotechnology) are the main antibodies for epithelial cell biology. Anti-α-smooth muscle actin (α-SMA, muscle actin alexa fluorTM 488, CAT#53-9760-82, 1/1000 dilution, Invitrogen) and Ep-cam (E-10) CAT#sc-13160, 1/200 dilution, Santa Cruz Biotechnology. VEGF (CAT#sc-135560, 1/200 dilution, Santa Cruz Biotechnology) and cytokeratin 18 (DA-7) (CAT#sc-51583, 1/500 dilution, Santa Cruz Biotechnology) were employed.

2.2.16. Gene expression analysis

Tissue samples were carefully collected from three groups: diabetic wound models, burn wound models, and healthy control tissues. To preserve RNA integrity, all tissues were processed under sterile conditions immediately after collection. Total RNA was then extracted. Complementary DNA (cDNA) was synthesized from the isolated RNA using a downstream quantitative PCR (qPCR) analysis. For gene expression analysis, qPCR was performed using specific primers for the genes of interest: COMP, TNFα, MMP9, and IL-10. The reactions were conducted using a SYBR Green-based detection system, and β-actin or GapDH served as the internal reference gene for normalization. The amplification was carried out in a real-time PCR machine, ensuring

high sensitivity and specificity in detecting gene expression levels. To determine relative gene expression, the $2^{\Delta\Delta Ct}$ method was used to calculate fold changes in mRNA levels. All data were normalized to the housekeeping gene, and results were expressed as fold changes relative to the control group. This robust methodology provided a clear understanding of the differential expression of key genes involved in inflammation, tissue remodelling, and anti-inflammatory responses in the wound models.

2.2.17. Statistical Analysis

The data are expressed as mean ± standard deviation (SD). The Student's t-test was used for statistical comparisons, and a p-values of <0.05 was deemed statistically significant. Statistical significance is indicated for all images is $*$ for $p \leq 0.05$, $**$ for $p \leq 0.001$, and $***$ for $p \leq 0.0001$.

2.3. Results:

2.3.1. Scanning electron microscopy:

SEM pictures reveals a consistent porous structure of the nanopolymer (fig. 2.3.), which depicts the porous structure of the surface film and nanopolymer. With pores ranging from 2 to 20 µm.

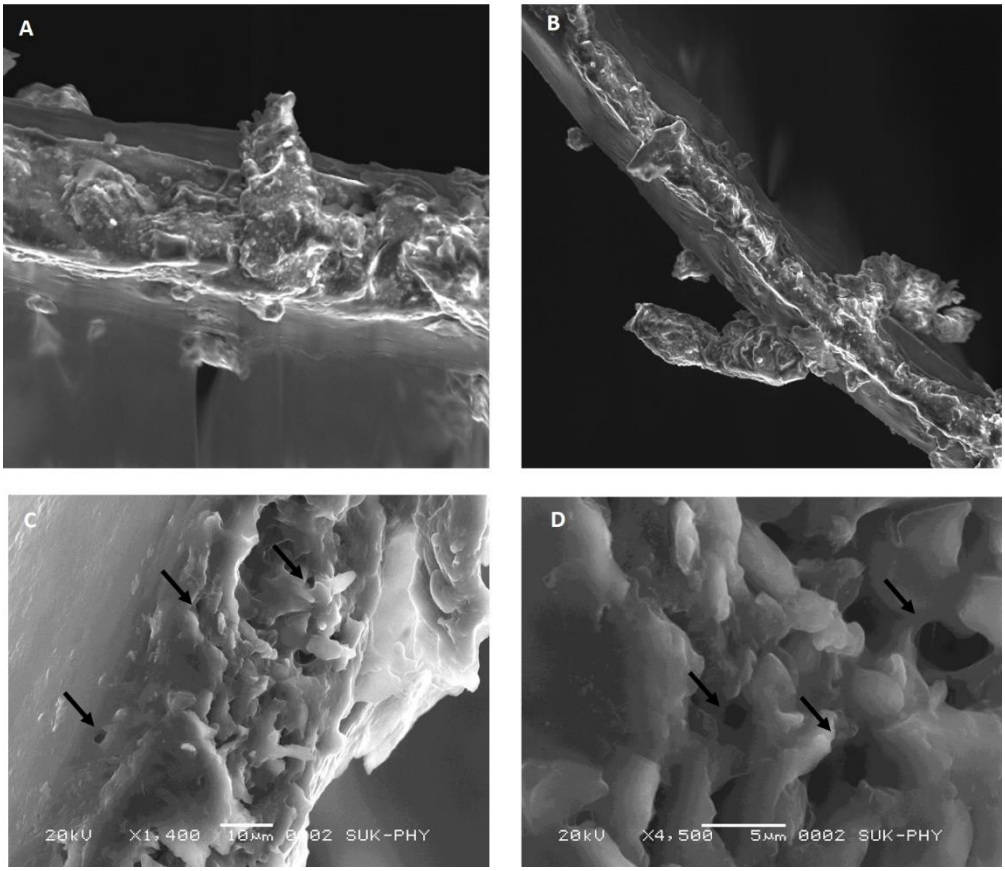


Figure 2.3: Scanning electron microscopy (SEM) analysis

2.3.2. Estimation of platelets and other growth factors:

The platelets obtained from normal whole blood content is 109.6 ± 7.4 µl/ml (Fig. 2.4.A). The freshly made nanopolymer's IGF concentration, according to the ELISA data, is 66.63 ± 9.79 ng/ml. Between the first and fourth months, there is a 6.15% drop in concentration (Fig. 2.4.B). The freshly made nanopolymer has a VEGF concentration of 78.86 ± 22.87 ng/ml, and the concentration dropped 13.61% from the first to the fourth month (Fig. 2.4.C). It is discovered that the freshly made nanopolymer contained 31.40 ± 8.4 ng/ml of BFGF. Between the first and fourth months, the concentration rate dropped by 20.38 percent (Fig. 2.4.D).

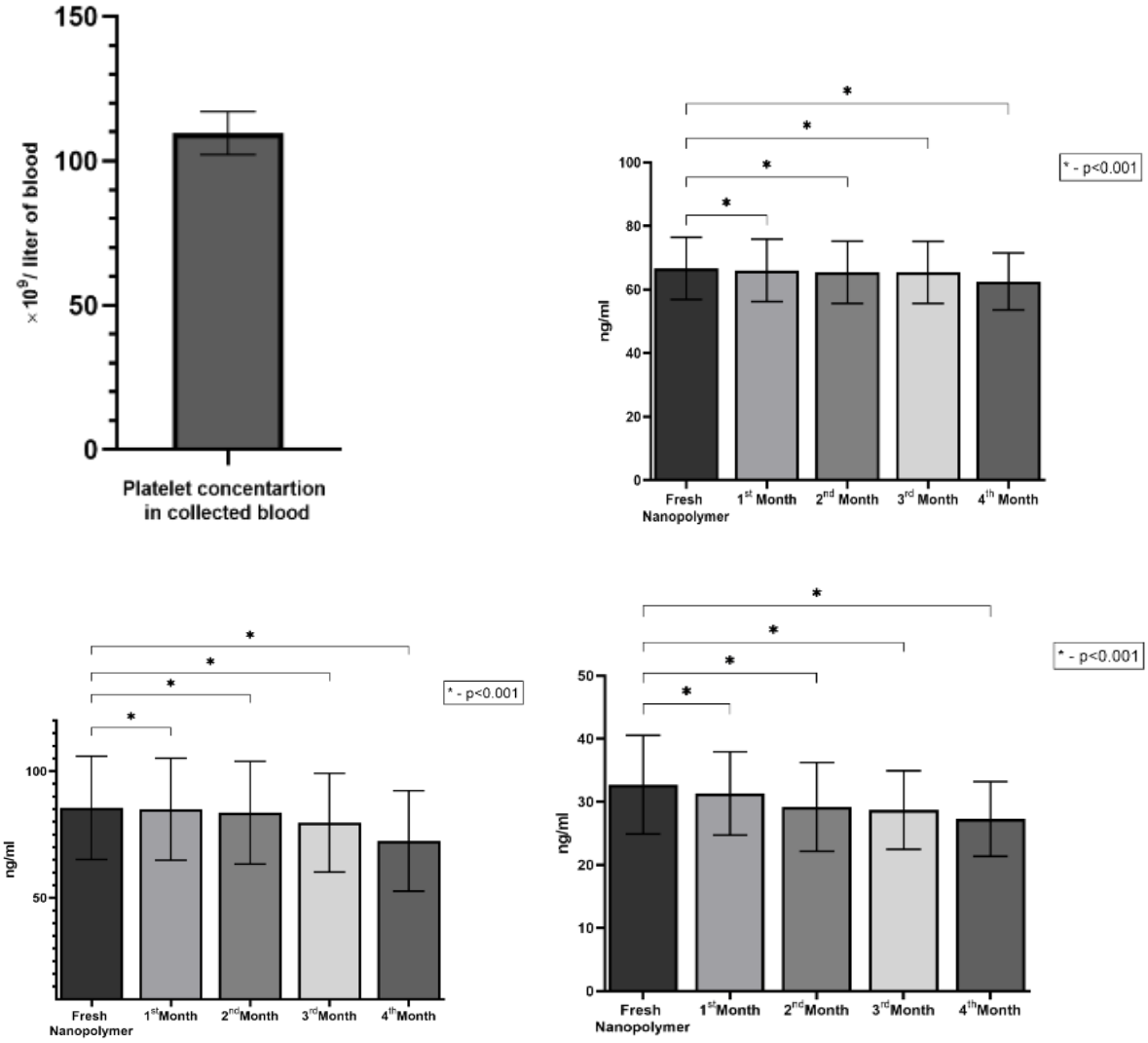


Figure 2.4.: Estimation of platelets and other growth factors

2.3.3. HPLC

The HPLC chromatogram of the PRP-incorporated nanopolymer sample is shown in Fig. 2.5. Two prominent peaks are observed in the chromatogram. The first peak appears at approximately 4.5 minutes with a sharp dip, indicating the possible presence of an early eluting compound or component. The second peak at approximately 5.8 minutes represents a major component with high intensity, likely corresponding to the PRP-derived growth factors or specific nanopolymer constituents. Beyond 6 minutes, no significant peaks are observed, suggesting a clean profile with no additional impurities or degradation products detected in the sample. The retention times and intensities observed are consistent with the expected components within the PRP-nanopolymer scaffold. Further identification and quantification of peaks may require comparison with appropriate standards or marker molecules. The chromatogram reveals a well-resolved peak for Collagen and HA at a retention time of 4 minutes. The presence of collagen in NP was confirmed with that of Standard.

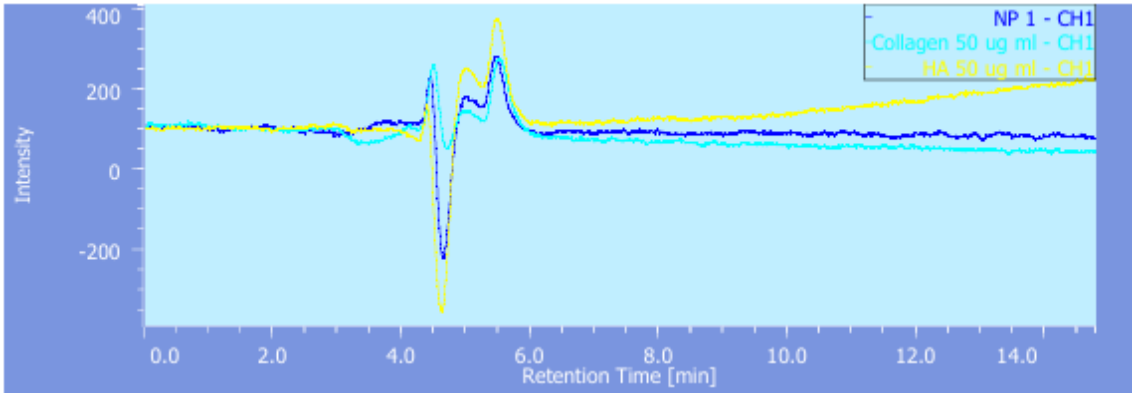


Figure 2.5: HPLC Analysis

2.3.4. FTIR

The FTIR spectrum of the nanopolymer is presented in Fig 2.6. The spectrum shows several prominent peaks indicating the presence of characteristic functional groups. FTIR spectra of the developed Nano-polymer Bio-ink were recorded using JASCO FTIR 4700 spectrometer (JASCO, Lisses,France). The peaks confined to contain functional groups as Hydroxyl group, Aldehyde, carboxyl. The peaks are obtained at 3727(OH) , 2359(C=C), 1500(C=O) and 628 (C-H). The absence of additional peaks or shifts suggests effective cross-linking within the polymer matrix without chemical degradation.

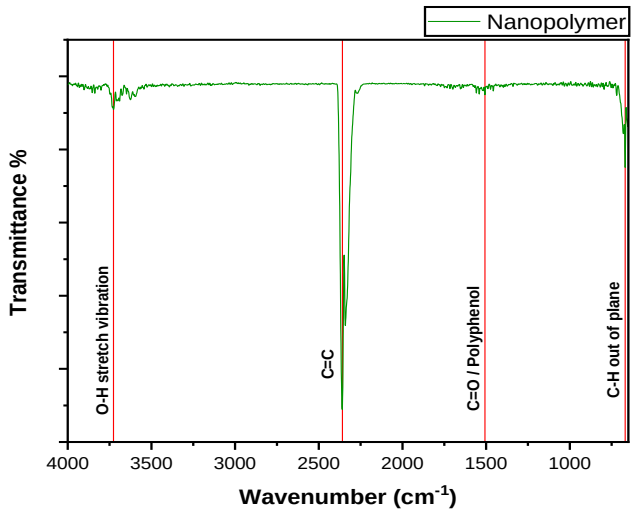


Figure 2.6: FTIR with prominent peaks of nanopolymer

2.3.5. XRD

X-ray diffraction (Fig. 2.7.) analysis showed the polymerization of PVA and PEG. The incorporation of polymers PVA and PEG has achieved and the sharp peaks indicate its polymorphic structure.

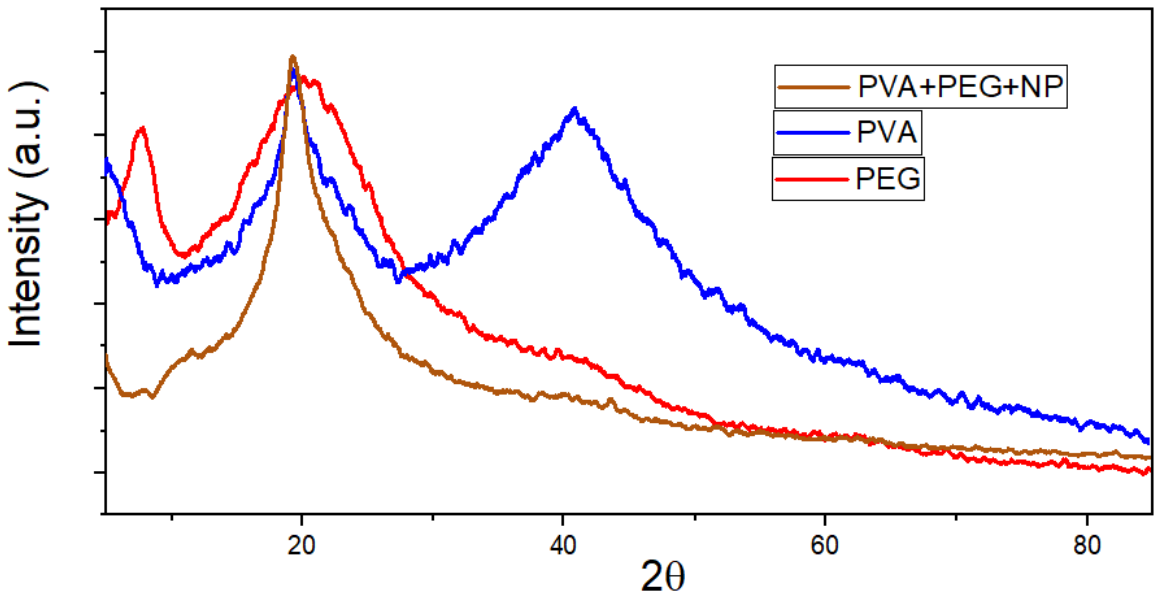


Figure 2.7: X-ray diffraction analysis

2.3.6. EDS

The EDS spectrum of the nanopolymer scaffold is shown in Figure 2.8.. The spectrum exhibits prominent peaks corresponding to carbon (C) and oxygen (O), which are key components of the polymer matrix. The sharp peak at approximately 0.2–0.3 keV confirms the presence of

carbon, while the peak near 0.5 keV corresponds to oxygen. These peaks validate the organic nature of the scaffold, primarily composed of carbon and oxygen-based compounds such as PVA, PEG, and other polymeric constituents. The absence of additional peaks in the spectrum indicates that no metallic contaminants or unwanted elements are present, confirming the purity of the scaffold.

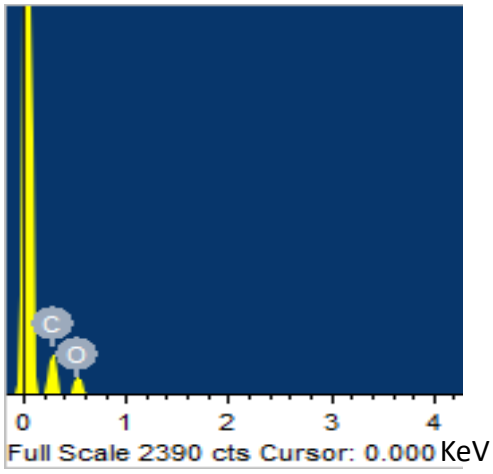


Figure 2.8: EDS Analysis

2.3.7. Spreadability assay

Plotting the nanopolymer spreadability on the graph reveals that it decreases with time. The graph indicates that the nanopolymer spreads at a steady rate for the first two minutes, but that the rate drops after five minutes. Spreading is 0.5 on average.

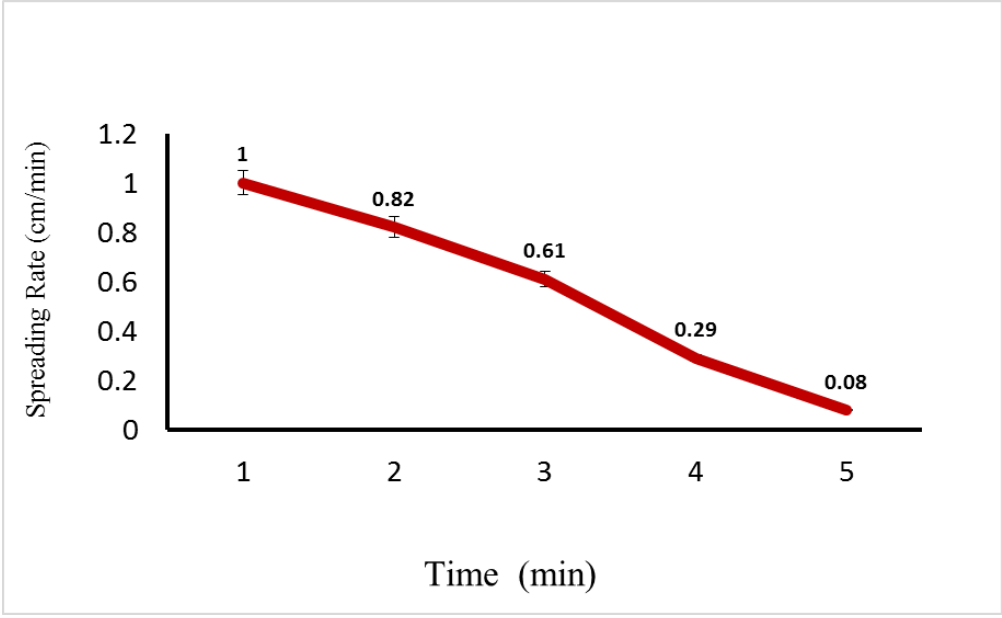


Figure 2.9: Spredability Rate showed it was maximum in the beginning and decreased after 2 minutes

2.3.8. Tensile strength

Tensile test was performed on the sample and the strength is 20kg/cm2. Speed is maintained at 50mm/min and the peak load and displacement is 1.95kgf and 23mm (Fig. 2.10.).

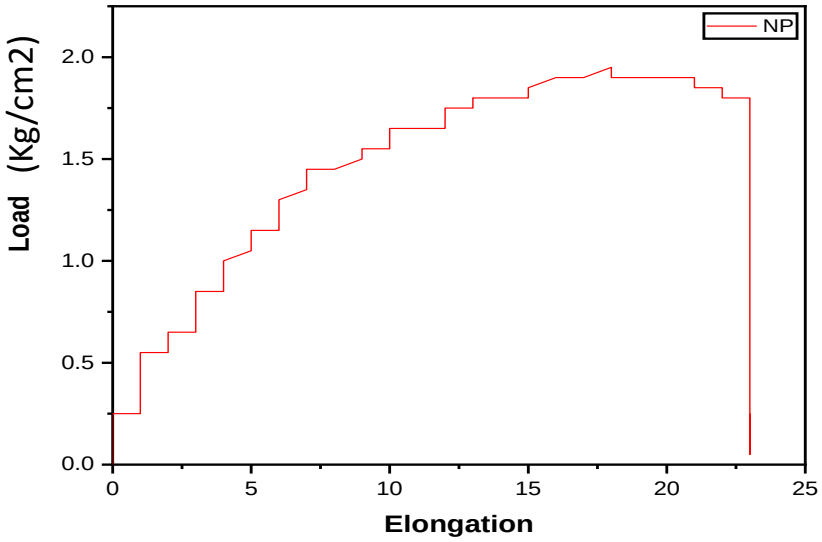


Figure 2.10 Tensile strength test

2.3.9. Antibacterial assay:

The zone of inhibitions are measured after 24 h of incubation. The Nanopolymer shows (Fig.2.11.) the highest zone of inhibition for all four organisms. The control shows no zone of inhibition and the silver sulfadizine cream shows less than 2 mm of diameter as a zone of inhibition.

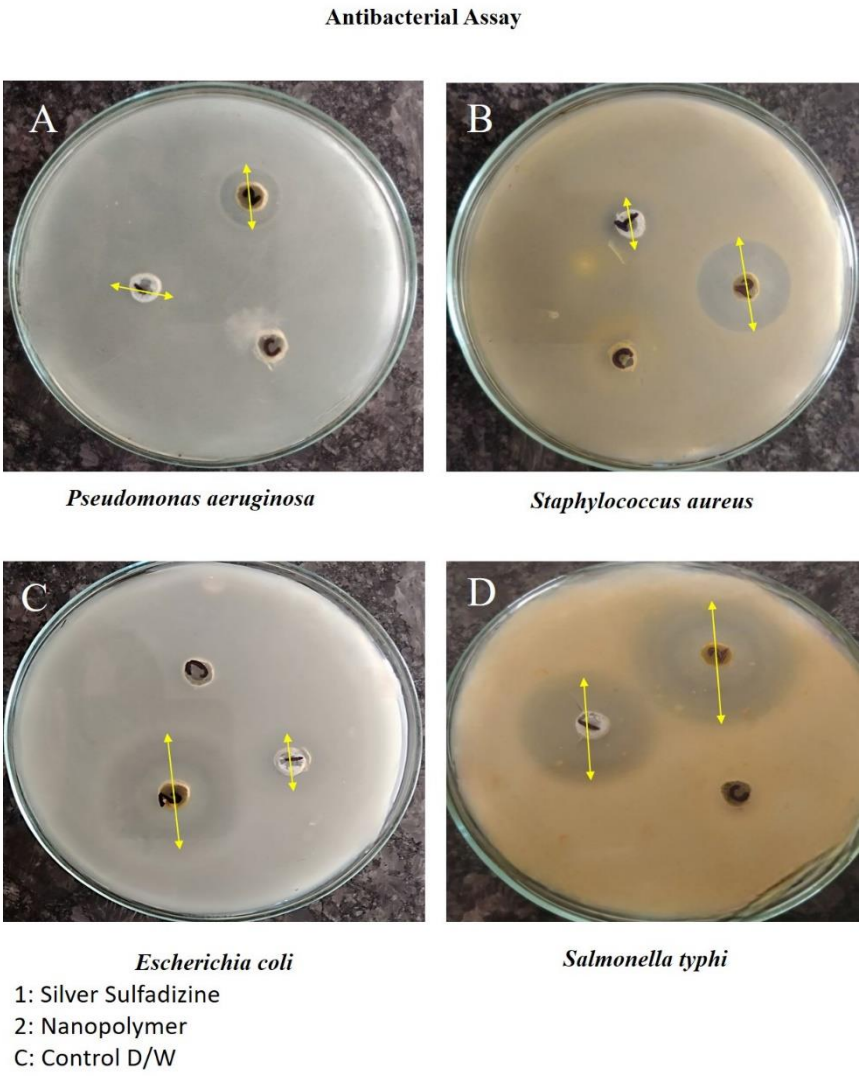


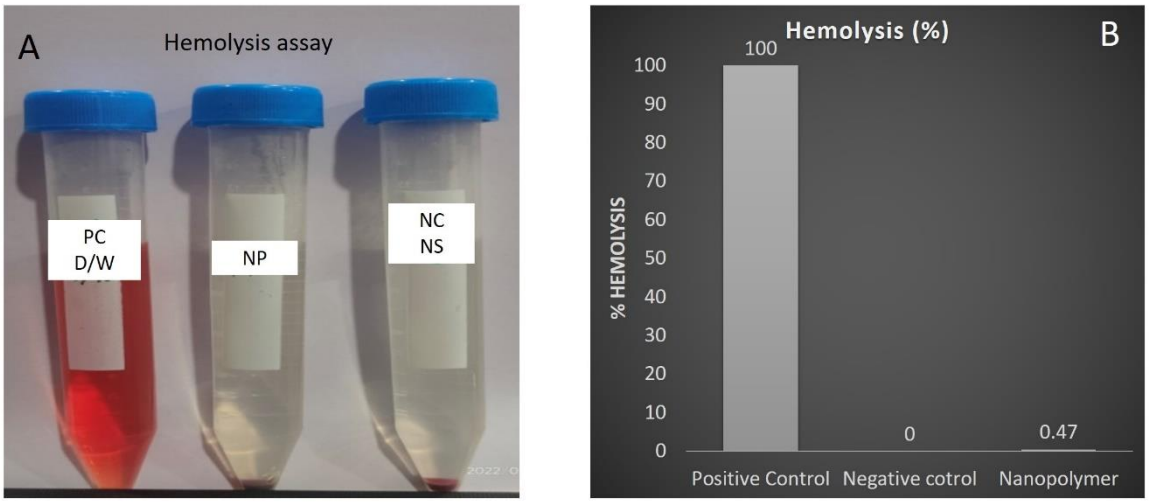
Figure 2.11: Zones of inhibition of nanopolymer on Gram positive and Gram Negative bacteria

Table 2.1: Zones of inhibition of nanopolymer in mm

Test organism	Control D/W	Nanopolymer	Silver Sulfadizine
P.aeruginosa	-	10 mm	3mm
S.aureus	-	11 mm	5mm
Salmonella typhi	-	13 mm	8mm
E.coli	-	9 mm	3mm

2.3.10. Hemolysis assay:

The hemolysis test is used to determine whether biomaterials are compatible with blood. Materials with a hemolysis value of less than 5% are regarded as safe. The 5% rate rise indicates poor blood biocompatibility. Results of a hemolytic study showing the nanopolymer biocompatibility with human red blood cells after two hours of incubation are displayed in Fig. 6. According to a study, the nanopolymer hemolysis rate is 0.47, which is less than 5.0 percent and suggests no hemolytic activity (Fig.2.12.A&B).



PC: Positive Control; NP: Nanopolymer; NC: Negative control

Figure 2.12: Hemolysis assay of nanopolymer

2.3.11. MTT assay

The nanopolymer's cytotoxicity is estimated by the resulting absorbance of control and increasing concentration of nanopolymer is plotted in graph (Fig.2.13.). From the graph, it can be concluded that over all the nanopolymer have a proliferative effect while achieving maximum at a concentration of 20 µl/100 µl of media.

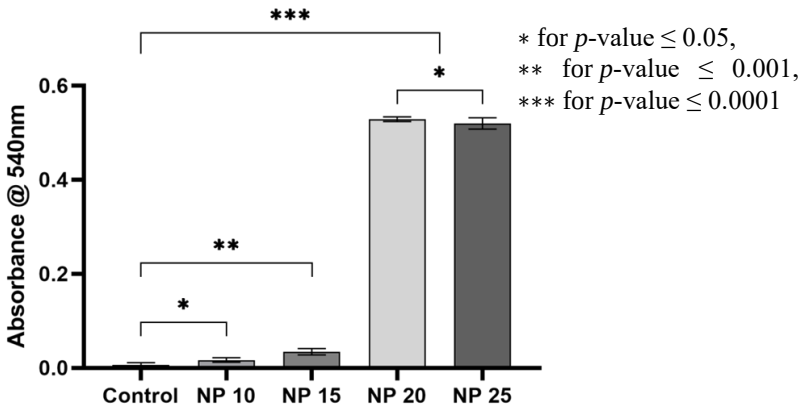


Figure 2.13: MTT assay for cell proliferation and cell toxicity.

2.3.12. Wound healing assessment in animal model:

2.3.12.1. Diabetic wound model:

The diabetic rats had HbA1c range of 7.5 to 9 %. Depending on the results of HbA1c values the selection of animals were performed for further studies.

To assess the extent of decline and the rate of recovery of the three groups (Control, Sham, and Nanopolymer), the animals are observed for 15 days (Fig.2.14) Control deep wounds treated with a conventional topical cream containing silver sulfadiazine only partially healed, in contrast to the nanopolymer treated wound that was entirely healed. The highest degree of reduction was found in wounds treated with nanopolymer. The results showed that applying nanopolymer topically to open wounds accelerated the healing process compared to commercially available topical treatments including silver sulfadiazine. It was confirmed using histological staining methods.

HE staining is used to show that the excised nanopolymer-treated wound sample has a nucleus (Fig.2.15). It also shows the growth of blood vessels, hair follicles, and other endothelial cells. The nucleus seems blue, yet the entire cytoplasm is pink. Using Masson's trichrome staining, the existence of collagen fibers is investigated. The pink color of the staining is caused by the collagen that has been deposited in the skin samples. Compared to wounds treated routinely, the rate of regeneration in test samples was greater.

