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Wound healing potential of collagen bilayer dressing on infected dermal wound- A study on rat animal model.

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Abstract: In the current study, a chemically modified collagen bilayer dressing with ciprofloxacin was prepared from type-I collagen. The modified collagen was reconstituted into a membrane and sponge and the drug Ciprofloxacin-HCl was incorporated and fabricated into a bilayer dressing. In vitro release pattern of the dressing was analysed in PBS which recorded release for 6 days. The efficacy of the dressing was checked on infected wound in rat model and compared with that of open infected wound and control group. The healing pattern was analyzed on days 3, 5, 7, 14 and 21 by wound healing rate, biochemical and histological examinations of granulation tissue samples. Integrin expression on the healing wound was analysed using immunohistochemistry and western blotting analysis. In vivo analysis showed significant wound closure, biochemical analysis such as protein, DNA, hydroxyproline, uronic acid, hexoseamine, SOD and catalase from the granulation tissue, showed enhanced healing in the group treated with collagen bilayer dressing with ciprofloxacin. Integrin expression was more in initial stages of healing than the other two groups confirming enhanced healing in treatment group. Histological analysis and wound closure further confirmed proper healing in this group. Our results suggest that sustained bilayer dressing with drug is efficient in controlling and eliminating the bacterial population at the wound site thereby enhancing the healing.

Key words: Succinylation; collagen bilayer dressing; drug delivery; ciprofloxacin; wound infection, integrins.

1. Introduction

Wound healing is a complex and dynamic cascade of events initiated by injury. The process involves coordinated cell activation, division, chemotaxis, migration and differentiation of many cell types. They are mediated by locally released growth factors and cytokines 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].

Chemotactic signals attract neutrophils and monocytes to wound sites [5]. The recruitment of fibroblasts in the wound area attracts fibroblasts from surrounding tissues to move into the area of injury and proliferate. 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 wound repair mechanism enters a period of transitional phase. The cells undergo marked phenotypic alteration and re-epithelialization of wound begins within hours after injury [6&7]. Fibroblasts deposit loose ECM initially composed of great quantities of fibronectin which activates integrin receptors. Integrins are heterodimers composed of non-covalently associated transmembrane α and β subunits. Most integrin receptors bind ligands that are components of the extracellular matrix including fibronectin, collagen and vitronectin. Certain integrins can also bind to soluble ligands such as fibrinogen, or to counter receptor on adjacent cells, such as Intracellular Adhesion Molecules (ICAMs) leading to aggregation of cells. Signals transduced by integrins play a role in many biological processes including cell growth, differentiation and migration.

In the present work, the expression of protein and DNA during healing and the histological analysis of the healing along with the expression of integrin-αV in the healing environment at various time intervals representing the granulation, inflammatory and tissue remodeling phase in the presence and absence of collagen and drug incorporated collagen matrix has been studied.

2. Materials and methods

2.1 Extraction Of pure Type I collagen

Collagen was extracted from Bovine source following the reported procedure [8].

2.2 Succinylation of Pure collagen

The pure collagen was succinylated using succinic anhydride at pH 9.0. The succinylated collagen was washed in water and swelled in milli-Q water [9].

2.3 Preparation of bilayer dressing

The pure collagen solution was converted to film and sponge by conventional air drying method [10].

2.4 Incorporation of drug

The Concentration of the drug incorporated into the dressing was 0.2 mg/cm^2. About 0.06 mg was mixed with 10% PVP and allowed to be absorbed over the sponge then remaining 0.14mg drug was mixed with 10% PVA solution and allowed to air dry. After drying the sponge and film were fabricated into bilayer dressing and the drug remains totally protected in the bilayer system, which acts as a seal.

2.5 In vitro release in phosphate buffer saline

The pattern of drug release from the dressing was done using Franz type diffusion cell. The dressing was immersed in PBS, pH 7.4 and the system was kept under constant stirring at slow speed. The release was calculated by collecting PBS every hour. The release pattern was confirmed using two systems which were run simultaneously. In one, the drug was collected every one hour and in the other system the drug was collected at 24 hrs intervals. The concentration was determined spectrophotometrically at 276 nm and concentration was compared with the standard of known concentrations.

