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Expression of pro-inflammatory cytokine (IL -1α) and collagenase (MMP-8) in infected dermal wound healing in a rat model.

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ABSTRACT: Wound infection is a major problem, which hinders the normal healing process. To overcome this problem in the current study, chemically modified collagen bilayer dressing with drug ciprofloxacin was designed and its efficacy was checked on infected wound healing. Therefore the author's objective was to evaluate the dressing on full thickness infected wound on rat animal model. *In vitro* drug release pattern, antimicrobial activity and *in vivo* efficacy of the dressing were studied. The healing pattern was analyzed on days 3, 5, 7, 14 and 21 by wound closure rate, bacterial population, collagen content, immunological and histological examinations of tissue samples. *In vitro* drug release pattern showed a release profile for 5 days with effective drug concentration confirmed by zone of inhibition. *In vivo* analysis showed significant wound closure, collagen content from the granulation tissue, enhanced healing in the treatment group. Immunological analysis of MMP-8 and IL-1α using western blot and immunohistochemistry confirms normal healing pattern in treatment group. Histological analysis and wound closure further confirmed proper healing in treatment group. Our results suggest that sustained release of ciprofloxacin from a collagen bilayer dressing eliminates bacterial infection, leaving a pathogen-free wound environment, and it can be used as a dressing for an on-site delivery system.

KEYWORDS: Wound healing, Bilayer dressing, MMP-8, IL-1α, histology

1. Introduction

Wound healing involves a complex and coordinated process of cell activation, cell division, chemotaxis, migration and differentiation of many cell types. It is mediated by locally released growth factors and cytokines/inflammatory mediators like interleukins and adhesion receptors like integrins, which may act in an autocrine or paracrine manner. All phases of wound healing are either directly or indirectly controlled by cytokines. It is the balance of these cytokines and other mediators, rather than the mere presence or absence of one or more cytokines, which plays a decisive role in regulating the initiation, progression and resolution of wounds [1-4].

The sequence of events begins at the moment of injury, which involves platelet aggregation, blood clotting, formation of fibrin, an inflammatory response to injury, alleviation in the ground substance, endothelial and capillary proliferation and surface covering, regeneration of certain cell types, variable contractures and remodeling. The neutrophil and macrophages are the initial types of cells infiltrating the wound tissue and start the process of phagocytosis. Macrophages continue to accumulate at the wound site by recruitment of blood-borne monocytes and are essential for effective wound healing and if macrophage infiltration is prevented, then healing is severely impaired [5]. Fibroblasts distant to the site begin to proliferate and follow along. The net result is an increased production of very active collagen producing cells. As the new matrix begins to accumulate and fill the void created by the injury, the phase of transitional repair ensues. The cells continue to reabsorb and redeposit collagen in an effort to regain the structural integrity that existed prior to injury. The inflammatory response to tissue injury is generally considered a protective mechanism, which prevents excessive blood loss and development of infections.

The prefabricated collagen matrix acts as a template for fibroblasts adherence and orientation. Although more collagen is present in the initial period than at later time points, wounds get greater tensile strength as remodeling of collagen occurs. These events are facilitated by collagenases, gelatinases, stromelysins and membrane type metalloproteinases all of which are responsible to reshape the initial collagen deposition. As a consequence of the inflammatory reaction there is a rapid catabolism of the existing collagen at the site. The speed and extent of the collagen removal can be corrected with the acuteness of the inflammation.

Tissue injury, whether full thickness or an incision has the risk of serious infection, if the number of organism passes a certain critical level when the wound becomes infected [6]. During wound healing, number of remodelling events takes place and is under the control of matrix metalloprotease and their inhibitors (TIMPs). Interleukins are also secreted by activated white blood cells, which stimulate activation, differentiation and proliferation of other white blood cells.

We report here the biochemical, histological pattern and production of proinflammatory species like IL-1~~alpha~~α and collagenase (MMP-8) in full thickness wound infected with *Pseudomonas aeruginosa* and *Staphylococcus aureus* during the early and later days of healing with chemically modified drug incorporated collagen bilayer dressing.

2. Materials and Methods

2.1 Preparation of Succinylated Collagen bilayer dressing

Pure collagen was extracted from bovine source following the reported procedure [7]. The obtained collagen was succinylated using succinic anhydride at pH 9. The succinylated collagen was precipitated at pH 4.2 and washed in water and allowed to swell in Milli-Q water. The succinylated collagen solution was converted into a bilayer dressing consisting of collagen film and sponge as reported earlier [8].

2.2 Incorporation of Drug

In this system the drug Ciprofloxacin-HCl (anionic) (HiMedia Laboratories-India) was mixed with 10% (w/v) of Poly (N-vinyl-2-pyrrolidione) (PVP) (Merck (Germany) solution and was made into a homogenous solution. Half the volume of the solution was allowed to diffuse into the sponge and half into the film. The concentration of the drug distributed between the two layers was 0.2 mg/cm^2. The drug remains totally protected in the bilayer system, which acts as a seal.