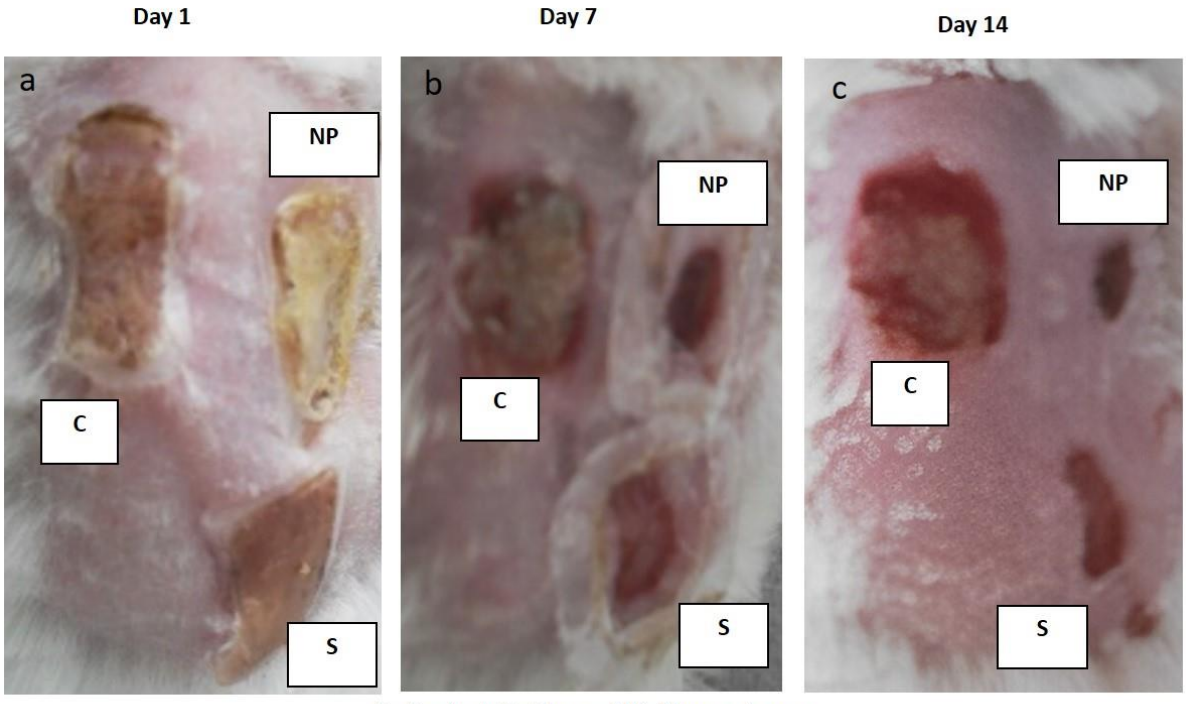


Figure 2.14: Wound healing analysis of nanopolymer on the diabetic rat model

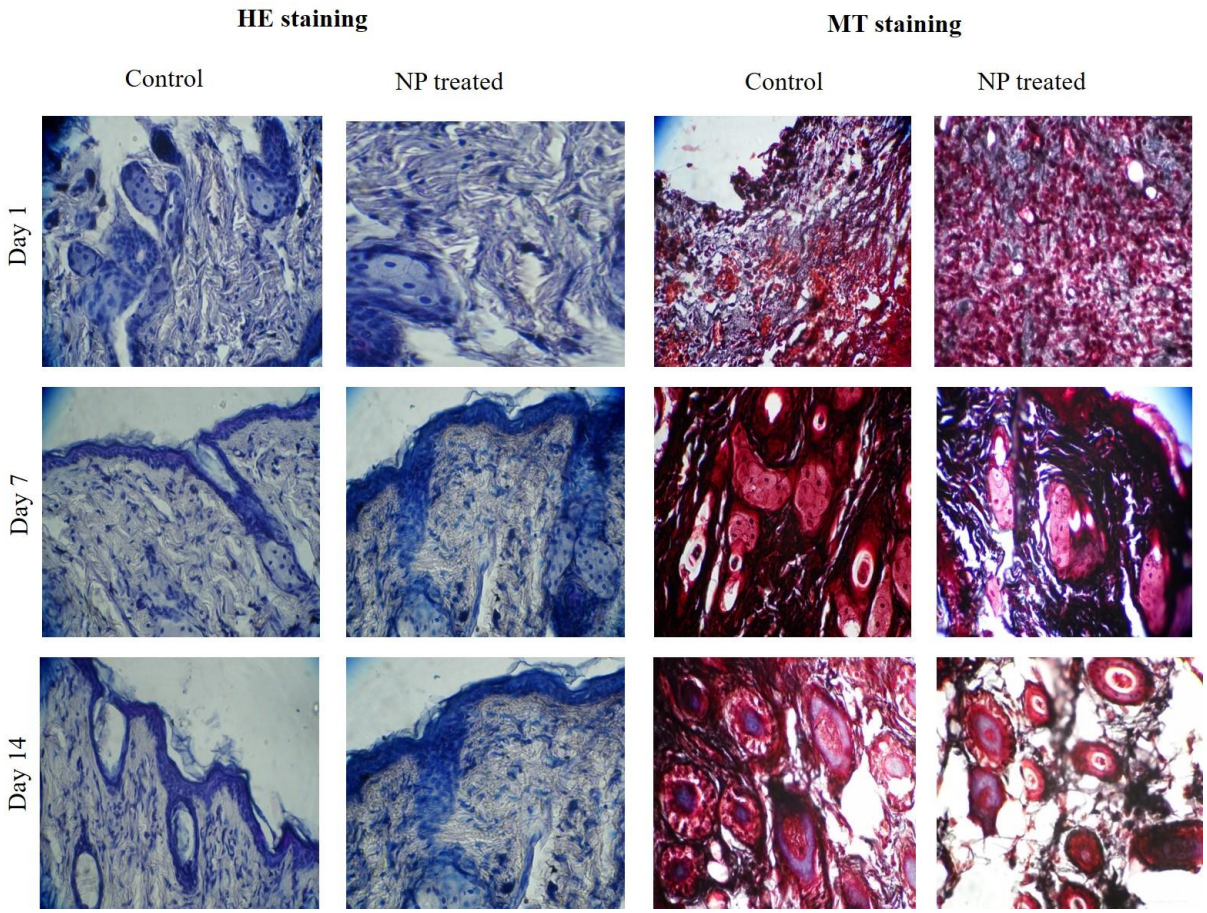


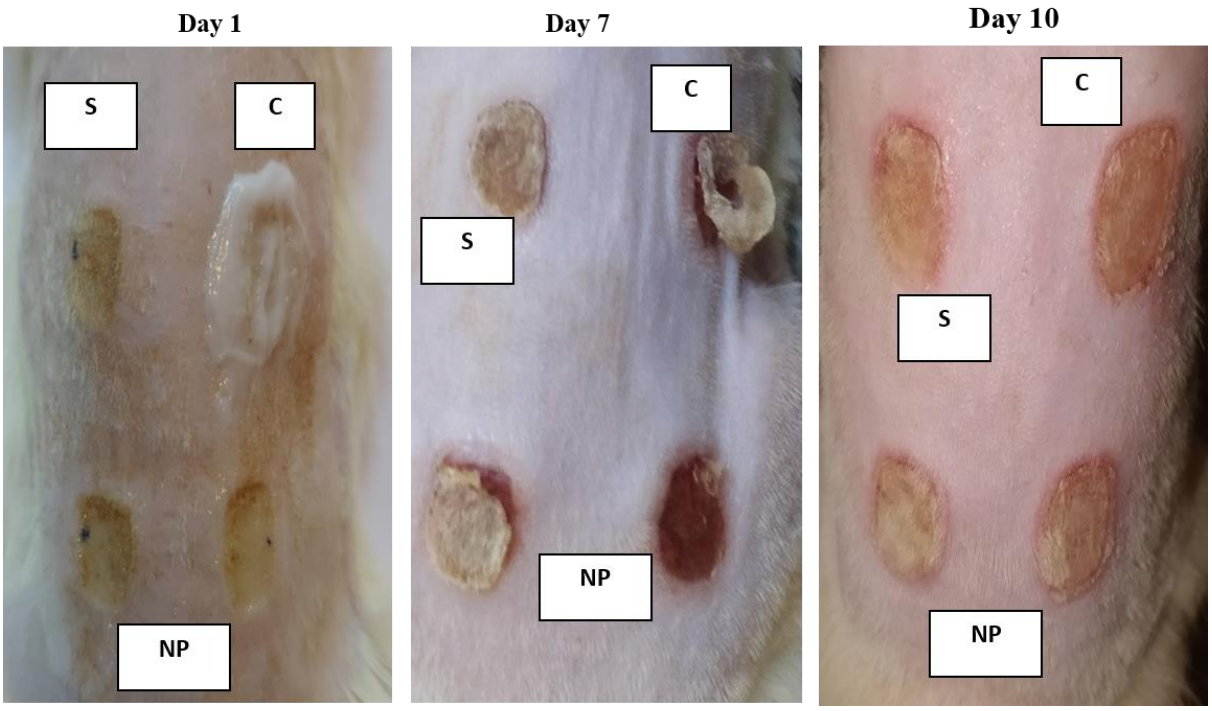
Figure 2.15: Analysis of wound healing by histochemical analysis

2.3.12.2. Burn wound model:

To assess the extent of decline and the rate of recovery of the three groups (Control, Sham, and Nanopolymer), the animals were observed for 15 days (Fig. 2.16). Control wounds treated with a conventional topical cream containing silver sulfadiazine only partially healed, in contrast to the test wound that was entirely healed. The highest level of reduction is found in wounds treated with nanopolymers. The results showed that applying nanopolymer topically to open wounds accelerated the healing process compared to commercially available topical treatments including silver sulfadiazine. It was confirmed using histological staining methods. The presence of a nucleus in the excised nanopolymer-treated wound sample is demonstrated by HE staining (Fig.2.17). Additionally, it displayed the development of hair follicles, blood vessels, and other endothelial cells. The entire cytoplasm is pink, while the nucleus appears blue. AB staining revealed that test samples has more GAGs than wounds that had received conventional treatment. The presence of collagen fibers was examined using Masson's trichrome staining. The collagen deposition in the skin samples is what gives the staining its

pink hue. In test samples, the rate of regeneration is higher than in wounds that are treated conventionally.

Figure 2.18. C illustrates the regeneration of smooth muscles in the epithelial region through the expression of α-SMA in the wound treated with nanopolyner. An epithelial marker called CK-18 is also highly expressed in wounds treated with nanopolymers. E-cad expression is found to be lower across all groups. However, there is no expression in the wound that is treated traditionally. In adherent junctions between epithelial cells, it is expressed. EpCAM is an adhesion element of epithelial cells that has been shown to promote re-epithelialization in wounds treated with nanopolymers. Vascular endothelial growth factor VEGF verifies neo-vascularization in wounds treated with nanopolymers. In comparison to wounds treated with silver nitrate, the expression of all antibodies in the nanopolymer-treated wounds demonstrated a higher degree of healing in the test and an increased pace of regeneration.



C: Control; S: Sham; NP: Nanopolymer

Figure 2.16: Analysis of wound healing of nanopolymer on burn wound model

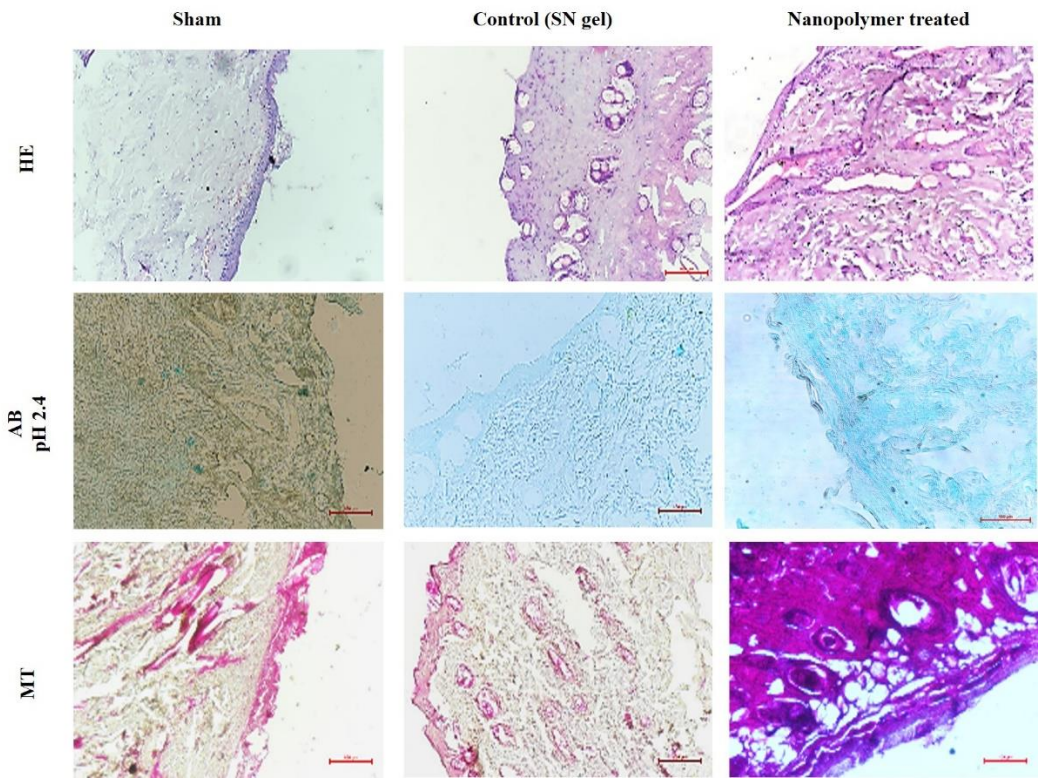


Figure 2.17: Histochemical analysis of the control, SN gel and nanopolymer treated wounds of nanopolymer treated burn wound model

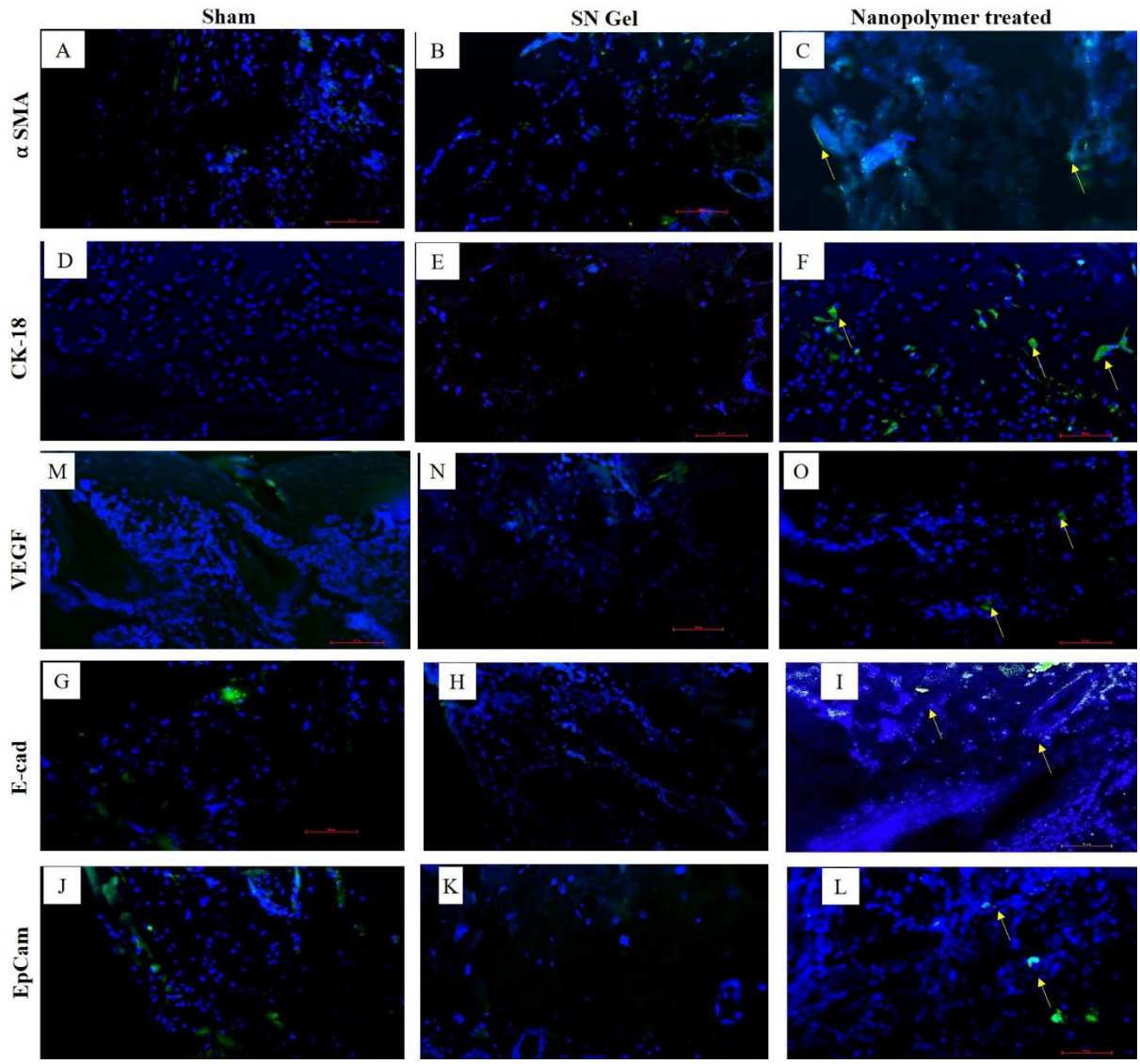


Figure 2.18: Immunohistochemical Analysis of nanopolymer treated wounds of burn wound model; expression of the α SMA, CK-18, E-cad, EpCam, VEGF

2.3.12.3. Gene expression studies

The results of the mRNA analysis (Fig. 2.19.) reveal distinct patterns of gene expression across the diabetic wound model, burn wound model, and control group. COMP expression is notably higher in the diabetic wound model compared to the burn wound model, while the control group shows minimal activity. Both wound models exhibit elevated levels of TNFα, an inflammatory marker, though the levels are similar between them and significantly higher than the control group. For MMP9, a marker associated with tissue remodelling, the burn wound model shows slightly higher expression compared to the diabetic wound model, with the control group displaying negligible expression. IL-10, an anti-inflammatory cytokine, is expressed prominently in the diabetic wound model, followed by the burn wound model, while the control

group shows very little activity. These findings highlight the differing molecular responses in diabetic and burn wounds, emphasizing the heightened inflammatory and regenerative activity in both wound types compared to the control.

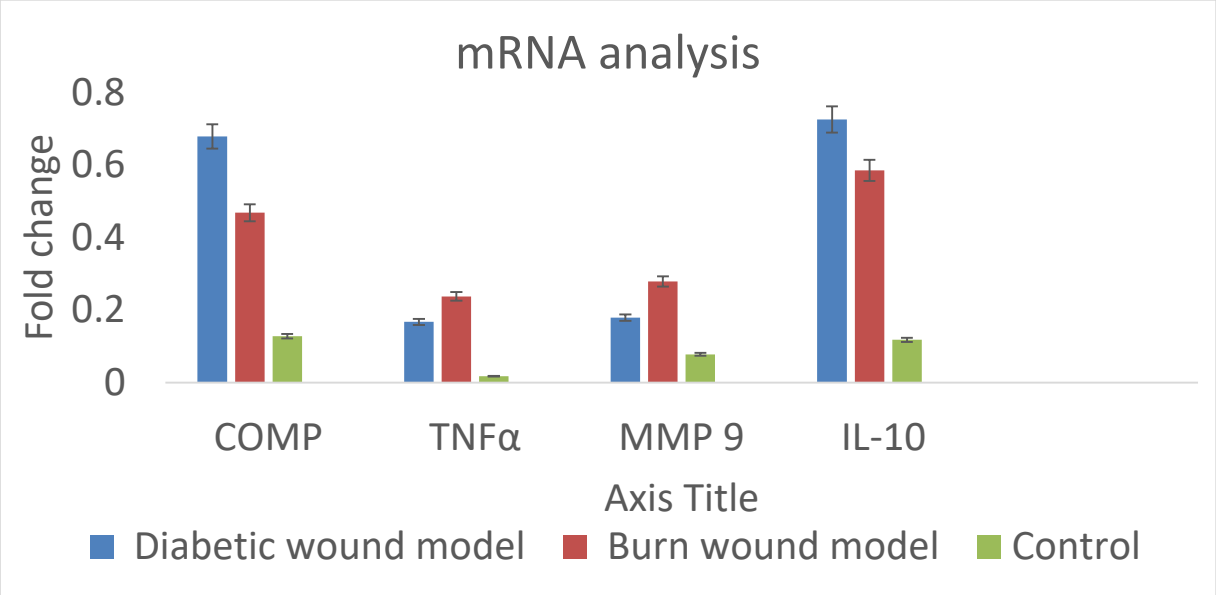


Figure 2.19: Gene expression studies

2.4. Discussion:

Wound healing is a dynamic process involves inflammation, proliferation and remodelling phases (Tottoli EM. et al., 2020). Abnormal wound healing is usually not observed in healthy, normal persons, but is frequently connected to an underlying process, such as hunger, diabetes, or cancer. Diabetes is the major concern in the world, 15% of these individuals had diabetic foor ulcers, which are the cause of the great majority of amputations in this patient group (Pal K. et al.,2007). The incidences of chronic wounds are increasing in aging population and the people with diabetes.

Nanotechnology based therapeutics can trigger the specific biochemical event within the impaired healing process; can alter one or more phases of wound healing. The advantage of such nano-platforms is their adaptability and ability to tune with injury site. Nanotechnology can be used in controlled and sustained release of the active biomolecules over a period of days or weeks, while conventional delivery systems work for 1 to 2 days in the release of the therapeutic agent. Extended inflammatory phase along with persistent bacterial and fungal infections, increasing levels of proteases and reactive oxygen species (ROS) is the sign of impaired wound healing. The more elevated levels of proteases in constant injuries advance

the annihilation of extracellular matrix (ECM), Growth Factor (GF) receptors and GF like platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF), keratinocyte growth factor (KGF), and transforming growth factor-beta (TGF-b). Wound healing is facilitated by the moist environment of the wound. This prevents dehydration and enhances angiogenesis along with synthesis of collagen resulting in increased breakdown of fibrin and dead cells.

Soon after the injury, the procedure of repair is started by sequential release of growth factors, cytokines, and low-molecular-weight substances from degranulating platelets and serum of damaged blood arteries. In the moist and localized growth factor rich environment accelerate the wound healing process. This was achieved by incorporating PRP GFFs in the nanopolymer consisting of PEG and PVA which provided both the essential environment and tropical GF support for fast recovery.

Seven key growth factors are present in PRP's α, including 3 isomers of platelet-derived growth factor (PDGF-αα, PDGF-αβ, and PDGF-ββ), 2 isomers of transforming growth factor-β (TGF-β1 and TGF-β2), VEGF, and epithelial growth factor (EGF)(Qian Y. et al., 2017). Each of them functions as a growth factor or cytokine that aids in wound healing and is released by activated platelets during the healing process (Sridharan K and Sivaramakrishnan G. 2018). When cross-lnked into networks, the hydrophilic polymer poly (ethylene glycol) can contain a significant amount of water (Song A., et al., 2012). Polymerization of all the polymers with PRP is achieved by continuous freeze thaw cycles (Fig. 2.1).

Polymerization enhances the catalytic effect of polymer which can be confirmed with X-ray diffraction analysis (Fig.2.7). in addition to offering a superior physiological environment that promotes cell adhesion, proliferation, and/or differentiation, the optimal wound-healing scaffolds should have the right mechanical and physical characteristics to avoid secondary infection. Consequently, we created an artificial cell-adhesive polypeptide hydrogel with built-in antibacterial activity, all the physical and mechanical properties (Ganguly S et al., 2019).

The porous morphology of hydrogels is an outcome of internal cross-linking was confirmed with SEM imaging (Fig. 2.3) and XRD. Spredability analysis (Fig. 2.2 and 2.9) showed the nanopolymer has a property of covering the wound without runny nature. The hemolysis analysis (Fig. 4B,C) showed the nanopolymer is compatible to the blood. The nanopolymer is proven effective against pathogenic bacteria like *S. aureus* (Fig.2.11). This pathogen is the main cause of secondary infections in diabetic foot ulcers or any other wounds(Alavi M, Rai M, 2020).

The study was conducted to check the stability of the GFs concentrations in the nanopolymer for long term storage. It was confirmed that GFs were stable in -20^{0}C for 4 months (Fig. 2.4 A,B,D). We observed that decreased GF concentration did not reduce the efficacy of the nanopolymer after 4 months. MTT assay of nanopolymer showed increased proliferation of cells at 20 and 25 µl (Fig. 2.13). Importantly, it was observed that nanopolymer at higher concentration enhance the rate of proliferation. This could be important observation for clinical studies where repetition of nonopolymer application will provoke regenerative response.

In the diabetic wound model, nanopolymer (NP)-treated wounds demonstrated significantly faster wound closure compared to control (C) and silver nitrate (S)-treated wounds, as evident from visual assessment (Fig. 2.14 A). By day 14, NP-treated wounds achieved nearly complete closure, while C and S wounds displayed incomplete healing.

Histological analysis (Fig. 2.14 B) further validated these findings, with hematoxylin and eosin (HE) staining showing a thicker epidermis and more extensive cellular proliferation in NP-treated wounds compared to C and S groups across all time points. Masson's trichrome (MT) staining revealed densely packed and well-organized collagen fibrils in NP-treated wounds by day 14, resembling normal skin structure, while collagen deposition was less organized in the C and S groups. These results highlight the superior regenerative capacity of nanopolymer in diabetic wounds, likely due to its ability to facilitate a continuous and regulated delivery of growth factors., enhance extracellular matrix (ECM) remodelling, and promote effective tissue repair. In the animal study, it was observed that the size of the wound in nanopolymer treated group decreased faster with time compared to SN treated wounds (Fig. 2.14). Histochemistry study also confirmed ECM re-modulation in nanopolymer treated group. HE staining (Fig. 2.15B) showed presence of nuclei was more in nanopolymer treated wound than both control and SN treated wound. There was also presence of other glandular structure like hair follicle and vascular glands in nanopolymer treated wounds. AB staining (Fig.2.15 B) showed better arrangement of GAGs and Masson's trichrome staining (Fig.2.15 B) displayed the most densely packed collagen fibrils among all nanopolymer treated group, and these fibers were resembled like of normal skin. This positive effect provided further confirmation of our hypothesis that nanopolymer indeed possesses sustained-release capabilities for PRP GFs and facilitates ECM remodelling (Fig. 2.15B).

The IHC results confirmed the re-epithelization and neo-vasculogenesis. αSMA showed smooth muscle lining was better when compared with control and SN treated wounds (Fig. 2.16). The expression of the CK-18 proves the epithelial lining was restored (Fig. 2.16). E-cadherin is a calcium-dependent cell adhesion molecule that is normally expressed at adherent

junctions between epithelial cells (Fig. 2.16). Nanopolymer treated group showed improve E-cadherin expression between cells. Epithelial cell adhesion molecule (EpCam) expression was well preserved in nanopolymer treated wounds compared to control and SN treated group (Fig. 2.16). Expression of vascular endothelial growth factor (VEGF) in nanopolymer treated group confirmed neovascularization (Fig. 2.16).

The mRNA analysis highlights distinct molecular responses in diabetic and burn wounds, with elevated inflammatory (TNFα) and regenerative (IL-10, COMP, MMP9) markers compared to controls (Fig. 2.17). The diabetic wound model shows higher anti-inflammatory and extracellular matrix activity, suggesting a prolonged yet less efficient healing process compared to the burn wound model. These findings underscore the need for targeted therapies addressing specific wound pathologies. These results provide evidence that nanopolymer can enhance wound healing rates, making it an attractive treatment modality for patients. The further studies are required for gene analysis for jumping onto any conclusion.

2.5. Conclusion:

The study reveals that nanopolymer used on diabetic wounds helps to accelerate the wound healing which include both acute and chronic wounds. The angiogenesis was observed without any necrotic tissues on the wounds treated with nanopolymer. The further studies will help to understand the translational use of nanopolymer. Nanopolymer used in burn wound study showed faster healing rate than conventional and natural healing. The acceleration of the healing process is due to incorporation of growth factors in the hydrogels which shows slow and localize release of growth factors. These growth factors are responsible for the regeneration and epithelization triggering the overall wound healing process.

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CHAPTER 3: EFFICACY OF 3D PRINTED SKIN ON ANIMAL MODELS TO STUDY WOUND HEALING

3.1 Introduction

The skin is the largest organ of skin and accounts for 15 to 20 % of total body weight (Velasco D. et al., 2018). It is the primary defence mechanism against a variety of environmental threats, such as microorganisms, ultraviolet radiation, and toxic or mechanical agents, as well as all physical factors (Chen J. et al., 2019). The dermis layer of the skin comprises collagen, fibrin, and elastin. The inherent ability of body to recover from most wounds is rarely challenged, but some wounds may be complicated to treat due to the degree of severity, infection, or an underlying medical condition such as diabetes. These wounds necessitate continuous care and medications. Allogenic skin grafting continues to be frequently used to treat non-healing wounds, but the limitations of donor sites, immunological responses, and subsequent infections limit its use in skin applications (Weng T. et al., 2021). In past experiences, burn wounds and diabetic ulcers have been treated with decellularized extracellular matrix (dECM) patches or sheets generated from decellularized human skin (Jorgensen AM. Et al.,2020), amniotic membrane (Ramakrishnan R. et al., 2020), pig small intestine submucosa (Smandri A., et al., 2020), and porcine urinary bladder (Daneshfar C., et al., 2021).

In humans, wound healing can be divided into four phases: hemostasis, inflammation, proliferation (re-epithelization, granulation tissue formation, and neovascularization), and maturation and remodelling (Liao J. et al., 2020). The wound healing starts with blood clotting that attracts chemokines and growth factors to localize at the site of injury. ECM components present beneath the epidermal layer are important for this reformation of the basement membrane. Fibroblasts pulling on the ECM trigger wound contraction, resulting in the release of various growth factors like cytokines, matrix metalloproteinases (MMPs), and ECM components (Rousselle M. et al., 2019; Shaw TJ. And Martin P.,2009; Chang Y. et al., 2015; Redd MJ. Et al.,2004).

In conditions like diabetes, tobacco abuse, stress, malnutrition, and obesity, the normal process of wound healing is hampered (Avishai E., et al.,2017). The non-healing wounds have an extended inflammatory phase due to ischemia, hypoxic conditions, high levels of oxygen radicals, and inadequate tissue perfusion (Bowler PG., et al., 2002).

Tissue engineering and 3D bioprinting are the platforms where different biomaterials and cells are being used to construct biological dressings. In 3D bioprinting, tissue-specific bioink was built into the desired structure by the computer-assisted deposition method for clinical investigations and testing (Weng T. et al., 2021; Fayyazbakhsh F. and Leu M.,2002). 3D bioprinting allows for the fabrication of detailed microenvironments and architectural replicas of the original skin. In addition to natural polymer materials like alginate, fibrin, collagen,

gelatin, chitosan, and hyaluronic acid, biodegradable synthetic materials like thermoplastic polyester plastics (polylactic acid (PLA), polylactic-co-glycolic acid (PLGA), polycaprolactone (PCL), and poly ethylene glycol (PEG) are widely used in biomedical 3D printing (Zaszczynska C. et al., 2021). Although synthetic materials have biocompatibility challenges, they have better mechanical properties and need a high temperature to be 3D printed. Despite being biocompatible and appropriate for producing bioink, water-soluble natural polymer materials have inadequate mechanical properties and stability (Benwood C. et al., 2021).

This study hypothesized that skin from chicken or pig would make an excellent material for creating skin-specific ECM, which could then be mixed with polymers to create bioink for 3D printing. Through in vitro, in ovo, and in vivo methods, this study showed the practical use of 3D printed skin. We found that the bioink preserved the intrinsic ECM components, suggesting that a 3D-printed skin patch could serve as a potent skin substitute in the future.

3.2 Materials and methodology

3.2.1. Fabrication and characterization of bioink

3.2.1.1. Preparation of the bioink

Preparation of the skin bioink was conducted according to a patented protocol (Patent Grant Number: 550247). Shortly, the chicken skin is collected from a nearby slaughterhouse. Chicken skin is cleaned in deionized water containing antibiotics and antifungal agents. Feathers, or hair, are removed from the skin. An additional wash of antibiotics and antifungal agents is given for 4 h on a shaker. Skin is minced into fine pieces and then transferred for digestion. Digestion was carried out using 1 N NaOH (2 g skin/ml) at 60 ˚C for 48 h as shown in fig. 3.1. After complete digestion, the slurry is filtered using a sieve to separate any unwanted remnants. The pH of the slurry is initially checked and is adjusted to 7.4. Following this, 10% PVA is mixed into the slurry and dissolved at 60°C over a period of 72 hours, then 1%gelatin is added and allowed to dissolve at 60°C for 48 hours. Skin bioink is stored in the temperature range of 4 to 8 ˚C (Fig. 3.2.1.1.).

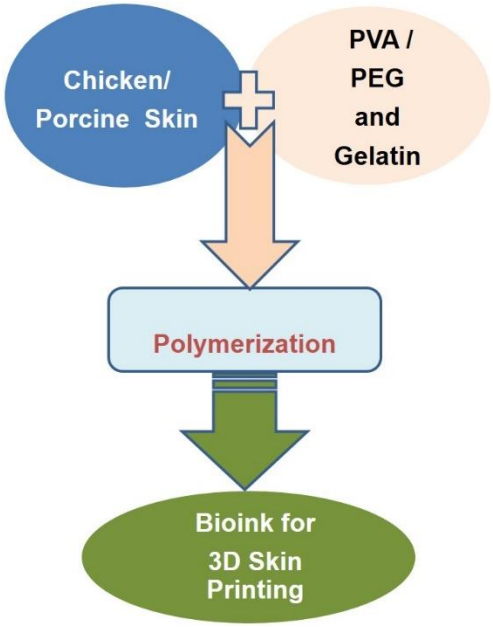


Figure 3.1: Preparation of Bioink of skin

3.2.1.2. Protein estimation in bioink

The Lowry assay was used for protein estimation in bioink. It is based on a reaction between protein and the folin-Ciocalteu reagent, which produces a blue color in the presence of protein. Protein estimation of digested tissue slurry and bioink was estimated by using the standard curve of bovine serum albumin (BSA). The standard concentration of BSA was 200 mg/ml.