2.6 Creation of Infected Wound

Male wistar albino rats weighing 200–250g 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. To generate infected wounds in rats, a full thickness wound of 2×2 cm^2 dimension was created and mixed culture of *Pseudomonas aeruginosa* and *Staphylococcus aureus* was injected between the very thin muscle and the paraspinus muscle and allowed the infection to develop for 24 hrs. The collagen dressing were applied over the wound and then sutured to the surrounding skin. Evaluations were performed after days 3, 5, 7, 14 and 21by collecting the granulation tissue from the wound site.

2.7 Treatment Groups

Group 1: Animals with open infected wound
Group 2: Animals with infected wound covered with bilayer collagen dressing without drug
Group 3: Animals with infected wounds covered with bilayer collagen dressing with drug

2.8 Wound Reduction

The reduction in wound area was calculated using the following formula

$$\%\ \text{Wound reduction} = \frac{\text{Wound Area on 0 day} - \text{Wound Area on Analysis}}{\text{Wound Area on Analysis}} \times 100$$

2.9 Colony forming Units (CFU)

Colony Forming Units (CFU) from biopsy of granulation tissue was calculated by plating out the homogenized, serially diluted tissue samples. Since the swab culture from wound surface collect the surface contaminating organisms tissue biopsy from the infected area will reproduce the concentration of infecting organisms.

2.10 Biochemical analysis

Protein content present in the granulation tissue was assayed using method of Lowry et al [11], DNA content by Burton [12], Hydroxyproline following Neuman and Logan method [13], Uronic acid by Bitter and

Muir [14], Hexoseamine content by the method of Elson and Morgan [15], Superoxide dismutase by Misra and Fridovich [16] and catalase content of the granulation tissue was assayed according to the method of Bergmeyer [17].

2.11 Histological Analysis

The histological analyses were carried out after day 3, 5, 7, 14 and 21 following wound creation. The paraffin embedded tissues was sectioned at 10μm thickness and were stained with Hematoxylin & Eosin Staining.

2.12 Masson's Trichrome Staining Protocol

Paraffin embedded tissues sectioned at 10μm thickness was used for masson's trichrome staining.

2.13Tissue Processing for Immunohistochemistry

The granulation tissues 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 was 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 primary antibody Integrin-αV (Santa Cruz Biotechnology, Cat No. Sc-6593) 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.

2.14 Western Blot Analysis

Supernatant of the granulation tissue homogenate were treated with equal volume of sample buffer free from β-Mercaptoethanol and subjected to native polyacrylamide slab gel electrophoresis. The protein separated 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 4°C. The membranes were washed twice in TTBS (50 μl of Tween-20 in 100 ml of TBS) and then with TBS twice. The membranes were then incubated with goat polyclonal antibody Integrin-αV (Santa Cruz Biotechnology, Cat No. Sc-6593) diluted 1:1000 with blocking buffer for 4 hrs at room temperature or overnight at 4°C. The membranes were again washed exhaustively with TTBS and TBS and 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).

2.15 Statistical Analysis

All statistical evaluations were 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 and Discussion

3.1Release kinetics

The concentration of drug released on day 1 was 10% followed by an increase in release rate which continued up to day 6 (Fig. 1). The drug release was very much regulated in the system since 80% of the drug is coated in the film, the drug initially releases from the sponge, which contains rest of the drug. After 2 days, as the film gets wet through the sponge further release of the drug ensues for another 4 days. The collagen sponge and film used in the bilayer system are chemically modified by succinylation. Succinylated collagen remains anionic and can be readily reconstituted at neutral pH. Dressings made from succinylated collagen do not require any neutralization to prevent acid leaching. Swelling rate of succinylated dressing at neutral pH is more

due to its anionic nature. Succinylated collagen sponge in the bilayer system behaves as an anion when applied on the wound. The drug (Ciprofloxacin) forms cations in the swollen network of succinylated sponge. The release of drug at the site i.e. at the wound depends on the nature of wound

3.2 Wound reduction

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. 2. 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 day 5. In the case of treated groups, the wound area was reduced to 56 % of the original wound area. The wounds reduced to 75% of the original wound area after day 7 of wound creation showing significant ($p<0.05$) wound reduction in the animals compared to the open and control groups (group 1 and 2) of animals. The same trend continued upto day 14, indicating more wound reduction in treated group. On day 21 the wound area were healed to 96% compared to control and open wound which showed 84 and 75% wound closure respectively.

