IN VITRO CHARACTERIZATION OF COLLAGEN PEPTIDE INCORPORATED FIBRIN SEALANT PATCHES FOR WOUND HEALING APPLICATIONS

THESIS SUBMITTED BY

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IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

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DECLARATION

I, Ms. ARYA.G, hereby declare that the thesis work entitled **'*In vitro* characterization of collagen peptide incorporated fibrin sealant patches for wound healing applications'** was done by me under the direct guidance of **Dr. Renjith P. Nair, Scientist C, Division of Thrombosis Research**, Biomedical Technology Wing, Sree Chitra Tirunal Institute for Medical Sciences and Technology, Poojappura, Thiruvananthapuram, Kerala, India. External help sought are acknowledged.

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CERTIFICATE

This is to certify that the thesis work entitled **'*In vitro* characterization of collagen peptide incorporated fibrin sealant patches for wound healing applications'** submitted by Ms. ARYA G (2020/MPhil/03) in partial fulfilment for the Degree of Mater of Philosophy in Biomedical Technology was done under my supervision and guidance at **Division of Thrombosis Research**, Biomedical Technology Wing, Sree Chitra Tirunal Institute for Medical Sciences and Technology, Poojappura, Thiruvananthapuram, Kerala, India.

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ABBREVIATIONS

CFC- Collagen peptide- Fibrin Chitosan matrix

CFG- Collagen peptide- Fibrin Gelatin matrix

DMEM/F-12- Dulbecco's Modified Essential/Ham's F-12 Medium

DS- Degree of swelling

FBS – Foetal Bovine serum

FDA - Fluorescein Diacetate

HA-Hyaluronic acid

MTT- (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide)

PBS- Phosphate Buffered Saline

WVTR- Water Vapour Transmission Rate

LIST OF FIGURES

CONTENTS

SYNOPSIS

Wound represents a major burden in the healthcare system. Although numerous strategies have been adopted, none of them proved to be ideal with all desired characters. In this study we evaluated the suitability of developing collagen peptide incorporated fibrin gelatin patch (CFG) and collagen peptide incorporated fibrin chitosan patch (CFC) as an advanced wound regeneration matrices for effective wound healing. The study mainly involves the optimization of collagen peptide concentration in CFG and CFC, preparation of CFG and CFC, physical and biological characterisation of prepared matrices.

The study is presented in five Chapters. Chapter I include an introduction to the research topic. Chapter II include review of literature. The review part gives a detailed account of mechanism of wound healing, current developments in biomaterials, an account of advanced wound care products, role of ECM in wound healing, ECM based products, a note on gelatin, chitosan and collagen peptide. Chapter III gives details of the materials and methods used in each study. This chapter mainly explains the optimization of collagen peptide concentration for fibrin sealant patch preparation, procedure for the preparation of collagen peptide incorporated gelatin/chitosan fibrin sealant patch, its physical and biological characterization. Chapter IV illustrates the results from the study using figures and graphs. Results are discussed in light of the literature; however, the importance of current study is highlighted. Chapter V summarises the results and consists of a conclusion and included identified limitations of the study and future prospects.

Chapter 1

INTRODUCTION

Wound is defined as damage or disruption to the normal anatomical structure and function of any part of body. Immediately after injury, body starts to heal the wound by natural mechanism. Wound healing involves multiple cell populations, the extracellular matrices and the action of soluble mediators such as growth factors, cytokines and the vascular system thus make it a complex process(Gantwerker & Hom, 2011; Singer & Boyce, 2017; Velnar *et al*., 2009). Wound healing is compromised in many pathological conditions. Today's modern world has made multiple progresses in the field of medicine, but effective wound management remains still as a major challenge. An overwhelming number of wound care products are now available in the market but none of them proved to be ideal (Sorg *et al*., 2017). The customary wound dressings such as cotton, bandages, and gauzes are dry, which fails to provide a moist and active environment for wound healing. Additionally, due to wound drainage, dressings tend to stick to the wound bed, and, when removed, they cause pain for patients. Substantial efforts have been made to deal with these drawbacks by discovering new strategies that promotes wound healing and the repair of damaged tissues(Negut *et al*., 2020; Radhakumary *et al*., 2011).

Wound healing matrices or scaffolds that mimic the properties of skin tissues or extracellular matrices components have gain more attention in the current scenario. The scaffolds comprising individual components of the ECM, can provide biochemical cues that alter the wound microenvironment and closely resembles

natural wound healing environment, thus facilitate rapid wound healing. (Zhao *et al*., 2017).

Collagen is one of the major extracellular matrices (ECM) component that can be isolated from tissues such as skin, tendons etc: Due to its unique features they are used as mechanical scaffolds that can provide specific binding sites for cell attachment and guides cell migration and cell growth during the proliferation and remodelling phase of the wound healing process. Native collagen provides the intact binding sites needed for interaction with platelets that enable platelets to adhere on the surface, induce its aggregation ,degranulation and initiate the formation of a physiological platelet that effectively reduces bleeding (Bohm *et al*., 2017).Collagen peptides are short chain proteins produced by the hydrolysis of collagen. Experimental studies have proven chemotactic property of collagen peptides in skin fibroblasts and shown that it increases the migration and growth, enhance cell proliferation and hyaluronic acid synthesis and thus helps in wound healing (Knefeli & Durani, 2017).But their allogenic nature limits their extensive application. Moreover, the inability of collagen to form scaffolds by itself lead to its use in combination with other polymers which further increases the cost.

Fibrinogen is a soluble blood plasma protein which is converted to insoluble fibrin, in presence of thrombin, after an injury(Zuliani-Alvarez & Midwood, 2015). Upon the initial tissue damage, vasoconstriction occurs, causing the platelet aggregation and coagulation cascade activation to produce a platelet plug. The coagulation cascade converts fibrinogen to insoluble fibrin in presence of thrombin, concurrently with the conversion of factor XIII to factor XIIIa, that initiates the

cross-linking of fibrin monomers which results in the development of a stable clot which will stop blood loss and prevent microbial infection (Luyendyk *et al*., 2019). Fibroblasts use this fibrin matrix as a surface for migration and helps in tissue remodeling. Fibrin and its degradation products induce endothelial cells and monocytes to secrete interleukins, growth factors, chemokines etc:(TNFα, IL-1ß, IL-6, MIP-1, MIP-2) and thus modulates inflammation (Jennewein *et al*., 2011).

Fibrin is now widely used in wound healing products in the form of glue, gel, sealants and as scaffolds that mimic natural healing microenvironment. Fibrin alone scaffolds will not be effective as they have a faster degradation rate, poor mechanical strength and handling properties. Therefore, they are usually combined with other natural polymers or synthetic polymers for improving its mechanical strength (Heher *et al*., 2018). Gelatin is a natural polymer that can be produced by irreversible hydrolyzation of the triple helical structure of collagen through processes such as heat and enzymatic denaturation. It can be extracted from several sources such as cattle bones, fish, pig skins, and some insects (Bello *et al*., 2020).Its high abundance, low-cost, biocompatibility, biodegradability, and low antigenicity make it an ideal candidate for wound healing applications (C. Wang *et al*., 2016).They helps in cell adhesion by providing specific peptide sequences for the recognition of integrin receptors in the cell. Its high porosity, and good permeability, and ability to biomimic the extracellular matrices favours the cell adhesion, migration, and proliferation(Kang & Park, 2021; Zheng *et al*., 2018). Several gelatin patches are commercially available but these patches alone are not sufficient to provide all cues for enhancing wound healing.

Chitosan is a copolymer generally obtained by chitin deacetylation. Major sources of chitosan include exoskeleton of crustacean, molluscs, insects etc: It possesses some unique properties such as biodegradability, non-toxicity, anti-bacterial effect and biocompatibility (Matica *et al*., 2019). They are soft, flexible and microporous that helps to soak up wound exudates while helping tissue regeneration. The monomeric unit of chitosan, N-acetylglucosamine, occurs in an extracellular macromolecule called hyaluronic acid which is important in wound healing (Ahmed & Ikram, 2016; Alven & Aderibigbe, 2020).Chitosan promotes surface-induced thrombosis and blood coagulation. It accelerates coagulation by influencing the activation of platelets and also forms an appropriate inflammatory microenvironment conducive for healing (Liu *et al*., 2018). Even though chitosan act as a good haemostatic and anti-microbial agent, its biologic cues for wound healing is limited. Under general conditions chitosan materials have poor degradation performance which also hinders their application as wound healing scaffold (Kong *et al*., 2020).

In this study we propose a novel collagen peptide based gelatin and chitosan fibrin sealant patch intended for wound healing applications. As per the results from existing literatures it is hypothesized that the medical grade collagen peptide and fibrinogen will provide necessary biochemical cues for cell growth and avoid problems of allogenic sources. Moreover, the gelatin/chitosan in the novel combination functions as a porous backbone to the matrices that provide sufficient mechanical strength and suitable microenvironment for cell attachment and proliferation. It will be also patient friendly as its adhesive and hemostatic property

avoids problems like suturing and will be convenient to use. Thus, the novel combination of collagen peptide-fibrin-Gelatin/Chitosan is expected to act together to regenerate the integrity of damaged tissue and will meet the requirements of expedited wound healing.