2.3 *In vitro* drug release

The pattern of ciprofloxacin release from the bilayer dressing was performed in Franz-type diffusion cells at 37° C. The receiver compartment was filled with phosphate buffer solution (PBS, pH 7.4), which was stirred with a magnetic stirring bar. At regular time intervals, an aliquot (1 ml) was removed from the receiver compartment and replaced with an equal quantity of PBS to maintain constant volume. The drug concentration was determined by high performance liquid chromatography (HPLC) (515 pump (Waters, USA) and SPD 10A Shimadzu UV-Visible detector (Shimadzu, Japan). The stationary phase was a Phenomenex C18 column (5 µm, 250×4.6 mm) and the mobile phase was CH3OH/Na2HPO4 (30:60), pH 3.0. The flow rate was 1.0 ml/min and the detector was set at 276 nm. A ciprofloxacin standard curve ranging from 0.1 to 400 µg/ml was established and p-hydroxybenzoic acid was used as an internal standard.

2.4 Agar diffusion

Antimicrobial properties of ciprofloxacin-incorporated dressings (11mmdiameter) were tested on Agar plates inoculated with a mixed culture of Staphylococcus aureus ATCC 29213 and Pseudomonas aeruginosa ATCC 27853 following the Kirby–Bauer disk diffusion test [9].

2.5 Creation of Infected Wound

Male wistar albino rats weighing 200–250 g were obtained from Tamil Nadu Veterinary Medical University (Chennai, India) and caged individually in aseptic conditions. Animal experiment was performed according to the Institute's Ethical Committee guidelines registered under CPCSEA (Reg. No. 466/01/a CPCSEA). Animals were anaesthetized by intraperitoneal injection of pentothal sodium (50 mg/kg body weight) and the hair from the dorsal region was removed with a razor. A full thickness wound with a surface area of 2 × 2 cm^2 was excised on the back of rat using sterile scalpel blade to the depth of loose subcutaneous tissues. The mixed culture (105 CFU/0.1 ml) of standard strains (Pseudomonas aeruginosa (ATCC 27853) and Staphylococcus aureus (ATCC 29213) were injected between the thin and paraspinus muscles using a sterile syringe and allowed the wound to develop infection for 24 h. The rats were divided into three groups with 25 animals per group. Group 1 comprised animals which had open infected wounds (open wound group). The animals belonging to groups 2 and 3 had collagen bilayer (control group) and drug incorporated bilayer dressings (treatment group) respectively over the infected wounds. The bilayer dressings were secured to the infected wounds using sutures. Evaluations were performed after 3, 5, 7, 14 and 21 days by collecting the granulation tissue from the wound site.

2.6 Wound healing rates

Wound closure was determined by using the initial and final areas drawn onto glass slides during the experiments, with percentage wound closure calculated as (initial−final)/initial)×100 [10].

2.7 Total bacterial count

A biopsy of the granulation tissue was taken on days 3, 5, 7, 14 and 21 to analyse the total number of bacteria. In brief, 10 mg excised granulated tissue was placed in 10 ml sterile saline, followed by vortexing for a few minutes and the total bacterial population was analyzed using the serial dilution method.

2.8 Collagen estimation

In addition to wound closure parameters as described above, the healing pattern was examined by the amount of collagen present in the granulated tissue which was measured by hydroxyproline estimation using the Neuman and Logan method [11].

2.9 Western Blot Analysis

Supernatant of the granulation tissue homogenate from each group was treated with equal volume of sample buffer free from β-Mercaptoethanol and subjected to native polyacrylamide slab gel electrophoresis. The separated protein was transferred to nitrocellulose membrane (Hybond ECL, Pharmacia Biotech, Amersham) at 25 volts (constant voltage) current settings for 1 hr. The membranes were then blocked using 5% Skim milk powder in Tris buffered saline (TBS, pH 7.4) for 1 hr at room temperature or overnight at 4º C. The membranes were washed twice successively in TTBS (50 µl of Tween-20 in 100 ml of TBS) and TBS. The washed membranes were incubated with goat polyclonal antibodies against MMP-8 (Santa Cruz Biotechnology, Cat No.Sc-8848) and Interleukin1α (Santa Cruz Biotechnology, Cat No. Sc-1254) diluted 1:1000 with blocking buffer overnight at 4°C. The membranes were again washed exhaustively with TTBS, TBS and then treated with respective secondary antibodies conjugated with alkaline phosphatase diluted 1: 500 with blocking buffer for 1 hr at room temperature. The membranes were further washed with TBS twice and visualized using BCIP/NBT reagent (Genei, Bangalore, India).

