REGENERATION OF SOFT TISSUE USING POROUS BOVINE COLLAGEN SCAFFOLD

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Skin, the protective organ consists of three layers namely Epidermis, Dermis and Hypodermis .Normally Epidermis regenerates easily, but when an injury is caused in the skin the Dermis and Hypodermis do not regenerate at ease. By providing the suitable biomaterials at the wound site, the material might influence the formation of extra cellular matrix in skin. Natural polymers have been used as one of the materials in the scaffolds for soft tissue augmentation. Collagen is one of the predominant protein in extra cellular matrix, used as biomaterial for regeneration of tissue, either skin or bone. The objective of this study is to investigate the use of porous bovine collagen scaffold in the dermal wound healing process. The Type 1 Collagen was extracted from Bovine tendons. This was characterized by FTIR, CD spectroscopy, SDS-PAGE, and hydroxyproline content analysis. The 1% Collagen solution was prepared in acidified water and to this, 400 µl of non-ionic wetting agent was added, agitated for few minutes to get a homogenized solution and then poured into the trough. This was allowed to dry in a dust free chamber. The animal experiment was performed for testing the biomaterial according to the Institute's ethical committee approval and guidelines (466/01/a/CPCSEA). Full thickness wounds (1.5 X 1.5 cm) were created on the shaved dorsal side of rats using sterile surgical blade. All surgical procedures were carried out under anesthesia using Thiopentone sodium (40 mg/kg body weight, intramuscular).The percentage of wound closure was calculated using the initial and final area drawn on glass slides during the experiments. The porous scaffold prepared from bovine Collagen that was extracted from the bovine tendons contains heterogeneous pores and its size varies from 500 – 800 µm. The high water uptake and swelling studies of the scaffold proves the efficiency of the biomaterial for wound healing purposes. The *in vivo* studies shows that animals treated with Collagen scaffold showed better wound closure and open wound groups showed moderate wound closure at the end of 16th day. The histological analysis of granulated tissue proves the effective regeneration of skin using porous Collagen scaffold. The porous Collagen scaffold has shown better wound reduction by regenerating effectively and supports tissue regeneration. This scaffold could be used in Skin Tissue Engineering.

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1. Introduction

The skin is the protective organ in the vertebrates against toxins and microorganism from environment. It is the largest organ of the integumentary system made up of multiple layers of epithelial tissues, and guards the underlying muscles, bones, ligaments and internal organs. Skin is composed of three primary layers, the *epidermis*, which provides waterproofing and serves as a barrier to infection, the *dermis*, which serves as a location for the appendages of skin and the *hypodermis (subcutaneous adipose layer)*. Usually, Dermis and hypodermis could not regenerate easily after injury[1, 2].But the providing biomaterials at the wound site enhance regeneration of soft tissue and acts as template for the site of injury. Natural polymers have been used as one of the

materials in the scaffolds for soft tissue augmentation. Especially, Protein based biomaterials have the capacity to mimic the extra cellular matrix at the damaged site and also to induce the cell fate process such as cell migration, cell differentiation and cell proliferation. In protein based biomaterials, Collagen is the predominant material used as biomaterial for regeneration of tissue, either skin or bone. Furthermore, collagen is resorbable, it has high water affinity, low antigenacity, very good cell compatibility and ability to promote tissue regeneration [3, 4, 5]. The use of porous bovine collagen scaffold in the dermal wound healing process was investigated in this study.

2. Materials and methods

2.1. Extraction of Type I Collagen from Bovine Tendons

The preparation of collagen from bovine tendon was done[6] according to the method developed by Bioproducts lab, Central Leather Research Institute, Chennai, India. Purity of extracted collagen was confirmed by physicochemical characterization such as SDS PAGE [7], CD Spectrum, and FTIR Spectrum.

2.2. Preparation of collagen scaffolds from Type 1 collagen

One percent collagen solution was prepared in acidified water using acetic acid. To this, 400 µl of Triton X-100 non-ionic surfactant was added and agitated for a few minutes to attain homogeneity. It was poured into a trough and allowed to dry in air in a dust free chamber.

2.2.1. Sterilization of Plain collagen dressings

The sterilization of collagen biomaterial dressings is done by the ethylene oxide sterilization method [9].