Chapter 2

REVIEW OF LITERATURE

Wounds have become a big socioeconomic burden due to their high prevalence and recurrence. Wound healing is a complex, dynamic process supported by a myriad of cellular events that must be tightly coordinated to efficiently repair damaged tissue (Wilkinson & Hardman; 2020). This dynamic series begins at the moment of injury may continue from months to years in order to restore the cellular structure. It involves four overlapping phases termed as hemostasis, inflammation, proliferation and remodelling (Banerjee *et al*., 2015). Derangement in wound-linked cellular behaviours, as occurs with diabetes and ageing ,will lead to healing impairment and the formation of chronic, non-healing wounds (Mp, 2016; Han & Ceilley; 2017).

Wounds can be categorized as acute and chronic wounds based on healing time. Acute wounds commonly heal through the normal wound healing process and heal within 12 weeks. Chronic wounds heal gradually post-injury (12 weeks or more) and are accompanied by inflammation, depending on the patients' health, external stimulus and microbial infection (Wallace et al., 2021; P.-H. Wang et al., 2018) ; Rezaie *et al*., 2019 ; Gonzalez *et al*., 2016)

2.1. Mechanism of Wound healing

An injured tissue classically goes through four temporal phases/stages to repair a wound: hemostasis, inflammation, proliferation, and remodelling. These phases

occur one after the other in this sequence and may progress in different rates (Childs & Murthy, 2017).

Hemostasis

Hemostasis occurs immediately after injury which involves three major steps: constriction of blood vessels, formation of a temporary platelet plug and coagulation cascade activation that leads to fibrin clot formation.(Periayah *et al*, 2017) . Initially after injury, hemostasis is controlled via vasospasm, a process in which blood vessels constrict in response to injury (Hunt *et al*., 2000). But this spasm subsides rapidly and the injured blood vessels will subsequently relax, allowing additional bleeding if platelets do not become involved. During the initial period of vasoconstriction, platelets aggregate and adhere to exposed collagen, especially collagen in the basement membrane underlying injured endothelial cells. Platelets then secrete vasoconstrictive substances for maintaining the transfected vessels constriction, initiate platelet plug formation to prevent additional bleeding(Greer et al., 2012; Wang et al., 2018).

Inflammatory Phase

The inflammatory phase (acute inflammation) of wound healing is fully established after 24 hours of vascular injury. If the healing process is disrupted by infection, trauma or some other distress it can last for more than 96 hours. The "cardinal signs" of inflammation namely redness (rubor), swelling (tumour), pain (dolor) and loss of function (functio laesa) are observed. The first cells that appear at an injured site are neutrophils (Reinke *et al*; 2012). They clean-up cell debris and bacteria developed due to the tissue injury which provides a good environment for

wound healing. It is then followed by macrophage accumulation which facilitates phagocytosis of bacteria and damage tissue. Macrophages secrete a variety of chemotactic factors and growth factors that helps to establish a microenvironment for the granulation phase. CS3CL1, CCL2, PDGF, VEGF, epidermal growth factor (EGF), IL-1, TNF-α, TGF-α, etc: are major factors among them. Moreover, fragments of collagen, fibrin, and other molecules in wounds will induce chemotaxis, cell proliferation, and angiogenesis (Wang *et al*., 2018).

Proliferation phase

During the proliferation phase, in tissues which are lined by epithelium migration of basal cells from the overlying epithelium begins early in the healing process and does not require an underlying collagenous matrix. These cells arise from the transfected edges of epithelium that border the wound, which rapidly undergo hyperplasia in response to EGF, FGFs, IL-1, hepatocyte growth factor (HGF), VEGF, IL-1β, and TGF-β, released by epithelial cells, endothelial cells, and fibroblasts (Robson *et al*; 2001). The migrating basal cells proliferate and differentiate. Once it gets differentiated, they cease to proliferate and migrate. Depending on the size of the wound, the proliferation phase may last up to 3 to 4 weeks or longer .This phase is characterized by the generation of new endothelium (angiogenesis), epithelium (epithelialization), and connective tissue stroma (fibroplasia/desmoplasia) to restore normal structure and function to the injured tissue (Kumar *et al* ; 2005).

Remodelling phase

The remodelling (maturation, contraction) phase begins by approximately 3 to 4 weeks after injury, after the completion of inflammation and proliferation phases. This phase is characterised by remodelling of granulation tissue by immature connective tissue (Martin RF; 2020). In response to TGF-β, PDGF, FGF-2, MMPs, and tissue inhibitors of MMP (TIMPs) immature connective tissue will become mature through extracellular collagen formation. Remodelling can last for 2 or more years (Okur *et al*., 2020).

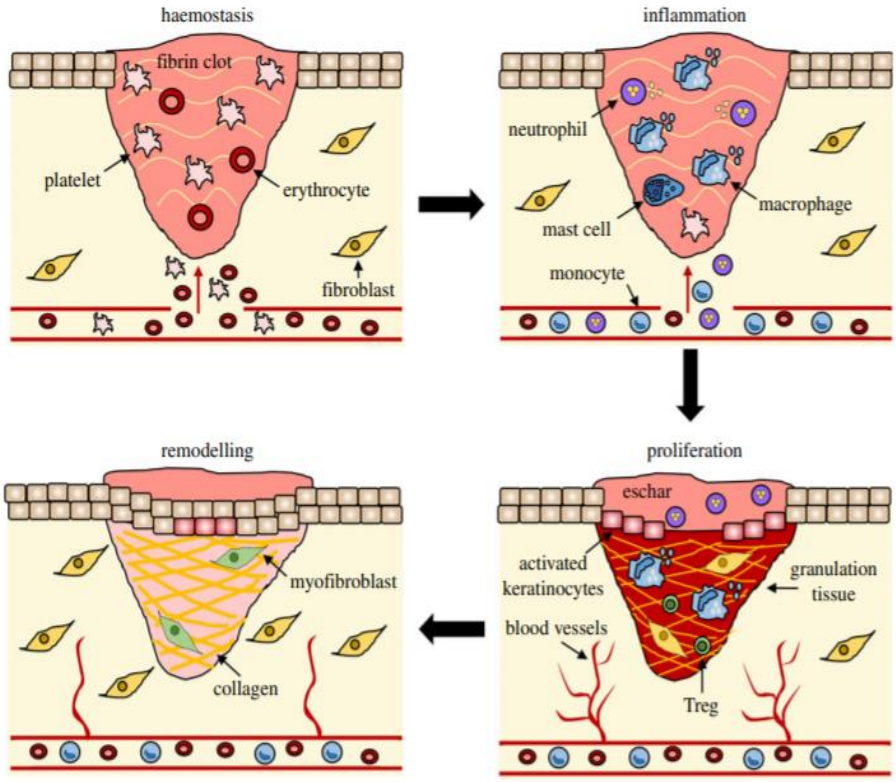

Fig: 1 Stages in wound healing process (adopted from Wilkinson & Hardman, 2020).

2.2. Factors affecting wound healing

Wound healing needs multiple factors affecting one or more phases of the healing process (Basu and Shukla; 2013). They can be broadly categorized into local factors and systemic factors. Local factors are those that directly influence the characteristics of the wound itself while systemic factors include the overall health or disease state of the individual that affect the ability to heal the wound. It is well known that there are many requirements for a proper wound healing to occur. Local factors include oxygenation, infection, foreign body, venous sufficiency, etc (Bishop; 2008; Rodriguez *et al*., 2008). Systemic factors include age, gender, sex hormones, stress, ischemia, diseases (diabetes, keloids, fibrosis, hereditary healing disorders, jaundice, uremia), obesity, medications (glucocorticoid steroids, non-steroidal anti-inflammatory drugs, chemotherapy) alcoholism and smoking, immuno compromised conditions like cancer, radiation therapy, AIDS, etc (Halim *et al*; 2012; Hess *et al*; 2011). The influences of both local factors and systemic factors are not mutually exclusive. Single or multiple factors may play a role in the overall outcome of healing process (Guo & DiPietro, 2010).

2.3. Biopolymers in wound healing

A biomaterial is defined as a natural or synthetic substance that has the ability to induce an intended host response. Polymeric biomaterials have a great impact on the advancement of modern medicine as they can break down and be removed after they have served their function. Natural polymers have been used in biomedical field for thousands of years. They generally include proteins (collagen, fibrin, silk, etc.) and polysaccharides (starch, alginate, chitin/chitosan, hyaluronic acid

derivatives, etc). They have demonstrated a potential advantage of supporting cell function and adhesion. Biopolymers that are usually utilized for the preparation of wound healing materials include gelatin, chitosan, cellulose, hyaluronic acid, alginate, elastin , dextran, fibrin, pectin and collagen(Alven & Aderibigbe, 2020; Gao *et al*., 2020; Pal *et al*., 2017; Pan *et al*., 2017).

2.4. Characteristics of an ideal wound healing product

The effective management of bleeding is necessary to achieve rapid hemostasis and thereby promoting positive outcomes. For this, various wound care products are now available. Among them wound healing matrices that mimic the properties of skin tissues are superior. An ideal wound healing product should be

- Biocompatible
- Bioresorbable
- Biodegradable
- Ready to use
- Non -immunogenic
- Maintain a moist wound environment
- Cost-effective
- Non-adsorbent
- Provide barrier to external contaminant

Although numerous products are commercially available, none of them proved to satisfy all these requirements. Therefore, the development of new wound healing

product with these desirable properties is highly demanded(Field & Kerstein, 1994; Wallace *et al*., 2021).