3.3 Colony forming units (CFU)

Biopsy of the granulation tissue was taken on day 3, 5, 7, 14 and 21of 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 Fig. 3. But the treated group showed 10^1 colonies on day 7 and almost no colonies on day 14. Treated group showed normal wound healing starting from day 14 whereas in the case of open and control (group 1 & 2), the CFU reduced to 10^5 on day 5 and 10^4 on day 7 causing incomplete healing in both the cases.

3.4 Biochemical analysis

i) Protein

The protein content in the granulation tissue gives information about the different kind of substrates produced by the infiltrating cells. Fig. 4 shows that there was a significant increase in protein level observed in treated animals (Group 3) when compared to other two groups (group 1 & 2) on day 3. This trend changed after day 5 with group 2 having protein content more than the other groups, which was statistically significant ($p<0.05$). The wound undergoes remodeling after 7 days wherein the proteins present in the granulation tissue are slowly replaced by fibronectin and type I collagen. Lesser protein content was observed on day 7 in all the groups than day 14, during which there is intense fibroblast proliferation and deposition of fibronectin is seen in the regenerating wounds. On day 7, the open wound animals had a significant increase in protein content than all the other groups still representing the inflammation phase in these animals. On day 14, all the animals had increased protein content representing high fibronectin deposition than the other days. During this time period treated animal (groups 3) had significantly increased ($p<0.05$) protein content than all the other groups. After day 21, the protein content returned to normal values in all the treatment groups with little difference among the experimental groups, which was statistically non-significant. The protein content increased significantly in the treatment group than the control indicating the synthesis of other extracellular matrix protein other than collagen in the granulation tissue by the in filtering cells.

ii) DNA

All the experimental groups had more are less same DNA content with statistically non-significant on day 3 (Fig. 5). Significant ($p<0.05$) increase in DNA content was observed in control (group 2) than all the other groups at day 5 of wound regeneration. This trend changed at day 7, wherein the open wound showed a significant increase over the treatment group. The DNA content dropped almost in all experimental groups on day 14. Conversely after day 21, due to tissue remodeling and rapid epithelialization, there was increased DNA content in the treatment group than open wound and control which had regenerated less efficiently than the treatment group. The synthesis of nuclear DNA exhibited a wave shape dynamics. Similar results were obtained in a study carried on synthesis of nucleic acid in which a wave shape dynamics was observed in DNA level of granulation tissue of rats, thus suggesting that the wave shaped increase is due to biosynthetic processes in the cells [18].

iii) Hydroxyproline

Treatment group showed statistically significant increase in wound granulation tissue hydroxyproline content than open wound and control groups on day 3, 5 and 7 (Fig. 6). As the wound is infiltrated by more

fibroblasts during the later stages of healing particularly in the remodeling phase collagen is deposited and as result there is increase in the hydroxyproline content in the treatment group undergoing proper healing.

iv) Uronic acid

In the case of uronic acid there was a decreasing expression trend observed than the early days of wound creation (day 3). On day 5, the treatment group had values lesser than control group animals. This trend changed significantly after day 7, wherein the treatment group had values more than the control and open wound groups (Fig. 7). This trend of increased expression in uronic acid content was maintained throughout the other experimental time periods as the wound was progressively healed.

v) Hexoseamine

The hexoseamine content in the early days of wound creation i.e. day 3 were increased than all the other experimental time periods with the levels returning to normal after complete healing at day 21 (Fig. 8). The open wound experimental animals had hexoseamine content which was significantly higher ($p<0.05$) than the other experimental groups. At day 5, there were fluctuations in the hexoseamine content in the granulation tissue of animals in treatment group having values similar to control and significantly higher than the group 1. During the day 7 and 14 experimental periods no major difference were observed among the groups except that the expression was lesser than the previous experimental periods. At day 21, the hexoseamine content reached normal levels.