2.2.2. Scanning Electron Microscopy studies of collagen scaffold

The SEM analysis was prepared by sprinkling collagen scaffold with gold material one side of double adhesive stub. The stub was then coated with gold using Jeol JFC 1100 sputter coater. The SEM analysis of the Collagen scaffold was carried out by using Jeol JSM 5300, Japan. The scaffold was viewed at an accelerating voltage of 15–20 kV.

2.2.3. Water Uptake Studies and Swelling Studies of Porous Collagen Scaffolds:

The water absorption was calculated by taking the porous collagen scaffold of 3 X 3cm 2 dimension. Maximum water uptake was determined by soaking the porous collagen scaffolds in phosphate buffer saline (pH 7.2) and monitoring the weight. The wet dressings were removed from the buffer and blotted with filter paper to remove excess water, and immediately weighed. Maximum water uptake was calculated from the equation,

$$W = \frac{(Mt - Mo)}{Mo,}$$

Where Mt represents the maximum weight and Mo, the original weight of the dressings.

2.3. *In vivo studies*

Male Wister albino rats weighing 150 to 200 g were used in this study. The animals were fed a commercial pellet diet (Hindustan Lever, Bangalore, India) and had free access to water. The animal experiment was performed according to the Institute's ethical committee approval and

guidelines (466/01/a/CPCSEA). For the study, they were housed individually in standardized environmental conditions. A total of 36 animals were taken in three groups (n = 6 per group) (treatment and two controls for this study).

Group 1 – Open wound covered with gauze dressing

Group 2 – Plain Collagen Scaffold

The animals were rehabilitated following experimentation.

Full thickness wounds (1.5 x 1.5 cm) were created on the shaved dorsal side of rats using sterile surgical blade. All surgical procedures were carried out under Sodium thiopentone (40 mg/kg body weight, intramuscular). The wounds were covered with collagen dressings and outer covered with gauze dressings.

2.3.1. Wound Healing Rate

The percentage of wound closure was calculated as follows by using the initial and final area drawn on glass slides during the experiments:

$$\% \text{ of wound contraction} = \frac{\text{Wound area day 0} - \text{wound area day (n)}}{\text{Wound Area day 0}}$$

n = number of days (8th, 12th, and 16th day).

Collection of Granulated Tissues

The granulated tissues from both treatment and control groups were excised on day 4, 8, 12, and 16 using sterile scissors and forceps.

2.3.3. Histological Analysis

Tissues collected at different intervals were transferred to 10% neutral buffered formalin for 24 h at 4°C. The formalin fixed tissues were dehydrated through grades of alcohol and cleared in xylene and then embedded in paraffin wax (58 to 60° mp). The molds were labeled and stored until use. The deparaffinized sections were stained with hematoxylin following counterstained with eosin. Masson's trichrome staining was done for all the samples of all the time points to observe collagen deposit in the granulated tissue. [10]

3. Results

3.1. Physicochemical measurements of Type 1 collagen

3.1.1. Type 1 collagen and its purity by SDS PAGE:

Separation of α, β, γ chains of collagen were achieved on the basis of molecular weight of each species of collagen on a separating gel of PAGE (Fig 1**).** In fig 1, the concentration of the α-1 band is twice that of the α-2 band. These results are consistent with the fact that known helical structure of type 1 collagen is composed of three-polypeptide chains β, α1 and α2. The molecular weight of type I collagen was 300 KDa.

Fig .1. SDS – PAGE OF NATIVE COLLAGEN

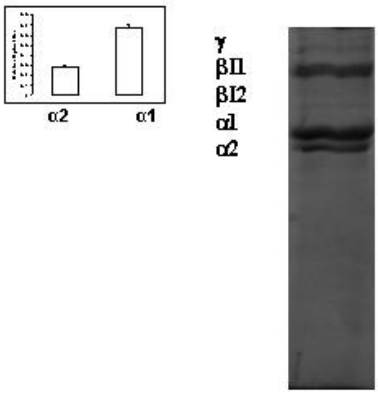


Fig 1. SDS PAGE of Collagen: The α-1 band is twice as large as that of the α2 band. The type 1 Collagen contains two α-1 and one α2 helix.

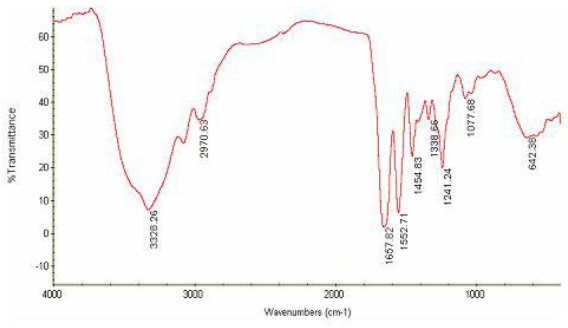
3.1.2. Hydroyproline Analysis of Collagen

Hydoxyproline estimation showed that 90% of hydroxyproline present in the extracted collagen from bovine tendons.

