International Journal of Genetic Engineering and Biotechnology.
ISSN 0974-3073 Volume 1, Number 1 (2010), pp. 11--22

http://www.irphouse.com

Regeneration of Soft Tissue with the Ciprofloxacin Incorporated Collagen Scaffold Delivery Vehicle

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Abstract

The method to regenerate tissue at the injured site is still challenging. Formation of extra cellular matrix in the damaged tissue could be influenced by providing materials as scaffold which acts as a template to grow the damaged tissue. Natural polymers have been used as one of the materials in the scaffolds for wound healing. The objective of this study is to investigate the use of ciprofloxacin- incorporated collagen based scaffold in the dermal wound healing process. The Type 1 collagen was extracted from bovine tendons. The 1% collagen solution was prepared in acidified water using acetic acid and to this, 400 µl of Triton X100, non-ionic wetting agent was added, agitated for homogenization for few minutes and poured into a trough. This preparation was allowed to dry in a dust free chamber. This preparation was incorporated with ciprofloxacin by physical entrapment method. The release of ciprofloxacin from ciprofloxacin incorporated collagen scaffold was studied. Antimicrobial efficacy of ciprofloxacin incorporated collagen scaffold (11 mm diameter) was tested on Mueller-Hinton agar (MHA) against wound pathogens following Kirby-Bauer disk diffusion test. An 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 a 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. A porous collagen sponge impregnated with ciprofloxacin showed a sustained release of ciprofloxacin with 37% of drug burst release within 5 hours followed by

controlled release up to 24 hrs. The *in vitro* antimicrobial efficiency of ciprofloxacin incorporated collagen scaffold against wound pathogens showed the zone of inhibition 38 ± 2mm whereas nil zone of inhibition for plain collagen scaffold. The *in vivo* studies shows that animals treated by drug-incorporated collagen scaffold provides 90% wound contraction at the end of 16^{th} day whereas plain collagen scaffold treated groups shows 65 % wound closure and open wound groups shows 50% wound closure at the end of 16^{th} day. The antimicrobial-agents incorporated collagen-based scaffolds shows the ability to regenerate tissue better than the scaffold without ciprofloxacin.

Introduction

The skin, the outer protective organ, protects against toxins and microorganisms that are present in the environment, and prevents dehydration of all the inner organs. It has self renewal capacity. Skin tissue repair normally involves systematic and coordinated process, resulting from vascular connective tissue genesis and epithelial cells generation. Interleukins and growth factors play a major role in the regulation of cellular processes in wound repair[1]. Wound caused by physical, chemical or biological factors leads to the loss of epidermis which can regenerate but, the loss of dermis does not regenerate. The loss of skin integrity due to injury or illness may result in substantial physiologic imbalance and ultimately leads to disability or even death. The most urgent need for skin regeneration is the quick reconstruction of epidermis and dermis at the wounded site [2].

Improved wound care products are needed to regenerate tissues with both the structural and functional properties in the wounded tissue. By providing a scaffold at the wound site, formation of extra cellular matrix and dermal regeneration are influenced at a faster wound healing rate. The scaffolds made from biomaterials function as primary closure in wounds. In addition, wound dressings should cover the wound surface and create and maintain a moist healing environment [3].

The infection at the site of injury delays wound healing and causes high inflammatory response. Moreover, infected pathogens secrete pathogenic enzymes such as collagenase and hyaluronidase that degrade extra cellular matrix. The infected dermal wound become a chronic wound and therefore wound healing is challenging [4, 5].

The wound healing biomaterial scaffold should mimic extra cellular matrix (ECM) which supports the wound healing processes. Scaffolds prepared from collagen, an important component of extra cellular matrix (ECM), may provide biological stimuli to support tissue growth. Collagen influences wound healing by regenerating a tissue by processes such as cell proliferation, cell migration and cell differentiation. Interactions between the different tissue components and collagen form an essential substrate for cellular adhesion [6]. Moreover, collagen–based biomaterials have several other advantages such as biocompatible and nontoxic materials to tissues. In addition, it enhances the deposition of oriented and organized, newly synthesized collagen fibers in the remodeling phase of wound healing. Furthermore, collagen functions as a substrate for haemostatisis of blood cells by

chemotaxis that promotes wound maturation by providing a scaffold for more rapid transition to normally at the injured site [7, 8].