vi) SOD

Superoxide dismutase (SOD) activity was significantly greater in the open wound representing free radical (superoxide) production and scavenging by SOD in these systems. As the wounds were proliferated with more and more neutrophils and macrophages, there was excessive scavenging of the tissue remnants and consequently more superoxide radicals were generated. This was counteracted with the production of SOD in the treatment group on day 5 (Fig. 9). The SOD activity was similar on day 5 and 7 and all the groups showed more or less similar expression. Once the tissue enters remodeling phase, there was more fibroblast proliferation seen, with an increased expression of SOD enzyme activity that was observed in the treatment group than control and open wound experimental groups at day 14. This level consequently changed and reached normal levels at day 21 during which complete skin regeneration was seen.

vii) Catalase

The rate of catalase activity was more or less similar in the groups with slight changes, which were statistically non-significant on day 3 (Fig.10). Conversely, the open wound experimental animals had catalase levels more ($p<0.05$) than the treatment groups. At day 5 and 7, the rate of catalase activity was more or less similar in groups 1 & 3 except group 2 on day 5, which had values significantly increased ($p<0.05$). Like SOD, an increased level of catalase was also observed on day 14 in all the groups which returned to normal levels on day 21. There was not much difference observed among the different groups in the case of this enzyme during the later study periods.

3.5 Histological Analysis

Histological observation shows heavy neutrophilic infiltration in wound surface on day 3 in all cases with fewer macrophages (Figures not shown). Bacterial colonies were found over the granulation tissue and dermal region was seen with fibroblasts (proliferating) with ECM deposition in treated groups. Day 5 analysis showed the retention of bacterial colonies with neutrophil infiltration. In case of treated group granulation tissue was seen extending into the muscle. Dermal region showed collagen deposition with maturing fibroblast on day 7 in treated animals where as in group 1&2 Bacterial colonies along with invading neutrophils were observed. Macrophages were seen below neutrophil infiltration in granulation tissue of animals with open wound. Treated group showed prominent angiogenesis with matured collagen bundles and wound edges showed 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. 11). But in case of treated group complete epithelialization was seen. Mature fibroblastic cells were seen in the dermal region with collagen deposition. 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 [19]. The appearance of collagen type I and VI were investigated in human skin wounds by immunohistochemisty [20]. 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.

3.5 Masson's Trichrome Staining

The process of collagen deposition during wound healing was observed by MTC stained sections. The treatment group shows a well organized collagen bundle compared to that of the groups 1&2 as shown in Fig. 11.