3.1.3. FTIR Studies of collagen:

Fig. 2 shows the FTIR spectrum of collagen. The major feature of the IR spectrum of collagen film is the amide I band between 1640 -1660 1/cm, which arises from the stretching vibration of C = O (carbonyl) groups of amide groups in the protein. The intense absorption between 1500 - 1600 1/cm is due to the amide II mode, observed at 1552.71 1/cm in the spectrum of collagen, which arises from N-H bending and strongly coupled to the C-N stretching vibration of collagen amide groups. Signals in the spectral region of 1200 – 1400 1/cm absorption are attributed to the amide III, arising due to the C-N stretching and N-H in bending from the amide linkages. The amide A band (NH stretching) as observed at 3328.26 1/cm, is almost symmetric, suggesting that the amount of water must be low. The band close to 1454.83 1/cm is probably associated with CH bending modes.

Fig 2 -FTIR SPECTRUM OF NATIVE COLLAGEN



3.1.4. CD spectra studies

Triple helix conformation of type I Collagen is confirmed by Circular dichroism spectrum. CD spectra of the samples are shown in Figure 3. Collagen is a sort of optically active protein and adopts the polypro line II-like helical conformation with a negative minimum absorption band

around 196nm and a weak positive maximum absorption band at 220nm. Collagen sample had a positive maximum peak at 220nm and a negative minimum peak at 196nm, suggesting a typical triple helical conformation.

Fig .3.CD SPECTRUM OF TYPE 1 COLLAGEN

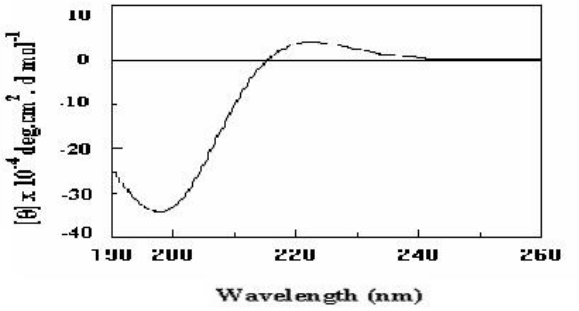


Fig 3.CD spectra of collagen

CD Spectrum of native collagen - π-π* transition peak at 196nm and positive n-π*transition at 220nm

3.2.1. Scanning Electron Microcopy

The SEM was done to determine the pore size of collagen scaffold. The Fig 4 shows SEM image of collagen scaffold. The scaffold contains the pores size vary from 500 -800 microns.

3.2.2. Water Uptake Studies of Porous Collagen Scaffold:

The porous collagen scaffold shows 310± 10% of water uptake

3.3. In Vivo Studies

3.3.1. Wound contraction

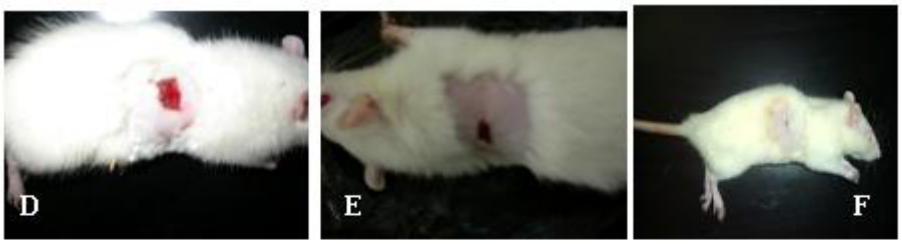
Fig 5 shows wound contraction in the animals treated by porous collagen biomaterial. In the group of porous collagen scaffold, the better wound closure was observed whereas, open wound group showed moderate wound contraction e at the end of 16^{th} day of the treatment, respectively.

Fig 5: Wound Reduction in Albino Rats:

Open Wound Group:

8th Day **12th Day** **16th Day**

Group Treated by porous Collagen Scaffold:



(A) Wound Contraction in animals at the end of 8th Day, (B) Wound Contraction in the animals at the end of 12th day,(C) Wound Contraction in the animals at the end of 16th day. The Group treated by porous Collagen Scaffold Showed better contraction, Collagen biomaterials enhances the epidermal regeneration via kertinocytes adhesion on it. (D), (E), (F) animals were treated by porous collagen scaffolds.