3.2.1.3. Fourier Transform Infrared Spectroscopy (FTIR)

For the identification of functional components in the bioink, Fourier transform Infrared Spectroscopy (FTIR) was used. The polymeric synthetic structures were evaluated using FTIR spectroscopy (Tensor II spectral region 450–4000 cm− 1), which also facilitated the investigation of bioink esterification (Xenikakis I. et al., 2021). Bioink, PVA, and gelatin samples were freshly prepared for FTIR analysis and deposited onto the crystal cell of the FTIR spectrophotometer.

3.2.1.4. Rheological analysis

The RST-CPS rheometer (7030107), designed with an active cone/plate system, was employed to investigate the rheological properties of the bioink (Possl A.et al., 2021). Prior to measurement, freshly prepared bioink was dispensed in small quantities onto the plate. The rheometer's rotational motor delivers high dynamic precision without the use of gearing or mechanical force transducers. The speed of the block or plate was set from 0.3300 to 33.3300 min− 1 for surface characterization. Shear stress (Pa) of 0.0000–4.3432 was provided by gradually increasing the torque (mNm) from 0.0000 to 0.4797 and the shear rate (1 s − 1)

0.9900–99.9900. Viscosity was measured to get a profile of viscosity vs. time by heating the plate from 1 ◦C to 11 ◦C at a rate of 1 ◦C min− 1 rise in temperature (Jang K.S., et al., 2021).

3.2.2. 3D printing of the skin using bioink

A TRIVIMA 3D bioprinter (Fig. 3.2 B) was used to manufacture 3D printed skin. A computer-aided Design (CAD) file of rectangular shape was created, and a further CAD file was stereolithographically (STL) converted, and G-code was created. Sliced CAD model G-codes was prepared on the computer using 3D printing CURA software. The nozzle attached to the bio-printer had an inner diameter of 0.5 mm to 1 mm. The bioink was loaded and attached to the 3D bioprinter nozzle. Parameters were set in the bioprinter for the desired 3D printed skin thickness. The ordered program was run to print with a speed of 2–4.5 mm/ s at 25 ◦C in a sterilized environment. The insulated printing plunger drives the syringe piston at a steady flow rate of 2–4 mm/s, depositing 2–4 layers of 250 µ average height and a distance of 200 µ of melted bioink that was extrude through nozzle (Fig. 3.2.A).

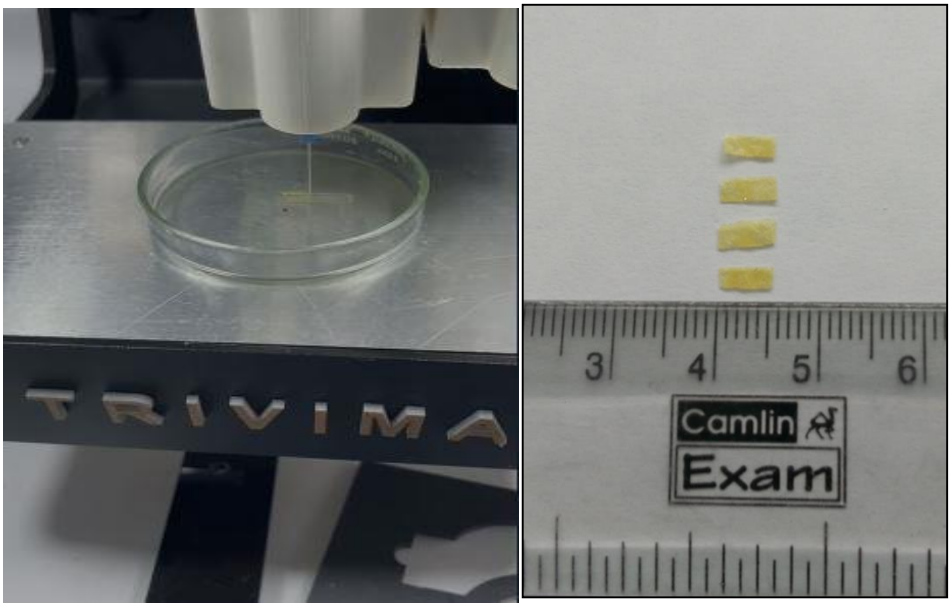


Figure 3.2: 3D Printer and the 3D Skin

3.2.2.1. Sterilization of 3D skin

The 3D skin is securely placed in self-sealing sterilization pouches before undergoing ethylene oxide sterilization at 321 g/m³, with environmental conditions set at 65% relative humidity and 50°C for 8 hours(RUGIKON Semi-Automatic KANC-125, Mumbai, India). The sterile 3D printed skin is stored in cool and dry conditions.

Figure 3.3: Sterilization of 3D printed skin

3.2.2.2. Mechanical analysis of 3D printed skin

For mechanical characterization of the samples, a uniaxial elongation test is performed on six samples using an Instron 544 and a 10-N load cell. The 3D printed skin is cut into rectangular shapes (5 mm × 20 mm). The elastic modulus (EM) is measured using Young's modulus based on the linear portion of the stress-strain curve. The ultimate tensile strength (UTS) is calculated using the maximum strength obtained from the stress-strain curve. 3D printed skin mimics the structure of the skin, recapitulating the ECM layers in the polymers, which provide mechanical strength.

3.2.2.3. DNA content analysis of artificial 3D skin

DNA was extracted from rabbit skin and a 3D printed skin scaffold. The extracted DNA samples were electrophoresed along with a DNA ladder to check the DNA content of both rabbit skin and 3D printed skin before transplantation studies.

3.2.2.4. Contact angle

Contact angle measurements were carried out by a contact angle goniometer. The needle of the microsyringe was placed in close proximity to the 3D-printed skin, with its tip immersed in the water droplet. The droplet was illuminated from behind, and a camera (Goniometer bench with F2 series digital camera) recorded the images. The obtained image was analyzed for surface wettability using software for contact angle measurement (Zoumaki M. et al., 2022).

3.2.2.5. Swelling analysis

The swelling analysis of the 3D printed skin was examined by examining the ability of the material to absorb the liquid with respect to time to reach saturation. For the swelling ability of 3D printed skin (n = 3), 0.035 g with 1 cm in length was immersed in 30 ml DW and incubated

at 37 ◦C for a period of five days. After soaking for 24 hours, the sample was removed from DW, its surface water was absorbed using tissue paper, and it was weighed repeatedly until equilibrium was reached. The percent swelling was then calculated using the designated formula (Zidari c M., et al.,2020).

3.2.2.6. Thermo-gravimetric analysis of 3D printed skin

The thermal degradation of 3D printed skin was assessed using a TGA instrument (SDT Q600 V20.9 Build 20) (Montero FE., et al., 2019). A dynamic mode skin analysis was conducted using TGA under a nitrogen atmosphere, employing a heating rate of 10°C min− 1 up to 800°C. The weight fluctuations with increasing temperature were plotted.

3.2.3. In vitro biocompatibility and biodegradability of 3D printed skin

3.2.3.1. Biodegradation test of 3D printed skin and SEM analysis

The 3.9 g (wt) of printed skin was incubated in PBS (pH 7.4) at 37°C for 28 days under 100 rpm shaking conditions to study its in vitro biodegradability. The buffer was changed daily. At intervals of 7, 14, 21, and 28 days, the 3D printed skin was retrieved, rinsed with deionized water, dried at 37°C for 12 hours until completely dry, and weighed. The biodegradability percentage was determined using the given formula (Horakova J., et al.,2018).

Weight loss% = Wi − Wf / Wi × 100 ………………………………………………….. (1)

where,

Wi: sample weight before biodegradation.

Wf: sample weight after biodegradation.

SEM analysis of the 3D printed skin was performed before and after in vitro biodegradation, with topographical images captured at a scale of 10 µm.

3.2.3.2. MTT assay

Small sterile scaffold sections each approximately 0.16 cm² (0.4 cm × 0.4 cm), were placed in the wells of a 96-well plate to evaluate cell proliferation on the 3D skin (n = 3). The scaffold was seeded with the isolated cord blood MNCs (1.3×10^4). The plate was placed in a CO_2 incubator at 37°C for 24 hours to support cell proliferation. After this period, MTT was introduced into each well, reaching a final concentration of 0.5 mg/mL in the culture medium. After addition of MTT, the plate was incubated for 5 h. The solution, along with the scaffold, was gently aspirated. 150 µl DMSO was added to dissolve formazan crystals and the absorbance was measured at 570 nm after half an hour. Cells seeded in wells without scaffolds served as control.

3.2.3.3. In vitro scratch assay

The in vitro wound healing capability of the 3D printed skin was accessed using a fibroblast cell line (L929) purchased from NCCS, Pune, India. In brief, 6-well plates with 2×10^5 cells/ml were seeded and cultivated until they reached full confluency. A scratch was made on the monolayer culture using a sterile 1000 µl tip following a wash with Delbucco's Phosphate Buffered Saline (DPBS). The wash of DPBS removes the detached cells and other cellular debris. Before starting the experiment, a sterilized extract of scaffold was obtained by placing scaffold in complete media for 48 h. The treatment group received scaffold extract media, while the control group received only complete media. Images captured by an inverted microscope fitted with a digital camera showed cell movement and morphological alterations. Three wells (n = 3) in each group were maintained for the experiments. The distance between the wound and scratch closure at different time intervals (0, 24, 48, and 72 h) was analyzed by Image J software.

3.2.3.4. Migration quantification of the wound scratch assay

One of the commonly employed approaches for quantification, known as the area method, indirectly evaluates migration. In this method, the experiment involves monitoring the percentage of wound area, denoted as (t), over time. The percentage of wound area can be calculated by using the given formula (Bobadilla A., et al., 2019).

$A_{(t)} = A_{(0)} \times 100\%$.....(2)

Where,

$A_{(t)}$ represents the wound area at time t, and $A_{(0)}$ represents its initial area.

By comparing the percentage of wound area at a specific time point, the migration rate can be assessed.

3.2.3.5 3D printed skin biocompatibility by CAM assay

Fertilized eggs (Gallus gallus) were procured from the local Government Hatchery in Kolhapur. Prior to incubation, the eggs were cleaned with 70% alcohol, and the egg incubator was sterilized using the same solution. The eggs were then incubated at 37°C under an 80% humidified atmosphere, with their air sacs positioned downward to ensure optimal heat distribution. The bowl of water was placed in the incubator to maintain 80 % humidity and monitoring it with a hygrometer. The two groups of fertilized eggs were maintained; the control group and the experimental group. The eggs were removed post 72-hour incubation and sterilized appropriately. Once the embryo was located, eggs were punctured with a 2.5 ml syringe needle no. 24 (Dispovan, Hindustan syringes, India) in the lower end to remove the albumin (2–3 ml). Then the eggs were transferred to incubator for 4 days. At day 4, a small

window was created in sterile condition and a sterile piece of scaffold (5 mm × 5 mm) was kept on the chorioallantoic membrane (CAM) area. A control was maintained, and photographs were taken for both groups. The window was secured and incubated. On days 6, 8, and 10, the transparent film covering the window was removed, and the CAM and scaffold were collected for histological examination. Photographic documentation of the CAM was performed for both the control and experimental groups at each time point.

Histological analysis of CAM area and an explant:

The scaffold and adjacent CAM tissue were harvested and fixed in 10% neutral buffered formalin for subsequent histological study. These samples were then isolated and processed for dehydration through gradients of alcohol, xylene and finally embedded in paraffin wax. The thin section of about 3 μm was taken using a microtome (YORCO sales Pvt. Ltd., India). These sections were then stained with HE for nuclear organization and cytoplasm according the standard procedure and alcian blue pH 2.5 stain. Bright-field images at 20× magnification were obtained using a Nikon Ts Eclipse microscope integrated with a Nikon camera.

Scanning electron microscope (SEM) analysis of a 3D printed skin explant:

SEM (JEOL JSM. 6360) was performed for the morphological analysis of the CAM samples. 3D printed skin graft implanted on the CAM area for 6, 8, 10, and 12 days was analyzed by SEM. The geometry of skin connective tissue is best displayed by the scanning electron microscope. 3D printed skin graft implanted on the CAM area for 6, 8, 10 and 12 days was collected and dehydrated in the incubator for 24 h. The height of the sample stage 25 need to be adjusted with sample preparation tool before placement of the vessel specimen and stage into the SEM. Proper height adjustment was important to make sure the stage and sample cleared the ceiling of the sample feed and was high enough for the SEM to bring an image of the vessel surface into focus. SEM was performed on cross sections of scaffolds to investigate morphology and porosity.

3.2.4. In vivo wound assessment study of 3D printed skin in rabbits

All animal experiments were performed as per guidelines of the Institutional Ethical Committee (IAEC) of National Research Centre on Equines, Hisar, Haryana, India (Reg.No.-193/GO/Re/SL/99/CPCSEA). New Zealand rabbits (Oryctolagus cuniculus) of either sex were used for studying wound healing application of 3D printed skin in vivo. The dorsal skin of the shoulder area of each rabbit was shaved and prepared aseptically using 70 % ethanol. Under lignocaine local anaesthesia approximately 8 mm thickness skin was excised by using punch biopsy. Three groups with four rabbit wounds each were used in wound healing experiments

for 3D printed skin as follows: Positive control group without any treatment, Standard topical ointment lorexaneT act as negative control group, and3D printed skin. The skin was applied once. Wounds were observed for a period of 18 days and healing was assessed based on parameters like rejection of skin, colour, exudates and granulation tissue. Additionally, two rabbits as a control were observed without any wound. Photography was followed until day 15 on the wound sites of animals. The degree of healing was evaluated by histopathology test on skin biopsy specimens obtained from the anesthetized rabbits on specific time period.

3.2.4.1. Histological and immunohistochemistry of 3D printed skin:

Skin biopsy samples were immediately stored in 10 % formalin solution for fixation for study of histochemistry and immunohistochemistry. The samples were fixed and processes through a series of dehydration with alcohol grades, enlightened in xylene, and finally embedded in paraffin wax. The thin sections of about 3–5 μm were taken using a microtome (YORCO sales Pvt. Ltd., India). These sections were treated with HE stain, Masson's trichrome (MT) stain and alcian blue pH 2.5 stain using standard protocol for imaging nuclear content and cytoplasm organization. Images were acquired under bright-field illumination at 40× magnification using a Nikon Ts Eclipse microscope with a Nikon camera.

For immunofluorescence analysis, 3 μm thickness tissue sections were obtained and sections were processed with xylene, followed by alcohol grading for rehydration. Heat induced epitope retrieval technique with Tris citrate buffers (pH 6) was used. Goat and rabbit serum samples were used for blocking. Then the samples were incubated with primary antibodies for overnight at 4 ◦C followed by washing and then incubated with fluorescence secondary antibodies for an hour. The primary antibodies for epithelial cell biology, cell adhesion markers E-cadherin(G-10) (CAT #sc-8426, 1/100 dilution, Santa Cruz Biotechnology), Vascular cell adhesion molecule-1 (VCAM-1 (E-10) CAT#sc-13,160, 1/200 dilution, Santa Cruz biotechnology), α-smooth muscle actin (α-SMA, muscle actin alexafluor™ 488, CAT#53-9760-82, 1/1000 dilution, Invitrogen), cytokeratin 18 (DA-7) (CAT#sc-51583, 1/500 dilution, Santa Cruz Biotechnology) were used. The sections were then incubated with the secondary antibodies for 1 h at room temperature. Fluorescent staining was counterstained with DAPI (Molecular Probes, USA) and mounted in an aqueous mounting medium (DACO, USA). Images were obtained on a Nikon Ti inverted microscope (Nikon Eclipse Ti, Japan) with Nikon Nis Elements software, ver. 5.2 (Nikon, Japan) (Bobadilla A., et al., 2019).

3.2.4.2. Hydroxyproline and collagen estimation:

The hydroxyproline and collagen estimation was performed for determination of wound healing. The concentration of hydroxyproline is a measure of the concentration of collagen.

Higher the concentration of hydroxyproline indicates faster rate of healing wound. Biochemical analysis showed increased hydroxyproline content, which is a reflection of increased cellular proliferation and there by increased collagen synthesis. It was tested on three different groups. The regenerated skin of control rabbits, 3D treated rabbits and control skin (naive skin) samples were hydrolyzed and assessed for hydroxyproline content by colorimetric assay using hydroxyproline kit (Sigma Aldrich). Hydroxyproline standards (0, 0.2, 0.4, 0.6, 0.8 and 1.0 mg/ml) were added into a 96-well plate. Quantity of 10 mg of each skin sample was homogenized and then hydrolyzed in a pressure-tight polypropylene vial with PTFE-lined cap in concentrated HCl for 3 h at 120 ◦C. After samples had cooled to room temperature, they were centrifuged for 2 min at 13,000 g and samples were dried in vacuum or in an oven at 60 ◦C. After adding the chloramine T/oxidation buffer combination for 5 min at room temperature, it was mixed with diluted 4-(dimethylamino) benzaldehyde reagent for 90 min at 60 ◦C. The absorbance was measured in a microplate reader (Power at 560 nm and the quantity of hydroxyproline was determined against a standard curve concentration. Collagen content was calculated from the above data and converted into percentage.

3.2.4.3. Gene expression analysis of MMP-9, IL-6, COMP, TNF-α by qPCR:

Total RNA was extracted from 25 mg of each regenerated skin tissue (artificial skin-treated rabbit wound and untreated control skin, as well as normal rabbit skin) using the RNeasy kit (Qiagen GmBH, Hilden, Germany), as per the manufacturer's instructions. RNA samples were further treated with DNase I (Fermentas, Lithuania) onto the column in order to remove genomic DNA. Total RNA in 1 µg quantity was used for first strand synthesis using oligo dT with MMLV reverse transcriptase enzyme as per manufacturer's instructions (Biorad). We further investigated mRNA gene expression of MMP-9 (matrix metalloproteinase-9), COMP-1 (Cartilage oligometric matrix protein-1) and proinflammatory cytokines (IL-6 and TNF-α) by Quantitative PCR (qPCR). qPCR was carried out with a 10 µl SYBR Green Supermix (qPCRMastermix) and 10 nm gene-specific forward and reverse primers of respective genes on Thermal cycler QuantStudio™ 3 '(Thermo Fisher Scientific, Massachusetts, USA') with conditions of one cycle of initial denaturation step of 5 min at 95 ◦C; 40 cycles of 15 s denaturation at 95 ◦C, 60 s annealing at 60 ◦C. The Threshold cycles (Ct) were determined and relative fold change in these genes were calculated as per the $2^{\Delta\Delta Ct}$ method with respect to normal skin and housekeeping gene GapDH (glyceraldehyde 3-phospahte dehydrogenase).

3.2.4.4. Statistical analysis:

The statistical analysis was conducted using the Tukey's multiple comparisons test for comparisons involving two groups. A p-value < 0.0001 were considered statistically significant.

The mean values are reported with their corresponding standard deviations (SD). The GraphPad Prism software was utilized for performing the statistical analysis.

3.3 Results

3.3.1. Fabrication and characterization of bioink

3.3.1.1. Protein estimation

The protein content of chicken or porcine skin, digested slurry, and bioink was calculated using the conventional BSA curve. The concentration of total protein per mg of the sample was found to be 185.33 ± 0.22 mg in chicken skin, 166.66 ± 0.83 mg in digested slurry, and 183.33 ± 0.69 mg in bioink. The results indicated that the protein content dropped by 10.07 % during the digestion process, and the final bioink formulation had a protein concentration that was nearly same as the native chicken or porcine skin.

3.3.1.2. Fourier Transform Infrared Spectroscopy (FTIR) study

The FTIR spectra of PVA showed a broad peak around 3275–3319 cm− 1 signifying stretching of hydroxyl groups. Chicken and or porcine skin matrix, amide band represents N–H stretching vibration and shifted to 1406–1640 cm− 1 as can be seen in the Fig. 1E. FTIR spectra of skin bioink, it is clear that the whole hydroxyl group of the polymers and amide groups play an important role in water uptake because of their hydrophilic nature. The physical bonding pattern throughout the bioink polymerization is shown in the Fig. 3.4. This confirms the polymerization was achieved perfectly.

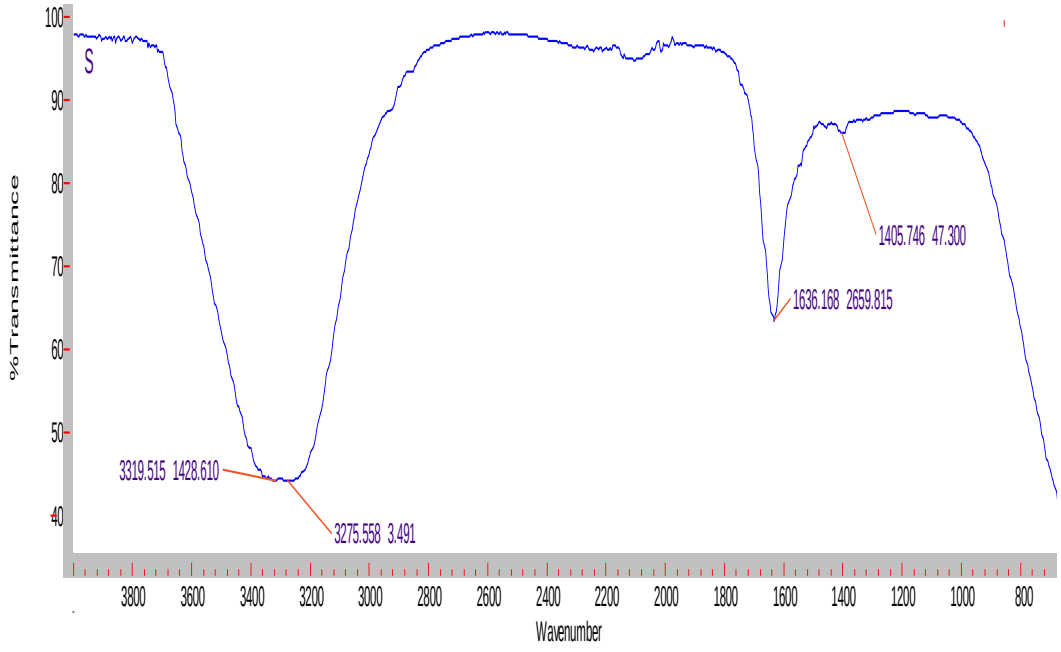


Figure 3.4: Fourier-transform infrared spectroscopy (FTIR) of the bioink

3.3.1.3 Rheological study

The results from the simulation are compared to photos of experimentally printed grid structures. The data indicates that the viscosity diverges at a finite value of the complex modulus or equivalently, the applied shear stress for the bioink with loading in excess of 0.05 wt%. On the basis of this data, it is clear that the onset for solid-like behavior occurs at the desire concentrations for the pristine bioink. The bioink prepared with the functionalized polymer exhibit by far the least change in the viscoelastic response at 0.05 wt% SWNT.

3.3.1.4 Viscosity study

Average viscosity of skin bioink is found to be 0.14 ± 0.01 and this significantly provided thick, sticky, and semi fluid consistency due to chicken and or porcine skin matrix and polymers intra-molecular bonding interaction (Fig. 3.5.).

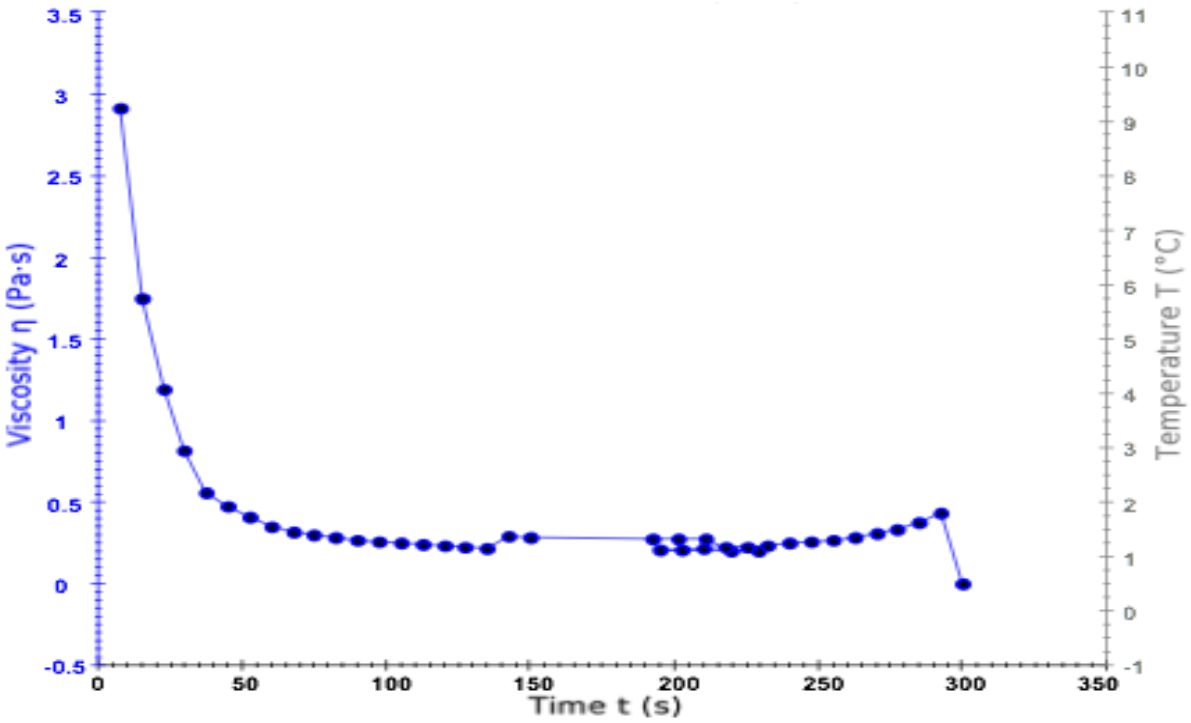


Figure 3.5: Viscosity of the bioink

3.3.1.5 3D printing of skin

The bioink was loaded into a 20 mL sterile syringe and connected to the nozzle of the 3D bioprinter, and a two-layer square of dimensions 20 × 10 cm was 3D-printed. The parameters were set in the bioprinter for the desired 3D printed skin thickness of 0.7 mm. Our 3D printing method has been improved to better replicate natural skin. The injected layer was left to form cross-liners under UV rays for 10 min. The shape was found to be well maintained, and no clumps or clogs were observed during the printing process.

3.3.1.6 Mechanical properties of 3D printed skin

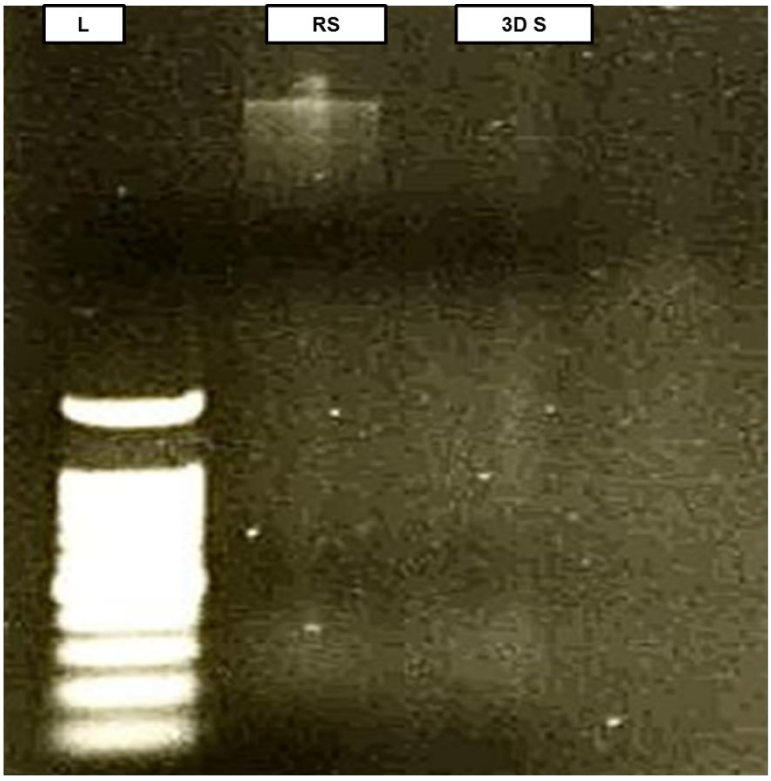
The compressive and mechanical properties of 3 printed skin were presented in Table 1. The ultimate tensile load was found to be 5.00 ± 2 N, ultimate tensile strength was 0.30 ± 1 N/mm^2 and, elongation of breaking load was 35.50 ± 2 % (Table 3.1).

Table 3.1: Mechanical properties of 3D printed skin

Output Parameter	3D printed skin
Ultimate tensile load (N)	5.00±2
Ultimate tensile strength (N/mm^2)	0.30±1
Elongation at breaking point (%)	35.50±2

3.3.1.7. DNA content analysis of artificial 3D skin

After electrophoresis (Fig. 3.6.) of the extracted DNA from rabbit skin and artificial skin, appeared bands confirmed the DNA content in rabbit skin whereas no appearance of bands proves the absence of nuclear content in the artificial 3D printed skin when compared with DNA ladder.



L: ladder, RS: Rabbit skin, 3D S: 3D skin

Figure 3.6: Gel electrophoresis of the DNA samples isolated from normal rabbit skin (RS) and 3D printed skin (AS) run along with ladder DNA (L).

3.3.1.8 Contact angle of 3D printed skin

The water droplet is nearly fully absorbed by the 3D printed skin. The angle of 22◦ shows hydrophilicity of the skin. 3D printed skin characterized as a super hydrophilic material (0–40◦ angle) (Fig. 3.7.) The hydrophilic component of 3D printed skin can readily absorb at the wound surface.

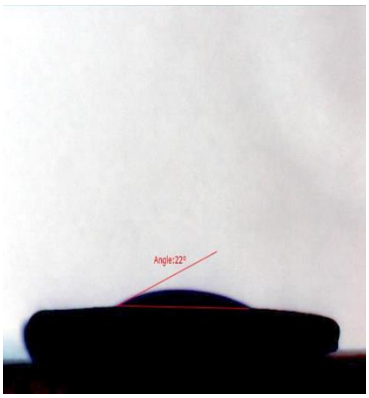


Figure 3.7: Contact angle of the 3D printed skin

3.3.1.9. Swelling analysis of 3D printed skin

The water retention capacity of the hydrogel membrane prepared is ~133.33 % of the dried weight, so it can be categorized as super absorbent/ super hydrophilic material (Fig. 3.8.).

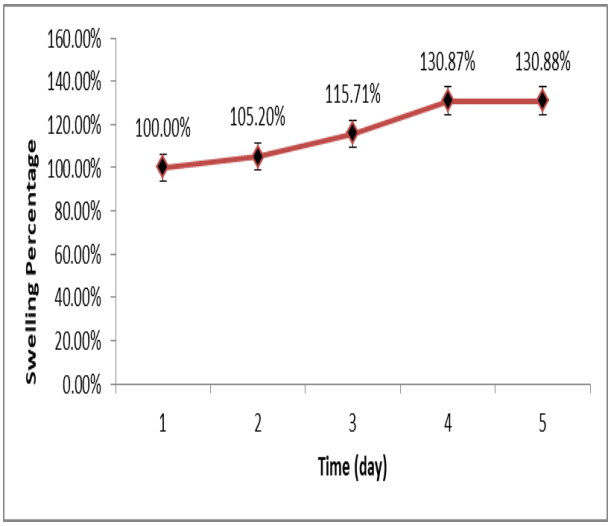


Figure 3.8: Swelling analysis of the 3D printed skin

3.3.1.10. Thermo gravimetric analysis (TGA) study of 3D printed skin

Thermal stability of the 3D printed skin is studied in this test. TGA test was carried out with Linseis (L81A1750-USA) at a heating rate of 8 ◦C min− 1 in nitrogen atmosphere from 20 ◦C to 800 ◦C. Skin starts to melt above 150 ◦C, at 400 ◦C almost 50 % melted and start to degrade at 800 ◦C. The final weight of 0.32 mg residue obtained as ash (Fig. 3.9.).