2.5. Current developments in biomaterials for wound management

Numerous studies have resulted in the development and commercialization of several wound management materials, such as gauzes, films, foams, hydrocolloids etc:

Gauze

Gauze or gauze-woven cotton dressing were used to protect the wound from the external environment that can easily absorb blood and exudates secreted during wounding. They are also cost effective, thereby making them readily accessible in most clinics to date (Gao *et al*., 2020; Murray *et al*., 2019)**.**It prevents the formation of a moist bacterial growth-promoting environment by absorbing wound exudates. But it can cause pain and secondary injury due to the excessive moisture absorption as they may adhere to the newly restored tissues. Moreover, the gauzes have to be changed frequently. In 1962, Winter first proposed the concept of a moist wound environment that helps in faster and better wound healing (Winter ;1962). Based on this concept, artificial dressings, such as films, foams, hydrocolloids, and hydrogels, have been developed.

Films

They are highly absorbent polyurethane dressings which is thin, transparent and flexible. They are semi-permeable to gaseous exchange and water vapor but will block bacterial infiltration. However, it results in the accumulation of excess exudates as it is non-adsorbent and when removed lead to secondary injury to the newly generated epidermis (Schoukens; 2019).

Foams

They are porous polymeric matrices that is absorbent, but is weakly adhesive and need a secondary film to fix it at the wound site. Additionally, when exudate is dried it is difficult to detach (Ulery *et al*., 2011)**.**

Wound dressings

They are developed mainly from biopolymers and synthetic polymers. The natural polymers that are commonly used include chitosan, cellulose, fibrin, elastin, hyaluronic acid, dextran, elastin, alginate, collagen, and gelatin etc: These polymers possess interesting properties such as good biocompatibility, non-toxicity, biodegradability, readily availability, and non-immunogenicity (Alven & Aderibigbe, 2020).

2.6.ECM in wound healing

The extracellular matrix (ECM) is a content of diverse active molecules secreted into the surrounding medium by the resident cells in the tissues or organs that provide biophysical and biochemical support to the surrounding cells (Agren *et al;* 2017). It

not only function as an architectural support but also play a significant role in cell regulation. It provides structure, organization, and orientation to cells and tissue and act as a template for cell migration, proliferation, differentiation, apoptosis etc. ECM components also regulate cell activity and function by binding to integrins and other cell surface receptors. It acts as a reservoir for growth factors and regulate their bioavailability (Sheng *et al*., 2017).

Major components of ECM

Collagen

Collagens is the most abundant proteins in the ECM which comprises 77% of the fat-free dry weight of human skin (Weinstein *et al*; 1960; Lauren *et al*; 2016). More than 28 different types of collagens are identified in vertebrates. Among them most important types are Type I, III, and V (Fibrillar collagen) and Type XII, XIV, XVI, and VI (Fibril associated collagens). Collagens are present in the dermis as fibrillar proteins which gives structural support to the cells, helps in cell adhesion, cell migration, tissue morphogenesis, tissue scaffolding, tissue repair, regulation of the resident and inflammatory cell functions etc (Tracy *et al*; 2016). Collagen is mainly composed of amino acids glycine, proline and hydroxyproline that forms aa triplex helical structure composed of three α chains (Shigemura *et al*., 2009). It has long been used in wound care and management as a wound dressing material in various forms such as powder, pastes, gel-impregnated dressing/pad etc: Bovine tendon or bovine dermal collagen are the major collagen sources. Bohm *et al*, (2017) conducted a comparative analysis to understand the influence of different collagen sources (bovine, porcine, and equine) and manufacturing processes on the

functionality of collagen for medical applications. The study reveals that collagen properties are more dependent on the differences in manufacturing process rather than different sources (Bohm *et al*., 2017).It has been reported that native collagen provides intact binding sites needed for interaction with the platelets that enable platelets to adhere on the surface, induce natural aggregation, degranulation and initiate the formation of physiological platelet plug that prevent blood loss. Upon heat treatment the triple-helical structure of collagen is converted into a globular structure which is referred to as gelatin. Gelatin undergo enzymatic hydrolysis to yield what is referred as collagen hydrolysate, gelatin hydrolysate, or collagen peptide which also has gained much attention in wound care(Sato *et al*., 2020).

Fibrin

Fibrin is a fibrillar protein derived from soluble plasma fibrinogen (Clark *et al*; 2001). During the hemostatic stage of wound healing, thrombin cleaves fibrinogen into fibrin monomers and activates factor XIII to factor XIIIa together with Ca2+. Fibrin monomers then form unstable and soluble fibrin polymers, which are then cross-linked by factor XIIIa to become stable and insoluble fibrin polymers, resulting in fibrin clots (Yoon HS *et al*.,2019). Fibroblasts utilize the contracted fibrin matrix as a surface for migration and tissue remodeling. They can also influence fibroblast responsiveness to TGF-β, which, in turn, activates collagen synthesis(Tracy *et al*., 2016).

Proteoglycans and GAGs

Proteoglycans are glycosylated proteins that comprises of a protein core covalently attached with glycosaminoglycan (GAG) chains. Proteoglycans and GAGs help to hold the growth factors and cytokines within the ECM, keeps the ECM hydrated and contribute to the overall mechanical properties by connecting to other ECM proteins. The most abundant proteoglycans in the skin include hyaluronan (HA), decorin, versican etc. (Xue & Jackson, 2015).

Hyaluronic acid (HA) is a high molecular weight polymer that consist of alternative residues of d-glucuronic acid and N-acetylglucosamine. They play key role in both fibrotic and regenerative wound healing (Neuman *et al*;2015). Depending on its size their functions will change. Large HA chains act as a space filler in granulation tissue and have structural functions during inflammation, while the short fragments appear to have stimulatory effect and attracting properties such as fibroblast migration and collagen production. They help in modulation of inflammation, chemotaxis, cell migration, collagen secretion and angiogenesis etc. (Frenkel *et al* 2012).Decorin is a most prevalent proteoglycan and have a higher binding affinity to TGF-β1 and thus negatively regulates TGF-β1 signalling.

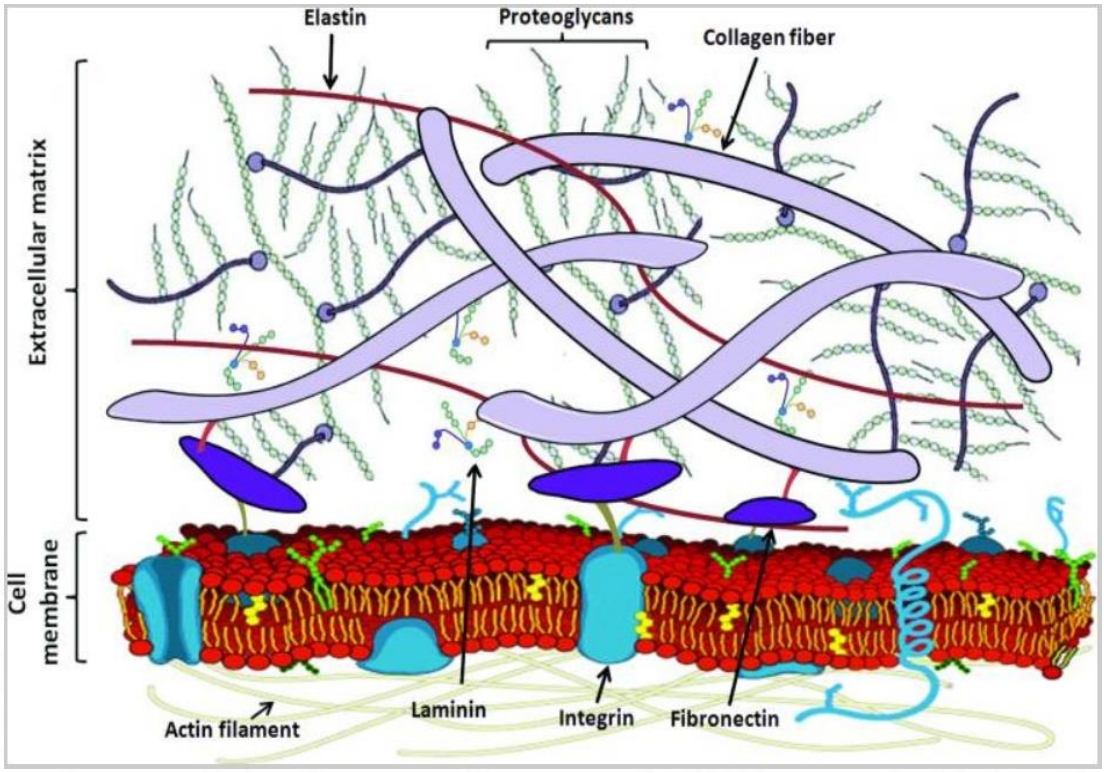

Fig:2. ECM components (Adopted from Xue and Jackson;2015)

2.7.ECM based products in wound healing

The potential use of ECM molecules as a source of wound healing products has recently received much attention. These products can act as scaffolds that mimic the properties of skin tissues or extracellular matrices components. They are safer to use as it is biocompatible, biodegradable and non-toxic, bioresorbable and non-immunogenic. It can also provide necessary biochemical cues that alter the wound microenvironment and, closely resembles natural wound healing environment, facilitate rapid restoration of normal skin architecture.