3.4. Histological analysis

3.4.1. H & E staining

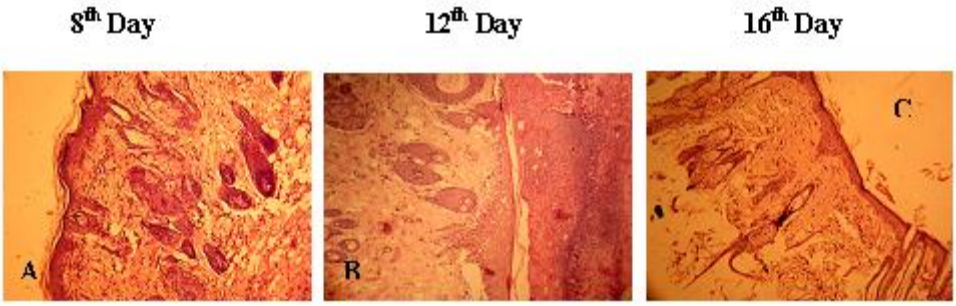
The following histology was observed in the Open wound group. In the 8th day, neutrophils found and bacterial colonies found in the injured area. Complete loss of epithelium with inflammatory infiltrates was observed. In 12th day, bacterial colonies were seen with angiogenesis started. In 16th day, slight formation of epidermis with angiogenesis, but complete healing was not happened.

In the group treated by collagen scaffold**,** in the 8th day, well formed epidermis and absence of bacterial colonies were observed. In 12th and 16th days, modest formation of epidermis and dermis was observed.

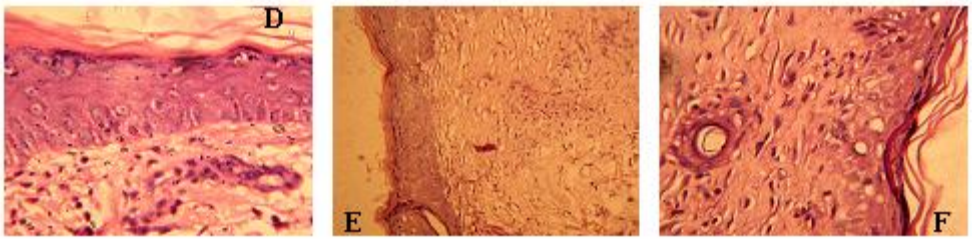
Fig 6: Histology Analysis of Granulated Tissue:

H&E Staining:

Open Wound Group:



Group Treated By Porous Collagen Scaffold:



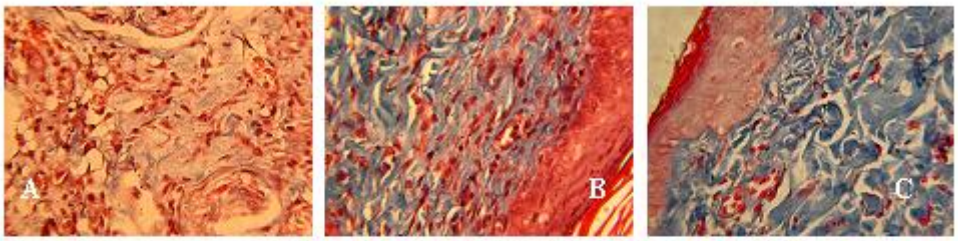
Hematoxylin and eosin stained sections of the granulation tissue at different time intervals. A, B, C, are control group on day, 8, 12, and 16 respectively. D, E, and F are treated group by plain collagen scaffold on day 8, 12, and 16 respectively. A, B, C, and E, are at similar magnification (150x) and D and F are at 400x. The well formed epidermis and dermis developed in the skin in the treated group. The Rat Skin was regenerated by porous collagen scaffold derived from animal sources

3.4.2. Collagen analysis in the tissue by Masson's Trichrome Staining:

In the open wound group**,** on the 8th day, (Fig 7A) neutrophils found and bacterial colonies found in the injured area. Complete loss of epithelium with inflammatory infiltrates was observed and less amount of collagen was seen due to infection. In 12th day and 16th day (Fig 7B and 7C), partially epidermis formed and a loose collagen fiber was observed. In Masson's Trichrome stained histological sections of tissue of 12th and 16th days of groups treated by collagen scaffold, bluish violet color indicates staining of well stretched and deposited collagen bundles formed in the tissue. In the case of open wound at the end of 12 and 16th days, loose collagenous matrix was also seen with proliferating fibroblast. In case of collagen dressing treated group at the end of 12 and 16th days, mature collagen bundles along were found.

