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Research Paper

Collagen-based wound dressing for doxycycline delivery: in-vivo evaluation in an infected excisional wound model in rats

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Abstract

Objectives A novel collagen-based dressing consisting of 2,3-dihydroxybenzoic-acid-modified gelatin microspheres loaded with doxycycline has previously been reported to address both infection and matrix degradation. In the present study the potential benefits of the dressing were investigated in an excisional wound model in rats challenged with *Pseudomonas aeruginosa*.

Methods A full-thick excisional wound (1.5 × 1.5 cm) was created on the dorsum of the rats and infection induced by injecting 10^5 colony-forming units (CFU) of *P. aeruginosa*. The healing pattern was assessed from wound reduction, matrix metalloprotease (MMP) levels, CFU reduction and histological and biochemical analysis.

Key findings The treated group exhibited complete healing by day 15, compared with day 24 in the control group. Early subsidence of infection (99.9% by day 9) resulted in faster epidermal resurfacing and fibroplasias, whereas the microbial load exceeded 10^3 CFU even on day 15 in the control group and caused severe inflammation. Biochemical analysis showed that the expression of both collagen and hexosamine was significantly increased in the treated group. Gelatin zymography revealed prolonged expression of MMPs 2, 8 and 9 in the control group compared with the treated group.

Conclusions The study indicates that the developed dressing attenuated both infection and metalloprotease levels, and may therefore have potential application in wound healing.

Keywords collagen; doxycycline; metalloproteases; wound dressing; wound healing

Introduction

Normal response to tissue injury is a compilation of sequential and overlapping events that ideally result in the restoration of biological and functional integrity and ultimately end with wound repair and closure. In contrast, in chronic wounds, a particular family of structurally related proteolytic enzymes, the matrix metalloproteases (MMPs), have been found to be persistently elevated and retard the progression towards closure. Persistent elevation of these enzymes may result in uncontrolled degradation of existing or newly deposited extracellular matrix components such as collagen, glycosaminoglycans and proteoglycans, as well as degradation of various growth factor proteins necessary to coordinate healing.[1] The excessive levels of MMPs are thought to have multiple causes, including high levels of bacteria (infection), the presence of non-viable tissues and repetitive mechanical trauma to the wound.[2] Furthermore, the exposure of subcutaneous tissue following loss of skin integrity (i.e. a wound) provides a moist, warm and nutritious environment that is conducive to microbial colonisation and proliferation. In particular, *Pseudomonas aeruginosa* and *Staphylococcus aureus* are opportunistic pathogens that can cause serious infection in wounds, leading to septicaemia, which is potentially fatal if left untreated.[2] Hence, therapeutic intervention to control infection and to positively regulate MMP balance is considered vital in achieving faster healing of wounds.

A wide range of broad-spectrum antibiotics have been reported to be effective in controlling infection at wound sites in the form of semisolid preparations as well as controlled delivery through polymeric hydrogels, sponges and membranes.[3–6] Various synthetic drugs that have been shown to inhibit MMP activity include *N*-hydroxyurea,[7] bisphosphonates,[8] minacycline, doxycycline[9] and iron-chelating siderophores such as ferrichrome, rhodotoric acid, desferoxamine mesylate and 2,3-dihydroxybenzoic acid

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(DHBA).[10] Recent advances in dressing technology have led to the emergence of topical products that do more than just cover and conceal; they address specific issues in non-healing wounds and facilitate the healing process. Active dressings that have emerged either control the infection by delivering antimicrobials[3–6] or modify the MMP levels in the wound site,[11,12] in addition to managing moisture levels and exudates. A clinical study into the treatment of human periodontal infrabony defects using oxidised regenerated cellulose membrane coated with 25% doxycycline demonstrated that the doxycycline was beneficial in reducing membrane-associated infection and could potentiate regeneration through host modulation.[13] Wenk *et al*[14] demonstrated a novel iron-chelating and reactive-oxygen scavenging dressing with deferoxamine-coupled cellulose and has subsequently evaluated its efficacy and functionality in several in-vitro assays. A new sulfonated polymer dressing with the ability to inactivate proteases has also been described.[15] Recently, a poly (2-hydroxy methacrylate) tethered with bisphosphonate in the form of hydrogel has been shown to inhibit proteases in chronic-wound fluid.[8] However, none of the dressings designed so far address both infection and protein degradation.