Avitene® (Davol Inc, Woburn, Massachusetts, USA) is a microfibrillar collagen-based product made from purified bovine collagen which is available as dry flour, sheets, sponges etc: It provides a large surface area and promotes platelet aggregation. High cost, short shelf life and the greater affinity for moist surfaces, such as gloves and any instruments limits their extensive use (Fujimoto *et al*., 2014; Resnik *et al*, 2018).

Kollagen resorb (Resorba Medical GmbH, Nürnberg, Germany) is a sterile 3-4mm thick, soft, dimensionally stable collagen sponge. This equine collagen fibrils (horse Achilles' tendon) are used as a hemostatic agent in cases of capillary, venous and diffuse bleeding and also used as a temporary skin cover (Bohm *et al*;2017).

Nobakoll (NOBA Verbandmittel Danz GmbH, Wetter, Germany, manufactured by MBP GmbH, Neustadt-Glewe, Germany) is a sterile collagen sponge made of porcine hide(corium)used for chronic wounds and wound healing by secondary intervention.

FloSeal® (Baxter Healthcare Corp, Fremont, California, USA) and Surgiflo® (Ethicon Inc., Johnson &Johnson Company, Somerville, New Jersey, USA) are two commercially available products that are composed of gelatin and thrombin. They exhibit a combination of effects of gelatin and thrombin. The gelatin matrices that comes in contact with bleeding site swells by 20% and seals the bleeding site together with the action of thrombin.

Tachosil® is another absorbable fibrin sealant patch that consist of an equine collagen fleece containing the fibrin glue components human thrombin and human

fibrinogen (Montorsi *et al*., 2012; Toro *et al*., 2011). It has the advantages of hemostasis, providing tissue sealing, supporting sutures, preventing adhesions, protecting nerves, slowly releasing medications, and promoting wound healing etc: It is clinically used for hemostasis, adherence purposes during skin grafting, nerve anastomosis, bone sealing, dura sealing, and mastectomy to reduce seroma formation, slowly releasing medications, including antibiotics, growth factors, and chemotherapeutic agents. Due to the presence of fibrin, Tachosil facilitates a faster wound healing (Yoon HS *et al*.,2019). But the allogenic source, higher costs etc: limits its extensive application.

2.8 Gelatin

Gelatin is the hydrolytic product of native collagen. Essential constituents of gelatin are proteins (80-95%) which exists as polymer chains of different lengths and exists as colloidal solutions or sols. They convert to gels on cooling and revert to sols on warming. They are cheap non-toxic, biocompatible material extracted from bones, cartilages, skin etc: (Hajosch *et al*., 2010; Yu *et al*., 2015).Numerous studies are documented so far indicating the role of gelatin in wound healing. Zhang *et al* investigated the possible beneficial role of Chum salmon skin gelatin and found that gelatin administration improves cutaneous wound healing of hyperglycemic diabetic rats by reducing inflammatory response, improving wound contraction, increasing collagen deposition and angiogenesis, and also by stimulating NO synthesis (Zhang *et al*., 2011)..

Several studies have used gelatin for wound healing applications. Takagi *et al*. developed a new topical hemostat agent composed of gelatin alone. This two layered hemostatic patch comprises a gelatin sheet and a gelatin film. The gelatin sponge layer absorb blood and activate autologous blood coagulating components rapidly while the gelatin film layer of the two-layer gelatin sheet inhibit the permeation of blood and strengthen adhesive bonding to the bleeding site. They have shown superior hemostatic property and fewer inflammatory reactions compared to commercially available Tachosil (Takagi *et al*., 2018).

Another study done by Kabiri *et al* aimed to synthesise an absorbable hemostat gelatin sponge crosslinked with a less toxic 1-ethyl-3- (3-dimethyl aminopropyl) carbodiimide (EDC). They found that crosslinking of gelatin sponges with EDC provides a means of easily tailoring a sponge biocompatibility and mechanical properties and also increase ease of handling in general. It was also observed that various gelatin contents and crosslinking agent concentrations and freezing temperature have an effect on the mechanical properties, rate of biodegradation etc:(Kabiri *et al*., 2011).

2.9. Chitosan

Chitosan is a copolymer obtained from the alkaline deacetylation of chitin which is the major component of exoskeleton of crustaceans. It is a cationic natural polymer composed of β-(1 → 4)-2-acetamido-2-deoxy-D-glucopyranose and 2-amino-2-deoxy-D-glucopyranose units. They possess intrinsic antimicrobial properties (Matica *et al*., 2019) and are used to effectively deliver extrinsic antimicrobial compounds into the infected area (Dai *et al*; 2011). Burkatovskaya *et*

al investigated its efficacy for treating wound infections in mice and found that chitosan acetate bandage effectively controlled the growth of bacteria in the wounds and prevented the development of systemic sepsis (Burkatovskaya *et al*., 2006). Chitosan also play a role in surface-induced thrombosis by influencing the activation of platelets and accelerates coagulation *in vivo* (Liu *et al*., 2018).Chitosan plays major roles in the first three stages of wound healing. They promote the aggregation of platelets and erythrocytes and inhibiting the dissolution of fibrin and thereby stops bleeding. During the inflammation stage they assist to clear bacteria from the wound. And in proliferation stage they accelerate skin proliferation via promoting the growth of granulation tissue (Feng *et al*,2019) .

2.10. Collagen Peptide

Collagen peptides are low molecular weight peptides produced by the enzymatic hydrolysis of collagen or gelatin (Sugihara *et al*, 2018). They can be prepared from skin, bones, and tendons, skin and scales of fish. It is an important raw material in medicine, cosmetics, food and health care industry, biomedical and leather industries. The degree of hydrolysis of collagen will determine the average molecular weight of the final product (Asai *et al*., 2019).Hydrolysed collagen or collagen peptides are unable to form scaffolds by itself because of its low molecular weight, therefore usually mixed with other biopolymers such as cellulose, chitosan etc: (Leon-Lopez *et al*., 2019).

Collagen peptides once thought to have an absence of function, have shown ability to potentiate and regulate a variety of cellular processes acting through integrin receptors. Collagen peptides isolated from different collagen types and have been shown to regulate processes such as cell proliferation, migration, apoptosis, and reduce angiogenesis (Sato *et al* ;2020).

Type I collagen peptides (CP), a kind of collagen hydrolysates, have exhibited numerous bioactivities, including antioxidant activity, reparative ability to skin and make it a popular regent for developing skin care product (Knefeli & Durani, 2017).

Bioactive peptides derived from collagen have been proven to show immunomodulatory, ACE (angiotensin-I converting enzyme) inhibitory properties, antibacterial, antioxidative etc. depending on their specific amino acid composition. Unlike collagen, collagen peptides can easily undergo gastric enzyme digestion, absorption and can be transported through human peptide transporter 1(PEPT-1) to systemic circulation. In the skin, they act as false collagen degradation peptides which send a false signal in the fibroblast cells to synthesize dermal extracellular matrices components. Experimental studies have proven chemotactic property of collagen peptides in skin fibroblasts and shown that it increases the migration and growth, enhance cell proliferation and hyaluronic acid synthesis (Xu et al,2019).

According to Mei *et al*, Pro−Hyp, Gly−Pro−Hyp, Pro−Ala, Glu, and Arg in the collagen peptide are the most important peptide entities to promote wound healing. The N-terminal proline dipeptides are more bioavailable for binding to the peptide transporter 1 (PepT1) and transported into the metabolic system and participate in

wound healing (Mei *et al*; 2020). Oral ingestion of collagen could lead to Pro−Hyp transportation either from collagen itself or from the hydrolysis of Gly−Pro−Hyp in the bloodstream and skin. Oral administration of the low-molecular-weight collagen peptides upregulate the content of nucleotide-binding oligomerization domain containing 2 (NOD2) and β-defensin (BD14) which were implicated in the regulation of microflora colonization into the wound tissues. It promoted wound healing by controlling the inflammatory reaction (Felician *et al*,2019).

Ramadass *et al*. fabricated a novel collagen hydrolysate composite scaffold (CHCS) using sol–gel transition procedure to use as an alternative to traditional collagen for wound healing therapy. They showed improved biostability, swelling, biocompatibility ,antimicrobial activity etc: compared to normal collagen scaffolds (Ramadass *et al*., 2014).

GAP AREA

Despite significant advancement in technology and scientific approaches, wound management still remains as a global health concern. After decades of research many features have been added to the wound healing products. But still there is no superior product that satisfy all the requirements to heal chronic wounds which often fail to achieve complete healing (Dhivya *et al*., 2015). Hence developing a wound healing material that can address the major interfering factors of normal process of wound healing will help patients and wound care practitioners. The potential use of ECM (extracellular matrix) molecules as a source of wound healing products has recently received much attention. These bioactive matrices based on ECM components are reported to be more preferable over traditional wound dressings because of its biocompatibility, lack of immunogenicity, bio- resorption, biodegradation. Moreover, the scaffolds based on ECM molecules can closely resemble the natural wound healing environment and provide necessary biochemical cues and thus facilitates rapid wound healing. Although numerous products are available based on major ECM components like collagen, fibrin, gelatin etc: the use of individual component alone have its own setbacks like poor mechanical strength and cannot provide all necessary biochemical cues for wound healing which limits the use of these individual components alone (Negut *et al*., 2020;Boerman *et al*., 2017).Thus, a combination of these ECM components (collagen peptide- fibrin- chitosan or collagen peptide- fibrin gelatin) is expected to induce a combinatorial effect and provide most of the biological cues to facilitate wound healing.