3.7 Immunohistochemistry

Epithelial cells (EC) from various tissues can produce important cytokines and chemokines when stimulated by pro-inflammatory cytokines. These EC also receive signals from cell surface integrins, like the α3 β1 integrin, which is important in cell migration and wound healing of epithelial monolayers. In unharmed skin, the basal layer of epithelium is attached to a specialized matrix, the basal lamina. Keratinocytes in the epithelium use the integrins to bind to laminin in the basal lamina. These integrins have intracellular links with the keratin cytoskeletal network. The keratinocytes at the edge of the surgical wound have to dissolve the hemidesmosome attachment and begin to express other integrins that are more suitable for the wound environment. The changes in the expression of integrins by cells are important for cell migration in the healing wound. During the months following dissolution of granulation tissue, the matrix is constantly altered, with relatively rapid elimination of most fibronectin from matrix and slow accumulation of large fibrous bundles of type I collagen. Once an abundant collagen matrix has been deposited in the wound, fibroblasts stop producing collagen and the fibroblast-rich granulation tissue is replaced by a relatively acellular scar. The expression of Integrin-αV, an adhesion receptor present in the cell surface was undertaken to explore the possibility of cell proliferation mediated via this receptor system along with ECM. The expression was seen from the day 3 of wound creation in all the experimental groups (Fig.12: A-C). The expression was more cytoplasmic in origin along the cells. The fibroblastic cells proliferating from the dermal region along with collagen bundles mostly expressed this cytokine. The expression was uniformly distributed during the day 3 and 5 of wound creation. The expression reached a maximum during the day 7 of healing (Fig.12: A-C). wherein the proliferation of fibroblasts and keratinocytes were maximum. Intense reaction was seen with control and treatment group on day 7 for this antigen. This was further confirmed by the western blot analysis, where intense bands were seen corresponding to lanes representing control, and treatment group (Fig. 12: A-C). During the days 14 and 21 the expression were normalized and seen more near the epidermal layers in almost all the experimental groups. Integrin receptors found on many cell surface acts as binding sites for many extracellular matrix proteins like collagen, fibronectin and vitronectin. Fibroblasts that migrate into a wound during the early stages of repair use cell surface integrins to interact with extracellular molecules as they move away from the interstitial matrix of normal tissue and into the provisional matrix of the wound [21]. Vitronectin is a glycoprotein released during inflammation and has been shown to regulate the phenotype of vascular smooth muscle cells via αV and β1 integrins [22]. Studies revealed that the αV β3, αV β5, and αV β6 integrins are up-regulated briefly during wound angiogenesis with different patterns of expression and that inhibition of the αV β3 integrin blocked new vessel formation during human wound healing [23]. In stratified epithelia, integrins play a fundamental role in mediating basal cell attachment to a variety of extracellular matrix molecules. The mechanisms that regulate the myofibroblast phenotype are unknown but are likely to involve signals from the extracellular matrix transmitted via specific integrins. The integrin αV β6 is undetectable on normal keratinocytes *in situ*, but is increased significantly in wound healing and in culture-established keratinocytes, suggesting that it may promote changes associated with cell motility. Previous reports suggest that cultured normal human oral keratinocytes express relatively high levels of αV β6 and this integrin has a functional role in both cell adhesion and migration towards fibronectin [24]. The integrins containing the αV subunit appear to be particularly important. Accumulating evidence indicates that endothelial cell integrins that bind to the matrix proteins associated with inflammation and wound healing are involved in the process of angiogenesis. In the present study the expression of integrin αV was seen during the early as well as later days of wound healing suggesting an important role of these adhesion molecules in orchestrating the proliferation and migration of cell types with special emphasis to fibroblasts in the early and keratinocytes in the later days of wound healing. Though the expression pattern varied among the different groups in the expression of adhesion receptor, the expression was seen mostly in the cytoplasmic region particularly in the cell membranes acting as anchorage points for better attachment of the cells with the ECM proteins. The overall effects lead to better cell attachment and proliferation using these receptors resulting in better and early epithelialization and wound closure in the experimental group.

3.8 Western blotting

The western blot analysis of integrins expression was observed on day 3, 5 and 7. On day 5, open wound showed more expression compared to that of groups 2 & 3. On day 7 the groups 2 & 3 showed more expression than group 1 (Fig. 13). The expression was uniformly distributed during the day 3 and 5 of wound creation. The expression reached a maximum during the day 7 of healing, wherein the proliferation of fibroblasts and keratinocytes were maximum. Intense reaction was seen with control and treatment groups on day 7 for this antigen. Integrins are important not only in cell adhesion but also in cell movement and migration. During wound healing, epidermal cells next to the wound site migrate into the wound by changing the number, type, and distribution of integrins present in the cells [25-27]. Thereafter, those migrating epidermal cells start dividing and proliferating to complete the wound coverage.

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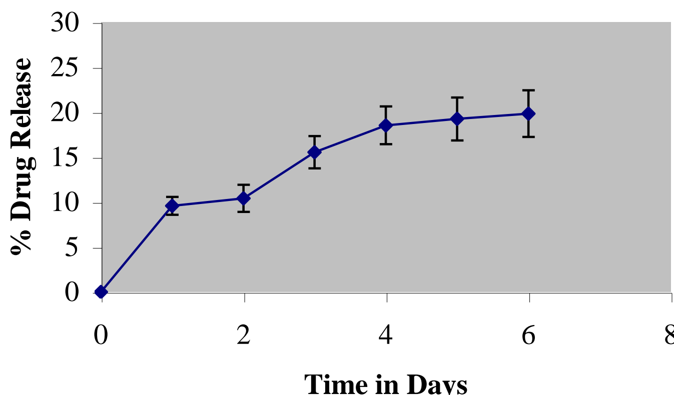


Figure 1: Ciprofloxacin hydrochloride release pattern of collagen bilayer dressing in Phosphate buffered saline (mean ± SE; n = 5).