2.10 Tissue Processing for Immunohistochemistry

The granulation tissues from each group were immediately transferred to 10% NBF fixative for 24 hrs at 4°C. The tissues were rinsed in cold PBS and the specimens were covered with 20% sucrose in PBS and left at 4°C overnight. The granulating/regenerated portion of the specimen from all the groups were processed for immunohistochemical localization of tissue antigens. The specimens were embedded in OCT (optimum cutting temperature, Tissuetek, USA) embedding medium and frozen. Serial transverse sections around the granulating/regenerating tissues were sectioned at 15 µm using a cryostat (JUNG CM 3000, Leica). The sectioned tissues were fixed with ice-cold acetone for 30 minutes. It was then washed twice with PBS (100mM, pH 7.4). The endogenous peroxidase activity was blocked using 10% H_2O_2 in methanol for 30 minutes and then washed twice with PBS. The slides were then blocked using 10% normal goat serum or BSA for 1 hr at room temperature and were washed twice with PBS. They were then incubated with the following primary antibodies; MMP-8, interleukin-1α for 4 hrs at room temperature and were washed twice with PBS at room temperature. The slides were incubated with rabbit anti-goat-IgG conjugated with HRP at the recommended dilutions (Genei, Bangalore, India) for 1 hr at room temperature and were washed twice in PBS. It was further incubated in DAB substrate system for 30 minutes to develop the color. The slides were monitored for color development and the reaction was stopped by washing the slides in water. The tissue sections were then counterstained with Harris hematoxylin to stain the nucleus.

2.11 Tissue Processing for Histological Analysis

The histological analyses were carried out after 3, 5, 7, 14 and 21 days, following wound creation. The granulating/regenerating tissues were immediately retrieved and transferred to 10% neutral buffered formalin (NBF) for 24 hours at 4°C. The formalin fixed tissues were dehydrated through grades of alcohol and cleared in xylene and then embedded in paraffin wax (58-60°mp). The moulds were labelled and stored until use. The tissues samples were sectioned at 10µm thickness, deparaffinized and stained with hematoxylin and counterstained with eosin.

2.12 Statistical analysis

All statistical analysis was performed using SPSS software (Version 7.5). Data were expressed as mean±SE. The difference between the control and treated groups were analysed using one-way ANOVA. The alpha level was set at 0.05.

3. Results

3.1 *In vitro* drug release

In vitro release study showed drug release for 5 days. The initial release was from the drug entrapped in the sponge. Once the dressing gets wet the drug coated in the film starts to release thereby maintaining the drug availability for 5 days (Fig. 1). Use of PVP – a biodegradable polymer helps in controlling the drug release and gets absorbed into the system without causing side effects.

3.2 Agar diffusion

The bilayer dressing containing ciprofloxacin showed a clear zone of inhibition (28 ± 2 mm) against the mixed culture (Staphylococcus aureus ATCC 29213 and Pseudomonas aeruginosa ATCC 27853) using the agar diffusion method and the zone was maintained for more than 5 days, but no zone was observed around the collagen dressing without drug (Fig. 2).

3.3 Wound healing rates

Visual inspection of the wound showed that all the animals had well formed granulation tissue by day 5. Following wounding an early increase in wound size was observed in infected animals, after which wound closure commenced. The percentage reduction of wounds in the different groups is shown in Fig. 3. Wound of group 1 rats (Open wound) were reduced to 43% of the original wound area, where as in group 2, it was around 51% of the original wound area after 5 days. In the case of group 3, the wound area was reduced to 55 % of the original wound area. The wound was reduced in groups 1, 2 and 3 to 52%, 66%, and 75% respectively of the original wound area after day 7 of wound creation. Among the experimental groups, animals having collagen matrices showed significant ($p<0.05$) wound reduction as shown in Table 4.1a than the control animals on day 7 after wound creation. The animals in group 3, which had drug incorporated collagen bilayer showed significant ($p<0.05$) wound reduction than the animals having open infections and bilayer collagen matrices (groups 1 and 2). The same trend continued after 14 and 21st days, indicating wound reduction in the group of collagen matrices containing drug (antibiotic). After 14 days wound healing showed more or less normal skin architecture in this group. On 21 days of wound healing, full regeneration of skin with normal skin architecture was observed.

3.4 Total bacterial count

Biopsy of the granulation tissue was taken on 3rd, 5th, 7th, 14th and 21st day of analysis and the number of colony forming units in the tissue was estimated. The number of bacteria dropped from the beginning of the experiment in all the cases including the open wound and control due to animal's immune system as shown in Figure 4. But the treated group showed 10^1 colonies on 7th day and almost no colonies on 14th day. Treated groups showed normal wound healing starting from 14th day whereas in the case of open and control (group 1 & 2), the CFU reduced to 10^5 on 5th day and 10^4 on 7th day causing incomplete healing in both the cases (Fig. 4).

3.5 Collagen estimation

There was an increase in wound granulation tissue hydroxyproline content, in treatment groups on all days of analysis when compared to open wound and control groups. As the wound is infiltrated by more fibroblasts during the later stages of healing particularly in the remodeling phase collagen is deposited resulting in an increase in the hydroxyproline content in the treatment group undergoing proper healing (Fig. 5).

3.6 MMP-8 expression in the regenerating wounds

The expression of MMP-8 was studied qualitatively by western blotting and Immunohistochemistry.

3.7 Western blot analysis

The western blot analysis shows intense reaction towards the protein during the early days showing more enzyme produced, which subsequently diminishes after progressive healing. At day 3 (figure not shown), increased expression of MMP-8 was observed in all groups. At day 5, 7 and 14, the band intensity was more in the open wound and control groups than treatment group demonstrating more MMP production concomitant

with moderate to heavy neutrophil infiltration as observed in the H&E stained sections. Similar expression pattern was found in groups I & II and in group III the intensity was very less on day 21 (Fig. 5).