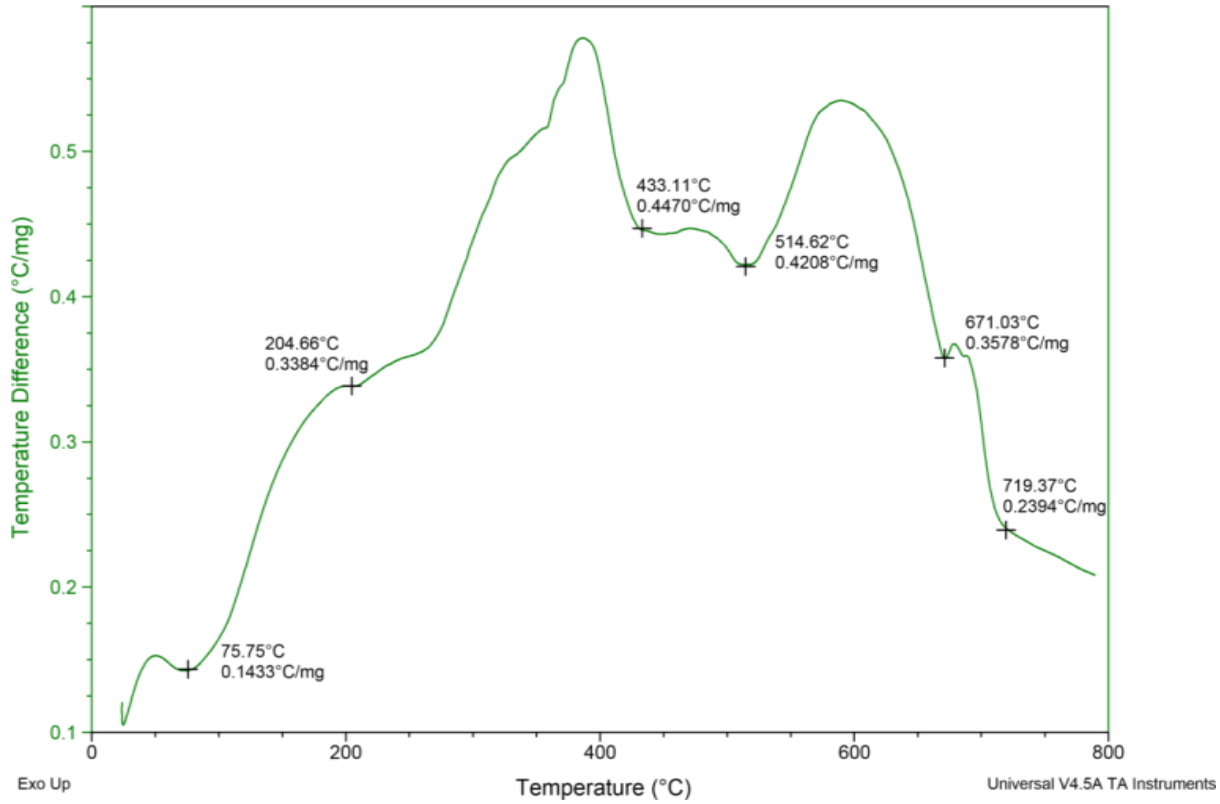


Figure 3.9: Thermogravimetric analysis (TGA) of the 3D printed skin

3.3.2. In vitro biocompatibility and biodegradability of 3D printed skin

3.3.2.1 SEM imaging to study degradation of graft

The SEM image (Fig. 3.10.) illustrated the degradation profile of the 3D printed skin incubated in phosphate buffer saline. The composite scaffold was incubated in PBS removes only a small amount of the polymers via surface degradation, leaving the fibre component completely embedded within the structure. These collagen fibres remained intact throughout the biodegradation process. This specialty could be useful in wound healing process. Ground substance showing the organization of ECM components and degradation mass ratio was 36.71%.

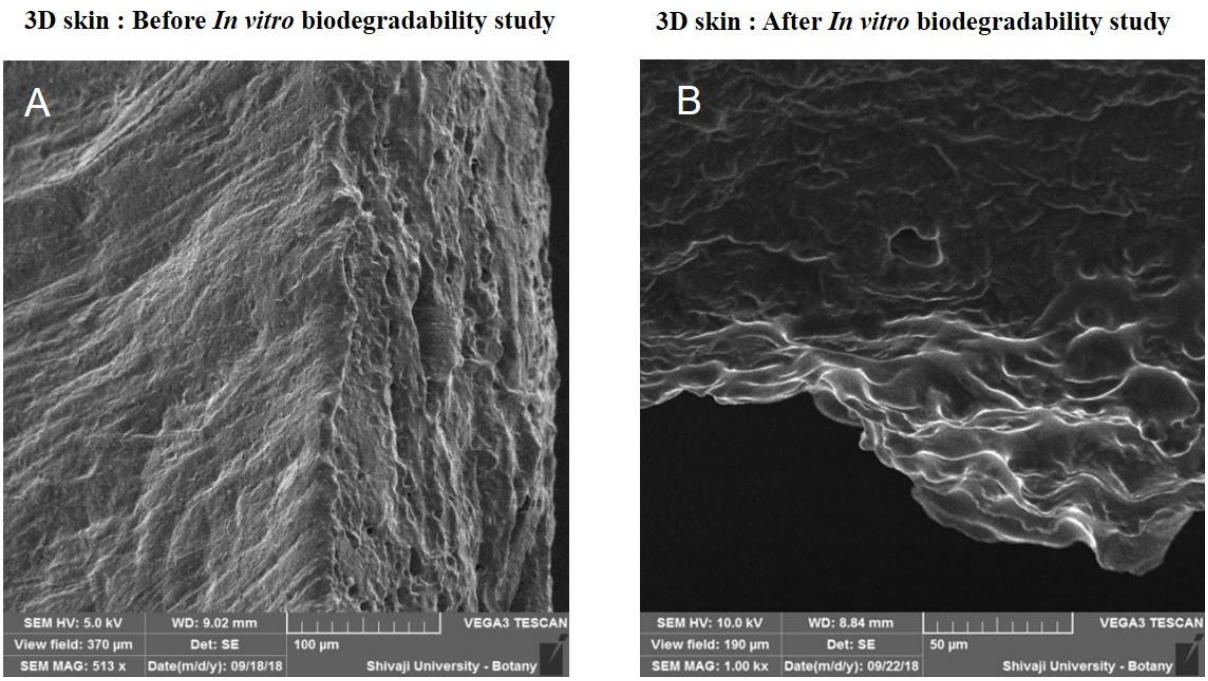


Figure 3.10: *In vitro* biodegradability testing of 3D printed skin in PBS; A: Before and B: after images by Scanning Electron Microscopy (SEM).

3.3.2.2 MTT analysis

The MTT assay was conducted to assess the cytotoxicity of the skin, revealing a higher absorbance value in comparison to the control. . As absorbance indicates the metabolic activity of live cells, it can be co-related to the number of living cells. Thus, indicating that the 3D skin helps in increasing the rate of proliferation of cord blood MNCs (Fig. 3.11.).

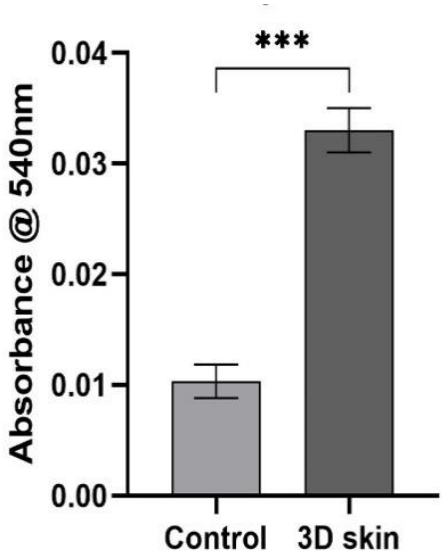


Figure 3.11: Biocompatibility study by MTT assay

3.3.2.3. In vitro scratch assay and migration quantification:

To evaluate the efficacy of 3D printed skin to heal wounds in vitro, a scratch experiment was carried out. The data showed that, compared to the control group, the scaffold had a positive impact on accelerating the healing process. In this investigation, the wound area percentage was calculated at various time intervals, such as 24 h, 48 h, and 72 h (Fig. 3.12).

Initial evaluation of a wound is 100 % at 0 h. The wound area in the control group is 91.30 ± 0.75 % after 24 h, however, the wound area in the treatment group is 67.14 ± 1.00 %. By 48 h, the control group has 63.68 ± 0.70 % of the original wound area left, whereas the treatment group has just 33.04 ± 1.44 %. The treatment group's wound area is reduced from 31.00 ± 0.71 % to 11.14 ± 0.74 %, which indicates that the wound has completely healed. This demonstrated that the scaffold aided cell migration, speeding up the healing of the wound ($p < 0.001$). Statistical analysis demonstrated a substantial ($p < 0.001$) decrease in the wound area percentage in the treatment group compared to the control group.

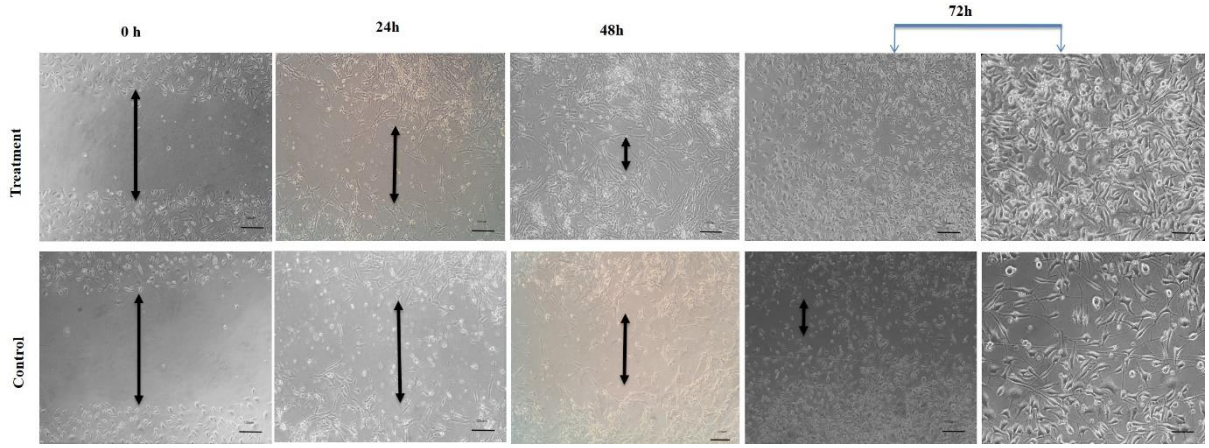


Figure 3.12: In vitro scratch assay 3D printed skin biocompatibility by CAM assay

Biocompatibility with living systems is a basic characteristic a bioink should exhibit. The biocompatibility study of 3D printed skin revealed that graft doesnot show any toxic effect and normal angiogenesis pattern is observed. Further, this graft was studied for the cellular recruitment and presence of ECM.

3.3.3.1. Histology of the CAM model:

3D printed skin graft implanted on the CAM area for 6, 8, and 10, days reveales the biocompatibility (Fig. 3.13A, A to F). It clearly suggests the grafted 3D printed skin had no toxic effect and normal angiogenesis pattern is seen with compared to control. The HE staining of CAM area reveals the uniform nuclear and cytoplasm structure with no observed necrosis. The H and E staining of grafted skin tissue harvested from CAM area showes normal recruitment of cells. On day 10, appearance of cells is found to be increased on the 3D printed graft (Fig. 3.13B, G, H, and I). Alcian blue stain for glycosaminoglycans (GAGs) (Fig. 3.13C,

J, K and L): 3D printed skin graft implanted on the CAM area for 6, 8, and 10, day revealed the biocompatibility and presence of GAGs. Alcian blue staining technique is widely used among development biologist to observe the viscose matrix of the extracellular glycoproteins usually have bound a large number of glycosaminoglycans (GAGs). Alcian blue stains both sulfated and carboxylated acid mucopolysaccharides and sulfated and carboxylated sialomucins (glycoproteins). Control 3D printed skin graft showed presence of GAGs which is an indicator of skin ECM in the polymer. CAM area of the egg showed normal expression of GAGs.

3.3.3.2. SEM analysis of 3D printed skin in CAM assay:

3D printed skin graft implanted on the CAM area for 6, 8, and 10 was analyzed by SEM (Fig. 3.13D — M, N, and O). The geometry of skin connective is tissue best displayed by the scanning electron microscope. The information can be obtained on the bulk organization of the collagen and elastic fibers are visualized simultaneously. Ultra structural studies show that a highly organized, striated, patterned, and networked structure. Porosity and pore-size are crucial to ensure cell colonization in the graft. Likewise, SEM micrographs showed a homogeneous distribution of equal sized pores within the entire area.

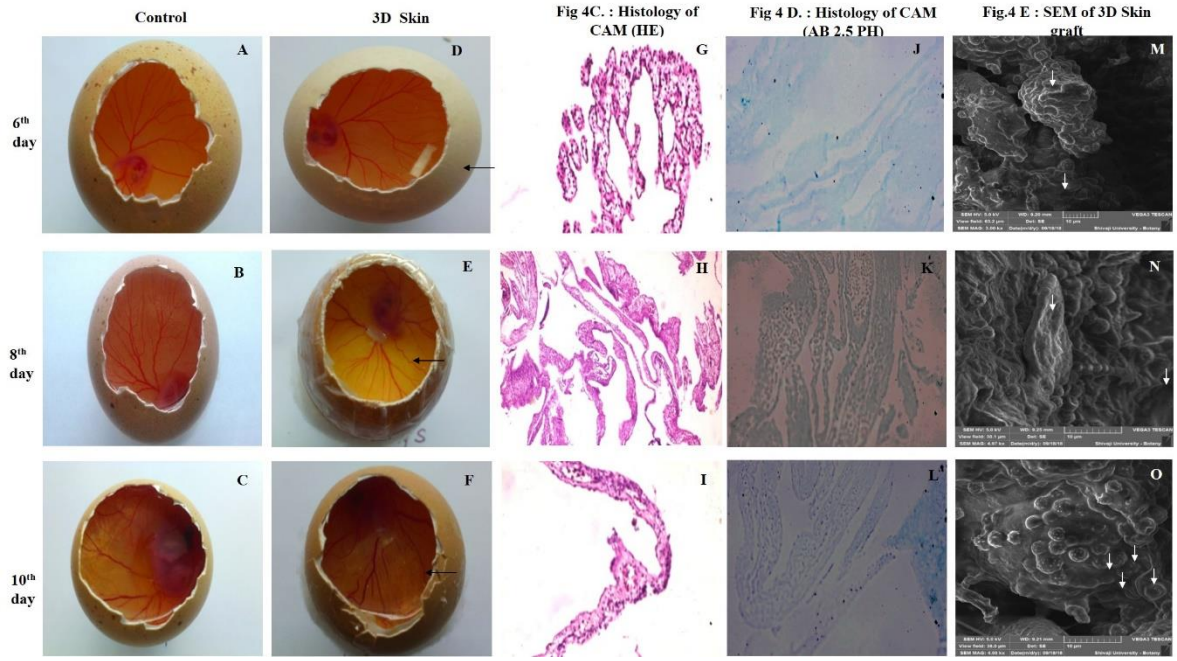


Figure 3.13: 3D Printed skin Biocompatibility by CAM Assay

3.3.3. The application of 3D printed skin for promoting wound healing

All rabbits accepted the 3D printed skin without exhibiting any xenogenic response. Analyzing the size of the wound was done by calculating its size against the number of days. In comparison to the control group of rabbit skin that had no treatment, it was observed that the healing rate in

3D printed skin group was higher. On day 2, the size of the wound in the 3D printed skin treatment was reduced to 7 mm, whereas that in the control measured at 10 mm. The size of the 3D printed skin-treated wound decreased to 5 mm on day 8. On the contrary, the control wound measured 8 mm. On day 15, the 3D printed skin-treated wound had fully recovered with minimal scarring, in contrast to the control wound, which had shrunk to 1 mm but had not fully recovered. The healing of the rabbit's wound was illustrated in Fig. 4 on days 0 (A), 8 (B), 12 (C), and 15 (D). Each group's wound reduction was evaluated as a percent reduction in the injured area. In the first week, 3D printed skin-treated skin showed a reduction of 30–40 % compared with a 20 % reduction in wound size in the control skin. In contrast to the control wound, which only showed a 90 % reduction on day 15, the artificial 3D-printed skin-treated wound took 15 days to completely heal (Fig. 3.14).

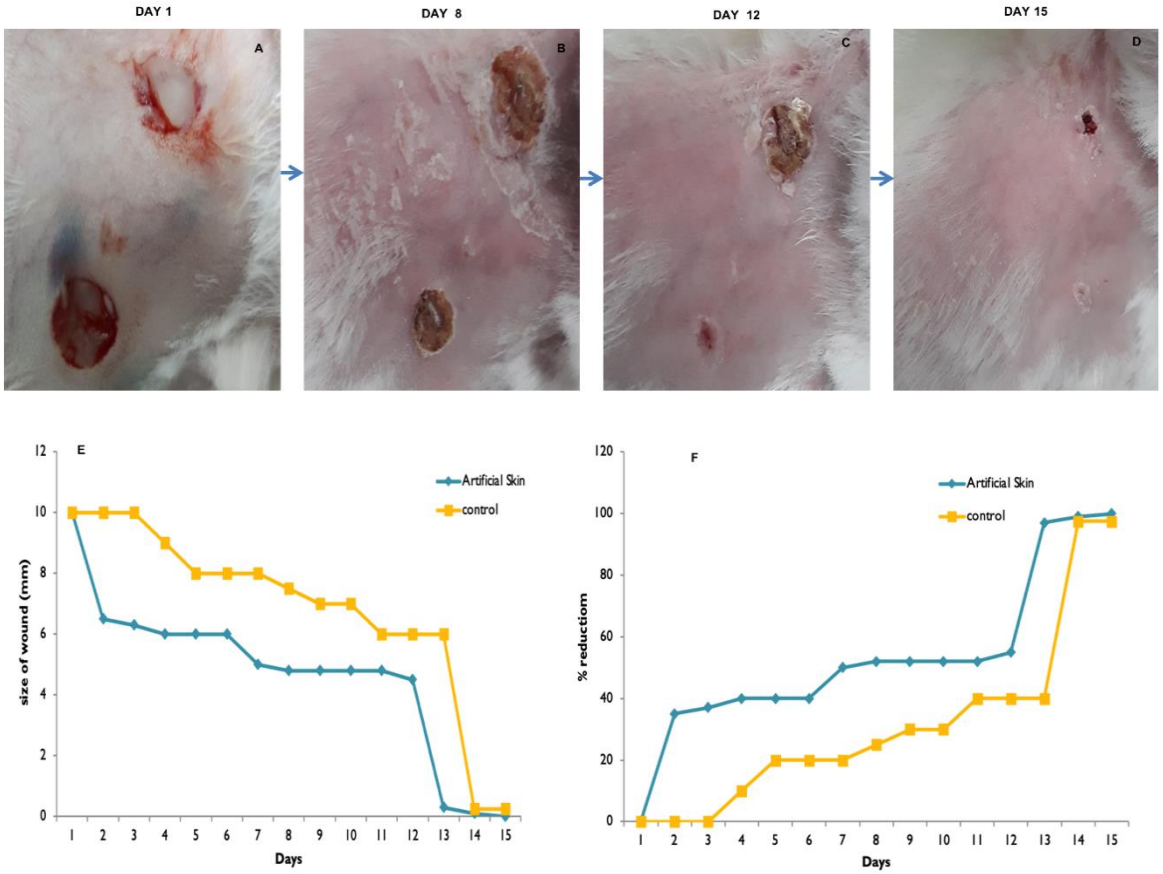


Figure 3.14: I*n vivo* wound healing assessment of 3D printed skin

3.3.4.1. Histology of animal models HE staining

Fig. 3.15 C — A to C showed the presence of nuclei in the transplanted skin tissue. This confirms that epithelialization and neovascularization were more prominent in transplanted skin samples than in controls. Blood vessel formation, hair follicle development, and recruitment of

endothelial cells are observed. Alcian blue staining Fig. 3.15C — D to F shows the presence of GAGs (glycosaminoglycans) in the transplanted 3D printed skin. It confirms epithelialization and neovascularization. The blue dots show the presence of GAGs in transplanted skin samples. Collagen detection is performed by Masson's trichrome staining. Collagen deposition is observed in pink. It is more prominent in the transplant skin than in the control (Fig. 3.15C — G to I).

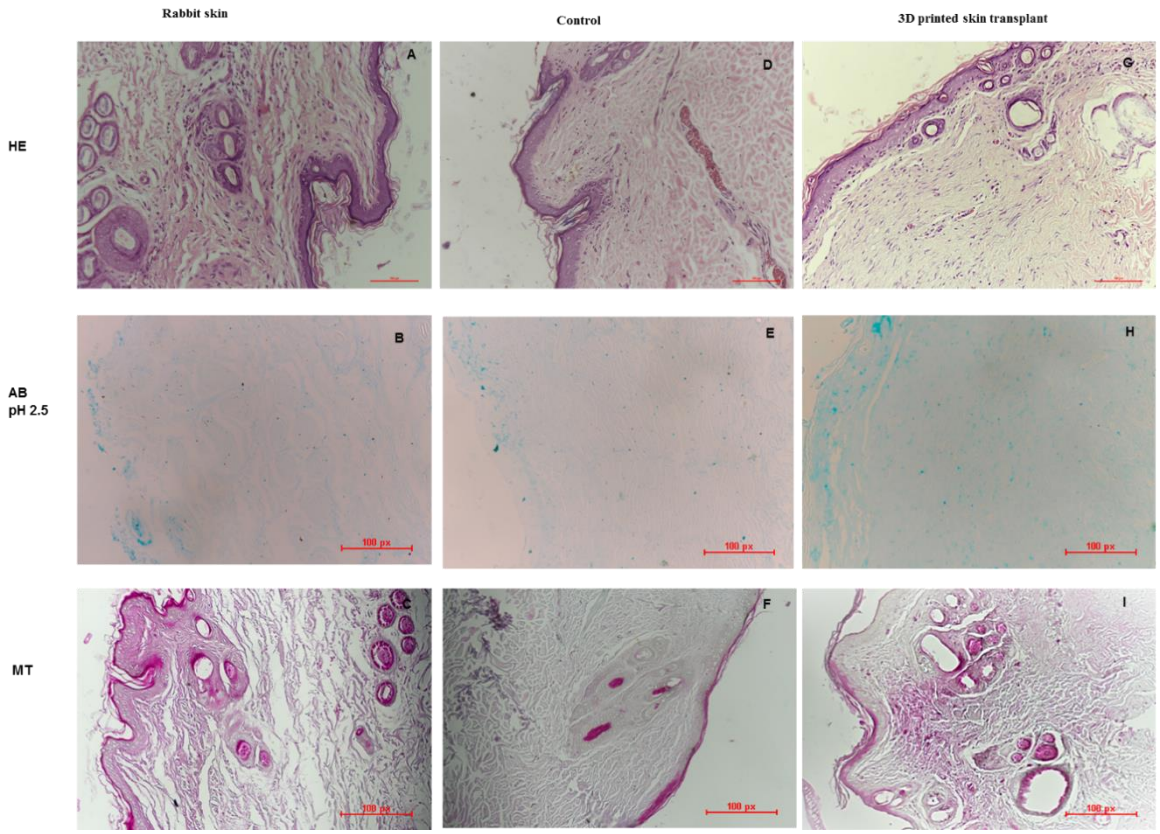


Figure 3.15: Histological wound healing analysis of the skin samples of animal models

3.3.4.2. IHC of the animal model samples

Immunohistochemistry was used to detect different biomarkers for the wound healing assessment such as α-smooth muscle actin (α-SMA), E-cadherin (E-CAD), Vascular cell adhesion molecule (V-CAM-1), and Cytokeratin 18 (CK-18) presented in Fig. 3.16D. Alexa 488 labelled secondary antibody was used for the detection of primary antibodies, which emit green fluorescence. α-SMA (smooth muscle actin) antibody was used for the detection of smooth muscle formation in transplanted 3D printed skin (Fig. 3.16D — I). The expression is more prominent in the transplanted 3D printed skin samples and considered to be an important component in tissue fibrogenesis. E-CAD expression confirms the formation of functional epithelial cells as it plays a critical part in epithelial cell adhesion (Fig. 3.16D — J). The expression of V-CAM-1 is more prominent compared with controls (Fig. 3.16D — K). It

confirms the deposition of epithelial cells, a result of normal wound healing. CK-18 expressed on epithelial cells and positive CK-18 confirms the presence of epithelial cells in transplanted 3D printed skin samples (Fig. 3.16D — L). The nucleus is counterstain with DAPI (D9542, Sigma), which emits blue fluorescence.

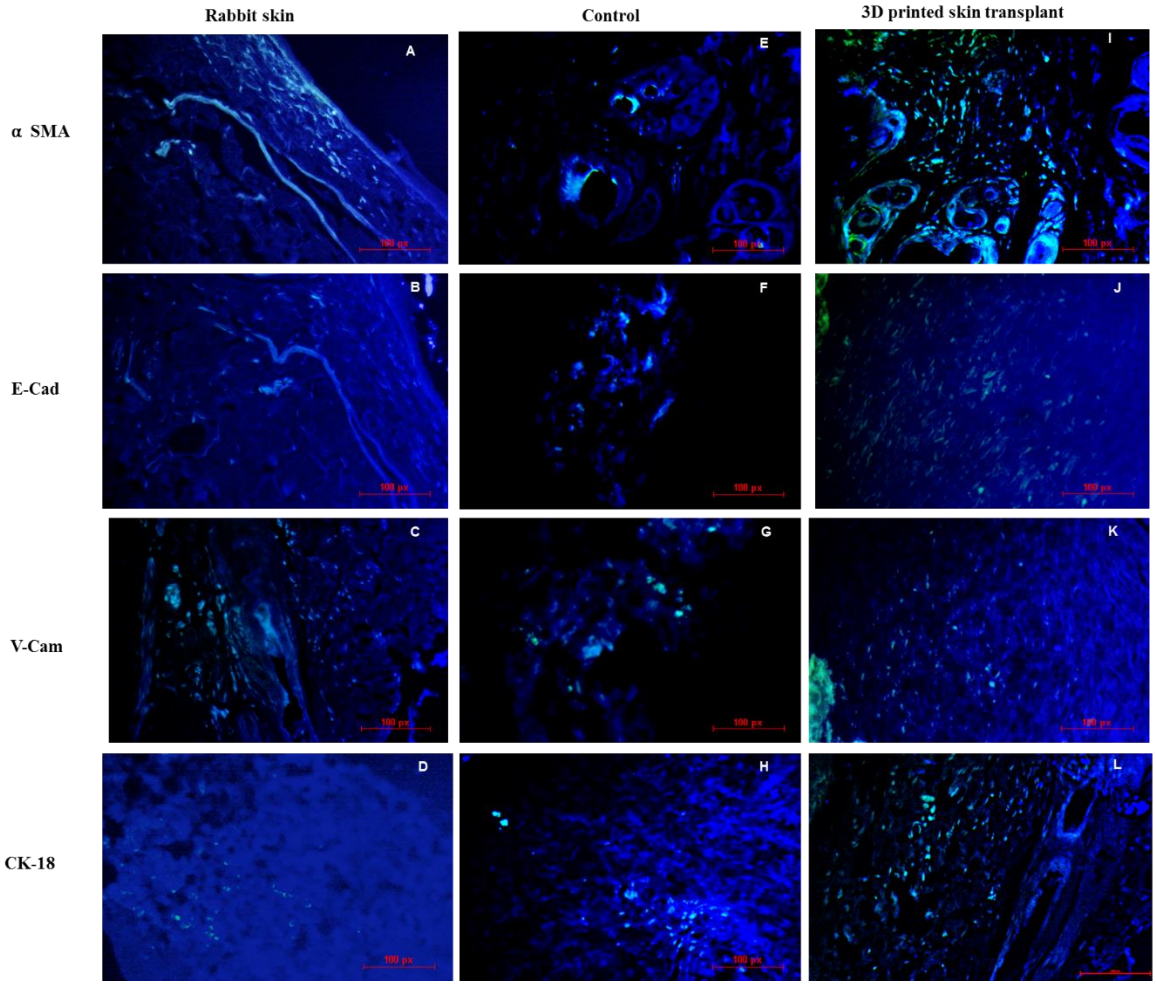


Figure 3.16: Immunofluorescence studies of skin samples of animal models

3.3.4.3. Hydroxyproline and collagen content estimation

The 3D printed skin-treated wound had the highest hydroxyproline and collagen content. The hydroxyproline content is about 0.9–1.2 mg/ml and the collagen content are 7.5 %. The control without any treatment has hydroxyproline of about 0.6–0.8 mg/ml and collagen of 5 %. Whereas in the third group of control (wound without healing) it is found to be least i.e., 0.2–0.4 mg/ml and 2 % of hydroxyproline and collagen, respectively (Fig. 5B).

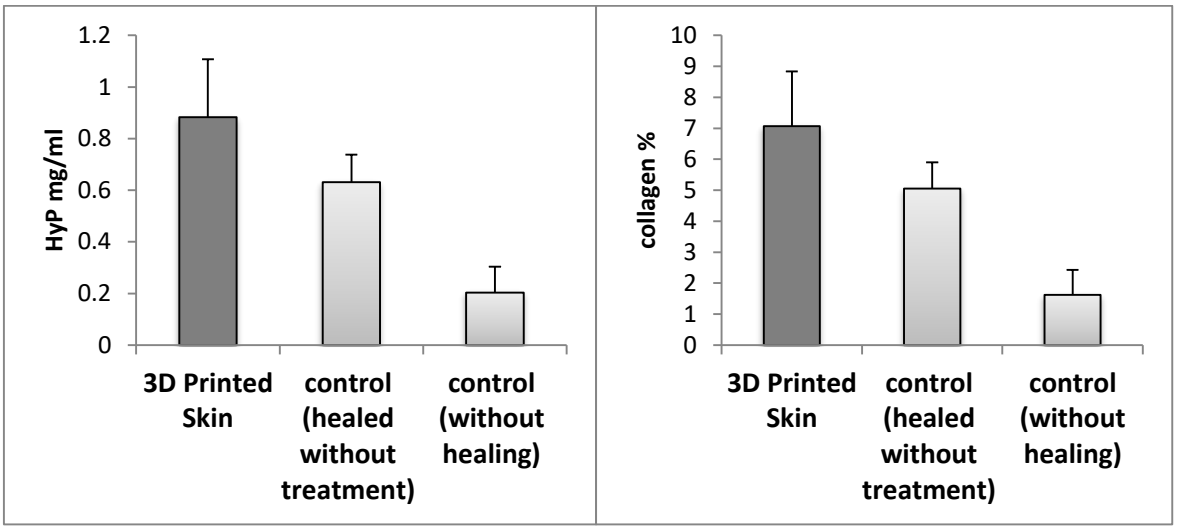
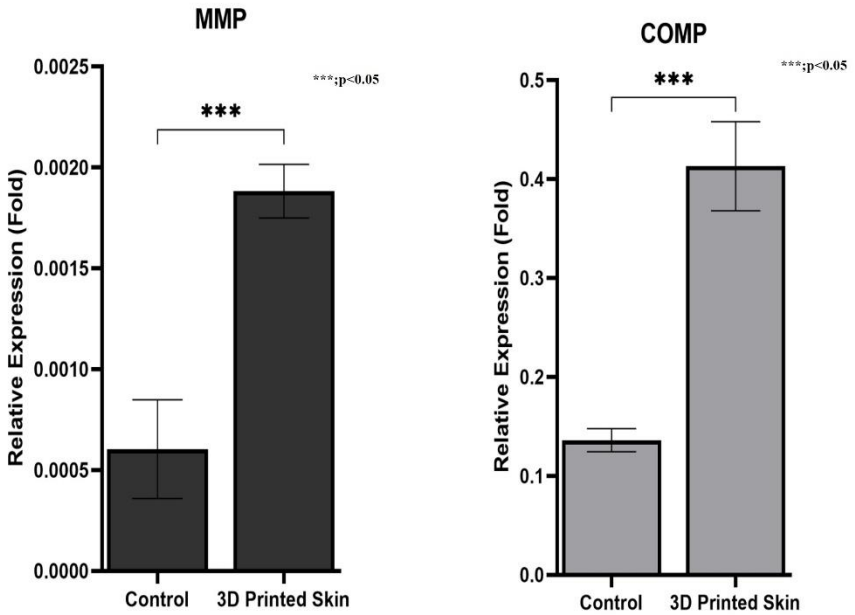


Figure 3.17 Hydroxyproline concentration of skin samples in mg/ml, and percentage of collagen in biopsy skin samples

3.3.4.4. Relative mRNA expression of MMP-9, IL-6, COMP, TNF-α by qPCR

The relative expression studies for the genes between control and 3D printed skin were carried out for four sets of genes. The mean values for the increase in the fold of the genes were plotted individually. MMP-9 shows >3-fold increase in expression compared to the control group. COMP shows >4-fold change, TNF-α shows >2-fold change, and IL-6 shows >4 fold change expression when compared with the control group (Fig. 3.17C, D, E, F).