Collagen-based materials formed into three dimensional scaffolds serve as a wound dressing since they contain large pores which enhance wound tissue infiltration *in vivo*. This wound dressing with burst release and followed sustained release of antibiotics to pathogens can cause rapid eradication of pathogens and subsequent prevention of bacterial infection. The sustained release of antimicrobial agents enhances healing of wounds rapidly[9], [10.] . In addition, the incorporation of antimicrobial agents to collagen dressings may help to maintain stability of collagen from microbial degradation of collagen at the infected wound site. A possible method to deliver antibiotics in a controlled manner is by physically entrapping it in the collagen polymer matrix.

Ciprofloxacin (1-cyclopropyl-6-fluro-1, 4-dihydro-4-oxo-7- (1-pipera Zinyl)-3-quinoline carboxylic acid) entrapped collagen scaffold was used in this study. The ciprofloxacin was used due to its broad spectrum antimicrobial activity as well as having good tissue penetration property. In this present work, we evaluated the tissue regeneration capacity of ciprofloxacin-incorporated collagen biomaterials using *in vivo* studies.

Materials and Methods

Extraction of Type I Collagen from Bovine Tendons

The preparation of collagen from bovine tendon was done [11,12] according to the method developed by Bioproducts lab, Central Leather Research Institute, Chennai, India.

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.

Incorporation of ciprofloxacin to porous collagen scaffold

After getting homogenized collagen preparation, a known amount of ciprofloxacin (0.5 mg) was added and stirred well and poured in the trough. Since the minimum inhibitory concentration of ciprofloxacin against *S. aureus* ATCC 29213 is 0.12 – 0.5 µg/mL and *P. aeruginosa* ATCC 27853 is 0.25 – 1.0 µg/mL, the amount of drug to be added in the collagen scaffold is 10 times of MIC value of drug per cm^2 of the scaffold. The thickness of prepared collagen scaffold is 2mm.

Sterilization of Plain collagen dressings and ciprofloxacin incorporated collagen dressings

The sterilization of collagen biomaterial dressings is done by the ethylene oxide sterilization method [13].

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.

***In vitro* release of ciprofloxacin from ciprofloxacin-incorporated Collagen Scaffold**

In vitro release of ciprofloxacin from ciprofloxacin incorporated collagen scaffold was carried out at 37.1°C in phosphate buffer saline (PBS) (50 ml) pH 7.4. The release medium PBS was collected at predetermined time intervals, and replaced with a fresh PBS (1 ml) each time. The collected samples were filtered through a 0.45 µm Millipore filter. The amount of ciprofloxacin released was then measured at 278nm using a shimadzu UV-2100S spectrophotometer.

***In Vitro* Antimicrobial Activity**

Antibacterial efficacy of ciprofloxacin-incorporated collagen scaffold (11 mm diameter) was tested on Mueller-Hinton agar (MHA) for the growth of *Staphylococcus aureus* ATCC 29213, *Pseudomonas aeruginosa* ATCC 27853 [12].

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 72 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
Group 3 – Ciprofloxacin - incorporated 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. Wounds were inoculated with the test organisms at 10^6 CFU (0.1 mL) between thin skin muscle and paraspinus muscle and allowed to infect for 24 h. All surgical procedures were carried out under thiopentone (40 mg/kg body weight, intramuscular). The infected wounds were covered with collagen dressings and outer covered with gauze dressings. An infected animal without dressings and an animal with dressings without drugs were also maintained in individual cages.

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 (4th, 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.

Gelatin Zymography

The presence of matrix metalloproteinases (MMPs) in the granulated tissues was analyzed by gelatin zymography.

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. [14]

Statistical Analysis

Statistical evaluations were performed using Origin Version 6 and the data were expressed as mean of six samples with standard deviation. The difference between groups was analyzed using one way ANOVA.

Results

Properties of Ciprofloxacin incorporated collagen scaffold

A known amount of ciprofloxacin was added to the collagen solution in the preparation of collagen scaffold, the ciprofloxacin bound to the collagen scaffold by physical entrapment. The FTIR confirms no interaction between ciprofloxacin and collagen.