3.8 Immunohistochemical analysis

In the immunohistochemical preparations at day 3, the antigen reaction was seen more in the cytoplasmic regions in the open wound and control groups, whereas in treatment group the expression was seen both at the nuclear and cytoplasmic regions. In the same way the immunohistochemical preparation shows cytoplasmic localization of MMP-8 in all the experimental groups on day 5. The expression is more in treatment compared to open and control groups on day 14. At day 21, localized intense reactivity was seen in the open wound and control experimental groups than treatment group which showed rather less and scattered expression of MMP-8 (Fig. 6).

3.9 IL-1α Expression in the Regenerating Wounds

The expression of IL-1α was studied qualitatively by western blotting and Immunohistochemistry.

3.10 Western blot analysis

The intensity produced due to the antigen antibody reaction was almost similar in all the experimental groups on day 3 and 5 after wound creation with only slight increase in the level of this pro-inflammatory cytokine (Fig.7). The level of IL-1α was more in treatment group on day 7 compared to the other experimental groups.

3.11 Immunohistochemical analysis

The immunohistochemical analysis of interleukin-1α was performed on the granulation and healing tissues of days 3, 5, 7, and 14 and were shown in Figure 8. There was nominal expression seen in treatment and control groups on days 3 and 5 whereas, open wound showed more expression. The expression was more localized to certain regions and cytoplasmic in origin. Predominantly IL-1α was expressed by the proliferating keratinocytes during the later stages. During the early days they were found along with the invading macrophages. The treatment group shows very less expression on day 14 almost reaching to normal level whereas the open and control shows high expression of pro-inflammatory cytokine indicating high inflammatory response.

3.12 Histological analysis

Histological observation shows heavy neutrophilic infiltration in wound surface on day 3 in all cases with fewer macrophages. Bacterial colonies were found over the granulation tissue and dermal region was seen with fibroblasts (proliferating) with ECM deposition in treatment groups. Day 5 analysis shows the retention of bacterial colonies with neutrophil infiltration. In case of treatment group granulation tissue was seen extending into the muscle. Dermal region showed collagen deposition with maturing fibroblast on day 7 in treatment animals where as in groups 1&2 bacterial colonies along with invading neutrophils were observed. Macrophages were seen below neutrophil infiltration in granulation tissue of animals with open wound. Treatment group showed prominent angiogenesis with matured collagen bundles and wound edges showing proper epithelialization on day 14. Epithelial proliferation with well formed collagen bundles but healing was incomplete in case of open and control group due to incomplete closure of wound surface on day 21 (Fig. 9). In case of treatment group complete epithelialization was seen. Mature fibroblastic cells were seen in the dermal region with collagen deposition.

4. Discussion

The healing pattern of infected wounds was studied in experimentally infected rats (mixed culture of Pa & Sa). The rate of healing was compared between different groups in which one group was left as infected open wound and the another one as infected wound with collagen dressing without drug. The treatment group included wounds treated with collagen dressings with drug Ciprofloxacin ionically bound to the system with a polymer.

The well formed epithelial layer in the regenerated wounds using the collagen systems (Fig. 9) on day 21 may be related to the chemotactic nature of collagen, which attracts and helps in the proliferation of cells that provided a moist environment and enhanced epithelialization. Epithelialization provides a covering for wounds while contraction takes place, depending on the size of the wound differing amounts of epithelium will remain after healing is complete. This may be attributed to the increased activity of fibroblast in the presence of

extracellular matrix proteins. Further it is known that the surrounding extracellular matrix as well as the connective tissue deposited in the wound gel plays an important role in the contractile process. These matrices provide the anchoring points and connecting cables to which contractile cells [12] bind and attempt to reduce the wound volume through an active contraction process [13].

The inflammatory phase is characterised by the presence of neutrophils and monocytes in the wound. Neutrophils are present early but only transiently in the inflammatory phase, appearing to be primarily responsible for the destruction of bacteria by phagocytosis. If the wound is not contaminated, neutrophil infiltration ceases after a few days. If however, there is bacterial contamination the alternative complement cascade remains active and neutrophil chemotaxis continues [14]. Neutrophils are the first leukocyte observed to infiltrate an area of inflammation and injury followed by monocytes. The classic pattern of neutrophil involvement in acute inflammation is initiated with a chemotactic response and migration to the site of injury. During this process, neutrophils become primed, activated, and adherent to endothelial surfaces of the blood vessels adjacent to the site of inflammation. Following attachment, neutrophils transmigrate through an intracellular junction into the extra cellular matrix and then move on to the site of injury. To facilitate their egress from the circulation they penetrate to extracellular matrix, and degrade and digest devitalised tissue and pathogens. Thus neutrophils possess an armamentarium of proteolytic and free radical generating enzymes [15]. Unlike other cells that primarily express and secrete proteases on demand, the proteases of neutrophils are formed during myelopoietic development and stored within a variety of cytoplasmic granules and secretory residues.