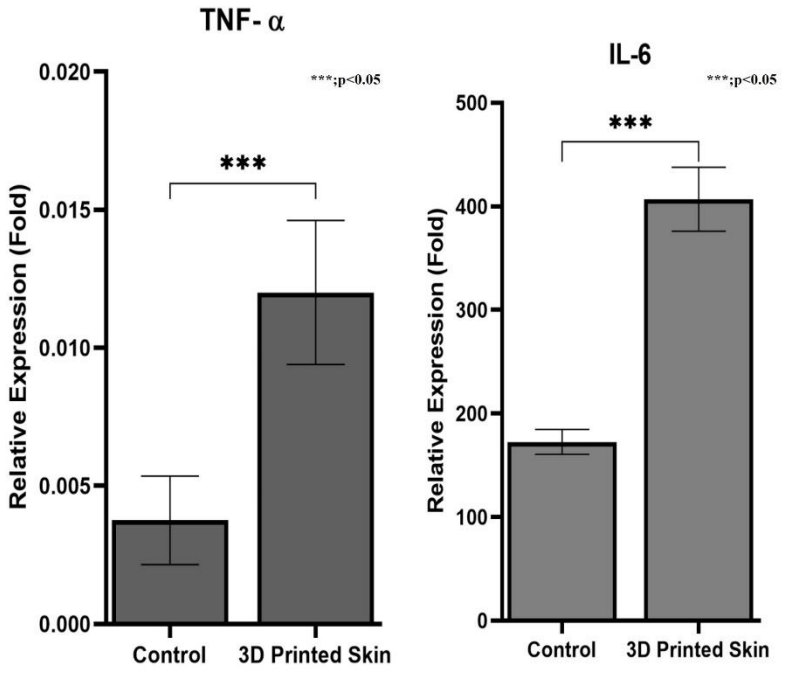


Figure 3.18: The mRNA expression of different genes MMP, COMP, TNF-α, IL-6 respectively

3.4 Discussion

The management of chronic and burn wounds presents a significant challenge in the healthcare sector, with prolonged healing contributing to substantial social and economic implications (Nischwitz S., et al., 2021; Luze H., et al., 2022). The primary objective of an ideal burn wound dressing is to effectively manage burns and chronic wounds through optimal wound dressing, providing pain relief and reducing the frequency of dressing changes (Luze H., et al., 2022). Utilizing 3D printing technology to produce biocompatible artificial skin emerges as a potential approach for comprehensive wound coverage.

In this study, 3D-printed artificial skin was developed using a slurry derived from chicken skin treated with NaOH. Gelatin and PVA were added to the mixture to strengthen it mechanically and polymerize it, which improved the bonding qualities and made up for the increased protein content (Jem K and Tan B, 2020). FTIR analysis revealed the incorporation of extracellular matrix (ECM) components into the skin bioink, crucial for cellular adhesion. The biodegradability study of 3D printed skin demonstrated stable collagen bundles on the surface, potentially influencing cellular behaviors at the wound site. For consistent skin printing, the bioink spreadability, injectability, and contact angle must be standardized. The bioink displayed proportional injectability during 3D printing, attributed to consistent polymerization, stability

at high temperatures, and suitability for clinical handling. Importantly, the 3D-printed skin lacks DNA, reducing the likelihood of an inflammatory response in animal testing. The study emphasizes the importance of enhanced bioink formulations featuring reliable printability, robust mechanical properties, and porous gradient skin scaffolds. Prior to the animal study, various in vitro and in ovo tests were conducted to assess the effectiveness of 3D printed skin grafts. The MTT cytocompatibility assay demonstrated increased metabolic activity of cord blood MNC in the presence of bioink compared to the control. In vitro scratch assays using a fibroblast cell line (L929) illustrated the 3D printed skin scaffold's potential as a facilitator of cell migration, leading to accelerated wound healing.

The 3D printed skin consistently showed a significantly reduced wound area percentage at all time points compared to the control group, indicating its potential in promoting faster and more efficient wound healing processes. Further evaluations in a CAM model of a chick embryo demonstrated the biocompatibility and non-toxic nature of 3D printed skin. Staining with alcian blue (AB) and HE revealed the presence of chick cells on the graft, with AB stain indicating glycosaminoglycans (GAGs).

SEM pictures showcased cellular structure formation during the graft's incubation in the CAM region, supporting successful engraftment and compatibility for subsequent animal experiments. The animal experiment involved New Zealand rabbits, and 8 mm thick skin was excised to create full-thickness wounds. Application of 3D printed skin significantly enhanced wound reduction compared to lorexane T and the untreated group, as observed through histological observations. Alcian Blue (AB) staining revealed connective tissue positive for GAGs, with higher levels of ECM conservation and epithelization in the 3D printed skin group. Masson's trichrome staining illustrated a significant increase in collagen fibers, including the restoration of various skin components.

Immunohistochemistry results confirmed accelerated epithelization, neovascularization, and smooth muscle cell recruitment in wounds treated with 3D printed skin. Preservation of α smooth muscle actin (α SMA) expression in the lining of smooth muscle and actin filaments was observed. Notably, the expression of E-cadherin (E cad) in deeper skin layers indicated the establishment of epithelial polarity. The study delved into the importance of E-cad for maintaining adhesive properties in keratinocytes and proper skin differentiation. Expression of VCAM-1, and CK-18 further supported active epithelization and successful recovery in the animal model.

Collagen analysis demonstrated higher percentages and hydroxyproline concentrations in groups treated with 3D printed skin, essential for tissue repair and wound healing. Matrix

metalloproteinases (MMPs) have a role in the wound healing stages, as evidenced by the three-fold increase in mRNA gene expression analysis for MMPs. The dermis of 3D printed skin wounds showed an ordered collagen network, as confirmed by COMP expression. Since TNF-α and IL-6 are essential for tissue repair processes, their mRNA expression indicated promising signs of wound healing. The study demonstrated the potential efficacy of 3D printed skin in wound treatment by using a bioink formulated with ECM elements for optimal cell migration and proliferation. It has been demonstrated to be achieve scarless wound healing but to validate its therapeutic potential, extensive research on large animal models will be needed. The technology might additionally be implemented into a bedside 3D printer for direct skin graft printing at the injury site, which would lead to promising medical advancements.

3.5 Conclusions

In conclusion, skin bioink was developed by combining skin-specific ECM collagen with PVA and gelatin. The 3D printed skin allowed cells to survive for an extended period as well as facilitated cell migration and proliferation. Our research demonstrated that incorporating skin extracellular matrix collagen into bioink increased its bioactivity in vitro, in ovo, and in vivo. The efficacy of 3D printed skin in wound healing applications will be verified by extending this to large animal models with long-term follow-up. We hope to use this technique in the future to apply customized 3D bioprinted skin at the bedside as a novel therapeutic approach.

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CHAPTER 4: STUDY OF NANOPOLYMER IN DIABETIC WOUNDS IN HUMAN SUBJECTS.

4.1 Introduction:

Diabetic foot ulcers (DFUs) are a serious and common problems of diabetes, affecting millions of people worldwide. Diabetic wounds are notoriously hard and slow to heal, often leading to severe infections, amputations, and a significant financial burden on healthcare systems (Baig et al., 2022). Due to the ongoing global increase in diabetes, there is a need for efficient and affordable treatments for DFUs becomes increasingly urgent (Sorber and Abularrage, 2021). Unfortunately, current treatment options often fail to fully heal these wounds or prevent them from recurring, highlighting the need for innovative solutions. Platelet-rich plasma (PRP) has gained recognition as an effective treatment for wound healing.

PRP is derived from the own blood of patient and contains high concentrations of growth factors such as vascular endothelial growth factor (VEGF), epidermal growth factor (EGF), and platelet-derived growth factor (PDGF). These growth factors are instrumental in wound healing by enhancing cell growth, blood vessel formation, and tissue repair (Cecerska-Heryć et al., 2022). However, PRP has limitations, including its rapid degradation and inability to allow for extended and controlled growth factor release, which reduces its effectiveness, particularly in treating chronic wounds like DFUs (Jiang et al., 2023).

To overcome these challenges, we have developed a nanopolymer-based system using polyvinyl alcohol (PVA). This innovative system combines PRP with a nanopolymer, enabling a slow and consistent release of growth factors over time. This ensures a longer-lasting supply of healing molecules to the wound, significantly enhancing the effectiveness of the treatment. Diabetic wounds are particularly hard to heal due to multiple factors. High blood sugar levels create toxic chemicals called advanced glycation end products (AGEs), which damage the extracellular matrix and weaken newly formed tissue. Diabetes also impairs the formation of new blood vessels, limiting oxygen and nutrient delivery to the wound site and slowing the healing process (Burgess et al., 2021).

Chronic inflammation is another major obstacle. In diabetic wounds, the immune system produces excessive inflammatory molecules while failing to generate enough pro-healing factors, which disrupts the normal wound-healing process (Pradhan et al., 2009). PRP addresses these challenges by delivering a concentrated mix of growth factors directly to the wound, stimulating new blood vessel formation and tissue repair. However, the rapid breakdown of PRP limits its therapeutic benefits. By combining PRP with our nanopolymer system, the treatment overcomes this limitation. The nanopolymer stabilizes the growth factors, ensures their sustained release, and targets them to the wound site, providing a more effective and reliable therapy (Patel et al., 2023). This approach holds great potential for improving the

treatment of DFUs and other chronic wounds, offering patients a faster and more complete path to healing.

4.2 Material and methodology:

4.2.1. Study Design and Participants

The study was designed as a prospective, experimental, observational study conducted at Dr. D.Y. Patil Medical College, Hospital, and Research Institute, Kolhapur.

4.2.2. Sample Size

A total sample size was decided by the following formula.

$n = [(Z_{\alpha/2} + Z_{\beta})^2 \times \{(p1\ (1\text{-}p1) + (p2\ (1\text{-}p2))\}]/(p1 - p2)^2$

where

n = sample size required in each group,

p1 = proportion of subject cured by drug

p2 = proportion of subject cured by Placebo

p1-p2 = clinically significant difference

$Z_{\alpha/2}$: This depends on level of significance (</5%),

Z_{β}: This depends on power (</80%)…….. (Sakpal TV., 2010)

Sample size of 27 patients who met the inclusion criteria were enrolled in the study. Patients were randomized into two groups: one group received nanopolymer gel dressing, while the other group received conventional dressing.

4.2.3. Eligibility Criteria

4.2.3.1.Inclusion Criteria

- All patients who visited OPD or are admitted to IPD with diabetic foot ulcers
- Eligible patients have ulcers larger than 5 cm
- HbA1C levels greater than 7.5
- Patient between the age of 18 to 65 years

4.2.3.2.Exclusion Criteria

- Patients are excluded if they failed to follow up in OPD, and undergoing amputation for diabetic foot ulcers
- Immunocompromised status
- Osteomyelitis or gangrenous ulcers

- Declined to provide informed consent

4.2.4. Preparation of Product for Application

The nanopolymer was prepared freshly. Sterility was checked and aliquot in sterile tube. The tubes were stored at -80^{o}C before the use.

4.2.5. Intervention

Patients in the nanopolymer group received daily dressing applications of the gel. Patients in the control group received only conventional dressing using EUSOL-soaked gauze. The treatment time is based on the size and degree of wound and wound healing.

4.2.6. Baseline Evaluations

All patients underwent a detailed history and physical examination. Initial evaluations included complete blood count, fasting and post-prandial blood sugar levels, HbA1C testing, and an initial edge biopsy to evaluate wound healing.

4.2.7. Biopsy Collection and Analysis

Biopsy samples were collected from each patient at two time points. The initial biopsy was taken before starting the treatment. The second biopsy was taken after two weeks of the treatment. The collected biopsy samples were processed for further analyses.

4.2.3.3.Histological Studies

Hematoxylin and Eosin (HE) staining was performed to evaluate tissue architecture and cellular activity. Masson's Trichrome (MT) staining to assess collagen deposition and organization. Elastin staining to examine the distribution and quality of elastic fibers in the regenerated tissue.

4.2.3.4.Molecular Studies

mRNA was isolated from biopsy samples for gene expression analysis. Complementary DNA (cDNA) was synthesized using the isolated mRNA. Specific primers were used to assess the expression of genes related to wound healing, such as those involved in inflammation, angiogenesis, and extracellular matrix remodelling.

4.2.8. Wound Dressing Protocol

Dressing was performed daily with strict aseptic precautions to ensure a sterile environment. Sterile pads or Gamgee were applied, followed by bandaging, in both study groups.

4.2.9. Post-Operative Measure:

Post-application care followed standard wound care protocols, ensuring that the nanopolymer remained in place and were not disturbed during the healing process.

4.2.10. Follow-Up and Monitoring:

Wound size was measured every alternate day using a digital wound measurement application and a wound camera. The graph was plotted for degree of reduction of wound. Granulation tissue formation was closely observed as a critical marker of healing. Edge biopsies were taken at baseline, after 2 weeks, for histochemical analysis.

4.2.11. Outcome Measures

4.2.11.1. Primary Outcomes

Wound healing rate and the incidence of complete wound closure were evaluated.

4.2.11.2. Secondary Outcomes

Safety, tolerability, quality of wound healing, scar assessment, pain reduction, patient satisfaction, and quality of life were analyzed.

4.2.12. Ethical Considerations

The study was approved by the Institutional Ethics Committee of D.Y. Patil Educational Society. All participants provided informed consent before being enrolled in the study. The study was also registered under Clinical Trials Registry of India (CTRI/2024/09/073475).

4.3 Results

4.3.1. Nanopolymer clinical Observations:

By Day 1, wounds in all subjects were open, with varying degrees of tissue exposure and inflammation. Granulation tissue formation began early, noticeable by Day 3 in some cases and by Day 5 in others, marking the onset of healing. Swelling and redness gradually reduced as the wounds progressed toward closure. By Day 10, significant wound contraction and epithelialization were evident in all cases, with clear signs of tissue regeneration. For most subjects, wounds were nearly healed by Day 12, showing reduced size and improved skin integrity. By Day 15 to Day 25, depending on the initial severity, complete healing was achieved, with wounds fully closed and regenerated tissue evident, indicating effective wound management and recovery (Fig 4.1.).

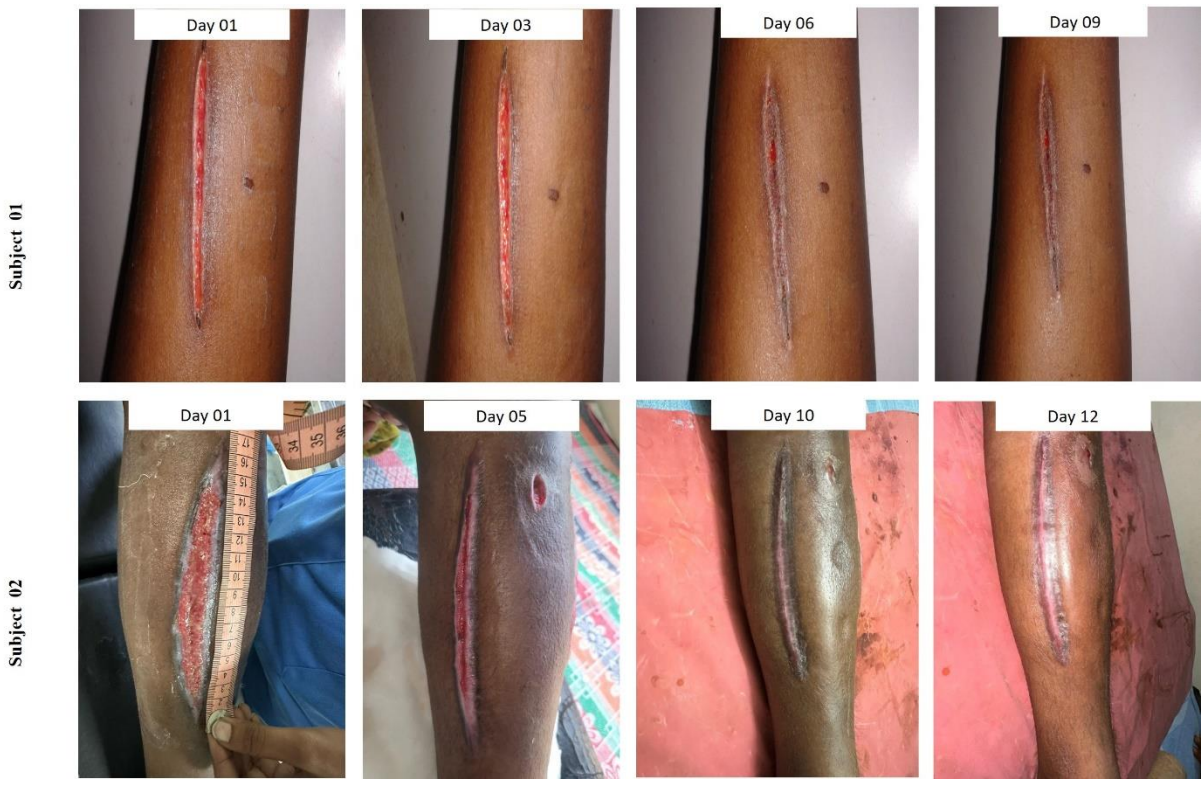

Figure 4.1: Subject 1 and Subject 2 represents the standard treatment dressing wound healing

4.3.2. Standard Treatment group clinical Observations:

The wounds initially appeared deep and infected, accompanied by significant inflammation. Standard care was implemented, including cleaning with antiseptic solutions, applying sterile dressings, and administering antibiotics and analgesics as needed to manage infection risk and pain. By Day 12, early granulation tissue development was observed in some cases, with the wounds showing a healthier appearance, indicating the initiation of the healing process. During Days 28–32, granulation tissue was observed in all patients, characterized by active fibroblast proliferation and angiogenesis, which contributed to wound bed preparation and contraction. But 6 of the patients were again subjected to the secondary infections resulting in the debridement of the tissue and opening of the wound. By Day 38, complete wound closure was achieved in 4 patients, with successful re-epithelialization and early scar formation marking the maturation phase. By Day 42, 6 additional patients exhibited complete wound closure, demonstrating significant tissue regeneration and fully re-epithelialized wound edges, restoring skin integrity. Post-Day 42 follow-up focused on scar management and maintaining skin hydration to support long-term recovery, along with patient education on preventing complications and preserving skin health.

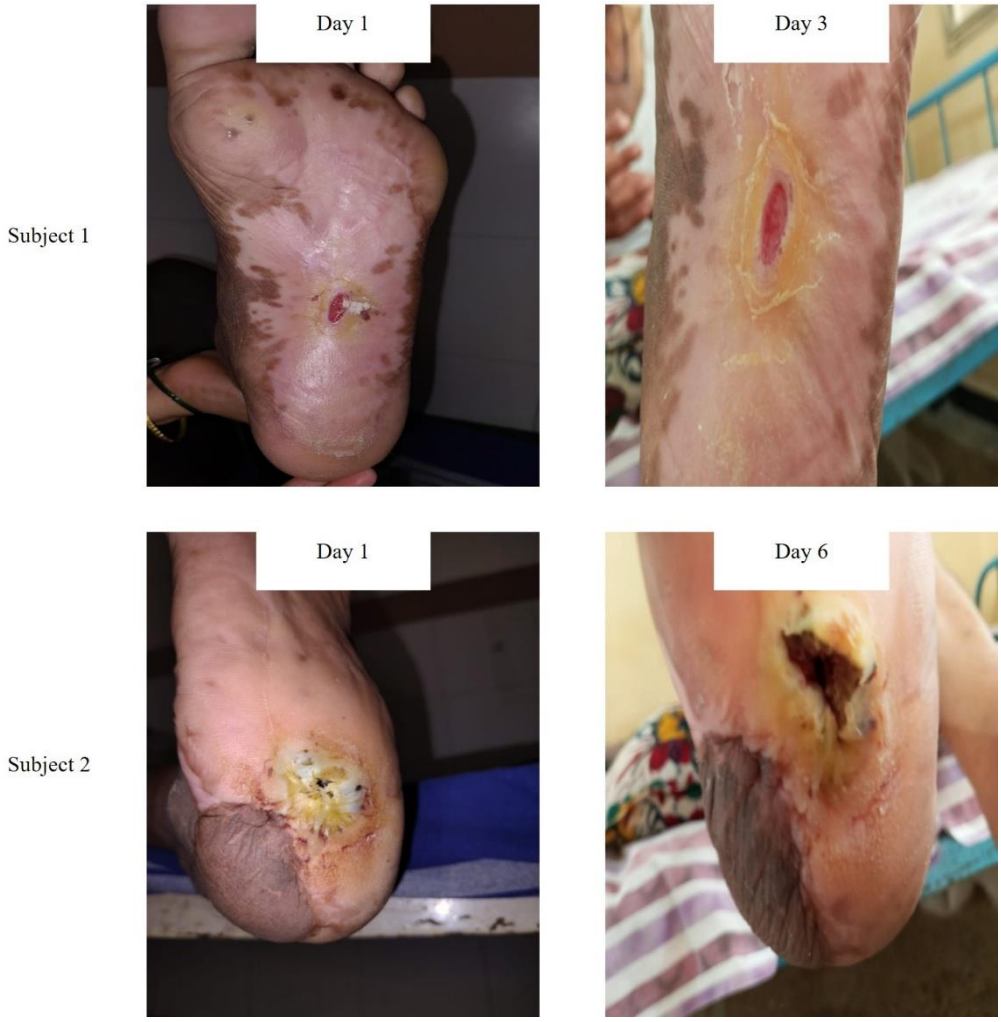

Figure 4.2: Subject 1 and Subject 2 represents the standard treatment dressing wound healing

4.3.3. Hematoxylin and Eosin (HE) Staining:

The HE staining (Fig 4.3) reveals significant tissue regeneration and granulation tissue formation in subjects treated with the nanopolymer compared to the standard treatment group. In all subjects, the nanopolymer treatment shows organized fibroblast proliferation, reduced inflammation, and well-developed epithelialization. The standard treatment group shows slower granulation tissue formation and delayed re-epithelialization.

4.3.4. Masson's Trichrome (MT) Staining

MT staining highlights (Fig 4.3.) collagen deposition, an important marker for tissue remodelling and wound healing. In the nanopolymer-treated group, collagen fibers appear denser and more organized compared to the standard treatment group, indicating superior tissue repair and structural integrity. In contrast, the standard treatment group shows loosely arranged collagen fibers, indicating incomplete wound healing.

4.3.5. Elastin Staining

Elastin staining (Fig 4.3.) further demonstrates the reorganization of extracellular matrix (ECM) components. The nanopolymer-treated group shows enhanced ECM remodelling with clear evidence of neo-angiogenesis, fibroblast proliferation, and proper epithelial alignment. The standard treatment group, however, shows delayed ECM organization, which corresponds to slower wound healing observed clinically.

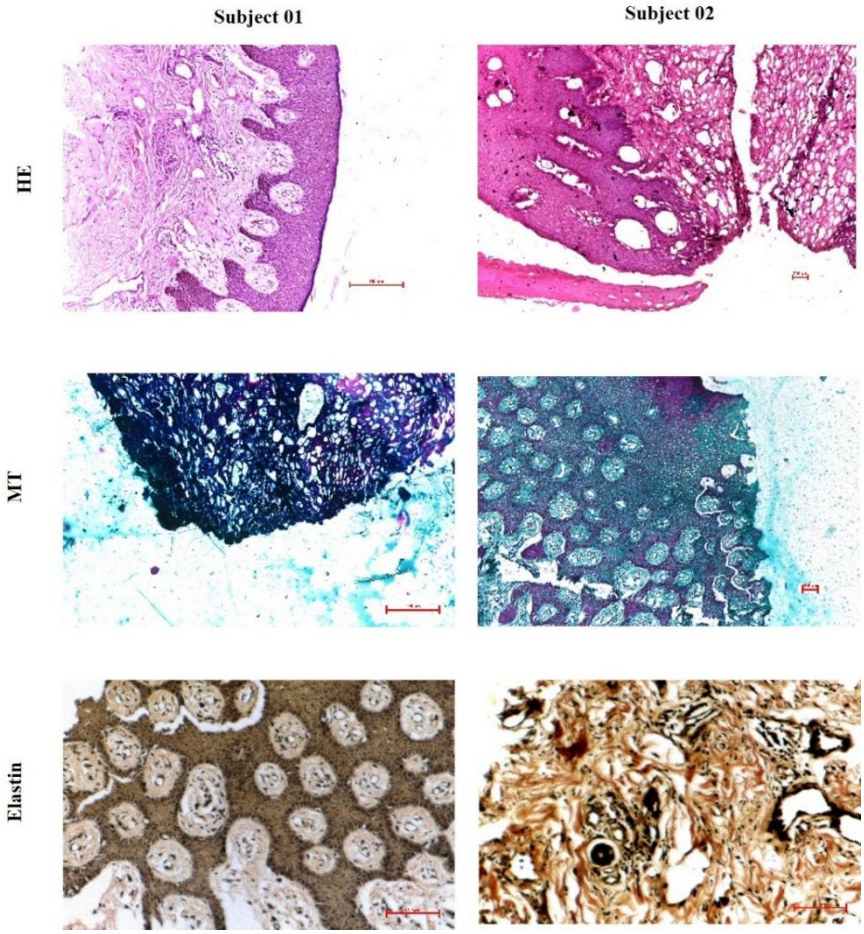

Figure 4.3: Histological evaluation of wound healing of nanopolymer treated subjects

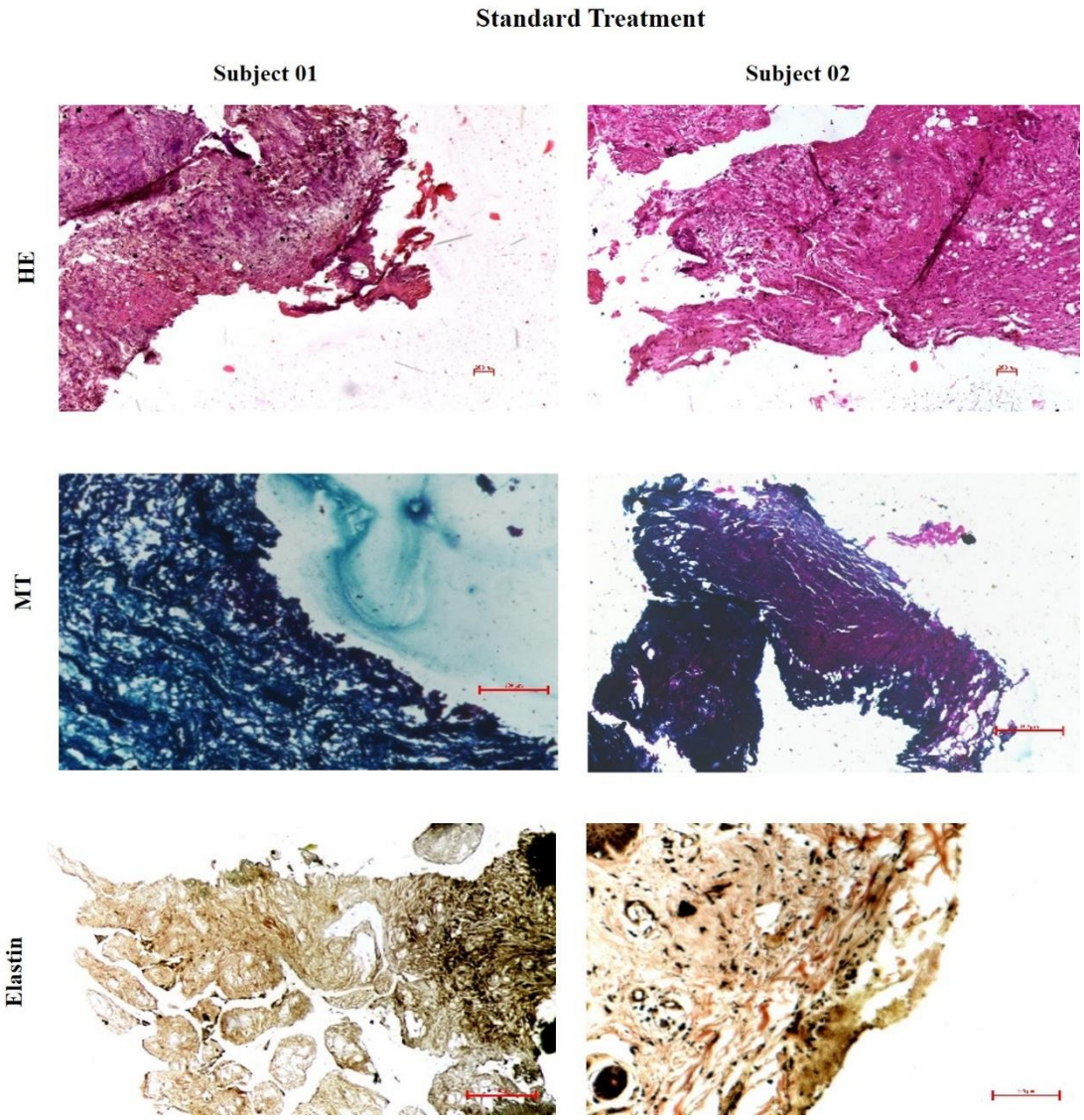

Figure 4.4: Histological evaluation of wound healing of standard treatment subjects

4.3.6. mRNA analysis

In the mRNA analysis of six patients treated with nanopolymer gel (Fig 3), significant gene expression changes indicative of enhanced tissue regeneration was observed across the cohort compared to standard treatment. In Patient 1, TGF-beta showed a 58.3-fold increase, highlighting its pivotal role in tissue repair, while IL-10, an anti-inflammatory marker, increased by 50.2-fold. COL-1, essential for collagen synthesis, rose by 12.1-fold, and VEGF, crucial for angiogenesis, exhibited a 3.78-fold increase. Matrix metalloproteinases (MMPs) also showed upregulation, with MMP-13 at 3.31-fold, and FOXM1, involved in cellular proliferation, increased by 2.85-fold. Patient 2 exhibited similar trends, with a 60.1-fold increase in TGF-beta, a 52.8-fold rise in IL-10, and a 13.2-fold increase in COL-1. VEGF was upregulated by 3.71-fold, and MMPs showed consistent increases. FOXM1 increased by 2.92-fold, suggesting improved cellular activities.