In our previous study, DHBA-modified gelatin microspheres were developed and showed potential to inhibit MMPs present in diabetic wound tissue extracts. In addition, a delivery system for antimicrobials was demonstrated *in vitro*. The functional microspheres were then incorporated into a reconstituted collagen membrane and were proposed as a novel wound dressing, playing a dual role in attenuating both MMP activity and bacterial growth.[16] In the present study, the in-vivo efficiency of the developed dressing was investigated in an excisional wound model in rats challenged with *P. aeruginosa*. The influence of early infection on various cascading phases during the healing process has been analysed with particular reference to efficient granulation tissue formation and effective remodelling. Biochemical parameters such as the levels of hydroxyproline, hexosamine and various MMPs in the granulation tissues were also measured.

Materials and Methods

Materials

Gelatin type-B from bovine skin (bloom strength ~225), doxycycline hyclate, soya oil, ethylenediamine, DHBA, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC), *N*-hydroxysuccinamide, acrylamide, *N*,*N*′-methylenebis-acrylamide, ammonium persulfate, sodium dodecyl sulfate (SDS), glycine, hydroxyproline, glucosamine HCl, *para*-dimethylaminobenzaldehyde, acetyl acetone, chloramine T, Coomassie Brilliant Blue G-250 and HEPES were obtained from Sigma (St Louis, MO, USA). *N*,*N*,*N*′,*N*′-tetramethylethylenediamine, Tris HCl, Tris buffer, Müller Hinton broth (MHB) and Müller Hinton agar were obtained from Hi-Media (Mumbai, India). *P. aeruginosa* (ATCC 25619) culture was procured from Imtech (Chandigarh, India). All other reagents were of analytical grade obtained from SD Fine Chemicals (Mumbai, India).

Preparation of DHBA-microsphere-impregnated collagen dressing

Collagen scaffold impregnated with doxycycline-loaded DHBA-modified gelatin microspheres was prepared as per our previous protocol.[16] Briefly, gelatin microspheres were prepared by a water/oil emulsification and solvent extraction method using soya oil as the oil phase and 15% aqueous gelatin solution as the aqueous phase. The prepared microspheres were crosslinked using water-soluble carbodiimide, EDC.[17] The microspheres were then functionalised with an MMP inhibitor, DHBA. The particle size of the DHBA microspheres were in the range 60–120 μm. The DHBA microspheres were then loaded with doxycycline by a swelling mechanism; the entrapment efficiency was 9.8 ± 0.4%.[16]

Pepsin-solubiliszed collagen was isolated from bovine skin according to the method reported by Shanmugasundaram *et al*.[6] For dressing development, a known amount (300 mg) of collagen was dissolved in 30 ml 0.05 mol/l acetic acid and 100 mg drug-loaded microspheres was added to the collagen solution and gently stirred to distribute the spheres homogenously throughout the solution. Fibril formation was initiated by adding an appropriate amount of 0.2 mol/l phosphate buffer (2 ml) and adjusting the pH to 6.9–7.2 using 1 mol/l NaOH, until turbidity appeared. After fibril formation, the viscous solution was uniformly cast over horizontal polypropylene platforms of 10×10 cm and allowed to dry at a constant temperature of 37°C until a thin scaffold was obtained, and then stored in desiccators until use.

Animals

Female Wistar rats (180–220 g) were from Venkateswara Enterprises (Bangalore, India). The rats were acclimatised to laboratory conditions and fed with standard rat chow and tap water *ad libitum*. The CLRI institutional animal ethical committee approved all the animal experimental protocols. Animal maintenance and care were according to the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) guidelines, India.

Wound creation and induction of infection

Rats were anaesthetised by intraperitoneal injection of thiopentone sodium, 40 mg/kg. The dorsal area of skin was shaved and disinfected using 70% alcohol. A full-thickness excisional wound (1.5×1.5 cm) was created on the dorsum of the animals using a sterile surgical blade.