Histological analysis illustrates that the collagenous matrix of the dermal scar is arranged into thick bundles that are parallel to the epidermis, whereas normal dermis lacks this type of organization [16]. The appearance of collagen type I and VI were investigated in human skin wounds by immunohistochemisty [17]. Result shows that both collagens appeared almost constantly after a wound age of 6 to 7 days and could also be found in wounds aged a few months.

Although the expression of MMP-8 has been reported to occur in atleast one other cell type, neutrophils serve as the principal source of this enzyme [18,19].The collagenolytic matrix metalloproteinase-8 (MMP-8) is essential for normal tissue repair but is often overexpressed in wounds with disrupted healing [20].

In Chronic pressure ulcers, there is a massive invasion of neutrophils and they release a very potent collagenase called MMP-8, which is responsible for connective tissue break down [21]. In collagen degradation, these enzymes can act after collagenases (MMP-1 and MMP-8) have degraded collagen types to gelatins, which are the main substrates for MMP-2 and MMP-9, whereas MMP-8 is expressed mostly by neutrophils and in the early phase of wound healing [22,23]. Both MMP-1 and MMP-8 are present at high levels during the inflammatory and early proliferative phases of wound repair. In a study carried out to test the pattern of appearance of MMP-8 and MMP-1 during normal dermal wound healing studies showed that wound exudates records peak expression of MMP-8 on day 4 and MMP-1 on day 7. Moreover at all times the MMP-8 were statistically higher than MMP-1. This study provides evidence implicating MMP-8 as major collagenase in healing dermal wounds [24].

Other studies investigated the role of collagenase in wound healing in a dorsal skin incision model implanted with polyvinyl alcohol sponge and given a dose of 10 mg/kg body weight GM6001, a novel inhibitor of MMPs. The results show that inhibition of MMP activity during wound healing enhances wound strength even though new collagen synthesis and the inflammatory response are significantly decreased. This could be achieved by decreasing collagen turnover or increasing collagen maturation and cross-linking or both [25].

Topical application of a chemically modified tetracycline to full thickness open wounds in streptozocin treated rats results in decreased collagenase and gelatinase levels and increased granulation tissue formation [26].[26] Some exciting new research suggests that members of the tetracycline family of antibiotics, such as doxycycline, when given systemically, may be useful to treat pressure ulcers, because at low doses, they inhibit MMP-8 [27].

In the present study the expression of MMP-8 is more during early inflammatory phases due to heavy neutrophil infiltration in all cases i.e. open wound, control and treatment groups. There is a gradual decrease in the level of expression in treatment group than the other two groups and this might be due to the reduction in neutrophils infiltration in treatment group at early stage of healing and was observed more during remodelling phase i.e on day 14.

After injury, pro-inflammatory cytokines act as important modulators of the inflammatory process. IL-1 expression has been regarded as necessary for healing; however, its effects have also been implicated in delayed wound repair [28]. Keratinocytes respond to injury by releasing the pro-inflammatory cytokine interleukin-1, which serves as the initial "alarm signal" to surrounding cells. Among the consequences of interleukin-1 release is the production of additional cytokines and their receptors by keratinocytes and other cells in the skin [29]. There was increased expression of IL-1α in all the three groups during the early days i.e

upto day 7 of wound healing. This may be due to the infiltration of cell types like macrophages and keratinocytes in response to tissue injury. From day 7, the treatment group showed less expression compared to other 2 groups thus confirming the normal healing phase. Keratinocytes produce and release inflammatory cytokines interleukins in response to physical or chemical stresses. Among the keratinocytes synthesised cytokines, IL-1α play critical role in the epidermis. IL-1α is constitutively produced and retained in keratinocytes, and it responds to several stimuli. IL-1α is released [30] which is an essentially primary event of inflammation [31]. IL-1α stimulates further release of secondary mediators, including IL-8, IL-8 promotes dendritic cell migration and recruitment of monocytes and neutrophils as key steps in the initiation phase of cutaneous inflammation [32]. Inflammatory processes that occur after injury contribute to wound closure. Previous studies showed that wounds of restraint-stressed (RST) mice had a reduced number of inflammatory cells and healed more slowly compared to controls [33]. It is hypothesized that impaired production of keratinocyte-derived growth factors, such as IL-1α, leads to a decrease in the catabolism of the dermal matrix, whereas augmented epidermal PDGF production leads to increased formation of the dermal matrix in hypertrophic scars. These observations support the possibility that the epidermis is involved in preventing the formation of hypertrophic scars [34]. The wound healing process concludes with down regulation of fibroblast activity. An important regulator of fibroblast activity is the fibrogenic cytokine connective tissue growth factor. These reports indicate that interleukin-1α secretion by keratinocytes provides a mechanism for the down regulation of connective tissue activity during the end-stage of wound healing, when epithelia coverage has developed over the wound area [35]. Taken together, these data indicate that constitutive keratinocyte-derived IL-1 α is a stimulus for IL-6 production in wounded epidermis, the response involves NF kappa B and C/EBP beta transcription factors, and IL-6 may be associated with modulation of keratinocyte differentiation rather than proliferation [36] leading to normal wound healing process.