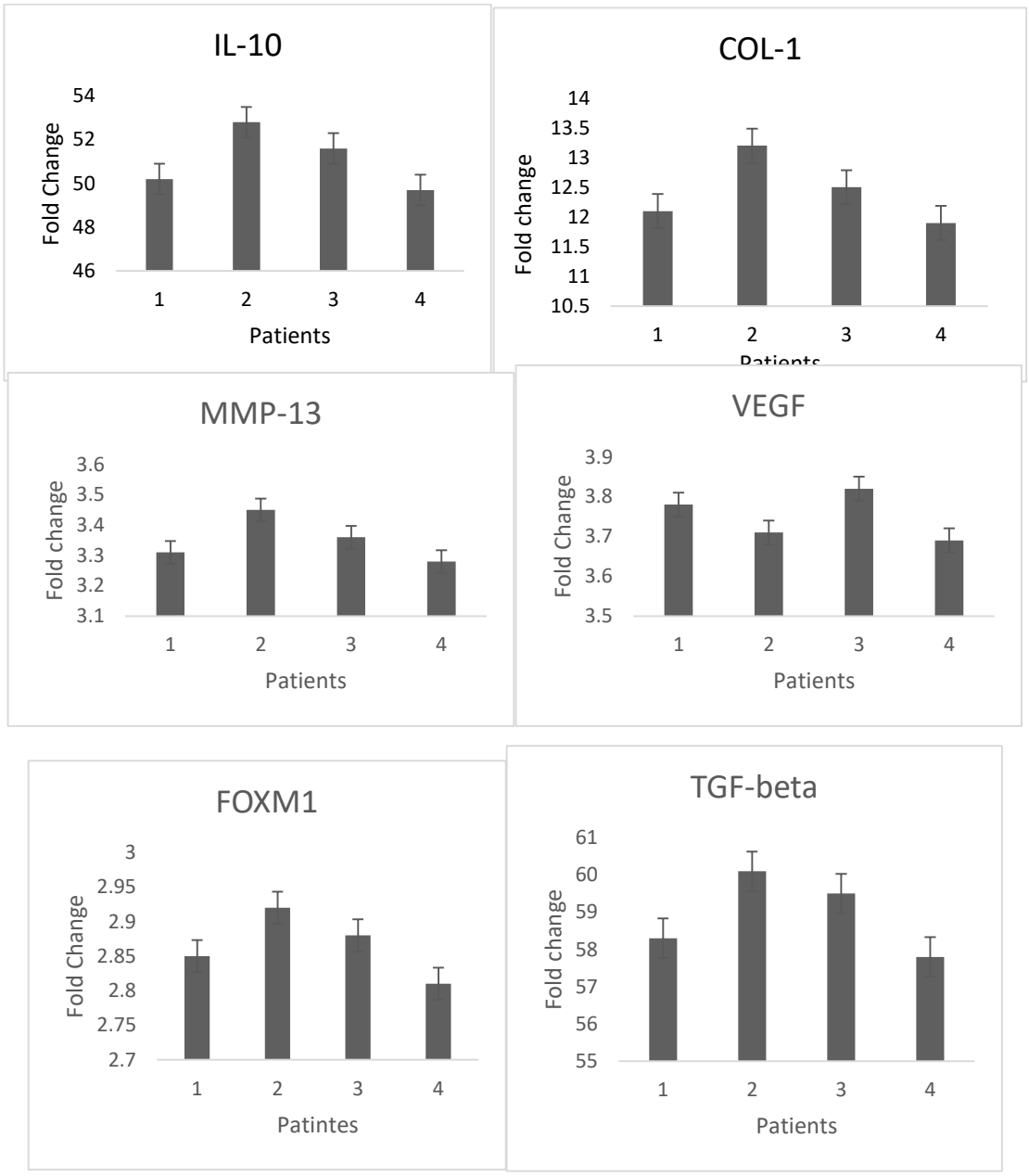

Figure 4.5: Gene expression during wound healing

4.4 Discussion

This study investigated the effectiveness of a nanopolymer gel dressing in healing diabetic foot ulcers (DFUs) and found remarkable results. The gel significantly improved tissue regeneration by encouraging fibroblast growth and collagen production, which are essential for building strong and healthy tissue. Masson's Trichrome staining revealed dense and well-organized collagen fibers aligned with elastin, creating a durable and supportive tissue matrix. This improved structural support ensured that the wounds healed better and stronger compared to traditional conventional dressing methods.

The results highlight distinct time points in diabetic wound healing progression between nanopolymer treatment and standard care. In nanopolymer-treated wounds, early granulation tissue formation was observed consistently by Day 03–05, leading to rapid wound contraction and reduced inflammation by Day 10. Epithelialization and significant tissue regeneration were evident by Day 12, with complete healing typically achieved by Day 25. In contrast, wounds treated with standard care demonstrated delayed granulation tissue formation, beginning around Day 05–10, with wound contraction and epithelialization noted by Day 15. Complete healing was observed later, typically between Day 38–42. Key time points in the nanopolymer group, such as earlier granulation tissue development and epithelialization, suggest its efficacy in accelerating the healing process compared to standard treatment.

The expedited wound closure and tissue regeneration in nanopolymer-treated wounds underscore its potential for improving clinical outcomes, particularly in cases requiring prompt recovery. These findings emphasize the importance of innovative materials like nanopolymer in enhancing wound management strategies. The histological results confirm the observations with the clinical findings in Fig. 4.2. Subjects treated with the nanopolymer exhibit faster wound contraction, thicker granulation tissue, and more organized re-epithelialization compared to the standard treatment group. The improved collagen deposition and ECM remodelling seen in MT and Elastin staining further validate the nanopolymer superior efficacy in promoting wound healing.

Inflammation, a major problem in diabetic wound healing, was significantly reduced in wounds treated with the nanopolymer gel. Chronic inflammation often delays the healing process, but the gel helped create a favourable environment for the wound to progress through the normal phases of healing. VEGF staining confirmed the growth of new blood vessels, improving oxygen and nutrient delivery to the wound site—a critical factor for diabetic wounds, which often suffer from poor blood circulation. The nanopolymer gel also promoted skin repair by boosting keratinocyte activity, a key factor in re-epithelialization. This process is essential for closing the wound and restoring the skin's protective barrier.

Molecular analysis revealed an increase in important healing factors such as TGF-β, VEGF, and IL-10. These molecules worked together to balance inflammation, encourage blood vessel growth, and repair the extracellular matrix (ECM). IL-10 reduced excessive inflammation, while TGF-β and VEGF supported collagen production and vascularization, creating the ideal conditions for tissue repair. The gel also played a vital role in remodelling the ECM. It increased the levels of MMP-13, an enzyme that breaks down old collagen and helps form new, organized fibers. This process allowed the wound to contract and the newly formed skin to become

stronger. Overall, the gel addressed multiple aspects of the healing process, including reducing inflammation, promoting angiogenesis, remodelling the ECM, and re-epithelializing the wound. These combined effects resulted in faster healing and better-quality tissue. When compared to traditional dressings, the nanopolymer gel showed significantly better results. Traditional dressings often fail to overcome the multifaceted obstacles of DFUs, like persistent inflammation and poor blood flow. In contrast, the nanopolymer gel worked on multiple levels, tackling these issues simultaneously. This led to quicker wound closure, stronger tissue structure, and improved molecular healing markers.

These findings are particularly significant for diabetic patients, who often face delayed wound healing and a high likelihood of adverse effects like infections or amputations. By reducing inflammation, improving blood flow, and strengthening new tissue, the nanopolymer gel offers a promising alternative for managing chronic wounds. However, while these results are encouraging, further studies are needed to confirm its long-term benefits and effectiveness in a wider diverse healthcare environment. If validated, this treatment could revolutionize how chronic wounds like DFUs are managed, improving patient outcomes and well-being.

4.5 Conclusions:

The nanopolymer gel looks very promising for healing diabetic foot ulcers (DFUs). It helps wounds heal by boosting collagen production, growing new blood vessels, and reducing harmful inflammation. This creates the perfect environment for the skin to repair itself. The gel also helps close wounds faster and makes the new skin stronger and healthier. It supports the growth of new skin cells and rebuilds the framework needed for tissue to grow properly. Compared to conventional dressings, the gel works better by speeding up healing and improving the quality of the repaired skin. This makes it a great option for diabetic patients who often struggle with slow healing and infections. However, more research is needed to test the gel in everyday use, improve how it made, and check its long-term benefits. Even so, this gel shows a lot of potential to help people with chronic wounds heal better and faster.

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CHAPTER 5: STUDY OF 3D-PRINTED SKIN IN DIABETIC WOUNDS IN HUMAN SUBJECTS

5.1 Introduction:

Recent advancements in 3D bioprinting technology have revolutionized wound care by enabling the creation of skin substitutes which nearly replicate the morphology and physiological properties of the skin (Zhu et al., 2023). These substitutes are engineered using bioinks composed of biomaterials like collagen, hyaluronic acid, and other extracellular matrix components, which are essential for promoting cell growth, migration, and tissue repair (Liu et al., 2024). These materials provide an optimal environment for cells to thrive, aiding in the regeneration of new tissue that mimics the properties of healthy skin. Such innovations have paved the way for treating complex and chronic wounds, including diabetic foot ulcers (DFUs), burns, and surgical wounds, which often fail to respond adequately to traditional treatment methods (Smith et al., 2021).

One of the key findings in the 3D-printed skin-treated group was the superior quality of the healed tissue. Collagen and elastin, the two primary proteins responsible for skin strength and elasticity, were more organized and abundant in this group. This resulted in the formation of a durable and flexible tissue matrix, which is crucial for restoring the functional properties of skin. Masson's Trichrome staining revealed dense and well-aligned collagen fibers, while elastin staining confirmed the presence of organized elastic fibers, contributing to the mechanical properties of skin and resilience.

Additionally, wounds treated with 3D-printed skin demonstrated elevated levels of biological markers critical for healing. VEGF, a growth factor responsible for promoting blood vessel formation, was significantly increased, facilitating improved oxygen and nutrient delivery to the wound site. Similarly, TGF-β, a cytokine involved in tissue repair and fibrosis, was also elevated, supporting the remodelling of the extracellular matrix and enhancing the quality of the repaired tissue (Davis et al., 2022). These markers indicate that 3D-printed skin not only accelerates the healing process but also enhances the overall quality of tissue regeneration.

Beyond the biological benefits, 3D-printed skin offers a more controlled and precise approach to wound care. By utilizing bioinks tailored to specific wound types and patient needs, this technology provides a personalized treatment option. For example, bioinks can be modified to include antimicrobial agents, growth factors, or immune-modulating components, making them highly adaptable to various clinical scenarios. This flexibility positions 3D-bioprinting technology as a versatile solution capable of addressing the unique challenges of different wound types, particularly chronic and complex wounds like DFUs.

If these challenges are addressed, 3D-printed skin has the potential to transform the standard of care for chronic wounds. Its ability to enhance healing speed, improve tissue quality, and offer

tailored solutions makes it a groundbreaking advancement in wound care. The continued development and refinement of this technology could revolutionize how wounds are treated, offering new hope to patients suffering from debilitating conditions like DFUs and burns.

5.2 Material and methodology:

5.2.1. Study Design:

The study was conducted as a non-randomized, active-controlled interventional trial over two years at Dr. D.Y. Patil Medical College, Hospital, and Research Centre in Kolhapur. The purpose was to evaluate the efficacy and safety of 3D-printed skin grafts in diabetic foot ulcers (DFUs) compared to autologous skin grafts, which served as the control. The study sought to address challenges in wound healing by leveraging the unique properties of 3D-printed skin.

5.2.2. Sample Size:

A total sample size was decided by the following formula.

$n = [(Z_{\alpha/2} + Z_{\beta})^2 \times \{(p1\ (1-p1) + (p2\ (1-p2))\}]/(p1 - p2)^2$

where

n = sample size required in each group,

p1 = proportion of subject cured by drug

p2 = proportion of subject cured by Placebo

p1-p2 = clinically significant difference

$Z_{\alpha/2}$: This depends on level of significance (</5%),

Z_{β}: This depends on power (</80%)…….. (TV Sakpal, 2010)

A total sample size was calculated by the above formula and 23 patients were enrolled in the study, meeting the inclusion criteria. Participants were divided into two groups: the intervention group, which received 3D-printed skin grafts, and the control group, which received autologous skin grafts.

5.2.3. Eligibility Criteria:

5.2.3.1 Inclusion Criteria

- All patients that come to OPD and IPD with foot and leg ulcer/ burn
- Patients having ulcer of size >5cm
- Patients with HbA1C >7.5
- Patients of age 18 to 65 years

5.2.3.2 Exclusion Criteria

- All patients who do not follow up in OPD
- Patients with diabetic foot ulcer undergoing amputation

- Patients with immunocompromised status
- Patients with osteomyelitis or gangrenous ulcers
- All patients who do not give consent for the study

5.2.4. Preparation of 3D printed skin for application

The 3D printed skin was sterilized and packed for application. The 3D printed skin was sterilized by using ethylene oxide gas and stored at cool place.

5.2.5. Intervention:

3D printed skin graft

The intervention utilized 3D-printed skin grafts made from xenogenic skin and polymerized with polyvinyl alcohol (PVA) to create a bioink. These grafts contained ECM components designed to attract cells, promote granulation tissue formation, and accelerate wound healing. The grafts were sterilized before application and carefully placed on the wound site under sterile conditions.

5.2.6. Comparator

Patients in the control group received autologous skin grafts. These grafts were harvested from the patient's own skin and applied to the wound following established protocols for autologous grafting.

5.2.7. Baseline Evaluations

All participants underwent a comprehensive evaluation before the intervention. This included recording detailed medical histories, conducting thorough physical examinations, and performing laboratory tests such as complete blood count, fasting and post-prandial blood sugar levels, and HbA1C. These assessments ensured that all patients met the inclusion criteria and provided a baseline for monitoring progress.

5.2.7.1 Biopsy collection

Biopsy samples were collected from each patient at two time points. First biopsy was taken at baseline or before graft application. The second biopsy collected at second week. The collected biopsy samples were processed for further analyses, including:

5.2.7.2 Histological Studies:

Hematoxylin and Eosin (HE) staining was performed to confirm the cellular tissue architecture and cellular activity. Masson's Trichrome (MT) staining was performed to assess collagen deposition and organization. Elastin staining was performed to evaluate the distribution and quality of elastic fibers in the regenerated tissue.

5.2.7.3 Molecular Studies:

mRNA was isolated from biopsy samples for gene expression analysis. Complementary DNA (cDNA) was synthesized using the isolated mRNA. Specific primers were used to assess the expression of genes related to wound healing, such as those involved in inflammation, angiogenesis, and extracellular matrix remodelling.

5.2.8. Application Protocol:

The 3D-printed skin grafts and autologous grafts were applied under strict aseptic conditions. The grafted areas were covered with sterile dressings and monitored daily for signs of infection, rejection, or other complications.

5.2.9. Postoperative measure

Post-application care followed standard wound care protocols, ensuring that the grafts remained in place and were not disturbed during the healing process.

5.2.10. Follow-Up and Monitoring:

The acceptance of the skin grafts and the wound healing rate were evaluated over 4 to 8 weeks. Primary markers of healing included granulation tissue formation and the rate of wound closure. Secondary evaluations focused on safety, tolerability, scar quality, pain reduction, patient satisfaction and quality of life.

5.2.11. Outcome Measures

5.2.11.1 Primary Outcomes

The study assessed the acceptance rate of the 3D-printed skin grafts, the wound healing rate, and the incidence of complete wound closure.

5.2.11.2 Secondary Outcomes

Safety, tolerability, and quality of wound healing were measured, along with scar assessment, pain reduction, patient satisfaction, and overall cost-effectiveness.

5.2.12. Ethical Considerations

Institutional Ethics Committee of Dr. D.Y. Patil Medical College provided the approval for the study. Informed consent was obtained from all participants to ensure ethical compliance and participant understanding of the trial. Also the study is registered under Clinical Trial Registry of India. The CTRI number for this ongoing trial is CTRI/2024/11/076815.

5.3 Results:

A few representatives from all the patients are discussed in the result section.

5.3.1. 3D printed skin clinical observations:

Patients treated with 3D-printed skin showed clear progress in healing. Subject 1 had a large wound with dead tissue on Day 0. By Day 3, new tissue started forming at the edges. By Day 9, the wound bed showed new skin growth, and between Days 15 and 21, the wound size reduced significantly, with clear signs of closure and tissue formation. Subject 2 showed similar improvements, with new tissue forming by Day 3. By Day 9, blood flow to the wound increased, and by Day 15, the wound had reduced in size with new skin covering it. For Subject 3, the wound was swollen and had dead tissue on Day 0, but by Day 3, there was less swelling, and new tissue started forming. By Day 9, blood flow improved, and tissue remodelling was visible. By Day 15, the wound was fully closed, with healthy new skin in place. Subject 4's wound showed improvement by Day 3, with new tissue forming. By Day 9, the wound size reduced significantly, and by Day 15, the wound was nearly closed. Subject 5 healed quickly, with a noticeable reduction in wound size by Day 7. By Day 14, new skin had grown, leaving minimal scarring and good recovery.

5.3.2. Standard Treatment group clinical observations

Standard treatment involved thoroughly cleaning the wound to remove debris, bacteria, and dead tissue. Sometimes, dead tissue was removed through a process called debridement to help healthy tissue grow. The wound was covered with a moist dressing to encourage healing. By Day 4, swelling and dead tissue usually reduced, and by Day 5, new tissue began forming at the wound edges. Between Days 5 and 8, blood vessels started growing, helping the wound heal. By Days 9 to 12, the wound began closing, and new skin started forming at the edges. Between Days 19 and 32, significant wound closure occurred, with tissue remodelling and reduced wound size. Over time, the tissue became stronger, and scars began to mature. During the treatment, the wound was monitored closely for any signs of infection or delayed healing. If needed, advanced treatments like 3D-printed skin were used to help the wound heal better.

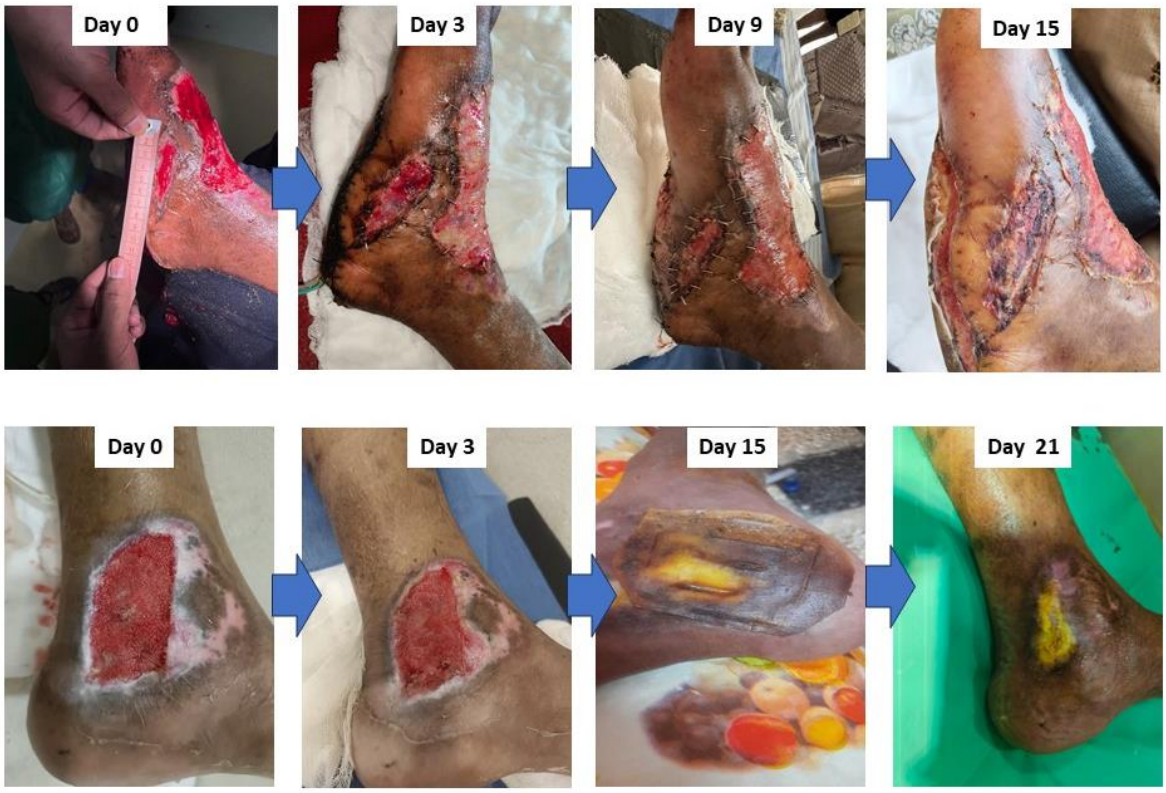

Figure 5.1: Representative photographs showing the progression of wound healing of the 3D printed skin in patient 1 and patient 2

Figure 5.2: Representative photographs of the standard treatment protocol of two patients

5.3.3. Histological studies:

5.3.3.1. Hematoxyline and Eosine staining:

The histological analysis shows clear differences between 3D-printed skin treatment and standard treatment. In the standard group, Day 0 reveals the significant damage, with disrupted skin layers, extensive necrosis, and the heavy inflammation. There was poor differentiation between the dermal and epidermal layers, and the inflammation slowed the healing process. By Day 15, re-epithelialization is observed, but the wound still had some inflammation, and the skin layers are only partially restored. Healing was slower, with incomplete regeneration of the outer skin and lingering signs of inflammation.

In the 3D-printed skin group, the results are more promising. On Day 0, there was still inflammation and tissue damage, but by Day 3, the granulation tissue has started forming, creating a more organized healing environment. By the end of Day 9, early signs of the new skin growth and reduced inflammation are visible. By Day 15, the wounds show clear re-epithelialization, with well-formed skin layers and the minimal inflammation. The epidermis look well-organized, and the dermis have the strong connective tissue structure, showing faster tissue regeneration and reduced scarring compared to the standard group as shown in the figure.

5.3.3.2. Masson's Trichrome (MT) staining:

Masson's Trichrome (MT) staining further highlighted differences in collagen deposition. In the standard treatment group, collagen fibers on Day 0 are fragmented and poorly organized, showing the minimal extracellular matrix remodelling. By Day 15, some organization of collagen is observed, but it is still less structured compared to the 3D-printed skin group.

In the 3D-printed skin group, Day 0 showed similar disorganization, but by Day 15, the collagen fibers are dense and well-aligned, reflecting efficient tissue remodelling and stronger skin structure. The collagen alignment in deeper dermal layers iss also more pronounced in the 3D group, indicating better mechanical support for tissue regeneration.

5.3.3.3. Elastin staining:

Elastin staining revealed further benefits of 3D-printed skin treatment. By Day 15, elastin fibers are visible in the dermal layers of 3D-treated wounds, improving skin elasticity and strength. In the standard treatment group, elastin formation is slower, and the regenerated skin shows less flexibility and mechanical integrity. This difference suggests that 3D-printed skin not only accelerates healing but also improves the functional properties of the new tissue.

The 3D-printed skin treatment showed faster re-epithelialization, better collagen organization, and enhanced elastin formation by Day 15. These results demonstrate stronger, more resilient

tissue with better functional recovery compared to the standard treatment, where healing was slower and less organized.

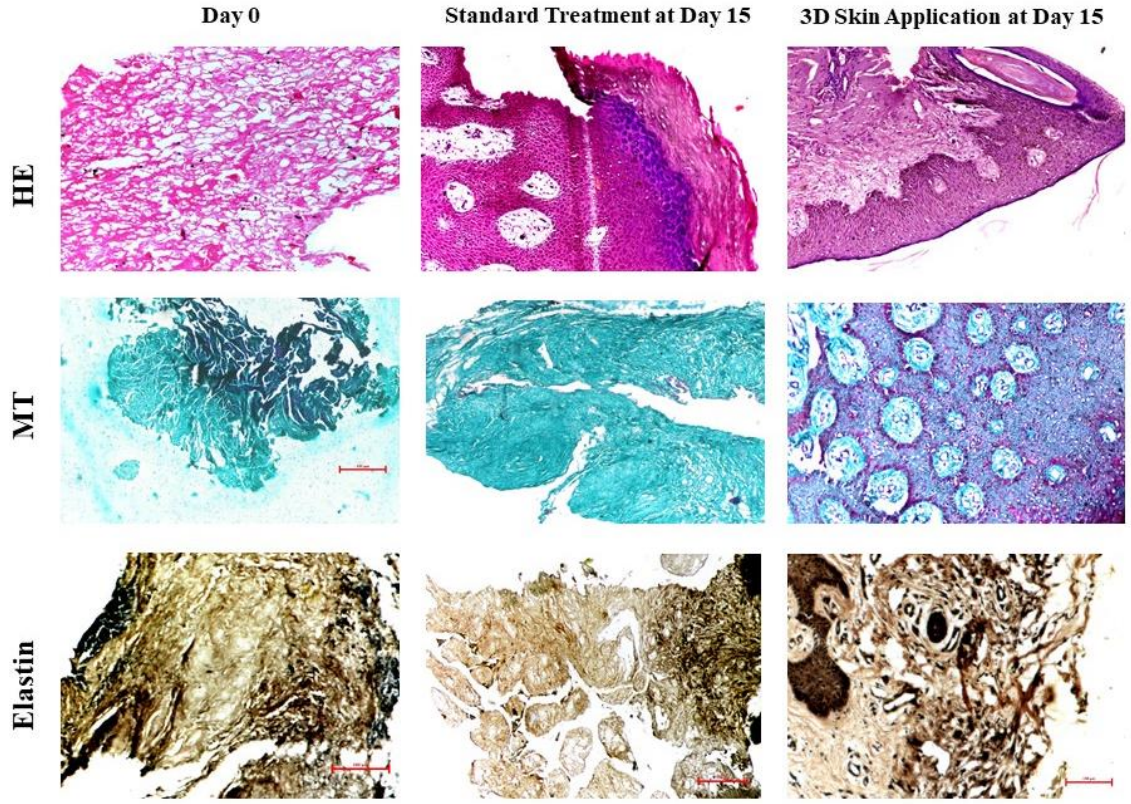

Figure 5.3:

Fig.5.3: Histological evaluation of wound healing with the application of 3D-printed skin compared to standard treatment at day 15 and the untreated wound at day 0. Hematoxylin and eosin (HE) staining (top row) ; Masson's trichrome (MT) staining (middle row); Elastin staining (bottom row)

5.3.4. mRNA Analysis:

The mRNA analysis for the 3D-printed skin treatment group revealed robust and consistent upregulation of key genes involved in tissue repair, inflammation regulation, collagen synthesis, angiogenesis, and cellular activity, highlighting the effectiveness of this treatment for wound healing.

TGF-beta (Transforming Growth Factor-beta): TGF-beta plays a pivotal role in tissue repair, cellular differentiation, and extracellular matrix production. The fold change for TGF-beta across all patients was substantial, ranging from 61.9 to 64.5. The highest fold change (64.5) was observed in Patient 4, suggesting the 3D-printed skin treatment triggered significant tissue regeneration and the restoration of the skin architecture. The robust expression of TGF-

beta indicates active wound healing processes, as TGF-beta facilitates fibrosis, wound contraction, and collagen deposition, critical for wound closure.

IL-10 (Interleukin-10): IL-10 is a potent anti-inflammatory cytokine that helps to reduce inflammation and create a favourable environment for tissue repair. All patients exhibited high fold changes in IL-10 expression (ranging from 54.1 to 56.3). This upregulation signifies that the 3D-printed skin treatment not only reduced inflammation at the wound site but also prevented excessive scarring and promoted a healing environment conducive to tissue regeneration. This suggests that IL-10 anti-inflammatory effects are crucial for maintaining a balance between inflammation and repair during wound healing.

COL-1 (Collagen Type 1): Collagen Type 1 is the main structural protein in the extracellular matrix and is essential for skin integrity and wound healing. The fold changes for COL-1 ranged from 11.3 to 12.7 across patients, indicating substantial collagen synthesis. Patient 2, with a fold change of 12.7, showed the most prominent increase, reflecting robust collagen deposition, which is vital for strengthening the wound area and improving the structural integrity of the newly formed tissue. This enhanced collagen synthesis suggests that 3D-printed skin treatment promotes efficient extracellular matrix remodelling, leading to better wound healing outcomes.

VEGF (Vascular Endothelial Growth Factor): VEGF is a fundamental growth factor that drives angiogenesis, enabling the formation of new blood vessels from the existing vascular system. Its upregulation is essential for supplying nutrients and oxygen to the regenerating tissue. VEGF fold changes ranged from 3.62 to 3.70 across all patients, indicating effective vascularization of the wound site. The significant increase in VEGF expression demonstrates that the 3D-printed skin treatment promotes angiogenesis, ensuring that the newly formed tissue receives adequate blood supply to support its growth and function.

MMPs (Matrix Metalloproteinases): MMPs are enzymes that break down extracellular matrix components and are crucial for tissue remodelling during wound healing. The analysis showed significant upregulation of various MMPs, particularly **MMP-13**, which showed the highest fold change (3.42 to 3.49) among the MMPs studied. Other MMPs such as **MMP-3**, **MMP-9**, **MMP-1**, and **MMP-2** also exhibited significant increases, suggesting active tissue remodelling. This remodelling is critical for wound closure, proper collagen deposition, and tissue maturation. The upregulation of these MMPs indicates that the 3D-printed skin treatment not only accelerates wound healing but also facilitates the proper organization and integration of newly formed tissue into the existing wound site.

FOXM1 (Forkhead Box M1): FOXM1 is a transcription factor associated with cell proliferation, survival, and differentiation, and it plays an essential role in tissue regeneration.

The fold changes for FOXM1 ranged from 3.01 to 3.15, indicating increased cellular proliferation. This suggests that the 3D-printed skin treatment promoted enhanced cellular activity at the wound site, contributing to faster healing and tissue regeneration. FOXM1 upregulation further supports the idea that the treatment not only promotes extracellular matrix formation but also stimulates active cellular regeneration to restore skin functionality.

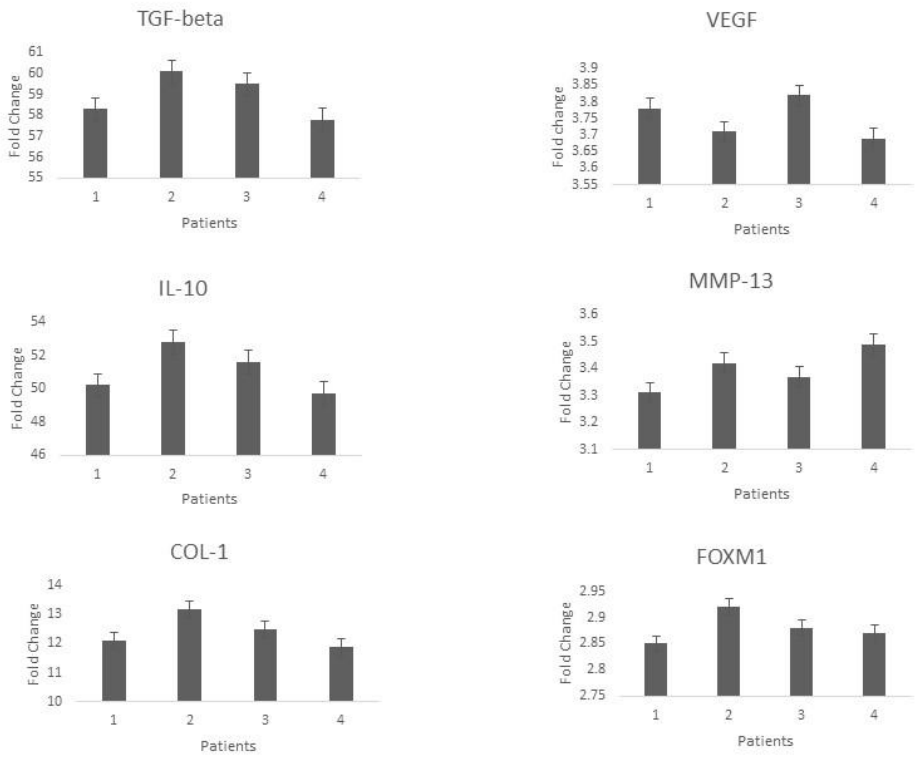

Figure 5.4:

Fig 5.4.: The mRNA analysis of the 3D-printed skin and standard treatment

5.4 Discussion

Advances in 3D bioprinting have revolutionized the field of wound care by enabling the development of skin substitutes that closely mimic the structure and function of natural skin. These skin substitutes are engineered using specialized bioinks containing biomaterials such as collagen and hyaluronic acid, which are known to support cell growth, migration, and tissue repair (Zhu et al., 2023). The ability to design and fabricate skin that behaves like natural tissue has opened up new possibilities for treating chronic and complex wounds, including burns, diabetic ulcers, and surgical wounds. In particular, 3D-printed skin has demonstrated its efficacy in promoting faster and better healing outcomes in clinical and experimental settings.