HYPOTHESIS

The study proposes a collagen peptide incorporated- fibrin gelatin patch and collagen peptide incorporated fibrin chitosan patch for wound healing applications. The cumulative effect of these extracellular matrices components is hypothesized to provide suitable biomimetic cues, microenvironment and support for cell growth and can be used as an effective strategy for wound healing.

OBJECTIVES

1. Optimization of collagen peptide concentration for the preparation of collagen peptide-fibrin gelatin/chitosan matrices.
2. Preparation and Characterization of collagen peptide-fibrin incorporated gelatin matrix.
3. Preparation and Characterization of collagen peptide-fibrin incorporated chitosan matrix.

Chapter 3

MATERIALS AND METHODS

3.1 MATERIALS

DMEM/F-12 (Hyclone - Dulbecco's Modified Essential/Ham's F-12 Medium, USA), FBS (Gibco, USA), 100X Antibiotics (Gibco, USA), Trypsin (Gibco, USA), Collagen Peptide (Nitta Gelatin, India), MTT((3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) Reagent (Sigma, USA), L929 cells (ATCC, USA). Gelatin patch (Eucare pharmaceuticals, India)) and Chitosan patches developed by Dr Lynda V Thomas, DTERT, SCTIMST was used for this study.

3.2METHODOLOGY

3.2.1 Cell culture

L929 cells were cultured in DMEM/F-12 complete medium (Hyclone - Dulbecco's Modified Essential/Ham's F-12 Medium, USA) with 10% FBS (Gibco, USA) and 1X antibiotic (Gibco, USA) in a 5% CO_2 incubator at 37^{o}C. Confluent cells were subcultured using 0.25% trypsin (Gibco, USA). Media change was given on alternate days.

3.2.2 Optimization of collagen peptide concentration.

To determine the optimum concentration of collagen peptide for incorporating the wound healing matrices, a 96 well plate was coated with gelatin (0.5%), fibrinogen

(5 mg/ml), thrombin (5 IU), and different concentrations of collagen peptide (5 µg/ml, 10 µg/ml, 20 µg /ml, 40 µg/ml). Coated plates were frozen in -80℃ for overnight and then lyophilized for 24 h.

Cell viability on different concentrations of collagen peptide was assessed by MTT Assay after 48h. Briefly, L929 cells were seeded on coated 96 well plates with a seeding density of $1x10^4$ cells/ 100µl and incubated at 37 ˚C at 5% CO_2 in a CO_2incubator. Cells seeded on uncoated wells are used as control. Media was removed on respective days and cells were incubated with 100 µl MTT (1mg/ml serum free DMEM/F-12 media) for 3 h. Thereafter,100 µl isopropanol was added and incubated for 30 minutes. The absorption was measured at 595 nm in a microplate reader (BIO-RAD, iMark, US) and percentage cell viability was calculated based on the following formula.

% Cell viability = (OD of Test/OD of control) *100

3.2.3Preparation of collagen peptide-fibrin gelatin/chitosan matrices

A mixture of Collagen peptide (20µg/ml), Fibrinogen(5mg/ml) and Thrombin (5 IU) was coated on both sides of gelatin patch or chitosan patch and allowed to clot for 30 minutes to prepare collagen peptide- fibrin gelatin (CFG) and collagen peptide- fibrin chitosan (CFC) matrices. Then it was freezed overnight at -80^0C. Next day matrices were lyophilized for 24 h and then stored at 4^0C until use.

3.2.4 Physical characterization of collagen peptide-fibrin-gelatin/chitosan matrices

3.2.4.1Tensile Test

Tensile strength of matrices was determined according to (Nair *et al*;2014). Briefly, the samples with 5cm length ,1cm width and 5mm thick were prepared. Tensile strength was then measured using Universal Testing Machine (Instron, USA). Both ends of the rectangular samples (Gelatin, Chitosan, CFG and CFC) were clamped into the gigs and the test was conducted at a cross head speed of 5 mm/min using a 10 N load cell.

3.2.4.2 Swelling study

Swelling study was carried out in phosphate buffered saline (PBS) as described by Kumar *et al* (Kumar *et al*., 2012).Briefly matrices were cut into a size of 1 cm^2, and immersed in 5 ml PBS solution. The samples were taken out at definite time intervals (10 min, 30 min, 1h ,2h, 4h), gently blotted to remove excess water and the wet weight was recorded. The degree of swelling was calculated by the following formula.

Degree of swelling (DS) = [(WW-DW)/ DW] *100

where WW is the wet weight of the matrices and WD represents the dry weight of the matrices respectively.

3.2.4.3Water vapour Transmission (WVTR)

The water vapour transmission rate of the matrices (CFG and CFC) was determined according to (Nair et al; 2014). Briefly, the samples were cut into small disc shape suitable to be mounted on the mouth of a cylindrical bottle containing 12 ml of distilled water. The matrices were then tightly sealed on the bottle mouth. Initial weight of the experimental setup was noted and then kept in a 37°C incubator at 50% relative humidity. Weight loss was measured and plotted against the elapsed time to get a slope. Water vapour transmission rate was then calculated by the following equation,

Water vapour transmission rate (WVTR) = (S*24)/A (g/day/m^2)

where S is the slope of the graph at 24 h and A is area of the mouth of bottle.

3.2.5 Biological characterization of collagen peptide-fibrin-gelatin/chitosan matrices

3.2.5.1 Test on Extract

Effect of the matrices and the components released from different matrices (Gelatin, Chitosan, CFG and CFC matrices) on cell viability was accessed by (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium-bromide) (MTT) assay. For this 1cm x 1 cm matrices were incubated with 1ml complete DMEM/F-12 medium at 37^o C for 24 h, after incubation the extract was diluted to 12.5% using complete DMEM/F-12 medium. Diluted test extract was incubated with sub-confluent monolayers of L929 cells at 37 ± 1^o C for 24 ± 2 h. Cells cultured in complete

DMEM/F-12 medium without extract were used as experimental control. The culture medium is removed after 24 h and 100µl MTT reagent (1mg/ml serum free DMEM/F-12 media) was added and kept for 3 h incubation. MTT reagent is then removed and 100 µl isopropanol was added and incubated for 30minutes.The absorption was measured using microplate reader at 595 nm and percentage cell viability was calculated.

3.2.5.2 Cell Viability on matrices

L929 fibroblast cells were seeded onto Gelatin, Chitosan, CFG and CFC matrices with a cell density of $2x10^5$cells and cultured at 37 ˚C at 5% CO_2 in a CO_2incubator for 24 h. Fluorescein Diacetate (FDA) stock at a concentration of 10mg/ml was prepared in acetone and a working solution was made by adding 10µl of FDA stock in serum free media (DMEM/F-12). FDA was added onto matrices and incubated for 2 minutes and then washed with HBSS buffer. The images were captured using an inverted fluorescent microscope [Leica DM IRB].

3.2.5.3 Cell Scratch Assay (*In vitro* wound healing Assay)

The cell scratch assay was done for assessing the wound-healing potential of collagen peptide incorporated wound healing matrices. For this assay extracts of matrices were used. For the preparation of extract matrices (Gelatin, Chitosan, CFG and CFC) of size 1cm x 1cm is incubated with 1ml media (DMEM/F12) for 24 h at 37 °C. L929 cells were plated in a 12-well plate ($1x10^5$ cells/ well) and cultured at 37 °C, 5% CO_2 for 24h to achieve 80% confluency. A sterile 200 µL pipette tip was used to make a straight scratch on the monolayer of cells, simulating an *in-vitro* wound and images were captured. Test extract from each matrix were added to the

cells and further cultured for 24 h. Cells incubated with culture media alone were used as control for comparison purposes. After incubation, the cells were washed with serum free-DMEM/F-12 three times, incubated with serum free-DMEM/F-12, and the images of cells were captured under an inverted microscope [Leica DM IRB].

3.2.6. Statistical Analysis:

For each test at least three replicate samples were used and average and standard deviation was calculated. Statistical significance was then determined using t-test. p-value less than 0.05 was considered as statistically significant**.**

Chapter 4
RESULTS AND DISCUSSION

4.1. Optimization of collagen peptide concentration

Optimum concentration of collagen peptide (CP) for incorporating in wound healing matrices was determined by MTT assay. L929 cells grown on different concentrations of collagen peptide (5 µg/ml, 10 µg/ml, 20µg/ml, 40 µg/ml) incorporated fibrin- gelatin matrices showed an increase in cell viability at 10µg/ml, 20 µg/ml and 40 µg/ml concentration. The viability was maximum at 20 µg/ml and then didn't showed much increase. Results are represented in (Figure.3). The results obtained from this study are similar to the study done by Felician *et al* who investigated the wound healing effects of collagen peptides isolated from jellyfish *Rhopilema esculentum* which showed a dose dependent wound closure effect and a concentration of 6.25µg/ml was found to be the minimum peptide concentration needed for *in vitro* scratch wound closure (Felican *et al* ; 2019).Collagen peptides are shown to have enhanced cell proliferation Mei *et al*; 2020). Previous study in our laboratory also showed enhanced cell proliferation when collagen peptide was used at a concentration of 20 µg/ml in the culture media. Based on the results obtained from our study collagen peptide at a concentration of 20 µg/ml is further used for the preparation of wound healing matrices.