5. Conclusion

The bilayer dressing designed for healing infected wound containing Ciprofloxacin as model drug was found effective in controlling wound infection with a drug release profile for 5 days. Moreover collagen in the dressing plays a major role in wound remodelling proteases so as to enhance the healing with normal healing morphology.

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Figures

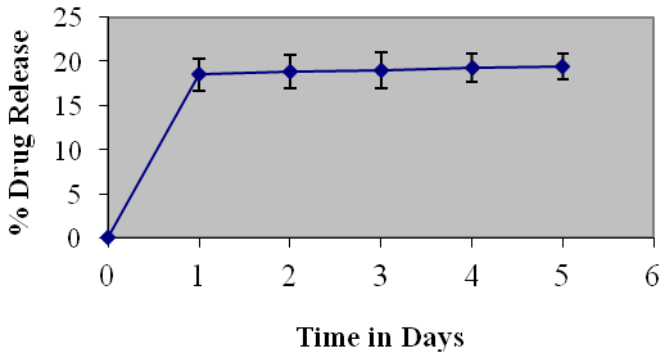

Figure 1: *In vitro* drug release pattern of the bilayer collagen dressing containing drug ciprofloxacin in PBS (mean±SE; n = 5)

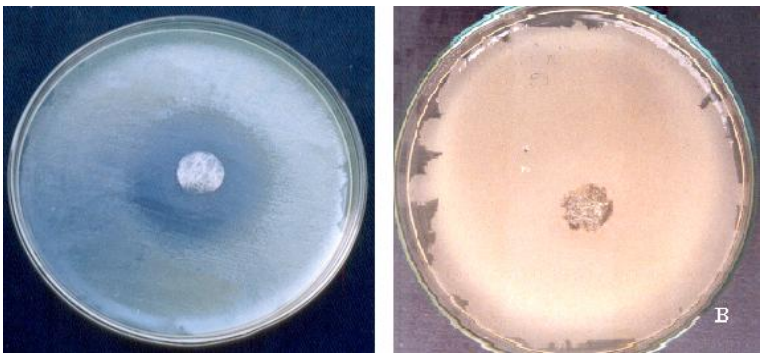

Figure 2: Agar disc diffusion of bilayer collagen dressing on agar plates inoculated with mixed cultures of Staphylococcus aureus ATCC 29213 and Pseudomonas aeruginosa ATCC 27853 (a) bilayer dressing containing drug Ciprofloxacin showing clear zone of inhibition (b) control dressing without drug showing full bacterial growth.

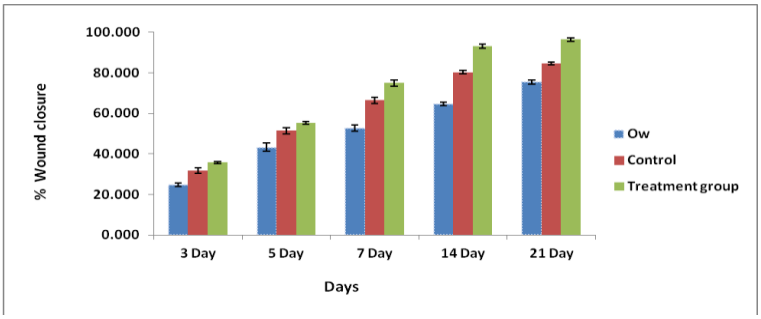

Figure 3: Wound healing rates of animals from three different groups (1) open wound (2) control and (3) treatment groups on different days of analysis.

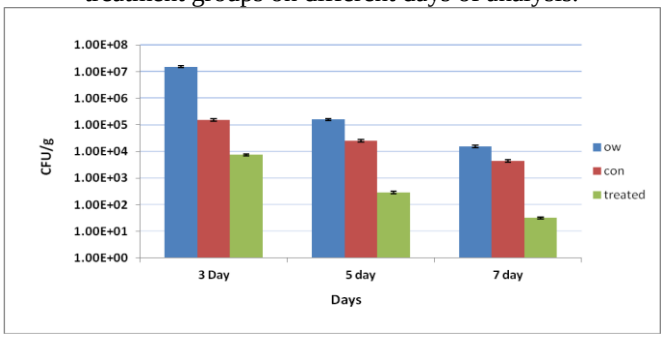

Figure 4: Total bacterial population present in the granulation tissue of experimental groups at different time intervals (n = 6).

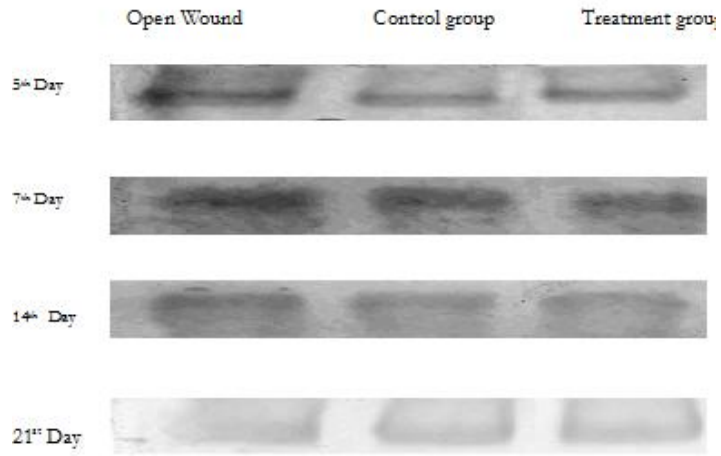

Figure 5: Western blot analysis of MMP-8 expression in the regenerating wounds.
Lanes 1-3 shows expression of tissue antigens in 1) Open wound 2) Control group 3) treatment group.