Scanning Electron Microcopy

The SEM was done to determine the pore size of ciprofloxacin incorporated collagen scaffold. The Fig 1 shows SEM image of ciprofloxacin collagen scaffold. The scaffold contains the pores size vary from 500 -600 microns.

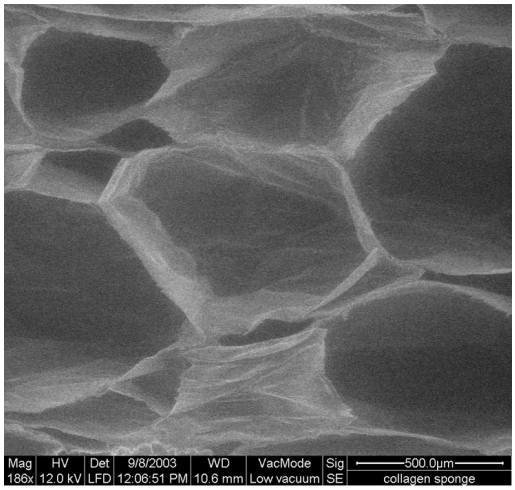

Figure 1 : Porous collagen scaffold
The porous collagen scaffold contains the pore size varies from 500 – 800µm

In Vitro Release of ciprofloxacin from ciprofloxacin incorporated collagen scaffold

In the present study, the collagen scaffold acts as a drug reservoir. Fig 2 shows the release profile of the ciprofloxacin from porous collagen scaffold. It releases the ciprofloxacin immediately and also in a sustained fashion as soon as it is exposed to the wound. The ciprofloxacin incorporated porous collagen scaffold showed a 37% of drug burst release within 5 hrs followed by controlled release of the remaining ciprofloxacin up to 24 hrs.

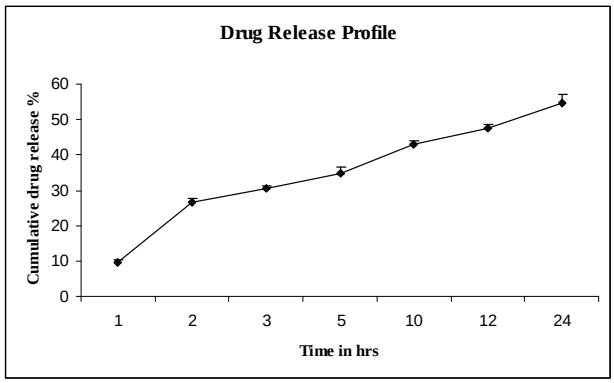

Figure 2 : In vitro Drug Release Profile

Agar Diffusion Test for *In vitro* antimicrobial activity

The activity of the released drug from the scaffold shows a clear zone of inhibition controlling the growth of both *staphylococcus aureus* and *pseudomonas aeroginosa*

inoculated separately. Ciprofloxacin-incorporated collagen scaffold showed a bacterial free zone of 38 ± 2 mm.

In Vivo Studies

Wound contraction

Fig 3 shows wound contraction in the animals treated by ciprofloxacin incorporated collagen biomaterial. In the group of ciprofloxacin incorporated collagen scaffold, wound contracted 40 %, 75 %, 82.5 %, 90 % at 4,8,12 and 16th day of the treatment, respectively, whereas ,plain collagen scaffold and open wound group showed wound contraction of 65% wound closure and 50% wound closure at the end of 16th day of the treatment, respectively.

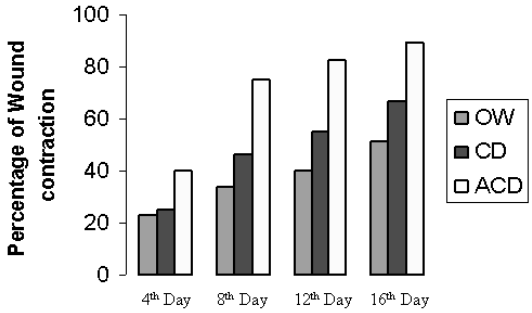

Figure 3 : Wound contraction in animals

Histological Analysis

The following histology was observed in the Open wound group. In the 4th day and 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 4th and the 8th day, well formed epidermis and absence of bacterial colonies were observed. In 12th and 16th days, complete formation of epidermis and dermis was observed.