Pathogenic strains of *P. aeruginosa* (ATCC 25619) were grown on MHB at 35°C until the logarithmic growth phase and then diluted to prepare the bacterial challenge inoculum (10^5 CFU/ml). To induce infection, 1 ml of the above inoculum was centrifuged and resuspended in 100 μl sterile saline; the suspension was then injected carefully between the subcutaneous skin and paraspinus muscle layer.[18] The infection was allowed to set for 24 h and the treatment protocol was carried out thereafter. The severity of the infection was assessed quantitatively at regular intervals by irrigating 100 μl sterile saline over the wound and taking a sample using a sterile swab (Himedia, Mumbai, India). The swab was then rinsed

with 1 ml saline and the CFU/ml determined using the spread plate method.

Treatment protocol

The infected rats were divided into two groups. Wounds of the treated group (n = 30) were covered with the developed dressing, which was then fixed with adhesive bandage. Dressings were sterilised with ethylene oxide before being cut to the size of the wound and soaked in sterile saline for 2 min before application. The wounds of the control group (n = 30) were covered with sterile gauze immersed in sterile saline and fixed in place with adhesive bandage. Dressings were changed every 3 days from day 1 until complete healing. At these time points the extent of wound closure was determined by tracing the margin of the wound area onto a transparent graph paper, and expressed as percentage reduction in surface area. Granulation tissue was collected at various time intervals after euthanisation of the rats, stored at −20°C and appropriately processed for various experimental protocols.

Histology

Granulation tissue biopsies collected at various time points were fixed in buffered formalin, embedded in paraffin, and 4–5 μm thick sections were cut using a microtome and mounted on glass slides. Histological sections were then de-paraffinised and stained with haematoxylin and eosin to detect changes in the dermal and connective tissues. Staining was performed according to a standard protocol.[19]

Determination of collagen and hexosamine content

A known amount of granulation tissue collected at various time points was freeze-dried to a constant weight and subjected to hydrolysis for 22 h with 6 mol/l HCl to determine the collagen content.[20] Total hexosamine content was determined by the method of Elson and Morgan[21] after subjecting the samples to hydrolysis for 6 h using 2 mol/l HCl. Collagen and hexosamine contents were expressed as mg/g dry weight granulation tissue.

Expression of matrix metalloproteases

Expression of active MMPs in granulation tissue of both groups at various time intervals was determined by gelatin zymography. The tissue was thoroughly rinsed with deionised water to remove adhering blood components and then homogenised in HEPES buffer (20 mmol/l, pH 7.2) at 4°C. The homogenate was centrifuged at 18 000 rev/min for 20 min and the supernatants stored in 0.5 ml aliquots at −80°C for further analysis. The protein concentration in the tissue lysate was determined by bicinchoninic acid protein assay.[22]

Samples of equimolar protein content (20 μg) were mixed with non-reducing Laemmli's buffer (0.125 mol/l Tris, pH 6.8, 4% SDS, 20% glycerol and 0.02% w/v bromophenol blue) and electrophoresed on a 10% polyacrylamide gel copolymerised with gelatin (1 mg/ml). Human diabetic wound tissue extract containing MMP 2 and MMP 9 was used as a marker for identifying these MMP types in the granulation tissue extract. The presence of MMP 2 and 9 in diabetic wound tissue extract was identified by resolving with standard MMP 2 and 9 from human fibroblasts (Sigma) in gelatin zymography[16] (data not shown). After electrophoresis, the gel was washed with 2.5% Triton X-100 for 1 h and then incubated with enzyme buffer (50 mmol/l Tris HCl, 150 mmol/l NaCl, 5 mmol/l $CaCl_2$ and 0.05% sodium azide) at 37°C for 18 h to allow reactivation of MMPs. Gel was then stained with 0.5% Coomassie Brilliant Blue R-250, and destained with 10% (v/v) acetic acid containing 30% (v/v) methanol. The MMPs were visualised as clarified bands corresponding to zones of digestion of gelatin substrate.

Statistical analysis

Values are expressed as mean ± SD. Statistical comparisons were performed using repeated-measures one-way analysis of variance with Bonferroni's multiple comparision test. using GraphPad Prism software. A P value < 0.05 was considered significant.

Results

The present investigation deals with several aspects of wound repair in a *P. aeruginosa*-challenged standard excisional wound model in rats. The collagen dressing developed was well adhered to the surface of the wound and remained intact until the next change, as shown in Figure 1.