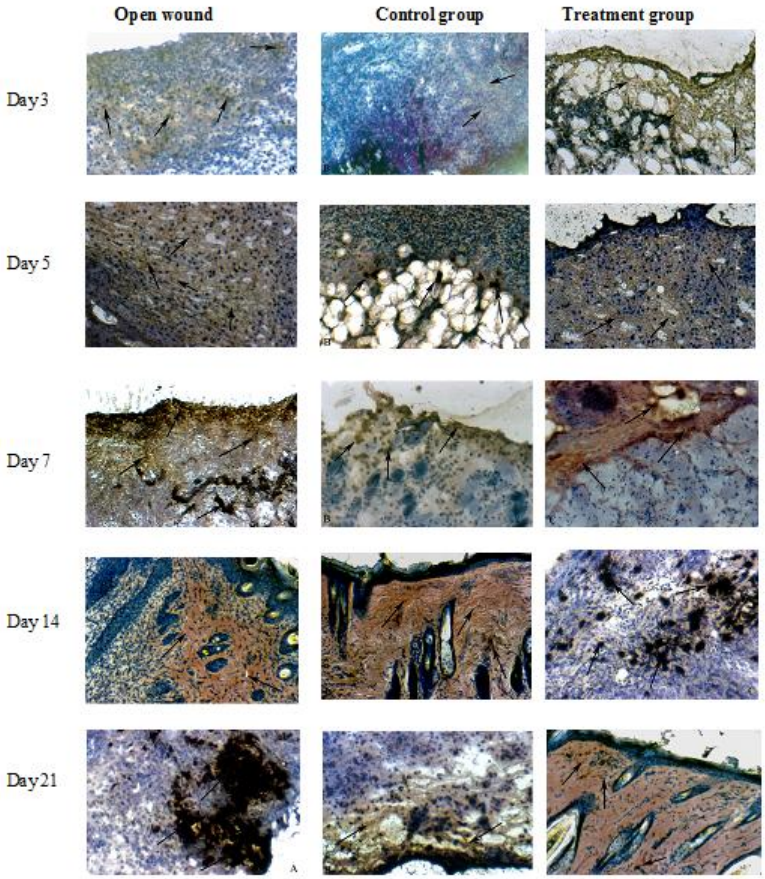

Figure 6: Photomicrograph showing immunohistochemical analysis of MMP-8 on day 3 – 21 of wound creation in the different groups. A - Open wound, B- Control collagen, C-Treated group. [Note: Arrows indicating regions positive for tissue antigens. Sections counterstained with Hematoxylin to show nucleus. Figures A-C are at similar magnifications (100 X)]

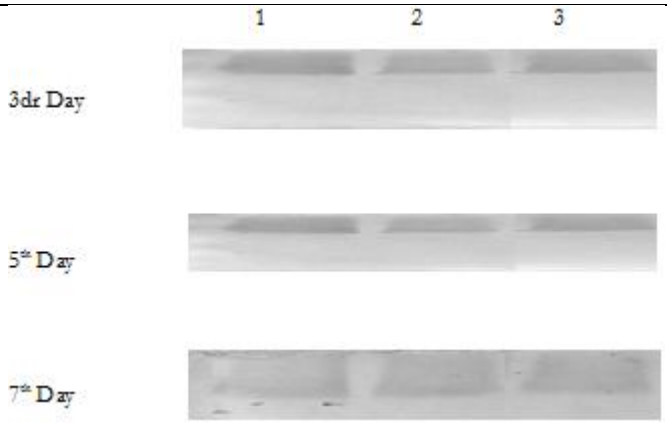

Figure 7: Western blot analysis of Interleukin-1a expression in the regenerating wounds. Lanes 1-3 shows expression of tissue antigens in 1) Open wound 2) Control 3) Treated group.