In the group treated by ciprofloxacin incorporated collagen scaffold, in the 4th day and the 8th day, epithilization with moderate extra cellular matrix was observed. On the 12th and 16th days, marked epithelialization with moderate amount of extra cellular matrix synthesis and new blood vessel formation were seen.

Histological analysis of wound healing by H&E staining

In the tissue samples of day 4 of ciprofloxacin incorporated collagen scaffold treated group, minimum neutrophilic infiltration was seen on the surface of the wound.

Fig 4I shows well formed epithilization in the tissue whereas in day 4 of open wound group (Fig 4A), heavy neutrophilic infiltration was observed in the surface of

the wound. A fewer macrophages were also seen. Bacterial colonies were found over the granulated tissue. In the plain collagen scaffold group, mild neutrophilic infiltration was seen on the surface of the wound (Figure 4E). Partial epithilization has also formed in this group.

In the 8th day of ciprofloxacin-incorporated collagen scaffold group, uniform granulation was formed in the tissue. In addition, angiogenesis was seen (Figure 4J) in this tissue. Maturing fibroblast was also seen in the dermal region with collagen deposition, whereas in day 8th of the open wound group, bacterial infection was still persistent along with heavy neutrophilic infiltration (Figure 4 B). In the plain collagen scaffold group, mild angiogenesis was observed. (Figure 4 F).

In 12th day of ciprofloxacin-incorporated collagen scaffold group, epithelialization was seen (Figure 4 K). Dermal region was seen to have good angiogenesis along with mature fibroblasts and collagen deposition, whereas in day 12th of open wound group, bacterial infection was still prominent along with invading neutrophils in the granulation tissue (Figure 4 C). Macrophages were also seen along with fibroblastic cells below the neutrophilic infiltration. Extra cellular matrix (ECM) formation at the dermal region was seen. Bridging of the wound surface was observed with plain collagen scaffold. Epithelial cell proliferation was also seen (Figure 4 G) in this group. Bacterial infection was lesser. The granulation tissue was well formed with collagen bundles.

In the 16th day of ciprofloxacin incorporated collagen scaffold group, complete healing was seen with good epithelialization (Figure 4 L). Dermal layer was seen to have mature fibroblasts with deposited collagen. Whereas in the 16th day of open wound, tissue remodeling was seen (Figure 4 D) and epithelial proliferation with well-formed collagen bundles was seen but healing was incomplete. Dermal region has mature fibroblasts i.e. surface of the wound was not covered by epithelium .In the plain collagen scaffold, complete epithelialization was seen (Figure 4 H). Mature fibroblastic cells were seen in the dermal region with collagen deposition.

Collagen analysis in the tissue by Masson's Trichrome Staining

In the open wound group, on the 4th day and the 8th day, (Fig 5A and 5B) 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 5C and 5D), partially epidermis formed and a loose collagen fiber was observed.

In the group treated by collagen scaffold, on the 4th day (Fig 5E), neutrophils found and bacterial colonies found in the injured area. Complete loss of epithelium and no collagen content in tissues was observed. In 8th day (Fig 5F), a bundle of collagen was seen .In 12th day and 16th day (Fig 5G and 5H), well formed epidermis and dermis was seen in the tissues. A loose collagen bundle in the tissues was observed.

In the ciprofloxacin incorporated collagen scaffold group, on the 4th day Fig 5I, 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 8th day Fig 5J, partially epidermis formed and a stretched

bundles collagen fiber in the dermis was observed. In the 12th, Fig 5K shows well formed epidermis and dermis was seen and stretched collagen bundles formed in the tissue.

Collagen analysis by Masson's Trichrome Staining: - Fig 5

In Masson's Trichrome stained histological sections of tissue of 12th and 16th days of groups treated by ciprofloxacin incorporated 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 with fibroblastic cells were found.