In our study, 3D-printed skin significantly accelerated wound healing compared to standard treatment methods. On Day 0, wounds were characterized by extensive tissue damage and the presence of necrotic tissue. However, by Day 3, granulation tissue had started forming, indicating the initiation of the healing process. By Day 9, there was evidence of angiogenesis, as new blood vessels developed, creating a better wound environment. By Days 15 to 21, wounds treated with 3D-printed skin showed significant size reduction and the formation of robust new skin layers. In contrast, wounds treated with standard methods exhibited slower progress, with complete closure often delayed until Days 19 to 32 (Damle et al., 2023).

Histological analysis further confirmed the superior healing outcomes with 3D-printed skin. Hematoxylin and Eosin (H&E) staining revealed early tissue formation by Day 3, accompanied by minimal inflammation. By Day 9, early signs of re-epithelialization were visible, and by Day 15, the wounds were fully closed with well-formed, organized skin layers. In contrast, the standard group showed slower healing, with persistent inflammation and incomplete restoration of skin layers even by the later stages of observation. Collagen organization, a critical factor for tissue strength and flexibility, was significantly better in the 3D-printed skin group. By Day 15, Masson's Trichrome staining demonstrated dense and well-aligned collagen fibers, which are essential for forming strong and flexible tissue. In comparison, collagen in the standard treatment group appeared scattered and disorganized, reflecting slower and less effective healing (Yang et al., 2023). Elastin, a protein that provides elasticity to the skin, also developed faster in the 3D-printed skin group, resulting in the formation of more durable and flexible tissue. In contrast, elastin deposition was slower in the standard group, contributing to weaker and less resilient skin (Yang et al., 2023).

Immunohistochemistry analyses provided further insights into the molecular mechanisms underlying the enhanced healing observed with 3D-printed skin. Levels of key markers such as VEGF, which is critical for angiogenesis, and TGF-beta, which supports ECM remodelling and tissue repair, were significantly elevated in the 3D-treated wounds. Additionally, anti-inflammatory markers like IL-10 were higher in the 3D-printed group, creating a favourable environment for healing by reducing chronic inflammation. In contrast, these markers were significantly lower in the standard treatment group, which contributed to slower healing and prolonged inflammatory phases (Yang et al., 2023).

Gene expression analyses corroborated these findings, revealing that the 3D-printed skin activated genes associated with tissue repair, angiogenesis, and inflammation control. This suggests that the 3D-printed skin not only provides a physical scaffold for tissue growth but also promotes a well-coordinated biological response that supports efficient wound healing.

The ability of 3D-printed skin to accelerate the healing process and improve the quality of regenerated tissue highlights its potential as a transformative therapeutic tool in wound care. By leveraging bioinks tailored to specific wound types, 3D bioprinting offers a personalized and versatile approach that addresses the unique challenges of chronic and complex wounds. However, while these findings are promising, further studies are needed to evaluate the long-term efficacy and safety of 3D-printed skin in various clinical scenarios. Future research should explore its scalability, cost-effectiveness, and performance in treating more severe and complicated wounds.

5.5 Conclusions

In conclusion, the advancement of 3D-printed skin substitutes represents a promising frontier in wound healing, with significant preclinical and clinical evidence supporting their potential to enhance tissue regeneration and accelerate wound closure. The ability of 3D-printed skin to mimic native skin architecture, facilitate rapid tissue repair, and improve the quality of the regenerated tissue underscores its clinical relevance, particularly for complex wounds such as burns, diabetic ulcers, and surgical defects. Key factors, like bioink composition, scaffold structure, and the ability to support angiogenesis and collagen organization, are critical to optimizing these constructs for real-world applications. While initial clinical trials have demonstrated promising results, further research is needed such as long-term durability, and expand the range of applications to different types of skin injuries. Ultimately, 3D-printed skin holds the potential to revolutionize wound care therapies, offering faster healing, reduced scarring, and improved functional and aesthetic outcomes for patients.

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CHAPTER 6: GENERAL DISCUSSION

The two studies detailed in this thesis present innovative advancements within the domain of regenerative medicine, emphasizing transformative approaches regarding wound healing using tissue engineering, platelet-rich plasma (PRP), and 3D bioprinting. This discussion aims to consolidate and critically analyse the findings, compare them with existing literature, and explore their implications for future research and clinical application.

The development of a nanopolymer scaffold incorporated with PRP growth factors represents a significant stride toward addressing challenges in chronic and acute wound management. Chronic wounds, such as diabetic ulcers and pressure sores, pose persistent medical obstacles owing to their prolonged healing phases, heightened infection risks, and significant socioeconomic burden. Acute wounds, while often less complex, can benefit from advanced interventions to expedite recovery and minimize complications. The clinical findings in this thesis underscored the multifaceted role of scaffold, including providing mechanical support, stimulating cellular proliferation, and offering antibacterial activity (Ahmed et al., 2022). A crucial innovation of the nanopolymer scaffold was its antibacterial properties, which addressed a critical gap in wound care by preventing secondary infections—a common complication in chronic wounds. Chronic wounds are particularly susceptible to microbial colonization due to the prolonged exposure of the wound bed and the compromised immune response of patients. By integrating functional nanomaterials with antimicrobial capabilities, the scaffold effectively mitigated infection risks while promoting regeneration. This dual-action approach—combining antimicrobial activity with regenerative potential—has garnered significant attention in contemporary wound management literature (Khosravimelal et al., 2023). The antibacterial properties were attributed to the incorporation of nanomaterials, such as silver nanoparticles or other bioactive agents, which disrupt bacterial membranes and inhibit microbial proliferation. These properties are especially critical in combating multidrug-resistant strains, which pose a growing threat in clinical settings. These attributes align well with the principles of tissue engineering, which advocate for materials that mimic the natural extracellular matrix (ECM), thereby facilitating tissue remodelling (Kim et al., 2023).

The porous architecture of the nanopolymer scaffold, with pore sizes ranging from 2 to 20 μm, was critical in enhancing cellular infiltration and vascularization (Zhou et al., 2023). These features are essential for optimizing the wound healing environment, as the pores not only provide space for cellular migration but also support the development of a robust capillary network. Oxygen and nutrient delivery—both vital for cellular metabolism and survival—are contingent upon effective vascularization. By mimicking the intricate network-like structures of native skin, the scaffold enhances angiogenesis and promotes efficient wound closure. This

biomimetic design is consistent with contemporary tissue engineering strategies, which prioritize replicating the hierarchical complexity of native tissues.

Furthermore, the incorporation of PRP—a concentrated source of growth factors such as vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), and insulin-like growth factor (IGF)—amplifies the regenerative potential of scaffold. These growth factors play pivotal roles in wound healing by stimulating angiogenesis, promoting fibroblast activity, and facilitating ECM deposition (Shah et al., 2022). VEGF, for instance, is integral to the formation of new blood vessels, ensuring adequate oxygenation and nutrient supply to the wound bed. PDGF aids in recruiting fibroblasts and smooth muscle cells, while IGF enhances cellular proliferation and differentiation. The sustained release kinetics of these bioactive molecules, observed over a four-month period, ensured prolonged bioactivity, a feature particularly advantageous in chronic wounds characterized by delayed healing phases (Rahman et al., 2023). Sustained delivery minimizes the need for repeated applications and maintains a conducive microenvironment for tissue regeneration.

The nanopolymer was perfectly efficient in promoting faster wound healing with reduced scarring apparently improved functional recovery. This was proven from clinical observations. Chronic wounds have become a substantial challenge as it is associated with prolonged inflammation, delayed healing, and excessive scarring. In the need of finding advanced solutions instead of traditional methods, we have used these scaffolds with significant potential of wound healing. The results of histology and PCR showed the scaffold's mode of action. The difference between treated and control wound samples was the rate of wound healing. The nanopolymer treated wound showed organized collagen deposition and enhanced epithelization, both are required for successful wound healing. The disorganized collagen is more often observed in vasculopathy. But in the nanopolymer treated group the alignment of collagen proved a natural healing. This is an indicator of vasculogenesis. The enhanced epithelialization observed also suggests that the nanpolymer promotes faster re-epithelialization, which is essential for wound closure and reducing the risk of infection. The results from PCR analysis proved the bioactive compounds present in the nanopolymer are responsible for wound environment at a cellular level. The upregulation of key genes promoted the inflammation process and reduction in wound while the pro-inflammatory cytokines were down-regulated. The nanopolymer promotes the wound healing from chronic to acute as well as modulates the inflammatory response. The chronic wounds have prolonged inflammatory reaction which hinders the wound healing. This damages the tissue. The nanopolymer controls the release of bioactive molecules which modulates the immune response, accelerating wound

healing process from prolonged inflammation to proliferative phase. The PRP present in the nanopolymer releases the fibroblast growth factor and other growth factors. The release of growth factors attracts the endothelial cells to the site of injury. This is the beginning of the proliferative phase. The shift is important for progression of wound healing phase which ultimately leads to faster times and reduced scar formation.

The clinical inferences of these findings are reflective. Chronic wounds are a major source of morbidity for patients, often leading to pain, loss of mobility, and decreased quality of life. The ability of nanopolymer to accelerate healing, reduce scarring, and enhance functional recovery could provide valuable therapeutic option for patients suffering from the chronic wounds. Furthermore, the scar-less healing could improve the aesthetic outcomes of wound healing, which is often a significant concern for patients, particularly those with visible wounds. This aligns with existing literature suggesting that advanced biomaterials, such as scaffolds, can significantly improve patient outcomes by addressing both the functional and aesthetic aspects of wound healing. For example, Tardalkar et al. (2023) demonstrated that advanced biomaterials not only promote faster tissue repair but also reduce the formation of excessive scars, ultimately enhancing the patient quality of life.

The results of this study highlight the potential of scaffolds in improving chronic wound healing outcomes. Through histological and PCR analyses, we have demonstrated that the scaffold promotes faster wound healing by reducing inflammation, supporting organized collagen deposition, and enhancing epithelialization. These effects are likely to result in reduced scarring and improved functional recovery, which are critical for the successful management of chronic wounds. Given the persistent challenges associated with chronic wound care, the findings from this study provide a promising avenue for developing more effective treatments for patients with difficult-to-heal wounds.

The mechanical robustness of the nanopolymer scaffold was instrumental in maintaining wound coverage and facilitating uniform healing. Mechanical stability is a crucial parameter in wound dressings, as it ensures the scaffold remains intact and adheres to the wound bed, providing consistent support throughout the healing process. The scaffold mechanical properties were tailored to withstand the stresses and strains encountered in dynamic environments, such as joint areas or areas prone to movement. This feature underscores its clinical utility, as mechanically robust scaffolds are less likely to fail or require frequent replacement, thereby reducing patient discomfort and healthcare costs (Liang et al., 2023).

The second study elucidated the potential of a biologically functional bioink derived from ECM components, primarily collagen, for 3D printing artificial skin. The bioink formulation, which

incorporated gelatin and PVA for enhanced mechanical strength, demonstrated superior spreadability and injectability—properties essential for consistent 3D printing and clinical handling (Jacob S., et al., 2021). The presence of collagen bundles on the surface of the bioink and the absence of DNA further highlighted its biocompatibility and minimized the risk of adverse inflammatory responses.

Biodegradability assessments revealed that the bioink scaffold provided structural integrity conducive to cellular adhesion, migration, and proliferation—core processes in tissue repair. FTIR analysis confirmed the presence of essential ECM components, validating its potential to replicate natural skin architecture and foster regenerative processes (Ewdah TM., et al., 2024). These findings align with recent advancements in bioinks that emphasize porosity, print fidelity, and biodegradability as critical parameters for effective wound healing applications (Wang Y., et al., 2021).

Extensive in vitro and in vivo studies supported the efficacy of 3D-printed skin scaffolds in promoting wound closure. MTT-cytocompatibility assays demonstrated increased metabolic activity of cord blood mononuclear cells (MNCs) in response to the bioink, while scratch assays revealed enhanced cell migration facilitated by the scaffold ECM-like properties. These results corroborate the growing body of research highlighting the pivotal role of ECM-derived bioinks in accelerating wound healing (Damle M., et al., 2023).

The scaffold's utility was further validated in animal models, where histological observations revealed increased collagen deposition, enhanced epithelialization, and robust neovascularization compared to control groups (Damle M., et al., 2023). Notably, the expression of critical markers like α-SMA, E-cadherin, and VCAM-1 confirmed the scaffold's ability to restore epithelial polarity and support keratinocyte adhesion, essential for scar-free wound repair.

Both the PRP-incorporated nanopolymer scaffold and the 3D-printed skin scaffold offer substantial advances in the field of wound healing, though each utilizes distinct mechanisms and strategies. The PRP-incorporated nanopolymer scaffold focuses on the controlled release of growth factors, combined with inherent antibacterial properties that reduce infection risks, thus potentially shortening hospital stays and minimizing complications (Ahmed et al., 2022). This approach has shown promise in promoting tissue regeneration, but it requires further optimization to cater to specific wound types and a more thorough evaluation of its long-term safety, especially in diverse patient populations (Rahman et al., 2023). Additionally, challenges related to scalability and cost-effectiveness need to be addressed before widespread clinical adoption can be realized.

Histologically, both scaffolds promoted significant improvements in wound healing compared to traditional methods. In our study, the 3D-printed skin scaffold accelerated wound closure, with granulation tissue forming early, angiogenesis occurring by Day 9, and near-complete wound closure by Day 15. In contrast, wounds treated with conventional methods exhibited delayed healing, with complete closure only achieved by Day 19 or later. Masson's Trichrome and H&E staining showed that the 3D-printed skin scaffold promoted better collagen organization and elastin formation—two critical components for tissue strength and flexibility. By Day 15, the 3D-treated wounds had dense, aligned collagen fibers and a more structured ECM, while the standard treatment group demonstrated scattered, disorganized collagen, suggesting delayed healing and weaker tissue formation. The faster collagen and elastin formation observed in the 3D group is indicative of enhanced tissue repair and suggests that this scaffold provides a more robust regenerative environment.

Immunohistochemical analysis further corroborated these findings, with increased expression of key markers involved in tissue regeneration, such as VEGF (vascular endothelial growth factor) and TGF-beta (transforming growth factor beta), in the 3D-printed skin scaffold group. These factors are essential for angiogenesis and tissue remodelling, both crucial stages of effective wound healing. Additionally, we observed elevated levels of anti-inflammatory cytokines, such as IL-10, in the 3D-treated wounds, promoting a favorable healing environment by reducing chronic inflammation. In contrast, the standard treatment group displayed lower expression of these markers, resulting in prolonged inflammation and delayed healing.

Gene expression analysis also supported the histological findings, revealing upregulation of genes involved in tissue repair, collagen synthesis, and immune modulation in the 3D-treated wounds. These results provide molecular evidence that the 3D-printed skin scaffold not only accelerates the physical aspects of healing but also promotes the activation of key genes that regulate the wound healing process.

Despite the promising outcomes of both technologies, several translational hurdles remain. For the nanopolymer scaffold, optimizing the growth factor release system for various wound types and further assessing its safety in diverse patient populations are key next steps. Additionally, the scalability and cost-effectiveness of its production remain concerns that must be addressed for broader clinical application (Rahman et al., 2023). Similarly, while the 3D-printed skin scaffold shows significant promise, large animal studies are needed to further explore its biocompatibility and long-term efficacy in human-like wound models. Furthermore, the development of portable 3D printers that can be used in real-time clinical settings is crucial for

enhancing the utility of this technology, especially in urgent or remote scenarios (Khosravimelal et al., 2023).

Both the PRP-incorporated nanopolymer scaffold and the 3D-printed skin scaffold represent exciting, complementary advancements in tissue engineering. These technologies address the multifactorial nature of wound healing, leveraging distinct mechanisms to promote tissue regeneration, control inflammation, enhance angiogenesis, and accelerate wound closure. While both hold significant promise, further optimization, large-scale clinical trials, and technological advancements are necessary to overcome current challenges and ensure their widespread clinical adoption. These studies exemplify the transformative potential of bioengineering and nanotechnology in regenerative medicine, with the possibility of improving patient outcomes and advancing the future of wound care.

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CHAPTER 7: GENERAL CONCLUSION

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This thesis has explored innovative strategies in regenerative medicine through the integration of nanotechnology, platelet-rich plasma (PRP), and 3D bioprinting, targeting significant challenges in wound healing. The presented studies underscore the potential of nanopolymer scaffolds and bioink-based 3D printing to revolutionize approaches to chronic and acute wound care.

The findings reveal that PRP-incorporated nanopolymer scaffolds provide a multifaceted solution by offering mechanical support, sustained release of growth factors, and antibacterial properties. These scaffolds not only facilitate cellular proliferation and tissue regeneration but also address critical issues such as infection control and extended healing times in chronic wounds. Clinical evaluations have demonstrated significant improvements in healing outcomes, reduced scarring, and enhanced functional recovery, marking a step forward in tissue engineering.

The development of biologically functional ECM-based bioinks for 3D bioprinting adds another dimension to the field by allowing customized solutions tailored to individual wound characteristics. The bioinks exhibited superior cytocompatibility, structural fidelity, and biodegradability while promoting cell adhesion, migration, and neovascularization. Preclinical studies validated their efficacy in wound closure and epithelial regeneration, demonstrating their readiness for further translational studies.

Both approaches align with the principles of regenerative medicine by addressing key aspects of the wound healing process, such as angiogenesis, inflammation regulation, and tissue remodelling. Their ability to harness biomimetic properties while integrating cutting-edge technology reflects the ongoing transition from conventional to personalized, patient-specific therapeutic solutions.

Despite their promise, challenges such as scalability, cost-effectiveness, regulatory hurdles, and long-term safety need to be addressed to enable widespread clinical adoption. Furthermore, collaborative efforts across interdisciplinary fields are essential for refining these technologies and ensuring their alignment with ethical and regulatory frameworks.

In conclusion, the innovations explored in this thesis have not only expanded the horizons of wound care technologies but have also laid the groundwork for future research in regenerative medicine. By bridging the gap between basic science and clinical application, these strategies hold the potential to significantly improve patient outcomes and redefine the standards of care for complex wounds. With continued advancements, these technologies promise to enhance global healthcare accessibility and impact, fulfilling a critical need in modern medicine.

CHAPTER 8: SUMMARY

The two studies explored in this thesis represent significant advancements in the field of regenerative medicine, particularly in the context of wound healing. Both studies focus on innovative approaches: the creation of a nanopolymer scaffold incorporated with platelet-rich plasma (PRP) and the formulation of a biologically functional bioink for 3D bioprinted skin. These approaches aim to tackle the complex challenges of chronic and acute wounds by employing state-of-the-art technologies in nanotechnology, bioengineering, and tissue engineering.

The first study concentrates on the development of a nanopolymer scaffold that incorporates PRP growth factors to enhance the healing process. This novel scaffold addresses several key issues in wound care, such as tissue regeneration, infection control, and the promotion of angiogenesis. A key strength of this scaffold is its ability to provide mechanical support, stimulate cellular proliferation, and deliver growth factors in a controlled manner. These characteristics are essential for accelerating wound healing, particularly in chronic wounds, which often face prolonged healing times and complications due to infections.

The scaffold features a porous nanopolymer structure with pores ranging from 2 to 20 μm. This architecture is crucial for improving cellular infiltration and blood vessel formation, both of which are vital for tissue repair. The design is in line with the principles of tissue engineering, which stress the importance of creating materials that mimic the natural extracellular matrix (ECM) to encourage tissue remodeling. The pore size is carefully optimized to facilitate oxygen and nutrient delivery, while also promoting the growth of capillary networks necessary for healing. Additionally, the incorporation of PRP into the scaffold, containing growth factors like vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), and insulin-like growth factor (IGF), enhances its regenerative properties. These growth factors are vital for stimulating blood vessel formation, facilitating ECM deposition, and promoting cellular proliferation.

Clinical observations show that the nanopolymer scaffold promotes faster wound healing, reduces scarring, and improves functional recovery. The sustained release of growth factors from the scaffold helps regulate the inflammatory response, accelerating tissue repair and improving patient outcomes. The mechanical stability of the scaffold is another significant feature, as it enables the scaffold to maintain its shape and provide consistent coverage during the healing process. This stability is particularly important for chronic wounds, where healing can often be delayed by instability at the wound site.

One of the most promising features of the nanopolymer scaffold is its antibacterial properties. Infection is a major concern in wound care, especially in chronic wounds where the immune

system may be compromised. The integration of functional nanomaterials with antimicrobial capabilities addresses this problem by reducing the risk of secondary infections, which are common in chronic wounds. This dual-action approach—fostering tissue regeneration while also preventing infection—has garnered significant attention in the field of wound care, with the potential to transform how we approach wound management.

The second study in this thesis focuses on the potential of a biologically functional bioink made from ECM components, primarily collagen, to create 3D-printed artificial skin. This approach builds on the principles of tissue engineering and regenerative medicine by using a bioink that closely resembles the natural ECM of human skin. The bioink formulation includes gelatin and polyvinyl alcohol (PVA) to improve its mechanical strength, spreadability, and injectability—all of which are essential for successful 3D printing and clinical use. The collagen-based bioink is designed to promote cellular adhesion, migration, and proliferation—key processes necessary for tissue repair.

Biodegradability assessments confirmed that the bioink scaffold provides the necessary structural integrity to support cellular processes, allowing cells to adhere and migrate across the scaffold. Fourier-transform infrared (FTIR) analysis also validated the presence of important ECM components, confirming the bioink's potential to replicate the natural structure of skin and promote regenerative processes. The presence of collagen bundles on the surface of the bioink and the absence of DNA further highlighted its biocompatibility and minimized the risk of adverse inflammatory reactions, making it a promising candidate for wound healing applications.

A series of in vitro and in vivo studies were conducted to assess the effectiveness of the 3D-printed skin scaffold in promoting wound closure. MTT-cytocompatibility assays demonstrated that the bioink supported increased metabolic activity of the cord blood mononuclear cells (MNCs), suggesting that it enhanced cell viability. Scratch assays, which measure cell migration, showed that the bioink facilitated enhanced migration, further supporting its potential to accelerate wound healing. These results align with the growing body of research emphasizing the role of ECM-derived bioinks in advancing tissue regeneration, especially in the context of wound healing.

The efficacy of the 3D-printed skin scaffold was further validated in animal models, where histological analysis revealed increased collagen deposition, improved epithelialization, and robust neovascularization compared to control groups. These findings indicate that the bioink scaffold plays an important role in tissue repair by enhancing key aspects of the healing process. The expression of critical markers such as alpha-smooth muscle actin (α-SMA), E-cadherin,

and vascular cell adhesion molecule 1 (VCAM-1) further confirmed the scaffold's ability to restore epithelial polarity and support keratinocyte adhesion—both essential for scar-free wound repair.

Both studies presented in this thesis represent important advancements in wound healing, though they take different approaches. The PRP-incorporated nanopolymer scaffold emphasizes sustained growth factor release and infection control, while the 3D-printed skin scaffold focuses on mimicking the ECM properties of natural skin and customizability. These innovations highlight complementary strategies that address the multifaceted nature of wound healing, including inflammation control, angiogenesis, and tissue remodeling.

A major advantage of the nanopolymer scaffold is its antibacterial properties, which help minimize the risk of sepsis and can potentially shorten hospital stays for patients. The scaffold's ability to release growth factors over a long period ensures its bioactivity and effectiveness, even in chronic wounds where healing is typically delayed. In contrast, the 3D-printed skin scaffold offers a unique solution for large or irregular wounds, particularly when integrated with bedside 3D printers for on-demand graft fabrication. The customizability of the 3D-printed skin scaffold allows for personalized treatment plans, catering to the individual needs of each patient, which aligns well with the increasing focus on personalized medicine in regenerative medicine.

While both technologies show great promise, there are still several challenges to overcome before they can be widely adopted in clinical practice. The nanopolymer scaffold requires further optimization to address the specific needs of different wound types, as not all chronic or acute wounds may respond similarly. Long-term safety assessments are needed to evaluate the scaffold's effectiveness and safety over extended periods, especially in diverse patient populations. Additionally, the scalability and cost-effectiveness of the nanopolymer scaffold must be thoroughly examined to ensure its accessibility for widespread use.

Similarly, the 3D-printed skin scaffold requires further research using large animal models to confirm its therapeutic efficacy and biocompatibility. While initial in vitro and small animal studies show promise, large animal studies are essential to better understand how the scaffold performs in more complex biological systems. The development of portable, clinically adaptable 3D printers could also enhance the utility of this technology, particularly in emergency or remote settings where immediate wound care is needed.

Both technologies have the potential to address critical healthcare challenges, including chronic wounds, diabetic ulcers, and burn injuries, which place significant social and economic burdens on healthcare systems. By reducing wound healing times, minimizing infection risks, and

lowering treatment costs, these technologies could improve patient outcomes and overall quality of life. The ability of the 3D-printed skin scaffold to be customized for individual patients is especially important in personalized medicine, allowing for more tailored treatments. Similarly, the versatility of the nanopolymer scaffold suggests that it could have applications beyond wound healing, such as in cartilage or vascular repair, further expanding its impact in regenerative medicine.

Both technologies will need to navigate rigorous regulatory pathways to ensure their safety and efficacy before they can be widely used in clinical practice. These regulatory processes will need to address product consistency, manufacturing standards, and long-term safety. Ethical considerations surrounding the sourcing of biological materials for bioinks will also need to be carefully managed to ensure that these technologies are widely accepted by society. Establishing standardized protocols for clinical trials and manufacturing processes will be essential for the successful integration of these technologies into healthcare settings.

In conclusion, the two studies presented in this thesis demonstrate the transformative potential of nanotechnology and bioengineering in regenerative medicine. By harnessing the unique mechanisms of action in the PRP-incorporated nanopolymer scaffold and the ECM-derived bioink for 3D-printed skin, these studies showcase how emerging technologies can address significant challenges in wound care. These innovations offer promising possibilities for improving patient outcomes and advancing clinical practice. Future research should focus on optimizing these technologies, expanding their applications, and ensuring their accessibility, paving the way for a new era in regenerative medicine that can significantly improve the healing process for both chronic and acute wounds.

CHAPTER 9: RECOMMENDATIONS

The research in this thesis has made major contributions to nanotechnology, tissue engineering, and regenerative medicine, focusing on wound healing. It takes an interdisciplinary approach, bringing together advanced biomaterials, cellular biology, and clinical observations. This work directly addresses the tough challenges that traditional treatments face when dealing with chronic and acute wounds.

Key Findings

1. **Nanopolymer Scaffolds with PRP Integration:**
   - The scaffolds were safe for the body, provided strong support, and fought bacteria, making them great for healing chronic wounds.
   - They slowly released growth factors like VEGF, PDGF, and IGF over months, helping new blood vessels grow, reducing swelling, and speeding up skin repair.
   - Their ability to fight infections solved a big problem that traditional treatments often struggle with the available material.
2. **Biologically Functional ECM-Based Bioinks for 3D Skin Printing:**
   - The bioinks made with collagen, gelatin, and PVA successfully mimicked the skin's natural structure, helping cells stick and move where needed.
   - Preclinical tests showed better wound healing, with more collagen production and less scarring compared to standard methods.

New Ways to Heal Wounds:

- Both technologies tackled key challenges like reducing inflammation, encouraging blood vessel growth, and helping tissues rebuild.
- Custom 3D-printed skin and nanopolymer scaffolds offer flexible solutions for different wound types, paving the way for personalized treatments.

These breakthroughs show how combining nanotechnology, platelet-derived growth factors, and bioprinting can revolutionize regenerative medicine. They provide powerful, adaptable tools to improve wound care and help patients heal faster, benefiting dermatology, plastic surgery, and trauma care.

Clinical Recommendations

- **Immediate Translation for Chronic Wound Management:**

  The PRP-based nanopolymer scaffolds, with their prolonged bioactivity and antimicrobial properties, can be immediately scaled for clinical trials targeting diabetic ulcers and non-healing chronic wounds.

- **Hospital-Integrated 3D Bioprinting Systems:**
  Bedside 3D printers using the bioinks developed in this study should be integrated into tertiary care centers and specialized burn units to provide tailored skin graft solutions in emergency cases or large-area wounds.
- **Combining Therapies:**
  Implementing both approaches—nanopolymer scaffolds for base preparation and 3D-printed skin grafts for surface repair—can address the entire spectrum of wound healing. For example, the scaffold could create a pro-regenerative environment, while the bioink scaffold completes skin restoration.
- **Streamlining Regulatory Approvals:**
  Encourage policymakers to develop specific frameworks for evaluating biomaterials and bioprinting platforms, enabling faster regulatory clearances and reducing timelines for clinical trials.
- **Promoting Subsidies for Innovative Technologies:**
  Facilitate funding and public-private partnerships for scaling regenerative medicine technologies to make them accessible to underprivileged populations, especially in rural or resource-limited settings.
- **Curriculum Integration:**
  Introduce dedicated modules on nanotechnology, 3D printing, and regenerative medicine in the medical and biomedical engineering curriculum to prepare future professionals for industry advancements.
- **Collaborative Research Programs:**
  Universities should establish interdisciplinary centers for nanotechnology and regenerative medicine, bridging gaps between basic science and clinical applications.
- **Expanding Scaffolding Applications:**
  Investigate the use of PRP-nanopolymer scaffolds for other tissue engineering applications, such as bone, cartilage, or nerve regeneration, based on their demonstrated compatibility and regenerative properties.
- **Exploring Diverse Bioink Formulations:**
  Leverage bioactive molecules, stem cells, and advanced ECM materials in bioink formulations to achieve next-generation therapeutic outcomes, particularly for complex wounds, burns, or diabetic complications.

- **Integrating Artificial Intelligence:**
  AI-based models should be developed to predict the behavior of cells in bioengineered scaffolds, accelerating the design of optimized biomaterials tailored to specific wounds.

Future Findings and Directions

While this study has achieved great progress in using biomaterials for wound healing, more work is needed to make these breakthroughs practical and widely useful in real-world healthcare.

1. **Scaling Up Production**
   - Develop cheaper ways to make nanopolymer scaffolds and bioinks without losing quality.
   - Automate 3D printing for reliable and consistent skin grafts.
   - Create strict quality checks to ensure the treatments work every time.
2. **Testing for the Long Run**
   - Run large clinical trials across different hospitals to study long-term safety and effectiveness.
   - Check how these materials work for people of different ages, genders, and with conditions like diabetes.
3. **Personalized Treatments**
   - Use a patient's own cells in bioinks to lower the risk of rejection and improve healing.
   - Upgrade 3D printing to include blood vessel networks for better healing of tough wounds.
4. **Exploring New Materials**
   - Try materials like silk, chitosan, or alginate to add new features and strengths to scaffolds.
   - Add nanoparticles to deliver medicine directly to wounds for infections or swelling.
5. **Connecting Research to Clinics**
   - Bring researchers, doctors, and engineers together to refine and test these ideas in real healthcare settings.
   - Set up labs in universities to show how these technologies can be used practically.
6. **Making it Sustainable and Accessible**

- Find cheaper alternatives to expensive materials, like plant-based or synthetic options.
- Create affordable systems that can work in low-resource areas, especially in places like rural India.

7. **Going Beyond Wound Healing**
    - Use PRP scaffolds to help repair cartilage, tendons, or blood vessels.
    - Adapt 3D bioinks to model or regenerate organs like livers or kidneys.
    - Explore these technologies for conditions like diabetic gangrene or other blood flow problems.

These innovations—PRP-loaded nanopolymer scaffolds and ECM-based bioinks—are changing the way we think about wound care. They provide real solutions for tough injuries, blending natural healing principles with advanced technology.

To make these ideas a reality, we need to close the gap between lab research and clinical practice. This means creating smoother paths to clinical trials, increasing funding for biomaterials, and fostering teamwork among researchers, doctors, and regulators.

Regenerative medicine is growing fast, and these findings highlight how personalized, integrative approaches can tackle not just wounds but many medical challenges. With continued effort, these innovations could reshape healthcare by improving outcomes, cutting costs, and making advanced treatments available to everyone.