Fig 7: Masson's Trichrome Staining

Open Wound Group:



Group Treated By Porous Collagen Scaffold:

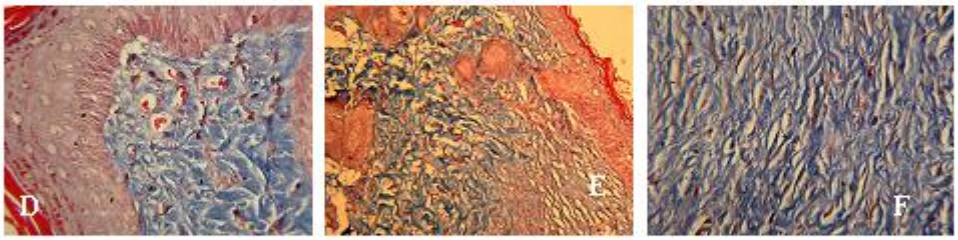


Fig 8. Masson's Trichrome stained sections of the granulation tissue at different time intervals. A, B, and C are control group on day 8, 12, and 16 respectively. D, E, and F are treated group by plain collagen scaffold on day 8, 12, and 16 respectively. A, B, C, D, E, and F, are at similar magnification (150x) .The bluish violet color indicates collagen deposits in the granulated tissue. The collagen formation is very less in the open wound group and also loose collagen bundles formed in the open wound group. The group treated by porous collagen scaffold shows that well formed and organized collagen bundles formed in the tissue.

4. Discussion and conclusion

The healing of dermal wound is challenging[2]. Generally, protein based biomaterials mimic extra cellular matrix of the site of injury and help skin regeneration. Among all the protein biomaterials, collagen is a potentially useful biomaterial as it is a major constituent of connective tissues. Its characteristics offer several advantages such as biocompatible and non-toxic in most tissues, cellular mobility and growth and porous nature. These properties allow a highly vascularized granulation bed formation on the wound. In addition, collagen enhances keratinocytes and fibroblast proliferation which are important in wound healing[3, 4, 5]. The collagen from bovine tendons is found to contain the monomeric, dimeric, trimeric and higher polymeric forms (α, β, γ) [6]. Scaffolds used as wound dressings for soft tissue repair should be reabsorbed into the body after successful tissue regeneration. Collagen–based scaffolds degrade in physiological pathway without induction of inflammatory response. The porosity of collagen scaffold directly influences cellular ingrowth. The modern tissue engineering task is to develop three-dimensional scaffolds of appropriate biological and biomechanical properties, at the same time mimicking the natural extra cellular matrix (ECM) and promoting tissue regeneration. The scaffold should permit cell adhesion, infiltration, and proliferation for ECM synthesis. Furthermore, it should be

biodegradable, bioresorbable and non-inflammatory, should provide sufficient nutrient supply and have appropriate viscoelasticity and strength. Attributed to collagen features mentioned above, collagen fibers represent an obvious appropriate material for tissue engineering scaffolds. Scaffold constructed from naturally occurring proteins in the extra cellular matrix (ECM) such as collagen allows much better infiltration of cells into the scaffold.

The porous collagen scaffold has the ability to absorb large quantities of wound fluid and also maintains moist environment at the wound site. The macro porosity present in collagen scaffold helps to encapsulate drugs efficiently and to enhance the cell fate process. The SEM observation shows that the pore size of the scaffold varies from 500 - 800 microns and these pores helps to encapsulate the drug effectively.

Wound contraction is also an element of wound healing, which occurs through the centripetal growth of tissues surrounding the wound [1]. *In vivo* studies showed that Porous collagen scaffold group had better wound closure than open wound group.

In the histological studies , porous collagen scaffold treated group shows epithelialization with moderate extra cellular matrix on the day 8 and the day 12 whereas in the control group, incomplete epithelialization with less extra cellular matrix synthesis and persistence of inflammatory exudates in the upper dermis with loss of epidermis were observed up to day 16.In Masson's trichrome staining, collagen scaffold treated group has shown well-formed collagen bundles and fibroblast proliferation. The porous collagen scaffold for treating dermal wound application was developed and the wound healing was observed in a shorter time.

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