Biochemical Studies

Role of MMPs in Tissue remodeling

Fig 6 shows gelatin zymography of MMP taken from tissues of all the groups. In the present study, MMP 2 and MMP 9 were detected in granulated tissue of all the groups on days 4, 8, 12, 16. MMPs were detectable in very low amount by gelatin zymography in the treated group than in the control groups. In the infected dermal wound, MMP -9 (gelatinase –B) is highly expressed in inflammatory period of wound healing .The MMPs secreted by inflammatory cells is highly excreted in the control groups than in the treated groups. The expression of MMP9 decreased in the treatment groups than in the control groups.

Discussion

The healing of infected dermal wound is exigent. Wound pathogens secrete enzymes which degrade extra cellular matrix at the site of injury and also form a biofilm which delays wound healing [1, 2, 3]. Biomaterials with antimicrobial agents enhance regeneration of dermal tissues and eradicate wound pathogens at the wound site. 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 [15, 16], 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. The cost of synthetic biomaterial such as Poly Lactic Glycolic Acid (PLGA), Poly Lactic Acid (PLA) and Poly Caprolactone (PCL) are very high and also cause inflammatory response to host tissue [7]. Therefore, natural biomaterials are studied as alternative materials to synthetic biomaterials. Type 1 collagen is cheap and it is present in higher order animals especially in the skin, tendon and bone, and is prepared from slaughter house. Scaffolds used as wound dressings for soft tissue repair should be reabsorbed into the body after successful tissue regeneration.

Collagen–based scaffold degrade in physiological pathway without induction of inflammatory response. The porosity of collagen scaffold directly influences cellular ingrowth. The porous nature of collagen scaffold helps to encapsulate the drug effectively. 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.[8, 9]

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.

Normally, collagen biomaterial is not suitable for infected dermal wounds, because wound pathogens utilize collagen as substrate for their growth and increase infection rate at the site of injury. The incorporation of antimicrobial agents to the collagen biomaterials prevents the above character. An ideal wound care system is the one that delivers sufficient quantity of drug at the site of action, decrease further bacterial proliferation and better dermal regeneration. The sustained release system facilitates it. [9]

In the ciprofloxacin-incorporated collagen scaffold, the same percentage of the drug is burst released as soon as it comes in contact with the wound. Moreover, the degradation of scaffold at the site of injury also causes sustained release of bound drug. The drug release during burst release showed much higher MIC value that can overcome the growth of the wound pathogens. This is confirmed by *In vitro* antibacterial test with significant zone of inhibition. 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 antibiotic incorporated collagen scaffold group had better wound closure than plain collagen scaffold group and open wound group.

In the histological studies, ciprofloxacin incorporated 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, in ciprofloxacin incorporated collagen scaffold treated group has shown well-formed collagen bundles and fibroblast proliferation.

Significant reduction of MMP 9 and MMP 8 in ciprofloxacin-incorporated collagen scaffold treated group supports the reduction of inflammatory phase. Significant reduction of inflammatory cells in the early phase of healing in

ciprofloxacin incorporated collagen scaffold treated group indirectly shows the reduction of bacterial population, thereby enhancing healing [18, 19]. The ciprofloxacin incorporated porous collagen scaffold for treating infected dermal wound application was developed and the wound healing was observed in a shorter time compared with other control scaffold that lacks ciprofloxacin.

Reference

[1] Adam J. Singer, M.D., Richard. Clark, M.D., Cutaneous wound healing New England Journal of Medicine. 1999: 2:738-746.

[2] R.A.F. Clark, Wound repairs Overview and general considerations. In: R.A.F. Clark, Editor, The Molecular Biology of Wound Repair, Plenum Publishing, New York, 1996:195–248.

[3] P.G .Bowlerl, B. I. Duerden, D. G. Armstrong, Wound Microbiology and Associated Approaches to Wound Management Clinical Microbiology Review. 2001: 14: 244 – 269.

[4] Schmidtchen, A., Holst, E., Tapper, H. et al., Elastase-producing Pseudomonas aeruginosa degrade plasma proteins and extracellular products of human skin and fibroblasts, and inhibit fibroblast growth Microbial pathogenesis. 2003: 34: 47 – 55.