Influence of infection on healing

Wounds infected with *P. aeruginosa* (10^5 CFU) exhibited differential microbial load during the course of healing in both the treated and the control groups. As shown in Figure 2, the number of CFU increased significantly by 100-fold in the control group (from 10^5 to 10^7 CFU) on day 3, while the treated rats showed a twofold decrease in CFU (from 10^5 to 5×10^4 CFU). On day 6, the treated group exhibited a significant decrease (52-fold) in microbial load (from 5×10^4 to 1×10^3 CFU), which steadily decreased and reached 99.9% decrease by day 9, whereas the infection persisted to day 15 in the control group (5×10^3 CFU).

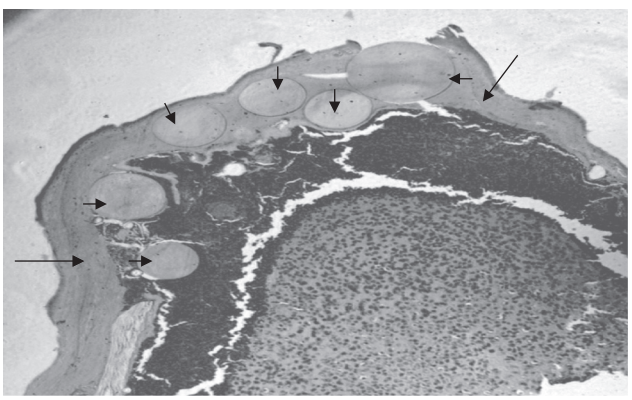

Figure 1 Photomicrograph of the haematoxylin-and-eosin-stained section of the dressing on the wound surface after 72 h. The swollen microspheres (short arrows) and the collagen scaffold (long arrows) are shown remaining intact on the wound surface.

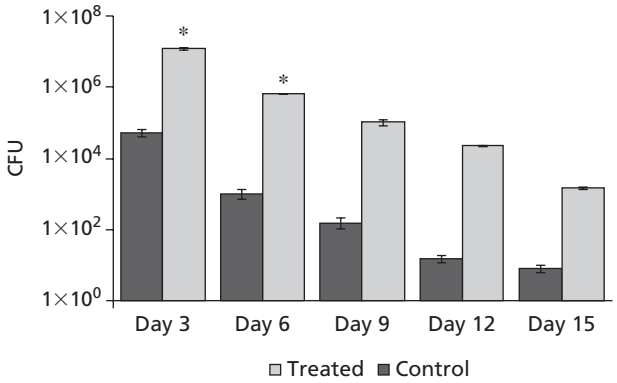

Figure 2 Microbial load during wound healing. Quantitative determination of microbial count in excision wound infected with 1×10^5 CFU *Pseudomonas aeruginosa* ($n = 6$). *$P < 0.05$.

Wound closure

The percentage wound closure at different time points is shown in Figure 3. Significant differences in wound closure between treated and control rats were observed. The treated group exhibited significantly faster healing, achieving complete healing by day 15, compared with day 24 in control rats. Wound closure was more extensive in the treated group at all time points ($P < 0.05$).

Collagen and hexosamine content

The level of collagen expressed during the process of healing is shown in Figure 4. A common pattern of collagen expression was observed in both groups, in that a steep increase in collagen content was followed by an equally steep decrease. Maximum deposition of 72 mg/g was observed in the treated group on day 6; this was significantly higher than in the control group, which exhibited maximum deposition on day 12 of about 52 mg/g.

Proteoglycan deposition, mainly hyaluronic acid, heparan sulfate, chondroitin sulfate and dermatan sulfate, in granulation tissue facilitates an environment for cell movement and collagen deposition. Hexosamine (marker for proteoglycan) expression during the healing process is shown in Figure 5.

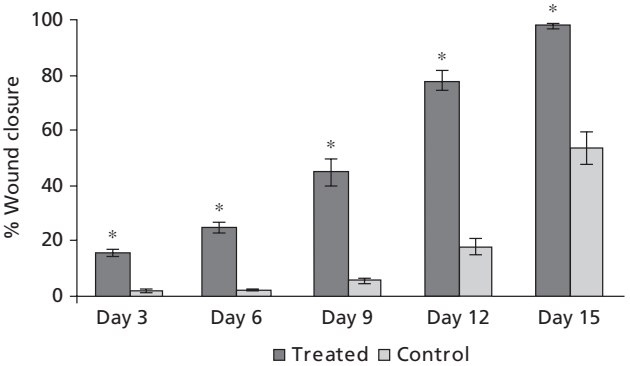

Figure 3 Progress of wound closure. Bars show means ± SD ($n = 6$). *$P < 0.05$.