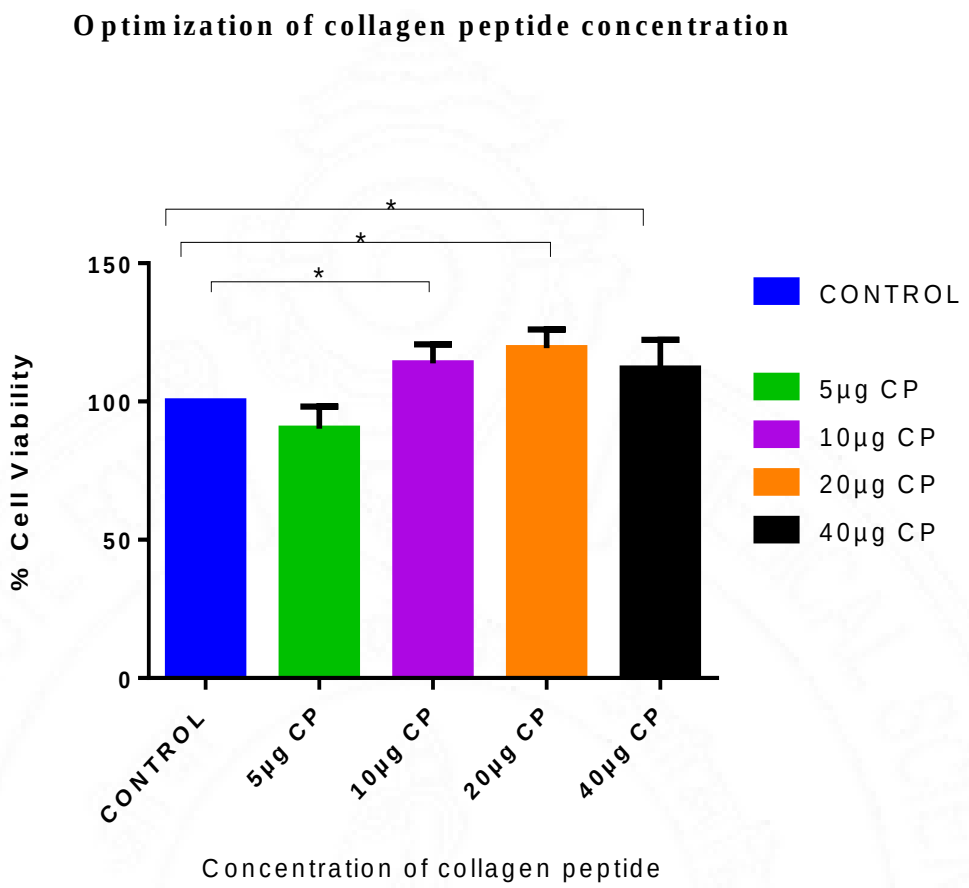

Fig:3. Optimization of collagen peptide (CP) concentration in matrices by MTT Assay. Bars represent average ± SD (n=4). *p < 0.05.

4.2. Physical Characterisation of matrices

4.2.1. Tensile Test

Tensile property is an important factor for a wound healing matrix to impart good handling property and also to prevent wound contraction (Negut *et al*; 2020; Talikowska *et al*; 2019). The developed CFG and CFC matrices have shown a

tensile strength of 0.146 ± 0.01 MPa and 0.32 ± 0.03 MPa respectively. The results are represented graphically (Fig: 4). From the figure, collagen peptide incorporated gelatin and chitosan matrices showed a decrease in tensile strength as compared to normal gelatin and chitosan matrices (0.216 ± 0.004 MPa and 0.404 ± 0.044 MPa respectively). As the collagen incorporated matrices have gone through additional procedures like lyophilization, it might have affected its mechanical strength and showed reduction in tensile strength. But all the scaffolds showed good handling property and it was easy to cut into different sizes.

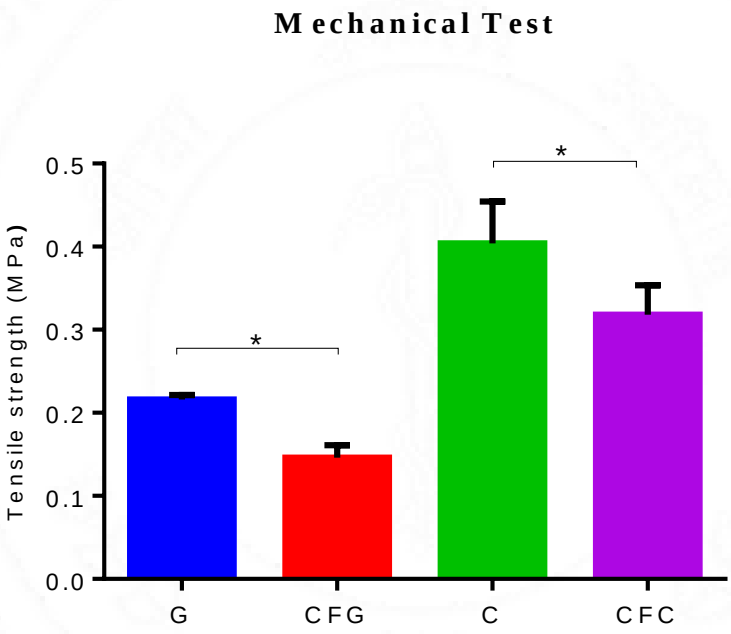

Fig:4. Relative tensile strength of wound healing matrices. Bars represent average ± SD (n=3). * $p < 0.05$. (**G**-Gelatin, **CFG**- Collagen peptide -Fibrin-Gelatin, **C**-Chitosan, **CFC**-Collagen peptide -Fibrin-Chitosan)

4.2.2. Swelling study

One of the most important properties of an ideal wound healing matrice is to prevent exudates accumulation and also to serve moist environment for wound healing. It will also be advantageous if it can prevent excessive bleeding, immediately absorb and reach swelling equilibrium and clotting within a short time. Swelling property of collagen peptide incorporated fibrin gelatin matrices (CFG) and fibrin chitosan matrices (CFC) was analysed and results from independent experiments are plotted graphically (Figure:4). From the results it is evident that gelatin and CFG reached swelling equilibrium within 30 minutes. Gelatin has showed maximum swelling (4164 ± 208%) compared to CFG (2855 ± 318%). which may be due to the presence of large interconnected pores which entrap and hold water through capillary action (Ahmad Abad *et al*., 2018).Chitosan and CFC reached swelling equilibrium within 60 min and showed maximum swelling in the range of 1328 ± 124% and 951 ± 40% respectively. The decrease in swelling capacity of collagen-fibrin incorporated matrices are likely to be due to the sealing of pores of underlying gelatin and chitosan matrices due to the deposition of collagen peptide- fibrinogen.

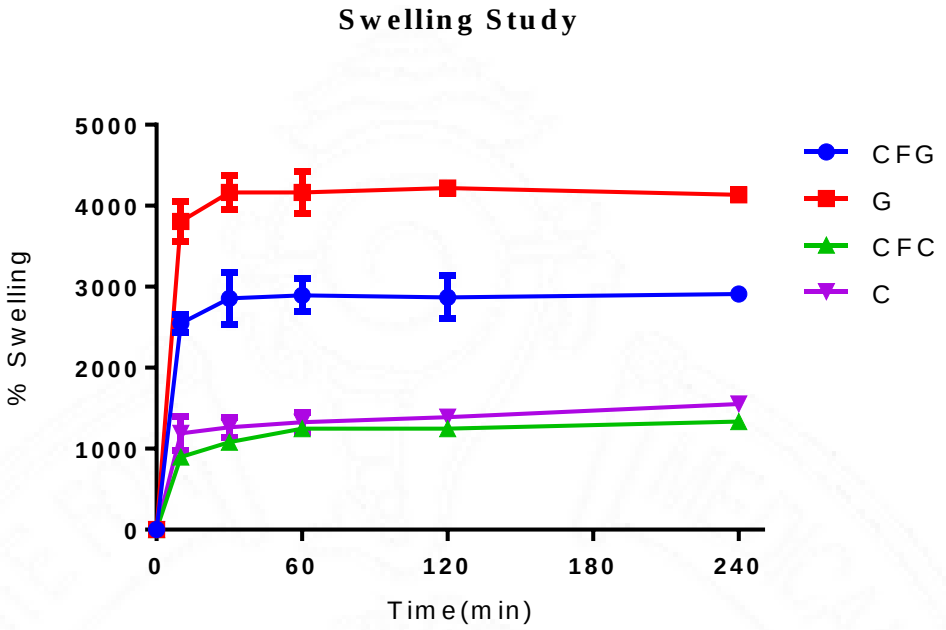

Fig:**5. Relative swelling ratio of wound healing matrices.**

Bars represent average ± SD (n=3). (**G**-Gelatin, **CFG**-Collagen peptide-fibrin - gelatin, **C**-Chitosan, **CFC**-Collagen peptide fibrin chitosan).