[5] James M.Pachence, Collagen –based Devices for Soft Tissue Repair J Biomed Mater Res Part B: Appl Biomaterials. 1996: 33:35 -40.

[6] Chi H.Lee, et al, Biomedical Application of collagen – Review, Int Journal of Pharm. 2001: 221:1 -22.

[7] Zbigniew Ruszczak Effect of collagen matrices on dermal wound healing Advanced Drug Delivery Reviews.2003: 55(12):1595-1611.

[8] Chvapil M. Collagen sponge: Theory and practice of medical applications J Biomed Mater Res.1977: 11: 721 -741.

[9] Loke WK, Lau SK, Yong LL, Khor E, Sum Ck. Wound dressing with sustained anti-microbial capability J Biomed Mater Res.2000: 53: 8 -17.

[10] Zbigniew Ruszczak and Wolfgang Friess, Collagen as a carrier for on-site delivery of antibacterial drugs Advanced Drug Delivery Reviews.2003: 55: 1679-1698.

[11] R. Sripriya, Md. Rafiuddin Ahmed, P. K. Sehgal, R. Jayakumar, Influence of laboratory ware related changes in conformational and mechanical properties of collagen J Appl Polym Sci.2003: 87: 2186–2192.

[12] Hsiu-O, H.; Lun-Huei, L.; Ming-Thau, S. Characterization of collagen isolation and application of collagen gel as a drug carrier, J Cont Release.1997: 44: 103–112.

[13] S. D. Gorham, S. Srivastava, D. A. French and R. Scott, The effect of gamma-ray and ethylene oxide sterilization on collagen-based wound-repair materials, Journal of Material Science: Materials in Medicine.1993: 4: 40-49.

[14] Luna .L et al., Manual of Histological Staining Methods., 3rd edition. 1968 McGraw hill publishers.

[15] K.Gelse. et al, Collagens—structure, function, and biosynthesis, Advanced Drug Delivery Reviews.2003: 55: 1531– 1546.
[16] Alberts B, Bray D, Lewis J, Raff M, Roberts K, Watson J. Molecular biology of the cell. 4th Ed. New York: Garland Publishing; 2002.
[17] Rao, K.P., Recent developments of collagen-based materials for medical and drug delivery systems J. Biomater. Sci.1995: 7: 623–645.
[18] G. David, D.P.M. Armstrong, B. Edward and M.D. Jude, The Role of Matrix Metalloproteinases in Wound Healing, J. Am. Pediatr. Med. Assoc.2002:92: 12
[19] Salo, M. Makela, M. Kylmaniemi, H. Autio-Harmainen and H. Larjava, Expression of matrix metalloproteinase-2 and-9 during early human wound healing Lab. Invest.1994:70: 176.

Figure 4 : Histology – H & E staining

Hematoxylin and eosin stained sections of the granulation tissue at different time intervals. A, B, C, D are control group on day 4, 8, 12, and 16 respectively. E, F, G and H are treated group by plain collagen scaffold on day 4, 8, 12, and 16 respectively. I, J, K and L are treated group by drug incorporated collagen scaffold on day 4, 8, 12, and 16 respectively. A, B, C, D, E, F and G are at similar magnification (150x) and H, I, J and L are at 400x.

Figure 5 : Histology –MT staining

Masson's Trichrome stained sections of the granulation tissue at different time intervals. A, B, C, D are control group on day 4, 8, 12, and 16 respectively. E, F, G and H are treated group by plain collagen scaffold on day 4, 8, 12, and 16 respectively. I, J, K and L are treated group by drug incorporated collagen scaffold on day 4, 8, 12, and 16 respectively. A, B, C, D, E, F, G,I and K are at similar magnification (150x) and H,J,L are at 400x.

Figure 6 : MMP in wound healing.

Gelatin Zymography shows Matrix Metalloproteinase expression in granulated tissue .Lane 1 – 4^{th} Day, Lane II-8^{th} Day,Lane III -12^{th} Day,Lane IV -16^{th} Day. In open wound Group, Gels shows high level of expression of MMPs due to high level inflammation and infections. In treated group, MMP 2 and MMP 9 expression is very less due to faster healing.n