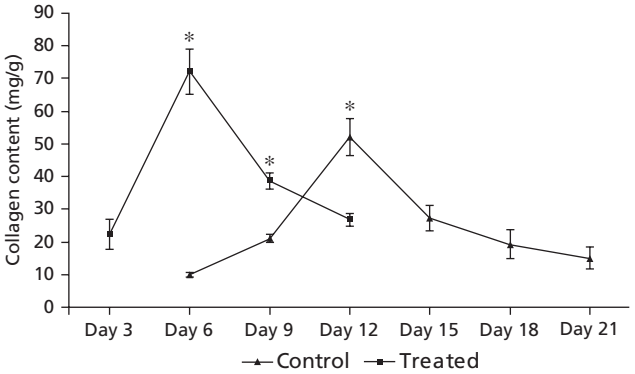

Figure 4 Collagen content in granulation tissue during wound healing. Bars show means ± SD ($n = 6$). *$P < 0.05$.

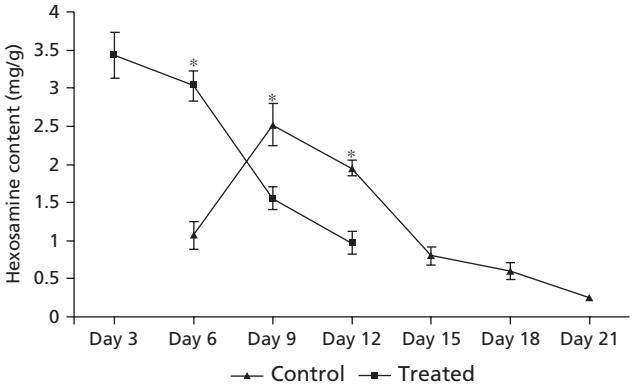

Figure 5 Hexosamine content in granulation tissue during wound healing. Bars show means ± SD ($n = 6$). *$P < 0.05$.

Peak concentrations were observed on day 3 (3.4 mg/g) in the treated groups and on day 9 (2.5 mg/g) in the control group. When compared with the control group, the treated group showed significantly higher deposition on day 6 and lower deposition on days 12 and 15 ($P < 0.05$).

Histological evaluation of wound healing

Figure 6 shows granulation tissue sections taken during the course of healing. Granulation tissue could not be collected from the control group on day 3 because of severe infection and the presence of minimal granulation tissue. Histological sections from the treated group on day 3 and the control group on days 6 and 9 showed extensive infiltration of inflammatory cells, which was found to have spread deep inside the muscle layer. Samples from the treated groups on day 9 had a well-defined dermis with appreciable infiltration of fibroblasts and thin epithelial layer along the borderline, whereas samples from the control group did not show any sign of defined matrix until day 9, after which there was loose matrix formation which showed prominent inflammatory cell infiltration even on day 15.

Detection of MMPs by gelatin zymography

The relative changes in expression of MMPs with time are shown in Figure 7. In the initial days of healing, the expression