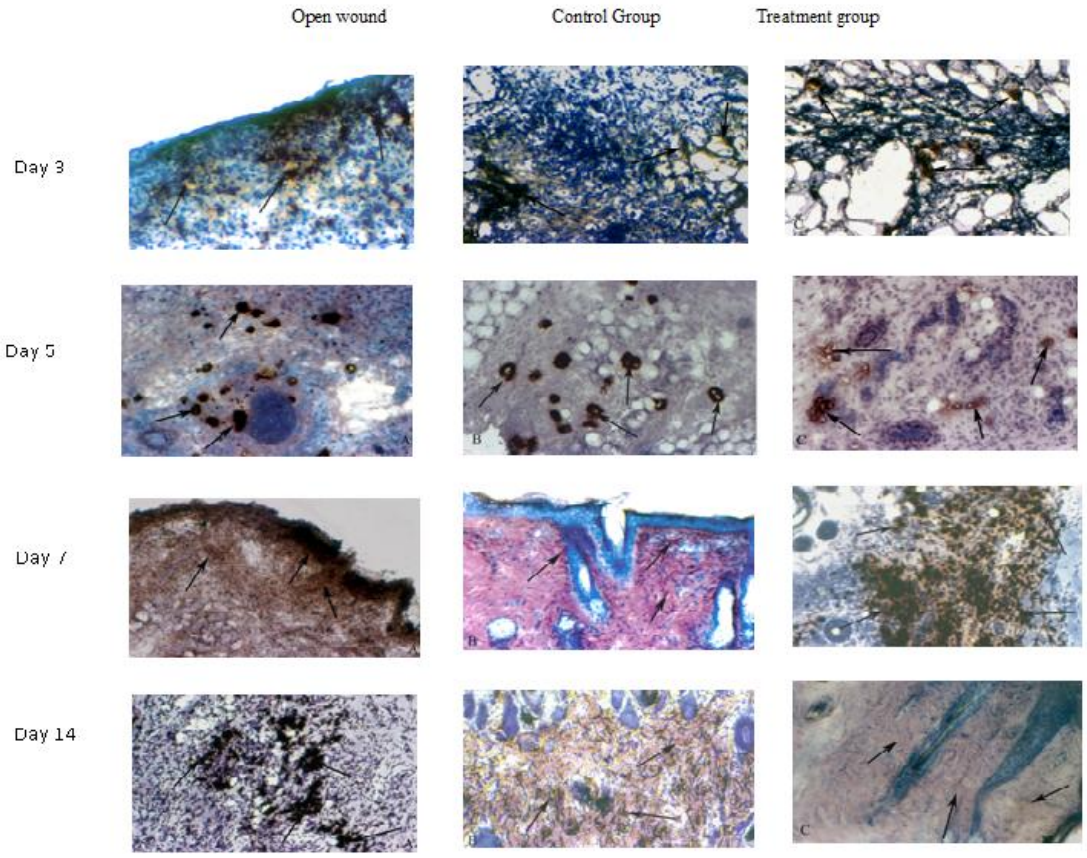

Figure 8: Photomicrograph showing immunohistochemical analysis of Interleukin-1α on day 3- 14 of wound creation in the different groups. A- Open wound, B- Control collagen, C-Treated group. [Arrows indicating regions positive for tissue antigens. Sections counterstained with Hematoxylin to show nucleus. Figures A-C are at similar magnifications (100 X)]

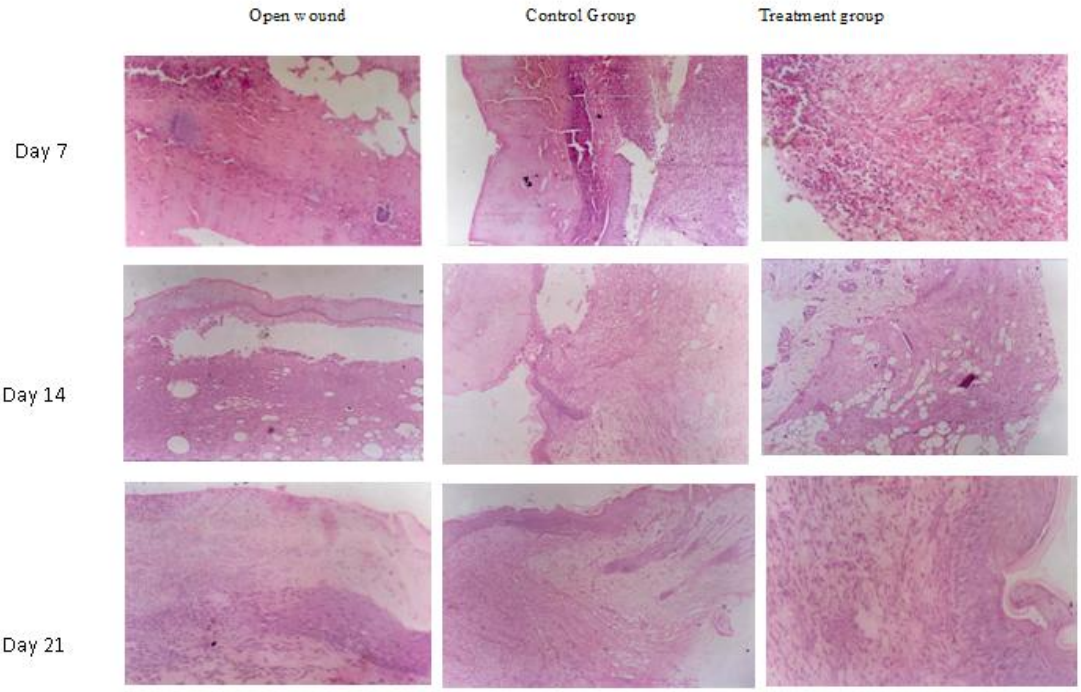

Figure 9: H&E stained section of the granulation tissue at day 7-21 of wound creation of different experimental groups. A-open wound, B-Control, C-treated. Figures A and B are at similar magnifications (80 X) and figure C at 320 X magnification.