4.2.3 Water vapour Transmission

One of the fundamental requirements for a wound healing matrix is to maintain an optimal moisture content at wound site and provide desirable microenvironment for natural healing process (Xu *et al*., 2016). The ability of a wound healing material to control water loss can be determined by water vapour transmission rate (WVTR). An optimum water transmission rate is required as a higher WVTR causes dehydration of the wound, whereas a lower WVTR will cause the accumulation of wound exudates which may promote microbial infection and also slow down healing process (Xu *et al*., 2016; Pan *et al*; 2017). The WVTR of normal skin is 204

g/m^2/day, which increases to 279 g/m^2/day for a first-degree burn and 5138 g/m^2/day for a granulating wound (Lamke *et al*;1977; Nair *et al*., 2014). In this study CFG and CFC showed WVTR; 4070 ± 125 g/m^2/day and 3603 ±63 g/m^2/day respectively that falls in the range of a granulating wound (Wu *et al*; 1995). The chitosan-only matrix exhibited a WVTR value of 4251 g/m^2/day, and this value is significantly decreased in the CFC matrix (3602 ± 63 g/m^2/day). This corelates with the study done by Deng *et al* (Deng *et al*., 2021) in which a thermoresponsive hydrogel formed by conjugating recombinant human collagen-peptide (RHC) with chitosan showed a lower WVTR compared to chitosan alone. However, a water vapour transmission rate of 2000–2500 g/m^2 day^{-1} is recommended for wound healing matrices as it helps to provide an adequate level of moisture without risk of wound dehydration and exudate leakage (Queen *et al*; 1985). The water vapour transmission rate of CFC was found closer to this range and other similar biopolymer based wound dressings (Akiyode, *et al*.; 2018; Pan *et al*; 2017). WVTR of the developed CFG and CFC matrices can be tailored to get an optimum range by changing the fibrin and collagen peptide concentration.

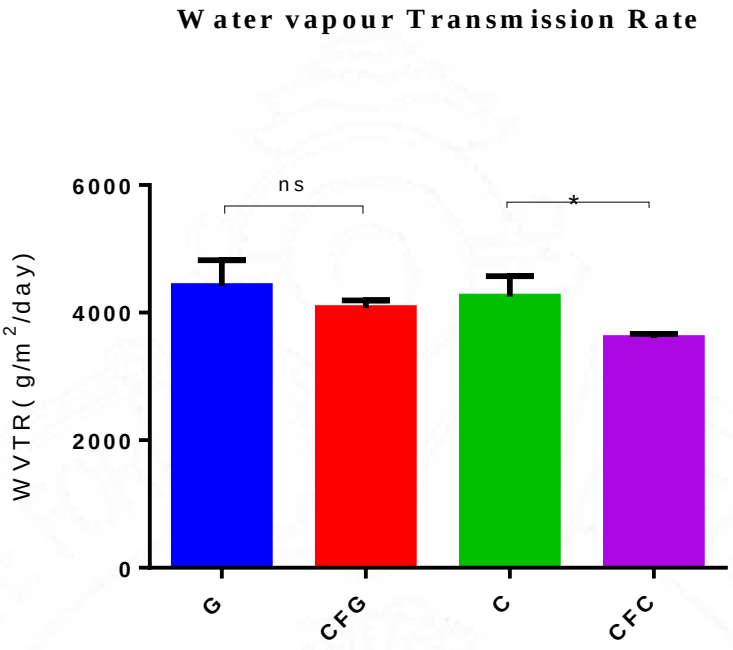

Fig:6. Relative water vapour transmission rate of wound healing matrices. (**G**-Gelatin, **CFG**- Collagen peptide -Fibrin-Gelatin, **C**-Chitosan, **CFC**-Collagen peptide -Fibrin-Chitosan). Bars represent average ± SD (n=3). * $p < 0.05$.

4.3. Biological characterisation of matrices

4.3.1. Test on Extract

MTT Assay was done to quantitatively evaluate the effect of releasates from the collagen peptide-fibrin incorporated wound healing matrices. L929 fibroblasts cells treated with test extract showed a higher percentage viability in collagen-fibrin incorporated matrices CFG and CFC. All tested matrices showed a higher percentage cell viability compared to the control (G-108 ± 6 %, CFG-128 ± 5 %, C-107 ± 1%, CFC-123 ± 7 %). This experiment clearly indicates that collagen

peptide-fibrin incorporated matrices supports cell growth and proliferation (Xiong *et al*., 2020 ;Mei *et al*; 2020) and will not induce any cytotoxic effect.

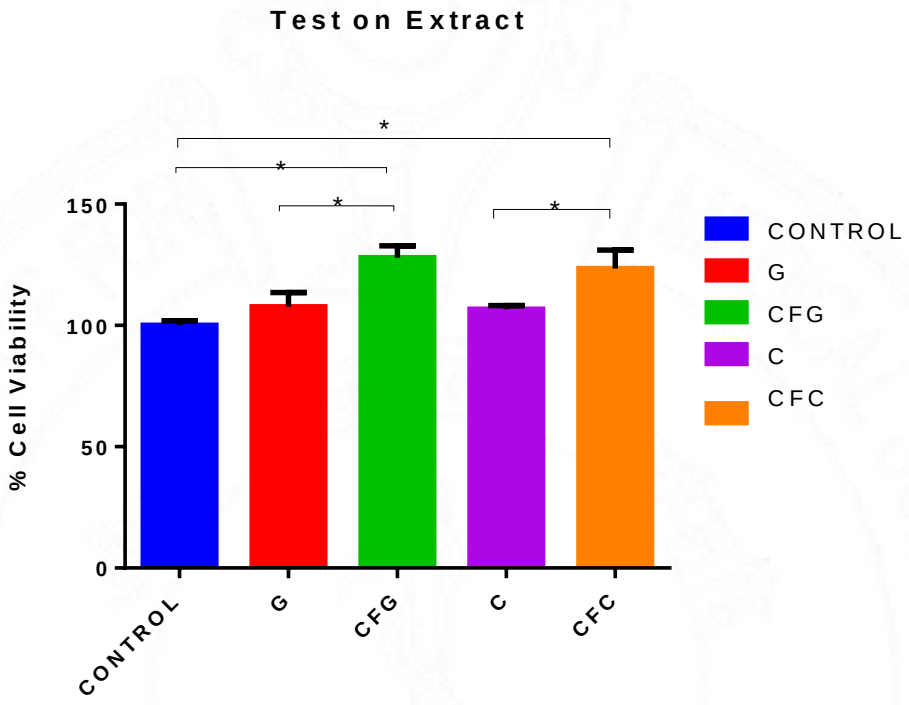

Fig:**7. Test on Extract**. Relative % cell viability of L929 cells in presence of test extracts of different matrices (**G**-Gelatin, **CFG**- Collagen peptide -Fibrin-Gelatin, **C**-Chitosan, **CFC**-Collagen peptide -Fibrin-Chitosan. Bars represent average ± SD (n=5). * $p < 0.05$.

4.3.2 Cell Viability Assay

To determine the growth, attachment and viability of cells on collagen-fibrin incorporated matrices, L929 cells were seeded on matrices and cultured for 24 hours. To visualise the cell attachment and evaluate the viability, the cells cultured on matrices were stained with FDA (Fluorescein diacetate). The representative results are shown in Figure 7. The results suggests that collagen peptide-fibrin incorporated gelatin and chitosan matrices supports more cell attachment, growth and viability than gelatin and chitosan alone which substantiate the MTT results of test on extract. Several previous studies suggest the suitability of fibrin coating for enhancing the cell attachment and proliferation in biomaterials (Gugerell *et al*., 2014; Jara *et al*., 2020; Wong *et al*., 2019).The ability of collagen peptides in promoting wound healing was also reported (Ouyang *et al*; 2018; Tingting *et al*; 2018). Marine collagen peptides isolated from the skin of *N. japonica* was shown to have potential wound healing-activity by promoting cell proliferation and migration through NF-κB signalling pathway (Yang *et al*; 2019). Collagen-derived peptides have also shown to increase the synthesis of structural ECM proteins like elastin, and decreases the synthesis of matrix degrading enzymes like MMP -1 and MMP -3 (Edgar *et al*; 2018). The enhanced cell attachment and viability in the CFC and CFG matrices may be due to the cumulative effect of collagen peptide and fibrin.