ANNEXURES

PUBLICATIONS AND CERTIFICATES

D. Y. PATIL MEDICAL COLLEGE KOLHAPUR

Constiuent Unit of D. Y. Patil Education Society (Deemed to be University),Kolhapur.
Re-accredited by NAAC with 'A' Grade

Dr. Rakesh Kumar Sharma
Dean & Professor (Obst. & Gyn)

Padmashree Dr. D. Y. Patil
Founder president

Dr. Sanjay D. Patil
President

No. DYPMCK/.....26...../2022/IEC

Date: 25 JUL 2022

INSTITUTIONAL ETHICS COMMITTEE, D. Y. PATIL MEDICAL COLLEGE, KOLHAPUR.

This is to certify that the research project titled,

"Feasibility of Nanopolymer and 3D Printed Skin in Lower Limb Wounds: Prospective Preclinical and Clinical Assessment."

Submitted by : **Ms. Mrunal N. Damle**

Under the supervision of appointed Guide (if any): **Dr. Meghnad. G. Joshi**-Guide,

Dr. Vaishali. V. Gaikwad-Co-Guide

Has been studied by the Institutional Ethics Committee (IEC) at its meeting held on **07/04/2022** and after corrected has granted approval for the study with due effect with the following caveats:

1. If you desire any change in the protocol or standard recording document at any time, please submit the same to the IEC for information and approval before the change is implemented.
2. As per recommendations of ICMR, you must register your study with the Central Trials Registry-India (CTRI), hosted at the ICMR's National Institute of Medical Statistics (http://icmr-nims.nic.in). The registration details as provided by the website are to be submitted to the Institutional Ethics Committee within a period of 3 months from issue of this letter.
3. All serious and/or unexpected adverse events due to the drug/procedures tested in the study must be informed to the IEC within 24 hours and steps for appropriate treatment must be immediately instituted.
4. In case of injury/disability/death of any participant attributable to the drug/procedure under study, all compensation is to be made by the sponsor of the study.
5. The Chief investigator/Researcher must inform the IEC immediately if the study is terminated earlier than planned with the reasons for the same.
6. The final results of the study must be communicated to the IEC within 3 months of the completion of data collection.
7. The researcher must take all precautions to safeguard the rights, safety, dignity and wellbeing of the participants in the study.
8. The researcher must be up to date about all information regarding the risk/benefit ratio of any drug/procedure being used and any new information must be conveyed to the IEC immediately. The IEC reserves the right to change a decision on the project in the light of any new knowledge.
9. Before publishing the results of the study, the researcher must take permission from the Dean of the Institution.
10. Annual progress report should be submitted for all sponsored projects to the committee.
11. Unethical conduct of research in non-sponsored projects will result in withdrawal of the ethics approval and negation of all data collected till that date.

Dr. Mrs. Shimpa R. Sharma
(Member Secretary, IEC)

Dr. (Mrs) Shimpa Sharma
Member Secretary,
Institutional Ethics Committee
D. Y. Patil Medical College,
Kolhapur - 416006

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D. Y. Patil Educational Society

Certificate of Plagiarism Check for Thesis

Author Name	Mrunal Narendra Damle
Course of Study	Ph.D.
Name of Guide	Dr. Meghnad G Joshi
Department	Department of Stem Cell and Regenerative Medicine and Medical Biotechnology, Centre for Interdisciplinary Research
Acceptable Maximum Limit	10
Submitted By	shivajikashte.cir@dypgroup.edu.in
Paper Title	Feasibility of Nanopolymer and 3D Printed Skin in Lower Limb Wounds: Prospective Preclinical and Clinical Assessment
Similarity	7%
Paper ID	3444280
Total Pages	114
Submission Date	2025-03-28 14:09:57

Signature of Student **Signature of Guide**

University Coordinator **Head of the Department**

* This report has been generated by DrillBit Anti-Plagiarism Software

Clinical Trial Details (PDF Generation Date :- Tue, 01 Oct 2024 08:28:13 GMT)

CTRI Number	CTRI/2024/09/073475 [Registered on: 05/09/2024] - **Trial Registered Prospectively**
Last Modified On	03/09/2024
Post Graduate Thesis	Yes
Type of Trial	Interventional
Type of Study	Biological
Study Design	Non-randomized, Active Controlled Trial
Public Title of Study	Use of nano polymer gel to cure diabetic foot ulcers
Scientific Title of Study	Use of nano polymer in wound healing in diabetic foot ulcer

Secondary IDs if Any

Secondary ID	Identifier
NIL	NIL

Details of Principal Investigator or overall Trial Coordinator (multi-center study)

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Source of Monetary or Material Support

Source of Monetary or Material Support
> Dr D Y Patil Education Society, Kadamwadi, Kolhapur, 416005, Maharashtra, India

Primary Sponsor

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Name	Self
Address	Dr D Y Patil Medical College Hospital and Research Institute, kadamwadi, Kolhapur
Type of Sponsor	Private medical college

Details of Secondary Sponsor

Name	Address
Dr Meghnad Joshi	Central Research Laboratory, 4th floor, Dr D Y Patil Medical College Hospital and Research Institute, kadamwadi, Kolhapur

Countries of Recruitment

List of Countries
India

Sites of Study

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Details of Ethics Committee

Name of Committee	Approval Status	Date of Approval	Is Independent Ethics Committee?
INSTITUTIONAL ETHICS COMMITTEE D Y PATIL MEDICAL COLLEGE	Approved	26/12/2022	No

Regulatory Clearance Status from DCGI

Status	Date
Not Applicable	No Date Specified

Health Condition / Problems Studied

Health Type	Condition
Patients	Type 2 diabetes mellitus with unspecified complications

Intervention / Comparator Agent

Type	Name	Details
Intervention	Nanopolymer gel	It is a composition of nanopolymers and growth factors which helps in healing diabetic foot ulcers.
Comparator Agent	Traditional conventional wound dressing	It is a standard wound dressing method used for diabetic foot ulcers, which will be followed. The method uses EUSOL-soaked gauze instead of nanopolymer covering on the site of the wound.

Inclusion Criteria

Inclusion Criteria	
Age From	18.00 Year(s)
Age To	65.00 Year(s)
Gender	Both
Details	all patients that come to OPD and IPD with diabetic foot ulcer. The patients with Hb1Ac less than 7.5. Diabetic patients with blood sugar control. Patient must be above 18 years.

Exclusion Criteria

Exclusion Criteria	
Details	All the patients who do not follow up in OPD. Patients with diabetic

	foot ulcer undergoing amputation. Patients with immunocompromised status. Patients with osteomyelitis or gangrenous ulcers. All the patients who do not give consent for the study.

Method of Generating Random Sequence	Not Applicable
Method of Concealment	Other
Blinding/Masking	Not Applicable

Primary Outcome

Outcome	Timepoints
1.Wound Healing Rate 2. Incidence of complete wound closure	4 weeks

Secondary Outcome

Outcome	Timepoints
Safety & Tolerability Quality of Wound Healing Assessment of scar Pain Reduction Patient Satisfaction & Quality of Life Cost-effectiveness	8 weeks

Target Sample Size	**Total Sample Size=**27 **Sample Size from India=**27 **Final Enrollment numbers achieved (Total)=**Applicable only for Completed/Terminated trials **Final Enrollment numbers achieved (India)=**Applicable only for Completed/Terminated trials
Phase of Trial	N/A
Date of First Enrollment (India)	01/10/2024
Date of First Enrollment (Global)	No Date Specified
Estimated Duration of Trial	**Years=**2 **Months=**0 **Days=**0
Recruitment Status of Trial (Global)	Not Applicable
Recruitment Status of Trial (India)	Not Yet Recruiting
Publication Details	N/A
Brief Summary	Nanopolymers enhance wound healing by promoting cell proliferation, reducing infection, and delivering therapeutic agents directly to the wound site. Their nanoscale size allows for better integration with biological tissues, facilitating faster and more effective tissue regeneration and repair. Hence this study is designed for evaluation of the efficacy of formulated nanopolymer for diabetic wound uclers.

Clinical Trial Details (PDF Generation Date :- Thu, 19 Dec 2024 08:23:58 GMT)

CTRI Number	CTRI/2024/11/076815 [Registered on: 14/11/2024] - **Trial Registered Prospectively**
Last Modified On	22/11/2024
Post Graduate Thesis	Yes
Type of Trial	Interventional
Type of Study	Biological
Study Design	Non-randomized, Active Controlled Trial
Public Title of Study	3D printed skin for diabetic wound healing
Scientific Title of Study	USE OF 3D PRINTED SKIN AS SKIN GRAFT IN CHRONIC NON-HEALING ULCERS

Secondary IDs if Any

Secondary ID	Identifier
NIL	NIL

Details of Principal Investigator or overall Trial Coordinator (multi-center study)

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Source of Monetary or Material Support

Source of Monetary or Material Support
> Dr. D.Y.Patil Education Society, Kadamwadi, Kolhapur, 416006, Maharashtra, India

Primary Sponsor

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Type of Sponsor	Other [SELF]

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Countries of Recruitment

List of Countries
India

Sites of Study

Name of Principal Investigator	Name of Site	Site Address	Phone/Fax/Email
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Details of Ethics Committee

Name of Committee	Approval Status	Date of Approval	Is Independent Ethics Committee?
Institutional Ethics Committee, D.Y.Pati. Medical College	Approved	26/12/2022	No

Regulatory Clearance Status from DCGI

Status	Date
Not Applicable	No Date Specified

Health Condition / Problems Studied

Health Type	Condition
Patients	Type 2 diabetes mellitus with unspecified complications

Intervention / Comparator Agent

Type	Name	Details
Intervention	3D printed skin	It is made up of xenogenic skin, polymerized with PVA to form a bioink. It has extracellular matrix which attracts cells and accelerate wound healing. The skin graft will be applied to the wounded area. The graft will have extracellular material which helps to heal the wound faster by attracting platelets to the site of injury

Comparator Agent	Autologous skin graft	Isografting is a standard procedure where the part of donar skin is applied to the wounded area. The autologous skin graft will be applied by following the standard protocol.

Inclusion Criteria

Inclusion Criteria	
Age From	18.00 Year(s)
Age To	65.00 Year(s)
Gender	Both
Details	The 3D-printed skin grafts are made up of xenogenic skin composed of extracellular matrix. This graft will be sterilized and used on the wounded area as a treatment.

Exclusion Criteria

Exclusion Criteria	
Details	All the patients who do not follow up in OPD. Patients with diabetic foot ulcers undergoing amputation. Patients with immunocompromised status. Patients with osteomyelitis or gangrenous ulcers. All the patients who do not give consent for the study.

Method of Generating Random Sequence	Not Applicable
Method of Concealment	Other
Blinding/Masking	Not Applicable

Primary Outcome

Outcome	Timepoints
The primary outcome of the study will be : 1. Acceptance of the skin graft 2. Wound healing rate 3. Incidence of wound closure	4 to 8 weeks

Secondary Outcome

Outcome	Timepoints
Safety & Tolerability Quality of Wound Healing Assessment of scar Pain Reduction Patient Satisfaction & Quality of Life Cost-effectiveness	7-8 weeks

Target Sample Size	**Total Sample Size=**26 **Sample Size from India=**26 **Final Enrollment numbers achieved (Total)=**Applicable only for Completed/Terminated trials **Final Enrollment numbers achieved (India)=**Applicable only for Completed/Terminated trials
Phase of Trial	N/A
Date of First Enrollment (India)	25/11/2024
Date of First Enrollment (Global)	No Date Specified
Estimated Duration of Trial	**Years=**2 **Months=**0 **Days=**0
Recruitment Status of Trial (Global)	Not Yet Recruiting
Recruitment Status of Trial (India)	Open to Recruitment
Publication Details	N/A

Brief Summary	The 3D printed skin grafts are made up of xenogenic skin polymerized with biological polymers lika Poly- Vinyl Alcohol. These grafts contains skin specific extracellular matrix which helps the healing of the wound if applied to site of injury. The extracellular matrix will accelerate the healing by attracting the cells and facilitating the binding of platelets to the injured site. As the diabetic wounds are most complicated wounds and have problems of healing due to different reasons; we are targeting these wounds to check the efficacy of the 3D printed skin graft. Hence this study is designed for evaluation of the efficacy of formulated artificial skin for diabetic wound uclers.

Proforma

Name: age: sex:

OPD no:

Address:

Contact:

Duration of diabetic foot ulcer:

Current mode of management:

History of diabetes:

Complete blood count:

Bsl fasting/pp

Hba1c:

Initial biopsy:

Biopsy on 2nd week

Biopsy on 4th week

Secondary Outcomes Data Collection Form

Study Title:

Patient ID: _____

Date of Treatment: _____

Follow-up Date(s): _____

1. Safety

- **Any adverse events observed? (Yes/No)**

  If Yes, describe the adverse event:
- **Severity (Mild/Moderate/Severe):**
- **Management and Outcome:**

2. Tolerability

- **Patient-reported discomfort during treatment**

  (None/Mild/Moderate/Severe):
- **Any signs of immune rejection or inflammation?**

  (Yes/No)
- **Other tolerability concerns:**

3. Quality of Wound Healing

- **Wound closure percentage at each follow-up:**
  - **Week 1: _____%**
  - **Week 2: _____%**
  - **Week 4: _____%**
  - **Week 8: _____%**
- **Presence of infection (Yes/No):**
- **Time taken for complete wound closure :**
- **Histological assessment (if performed):**

4. Scar Assessment

- **Scarring severity**

  (None/Mild/Moderate/Severe):
- **Visual Analog Scale (VAS) score for scar appearance (0-10):**
- **Patient satisfaction with scar (0-10):**

5. Pain Reduction

- **Pre-treatment pain score (0-10):**
- **Pain score at each follow-up:**
  - **Week 1: _____**
  - **Week 2: _____**
  - **Week 4: _____**
  - **Week 8: _____**
- **Use of analgesics (Yes/No):**

6. Patient Satisfaction

- **Overall satisfaction with treatment outcome (0-10):**
- **Would the patient recommend this treatment? (Yes/No):**
- **Additional comments from the patient:**

7. Cost-effectiveness

- **Estimated total cost of treatment:**
- **Comparison with conventional wound treatment cost:**
- **Additional costs incurred (if any):**
- **Overall economic feasibility assessment:**

Investigator Name: _____

Signature: _____

Date: _____

Baseline Evaluation Form

Project:

Patient Information:

- Name: _____
- Age: _____
- Gender: _____
- Contact Number: _____
- Address: _____

Medical History:

- Diabetes Mellitus: Yes / No
  - Duration: _____ years
  - Management: Diet / Oral Medications / Insulin
- Previous Wound History: Yes / No
  - If yes, details: _____
- Medications (Current): _____
- Allergies: Yes / No
  - If yes, details: _____
- Lifestyle Factors:
  - Smoking: Yes / No
  - Alcohol Consumption: Yes / No
  - Diet: _____
  - Exercise Routine: _____

Physical Examination:

- Vital Signs:
  - Blood Pressure: _____ mmHg
  - Heart Rate: _____ bpm
  - Respiratory Rate: _____ breaths/min
  - Temperature: _____ °C
- Wound Assessment:

    - Size: _____ cm
    - Depth: _____ cm
    - Exudate: Yes / No
    - Infection Signs: Yes / No
    - Surrounding Tissue Condition: _____
- Neurological Examination:
    - Peripheral Neuropathy: Yes / No
- Vascular Assessment:
    - Ankle-Brachial Index (ABI) (if applicable): _____

Laboratory Investigations:

- Complete Blood Count (CBC): _____
- Fasting Blood Sugar (FBS): _____ mg/dL
- Post-Prandial Blood Sugar (PPBS): _____ mg/dL
- Glycated Hemoglobin (HbA1C): _____ %
- Serum Creatinine: _____ mg/dL
- Blood Urea Nitrogen (BUN): _____ mg/dL
- Lipid Profile:
    - Total Cholesterol: _____ mg/dL
    - LDL: _____ mg/dL
    - HDL: _____ mg/dL
    - Triglycerides: _____ mg/dL
- Inflammatory Markers:
    - C-reactive Protein (CRP): _____ mg/L
    - Erythrocyte Sedimentation Rate (ESR): _____ mm/hr
- Wound Swab Culture (if applicable): _____

Additional Notes:

Physician's Name & Signature:

Date: _____

.

Case Report Form (CRF)

Study Title: Open-Label Multicenter Observational Study to Evaluate the Safety and Efficacy of Nanopolymer application in Male/Female Patients with Non-Healing Diabetic Wounds.

Section 1: Patient Information

1. **Patient ID: _____**
2. **Date of Birth (DD/MM/YYYY): _____**
3. **Gender:**
    - Male
    - Female
4. **Height (cm): _____**
5. **Weight (kg): _____**
6. **BMI: _____**

Section 2: Medical History

1. **Duration of Diabetes (years): _____**
2. **Type of Diabetes:**
    - Type 1
    - Type 2
    - Other (Specify): _____
3. **Current Medications:** (List all)
    - _____
    - _____
4. **Other Relevant Medical Conditions:**
    - _____
    - _____

Section 3: Wound Assessment (Baseline)

1. **Wound Location:**
    - Foot
    - Lower Leg

- Other (Specify): _____

2. **Wound Type:**
    - Ulcer
    - Surgical
    - Traumatic
    - Other (Specify): _____
3. **Wound Size (cm):**
    - **Length: _____**
    - **Width: _____**
    - **Depth: _____**
4. **Wound Duration (weeks): _____**
5. **Wound Stage:**
    - Stage 1
    - Stage 2
    - Stage 3
    - Stage 4
6. **Presence of Infection:**
    - Yes
    - No
    - If yes, specify type: _____
7. **Previous Wound Treatments:**
    -
    -

Section 4: Treatment Details

1. **Nanopolymer Treatment Start Date (DD/MM/YYYY):**

   _____
2. **Type of Nanopolymer Used:**
    - Topical Application: _____
3. **Application Frequency:**
    - (Specify): _____
4. **Concomitant Treatments Used:**
    -

Section 5: Follow-Up Visits

Follow-Up Visit 1 (Week 4):

1. **Date of Visit (DD/MM/YYYY): _____**
2. **Wound Size (cm):**
    - **Length: _____**
    - **Width: _____**
    - **Depth: _____**
3. **Wound Stage:**
    - Stage 1
    - Stage 2
    - Stage 3
    - Stage 4
4. **Presence of Infection:**
    - Yes
    - No
5. **Adverse Events Reported:**
    - Yes
    - No
    - If yes, describe: _____
6. **Patient Compliance:**
    - Yes
    - No

Follow-Up Visit 2 (Week 8):

1. **Date of Visit (DD/MM/YYYY): _____**
2. **Wound Size (cm):**
    - **Length: _____**
    - **Width: _____**
    - **Depth: _____**
3. **Wound Stage:**
    - Stage 1
    - Stage 2

- Stage 3
- Stage 4

4. **Presence of Infection:**
    - Yes
    - No
5. **Adverse Events Reported:**
    - Yes
    - No
    - If yes, describe: _____
6. **Patient Compliance:**
    - Yes
    - No

Follow-Up Visit 3 (Week 12):

1. **Date of Visit (DD/MM/YYYY): _____**
2. **Wound Size (cm):**
    - **Length: _____**
    - **Width: _____**
    - **Depth: _____**
3. **Wound Stage:**
    - Stage 1
    - Stage 2
    - Stage 3
    - Stage 4
4. **Presence of Infection:**
    - Yes
    - No
5. **Adverse Events Reported:**
    - Yes
    - No
    - If yes, describe: _____
6. **Patient Compliance:**
    - Yes

- No

Follow-Up Visit 4 (Week 16):

1. **Date of Visit (DD/MM/YYYY): _____**
2. **Wound Size (cm):**
   - **Length: _____**
   - **Width: _____**
   - **Depth: _____**
3. **Wound Stage:**
   - Stage 1
   - Stage 2
   - Stage 3
   - Stage 4
4. **Presence of Infection:**
   - Yes
   - No
5. **Adverse Events Reported:**
   - Yes
   - No
   - If yes, describe: _____
6. **Patient Compliance:**
   - Yes
   - No

Section 6: Study Completion

1. **Date of Completion (DD/MM/YYYY): _____**
2. **Overall Outcome:**
   - Healed
   - Improved
   - No Change
   - Worsened
3. **Additional Comments:**
   - _____

- _____

Investigator's Signature: _____

Date (DD/MM/YYYY): _____

Post study Follow-Up Visit 1 (--):

7. **Date of Visit (DD/MM/YYYY): _____**
8. **Wound Size (cm):**
    - **Length: _____**
    - **Width: _____**
    - **Depth: _____**
9. **Wound Stage:**
    - Stage 1
    - Stage 2
    - Stage 3
    - Stage 4
10. **Presence of Infection:**
    - Yes
    - No
11. **Adverse Events Reported:**
    - Yes
    - No
    - If yes, describe: _____
12. **Patient Compliance:**
    - Yes
    - No

Post study Follow-Up Visit 2 (--):

7. **Date of Visit (DD/MM/YYYY): _____**
8. **Wound Size (cm):**
    - **Length: _____**
    - **Width: _____**

- **Depth: _____**

9. **Wound Stage:**
    - Stage 1
    - Stage 2
    - Stage 3
    - Stage 4
10. **Presence of Infection:**
    - Yes
    - No
11. **Adverse Events Reported:**
    - Yes
    - No
    - If yes, describe: _____
12. **Patient Compliance:**
    - Yes
    - No

Post study Follow-Up Visit 3 (--):

7. **Date of Visit (DD/MM/YYYY): _____**
8. **Wound Size (cm):**
    - **Length: _____**
    - **Width: _____**
    - **Depth: _____**
9. **Wound Stage:**
    - Stage 1
    - Stage 2
    - Stage 3
    - Stage 4
10. **Presence of Infection:**
    - Yes
    - No
11. **Adverse Events Reported:**
    - Yes

- No
- If yes, describe: _____

12. **Patient Compliance:**
    - Yes
    - No

Post study Follow-Up Visit 4 (--):

7. **Date of Visit (DD/MM/YYYY): _____**
8. **Wound Size (cm):**
    - **Length: _____**
    - **Width: _____**
    - **Depth: _____**
9. **Wound Stage:**
    - Stage 1
    - Stage 2
    - Stage 3
    - Stage 4
10. **Presence of Infection:**
    - Yes
    - No
11. **Adverse Events Reported:**
    - Yes
    - No
    - If yes, describe: _____
12. **Patient Compliance:**
    - Yes
    - No

INFORMED CONSENT FORM

I, Mr/Mrs/Ms …….. Gender…….……..Age residing at…….

do hereby confirm that:

I have been asked by the student/researcher of D Y Patil Medical College, Hospital and Research Centre, Kolhapur ("the Medical College") whether I wish to participate in a study (research) under the aegis of the Medical College.: “Feasibility of nanopolymer and 3d printed skin in lower limb wounds: prospective preclinical and clinical assessment”

I. Purpose and methods of the research in simple language. (It should be written by investigator)
II. Expected duration of participation & frequency of contact, number of participant’s type of data collection & methods
III. Any alternate procedure or treatment should be informed
IV. The nature of the study being undertaken by the student/ researcher, as well as the extent of my participation in it, have been duly explained to me in a language that I understand;
V. The potential risks and consequences associated with this study have also, been duly explained to me in a language that I understand;
VI. I also understand that my participation in this study is only for the benefit of advancement in the field of medical research and that at no point in time is my participation being solicited for any pecuniary gain by the researcher or the Medical College;
VII. I have also been explained that I am in no way obliged to participate in the study and that, once I have agreed to participate in the study, I am still free to withdraw from participation in the study at any point in time upon notifying the Medical College in writing in the prescribed form without assigning any reason;
VIII. There will be no financial transaction between myself, the researcher and/or the D Y Patil Medical College for my participation in that study;
IX. I have been explained that any data collected out of my participation in the study will only be used for academic purposes and/or for further medical research;

X. I have also been reassured that any publication of the data collected during the course of the study or any publication of its conclusions, shall be done on a 'no names' basis and shall under no circumstances reveal my personal identity. Any personal details likely to reveal my personal identity shall at all times remain confidential;

XI. I understand that if any accident or undesirable medical complication arises out of a procedure or treatment done solely for the purpose of research, I will be offered treatment, free of cost, by the D Y Patil Hospital & Research Center, Kolhapur. Any additional compensation considered necessary by the Institutional Ethics Committee may also be given to me.

By affixing my signature/thumb print hereto, I am therefore freely and voluntarily signifying my consent, intent and willingness to participate in the study of the student researcher for the purposes of the dissertation under the egis of the D Y Patil Education society. I also certify that my right to privacy has not been infringed in any manner

[SIGNATURE/THUMB PRINT OF PARTICIPANT]
DATE

WITNESSED BY:

(1) NAME: TITLE/CAPACITY: SIGNATURE:	(2) NAME: TITLE/CAPACITY: SIGNATURE:

Name of Project investigator:

Address: Contact Number:

Signature of investigator:

Name of Project Co- investigator:

Address: Contact Number:

Signature of investigator:

Helpline Numbers:

Contact Details of Member Secretary:

Institutional Ethics Committee

रुग्ण समंती पत्र

डॉ डी वाय. पाटील मेडीकल कॉलेज अणि डाँ, डी. वाय. पाटील हॉस्पटल व रिसर्च इन्स्टिट्युट, कोल्हापूर

मी. श्री.।सौ.कु./श्रीमती ……लिंग..... वय ... ... राहणार या पत्राद्वरे खात्री देतो देते की,

१. मला डीं. वाय. पाटील मेडीकल कॉलेजच्या द मेडीकल कॉलेज वैदयकिय डॉक्टर संशोधक यांच्याकडून विचारले गेले की, मेडीकल कॉलेजच्या सहकार्याखाली संशोधन अभ्यासात माझी भाग घ्यायची इच्छा आहे का?

२. वैद्यकीय डॉक्टर संशोधक यांच्याकडून केल्या जाणाऱ्या संशोधन अभ्यासाचे स्वरूप व त्या मध्ये माझ्या सहभागाचा कालावधी याविषयी व्यवस्थितपणे मला समजणाऱ्या भाषेत सांगितले आहे.

३. संशोधन अभ्यासा दरम्यान उध्दभवणारे धोके आणि त्यांचे परिणाम मला समजावून व समजणाऱ्या भाषेत सांगितले आहेत.

४. मला हे सुध्दा माहित आहे की, माझा अभ्यासातील सहभाग हा फक्त वैद्यकीय संशोधन षेत्राच्या प्रगतीकरिता फायदा होण्यासाठी आहे, ना की मेडीकल कॉलेज किंवा संशोधन करत्याकडूनम पैश्याच्या फायद्याकरिता.

५. मला याची पण कल्पना दिली आहे की, मी कोणत्याही स्थितींत सहभागासाठी बांधील नाही आणि एकदा मी अभ्यासात सहभागासाठी सहमती दिली तरी मी माझा अभ्यासातील सहभाग कोणत्याही वेळी विहित नमुन्यात मेडिकल कॉलेज ला लेखी अर्ज करून कोणतेही कारण नमूद न देता रद करू शकतो,

६, माइयामध्य आणि संशोधनकर्ते आणि किंवा डी. वाय. पाटील मेडिकल कॉलेज यांच्यात अभ्यासात सहभागासाठी कोणताही आर्थिक व्यवहार असणार नाही.

७. मला याची पण कल्पना दिली आहे की, माइया अभ्यासातील सहभागातून जी काही माहिती गोळा केली जाईल त्याचा वापर फक्त शेक्षणिक हेतू आणि किंवा पुढील वैद्यकीय संशोधनाकरिताच होईल.

८. मला याचीपण खात्री दिली आहे की, अभ्यासाच्या काळात गोळा केलेल्या माहितीचे सार्वजनिक प्रसारण किंवा त्यांचा परिणामांचे सार्वजनिक प्रसारण नाव न जाहीर करता केले जाईल. कोणत्याही परिस्थीतीत माझी स्वतःची ओळख दाखवली जाणार नाही. कोणतीही वैयक्तिक माहिती माझी वैयक्तिक ओळख दाखवण्याची शक्यता असेल तर नेहमीच गुप्त राखली जाईल

९: मला माहित आहे की, संशोधनाच्या हेतूकरिता केला जाणाऱ्या एखाद्या उपचार किवा तपासणीमधून जरा एखादा अपघात किंवा काही अनपेक्षित वै्यकीय गुंतागुं निर्माण झाली तर, माइयावर डी. वाय.

पाटील हॉस्पिटल, कोल्हापूर कडून विना मोबदला उपचार केला जाईल. तसेच जादा भरपाईची आवश्यकता वाटल्यास इन्स्टिट्यूशनल एथिक्स कमिटीकडून मला दिलती जाईल.
१०. या संमती पत्रातील मजकूर आणि त्याचा परिणाम मला समजणाऱ्या भाषेत व्यवस्थित समजावून सांगितलं आहे.

रुग्णाचे नाव व पत्ता

साक्षीदार
१.
२.

Estd. 1960 P. B. No 131

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KWC/18/2022-23

Date: 02.06.2022

To,
Miss. Mrunal Narendra Damle.
Research Scholar.
Department of stem cell and Regenerative,
D.Y. Patil education society,
Kolhapur.

Subject: Invitation as an Examiner at HELIX-2022 on 3rd June, 2022.

Dear Madam,

Its gives me immense pleasure and honor to invite you as an Examiner for Rangoli competition at HELIX-2022 to be held on 3rd June, 2022 organized by Biotechnology department. The competition will be commenced from11.30 a.m.
So kindly accept this invitation and do the needful.

With thanks and regards.

Yours faithfully,

Dr. B .P. Ladgaonkar
Principal

PRINCIPAL
Smt. Kasturbai Walchand College
Arts-Science,Sangli.

Merck High-End Skill Development Centre,
a CSIR-IMTech Initiative
(Ministry of Science & Technology, Govt. of India)

Merck Innovation Lab
Bangalore Chandigarh

MERCK

CERTIFICATE
of Attendance

Presented to

Mrunal Damle

For attending a webinar on

2D Electrophoresis (2DE): Principles & Applications

Conducted on 22nd August, 2022

Wishing all the best for your future endeavor.

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Experience
Advance

Dr. Deepak Sharma
Principal Scientist - IMTech, Chandigarh

Dr. Pankaj Kumar Joshi
Head - Commercial Marketing
Science & Lab Solutions, Merck India

Merck High-End Skill Development Centre,
a CSIR-IMTech Initiative
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Merck Innovation Lab
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For attending a webinar on

Biosafety Levels in Research Laboratory

Conducted on 05th September, 2022

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Dr. Deepak Sharma
Principal Scientist - IMTech, Chandigarh

Dr. Pankaj Kumar Joshi
Head - Commercial Marketing
Science & Lab Solutions, Merck India

Merck High-End Skill Development Centre,
a CSIR-IMTech Initiative
(Ministry of Science & Technology, Govt. of India)

Merck Innovation Lab
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For attending a webinar on

Mass Spectrometry: Concept, Instrumentation and Applications

Conducted on 26th September, 2022

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Principal Scientist - IMTech, Chandigarh

Dr. Pankaj Kumar Joshi
Head - Commercial Marketing
Science & Lab Solutions, Merck, India

Merck High-End Skill Development Centre,
a CSIR-IMTech Initiative
(Ministry of Science & Technology, Govt. of India)

Merck Innovation Lab
Bangalore Chandigarh

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of Attendance

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Mrunal Damle

For attending a webinar on

Spectroscopy and Microscopy: Principles and Applications

Conducted on 29th June, 2022

Wishing all the best for your future endeavor.

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Dr. Deepak Sharma
Principal Scientist - IMTech, Chandigarh

Dr. Pankaj Kumar Joshi
Director, Head of Commercial Marketing
Research Solutions, Merck, India

KL (DEEMED TO BE UNIVERSITY)

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41 YEARS OF EDUCATIONAL LEADERSHIP

in association with
KL Technology Incubators Foundation and
Microbiologists Society of India

This is to certify that

Mrunal N Damle

of **Dr. D. Y. Patil Medical College**

has participated in a National conference on

BIOINNOVATION & ENTREPRENEURSHIP (NCBE-2022)

12th February 2022.

Dr. Praveen Kumar Vemuri
Co-Convener - NCBE-2022
Assoc. Professor, Dept. of Biotechnology

Dr. Giridhar Kanuri
Convener - NCBE-2022
Head, Dept. of Biotechnology

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