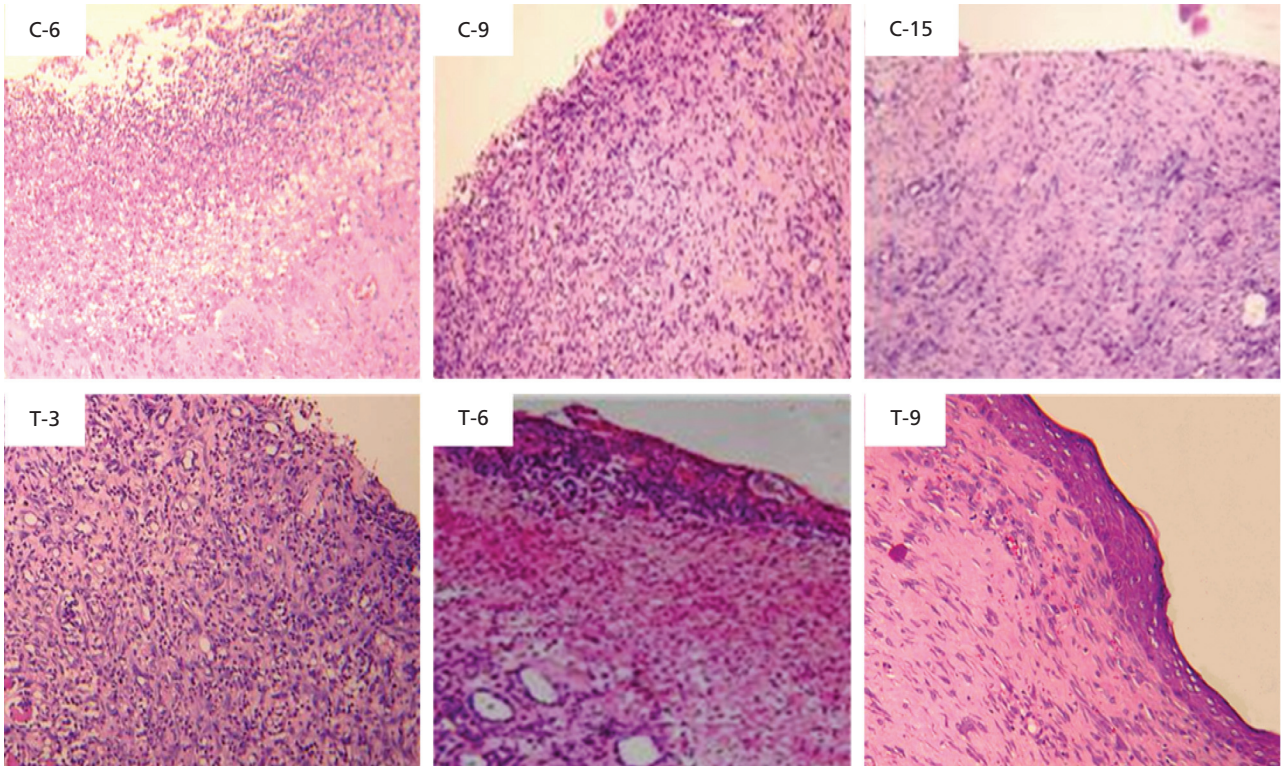

Figure 6 Photomicrographs of haematoxylin-and-eosin-stained sections of granulation tissue during wound healing. C represents the control group, T represents the treated group; numbers refer to the day of tissue collection.

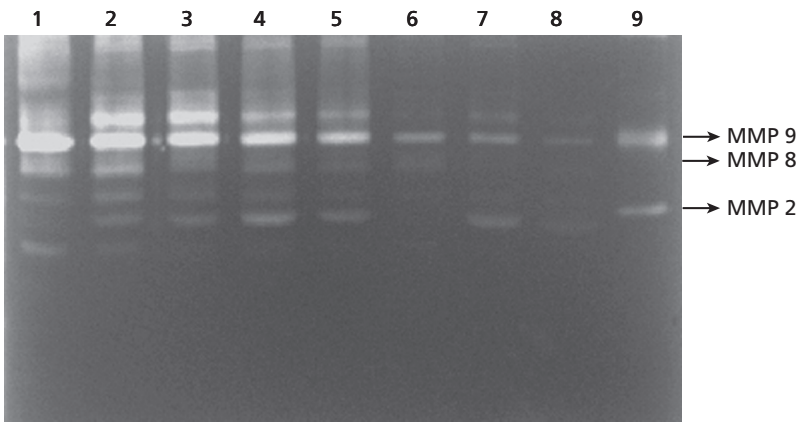

Figure 7 Gelatin zymogram of granulation tissue extract on various days of treatment course. Lanes 1–5, tissue extract from the control group on days 6, 9, 12, 15 and 18, respectively. Lanes 6–8, tissue extracts from the treated group on days 3, 6 and 9, respectively. Lane 9 is human diabetic wound tissue extract, used as a marker for MMP 9 and MMP 2.

of polymorphonuclear-neutrophil-derived MMPs such as MMP 8 (75 kDa) and MMP 9 (92 kDa) was high, followed by a reduction in later days of healing. The overall expression of MMP 9 was higher than other MMPs during the course of healing. However, the band representing MMP 9 decreased in intensity in later phases of healing and expression was higher in the control group than the treated group. MMP 8, which is secreted by inflammatory cells (lanes 1–4 representing day 6, 9, 12 and 15 in the control group, and lane 6, representing day 3 in the treated group) indicates that the wounds were at inflammatory phase during this time course. The expression of MMP 2 (72 kDa), which is secreted by fibroblasts in the wound site during remodelling, was seen from day 9 until complete healing in the control groups, whereas it was observed on day 6 in the treated group. The intensity of the band was much less on day 9, indicating faster remodelling.