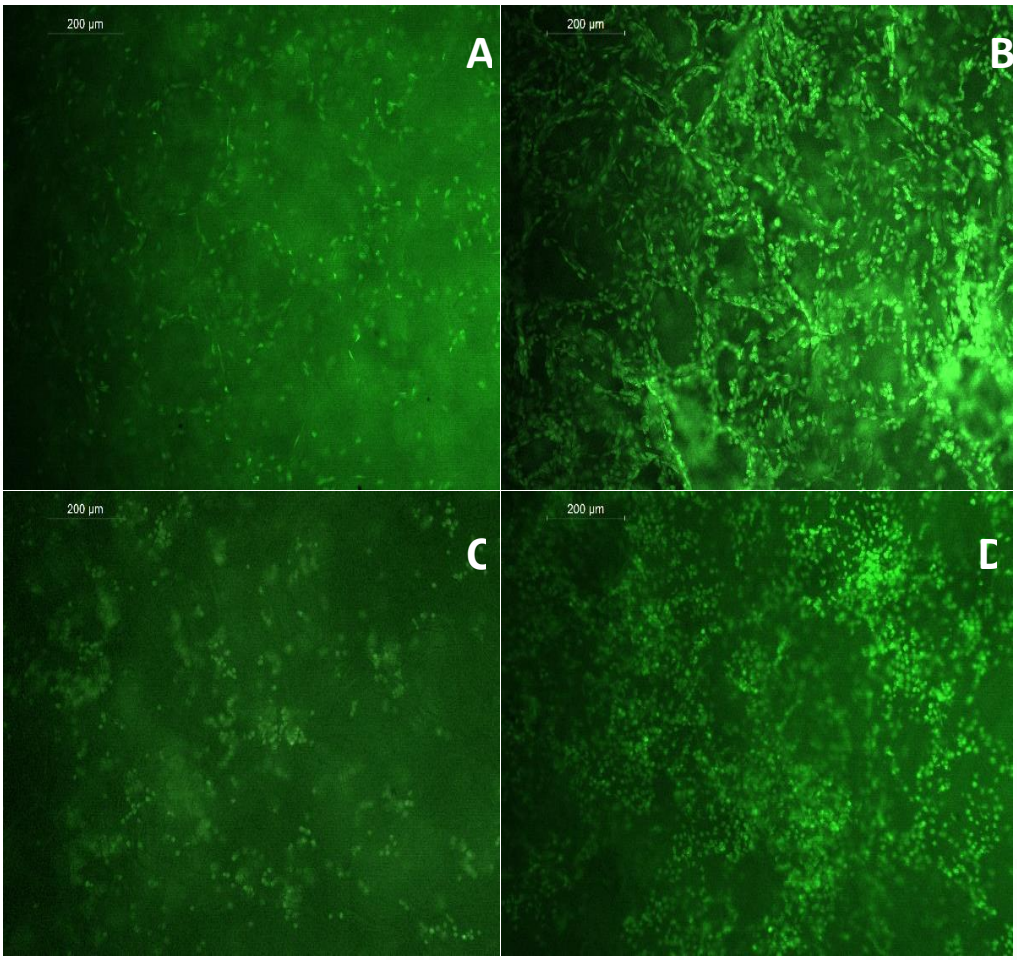

Fig:**8. Cell viability Assay.** Representative fluorescent images of L929 fibroblasts seeded on matrices stained with FDA after 24 h of culture. (**A-**cells on Gelatin matrix**, B-**cells on CFG**, C-**cells on Chitosan matrix**, D-**cells on CFC**)**. Scale bar 200µm.

4.3.3 Cell Scratch assay (*In vitro* wound healing)

The cell scratch assay was used for assessing the wound-healing potential of collagen peptide incorporated wound healing matrices *in vitro*. An *in vitro* scratch wound was generated on L929 cell monolayer and wound closure rate in the presence of extracts of collagen peptide-fibrin incorporated matrices (CFG and CFC) was monitored for 24 h.

It was observed that *in vitro* scratch wound closure was rapid with migration and cell proliferation in presence of collagen peptide-fibrin containing matrices (Fig:8). The results substantiate all our previous experiments and the observations of Felician *et al* (2019) that collagen peptide can induce cell migration, proliferation etc: The enhanced cell migration in CFC and CFG might be due to its chemotactic effects induced by collagen peptide (Felician *et al*., 2019; Hu *et al*., 2017). It is also reported that collagen peptides have a stimulating effect on recruiting and activating macrophages to produce chemotactic growth factors, fibroblast proliferation, and angiogenesis thus will be effective in cutaneous wound healing (Elbialy *et al*; 2020). Moreover, fibrin in the matrix is shown to enhance the cell adhesion and migration that helps in epidermal regeneration (Kubo *et al*; 2001; Geer *et al*; 2003). Thus, the scratch wound closure will be the additive effect of fibrin and collagen peptide in the matrices.

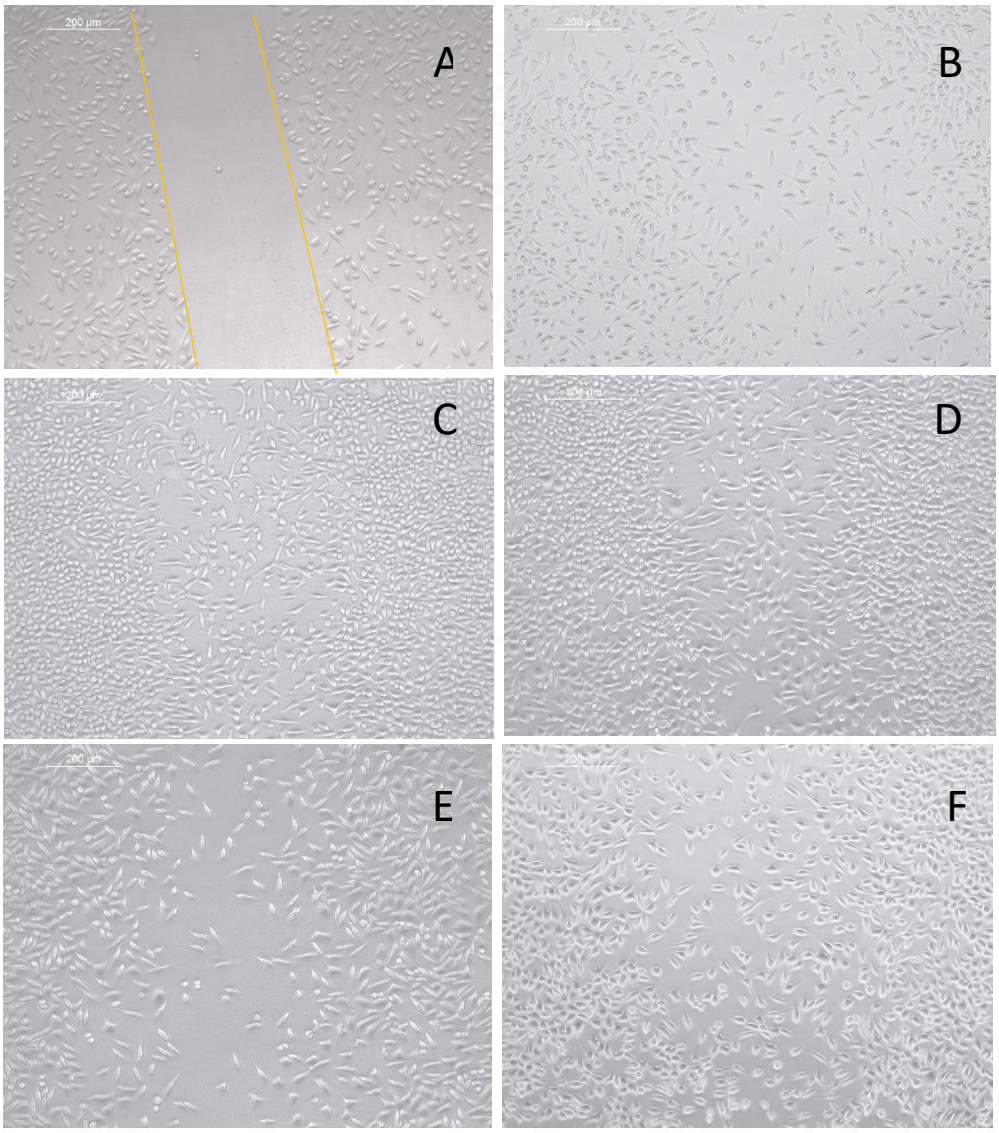

Fig:9. Cell Scratch Assay (**A**-Control after 0 h incubation, **B**-Control**, C**- Gelatin**, D**- GFC**, E** Chitosan**, F**- CFC respectively after 24 h incubation of test extract). Scale bar 200µm.

Chapter 5

SUMMARY AND CONCLUSION

In the present study we evaluated the suitability of collagen peptide- incorporated fibrin Gelatin matrix (CFG) and collagen peptide- incorporated fibrin-chitosan matrix (CFC) that can be used for wound healing applications.

- Collagen peptide concentration of 20µg/ml was found to be suitable for incorporating into wound healing matrices.
- Collagen incorporated fibrin gelatin (CFG) and Collagen incorporated fibrin chitosan matrices (CFC) have shown a tensile strength of 0.146 ± 0.01 MPa and 0.32 ± 0.03 MPa respectively.
- Swelling studies showed that CFG achieved maximum swelling within 30 min (2855 ± 318 %) and CFC took 60 min to achieve swelling equilibrium (951 ± 40%).
- The water vapour transmission rate of CFG and CFC was found to be 4070 ± 125 g/m^2/day and 3603 ± 63 g/m^2/day respectively.
- Test on Extract assay and growth of L929 on matrices suggests the suitability of CFG and CFC in cell attachment, cell viability and cytocompatibility.
- *In vitro* scratch wound assay confirms that collagen peptide-fibrin incorporated chitosan and gelatin matrices extracts enhances migration and proliferation of cells.

5.1. Limitations of the present study

Scratch wound assay and MTT assay needs to be done on different time periods using both test on extracts and matrices. Biodegradation rate of the matrices need to be analysed. Results of preliminary *in vitro* wound healing experiments were promising; however, *in vivo* wound healing efficacy needs to be evaluated in animal models.

5.2. Future prospects of the study

Clinical translation of the developed collagen peptide-fibrin- gelatin and collagen peptide-fibrin chitosan matrices for accelerating wound healing is expected to be the outcome of this study for which pre-clinical and clinical trial should be carried out.

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