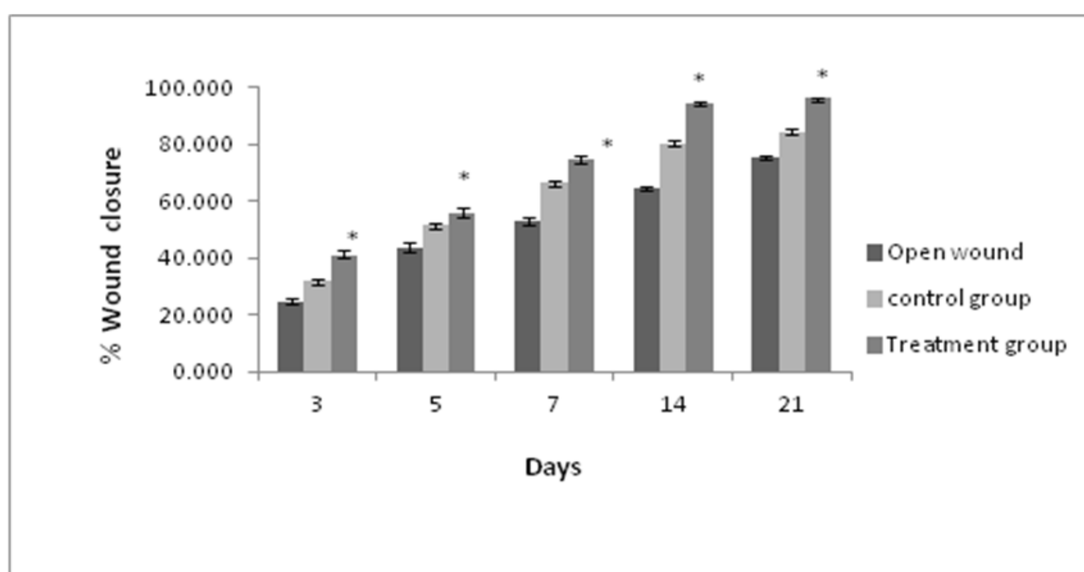


Figure 2: Percentage wound closure in the experimental groups at different time intervals (n = 6). Results are presented as mean ± SE. *Statistically significant, (P <0.05).

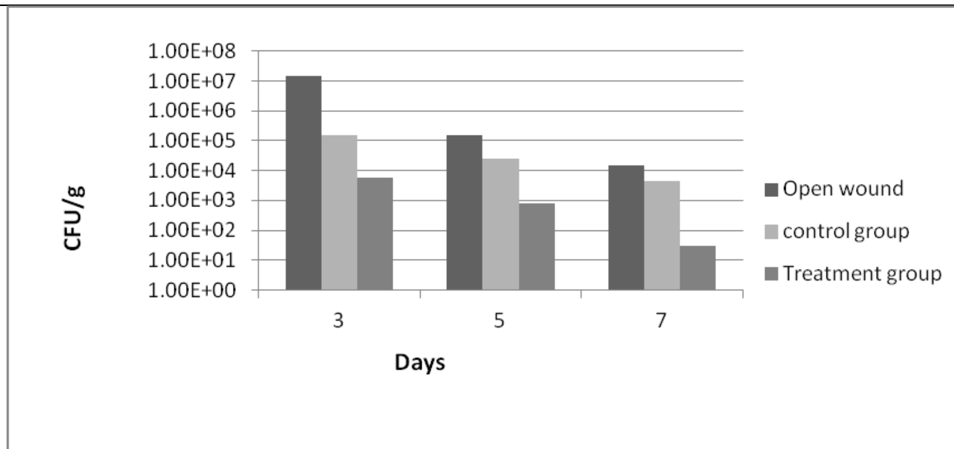


Figure 3: Number of bacteria present in the granulation tissues of experimental groups at different time intervals (n = 6).