Discussion

Despite major advances in wound management and other supportive care regimens, infection and elevated levels of proteases in wounds remain leading causes of morbidity and mortality. The ability of microorganisms to create massive damage in the wound bed depends on the virulence of the organism and the amount of inoculum present in the wound site, together with the host immune response. *S. aureus* and *P. aeruginosa* are the most common wound pathogens; infection is classified as at least 10^3 CFU/g tissue.[23] In humans, infection is spontaneous whereas in rat models the infection has to be induced. The influence of inoculum size mentioned above is crucial, and its importance was shown by Raju *et al*.[24] Wounds infected with 10^5 CFU of *P. aeruginosa* were found to exhibit noticeable and severe infection.

The existence of a large bacterial load in infected rats had a detrimental effect on the rate of granulation and re-epithelialisation. Bacterial infiltration was found in both control and treated groups on day 3, but it persisted deep in the dermis in control rats even on day 15. Persistent infection also delayed epidermal maturation. The treated group exhibited significant proliferation and migration of epidermal cells, resulting in faster granulation covering the entire wound surface. The slow rate of wound closure in control rats may be attributed to the presence of microorganisms and their metabolites, which inhibit wound contraction and impair healing. A noteworthy observation is that the developed dressing exerted a static antimicrobial effect during the initial days, reflecting the ability of the designed system to deliver doxycycline in a controlled manner.

Granulation tissue is a combination of cellular elements, fibroblasts and inflammatory cells, along with new capillaries embedded in a provisional matrix of collagen and glycosaminoglycan (GAG).[25] Quantitative assessment of collagen content provided insight into matrix deposition during healing. The decreased collagen content in control rats may be attributed to the presence of microorganisms, which prolonged the inflammatory phase, inhibiting both epithelial regeneration and proliferation of fibroblasts, leading to delayed healing. Collagen deposition was much faster in treated rats than control rats. Because of faster cellular infiltration and fibroplasias, GAG content also reached a maximum at a faster rate. GAG is an important component of the matrix molecules, acting as a ground substratum for the synthesis of new extracellular matrix. The increase in collagen content was associated with a concomitant decrease in hexosamine content.[26]

MMP 8, 9 and 2 are the major MMPs involved in remodelling analysed in the present study. These MMPs facilitate fibrin and eschar removal, and the resultant peptides are known to possess chemotactic and angiogenic properties.[27] Expression of MMP 9 and 8 early in the wound-healing process suggests that they may be involved in elimination of necrotic tissues, and may induce keratinocyte migration and granulation tissue remodelling.[28] During the later phases of wound repair, an increase in 72 kDa gelatinase and a persistence of MMP 2 suggest that these enzymes play important roles in the remodelling process[29] and in maintaining collagen homeostasis.

Active MMPs observed during initial days are required for debridement, paving the way for neo-dermis deposition. Early detection of MMPs in the treated group indicates the active clearance of dead tissue. Another important observation is the prolonged expression of MMP 8, 9 and 2 in the control group, which cause the destruction of the early provisional matrix, thus hindering the healing process. Healing was hastened in the treated group compared with the control groups, which can be attributed to the potential of the DHBA-microspheres to neutralise and/or inhibit MMPs, and doxycycline delivered from the wound dressing. One of the major factors that regulates MMP activity is the bacterial exotoxins,[30] along with excessive inflammatory cell infiltration at the wound site. The developed dressing reduces the bacterial load and was also found to positively modulate MMP activity and thus was able to stimulate healing at a much faster rate than in control rats.

Conclusions

The developed dressing was able to attenuate both infection and MMP levels and therefore has potential applications in wound healing.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

Funding

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