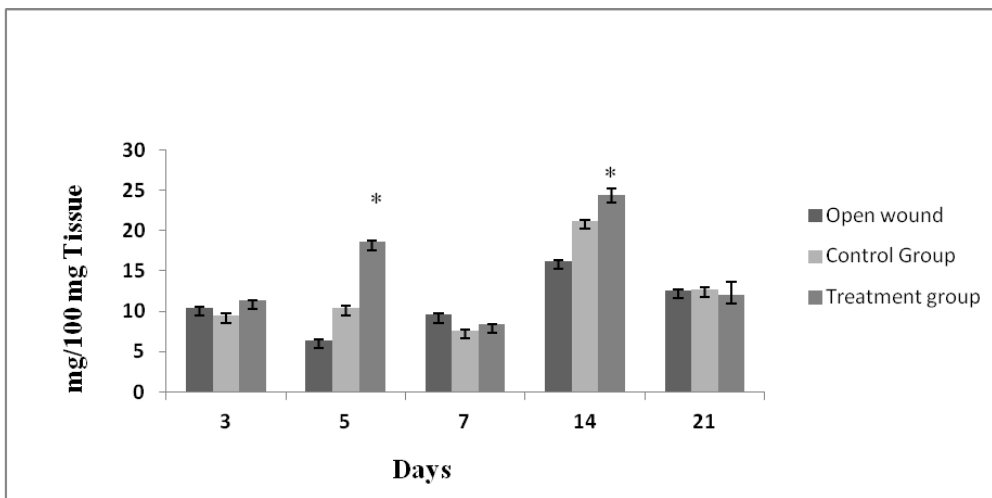


Figure 4: Protein content in the granulation tissues of experimental groups at different time intervals (n = 6). Results are presented as mean ± SE. *Statistically significant data. P <0.05.

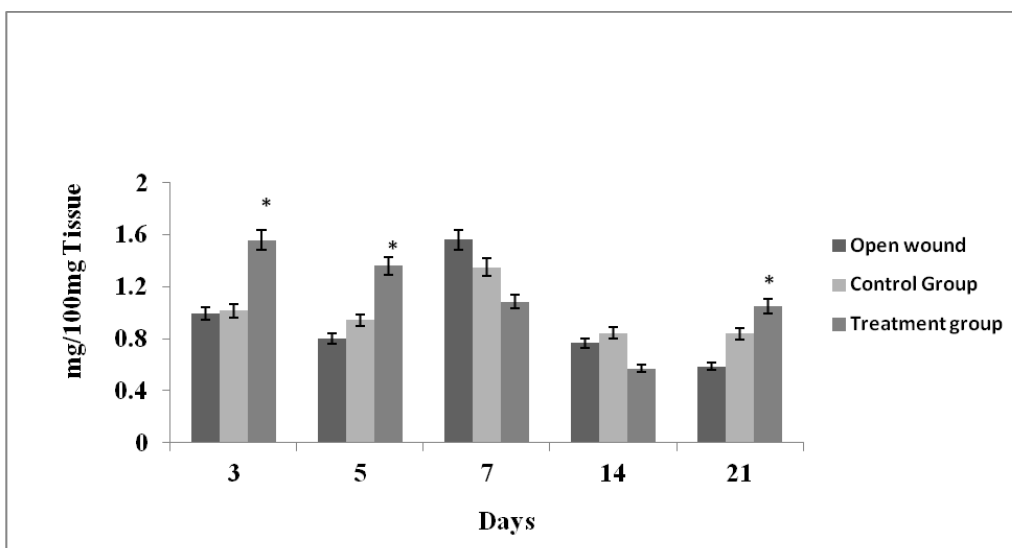


Figure 5: DNA content in the granulation tissues of experimental groups at different time intervals (n = 6). Results are presented as mean ± SE. *Statistically significant data, P <0.05.

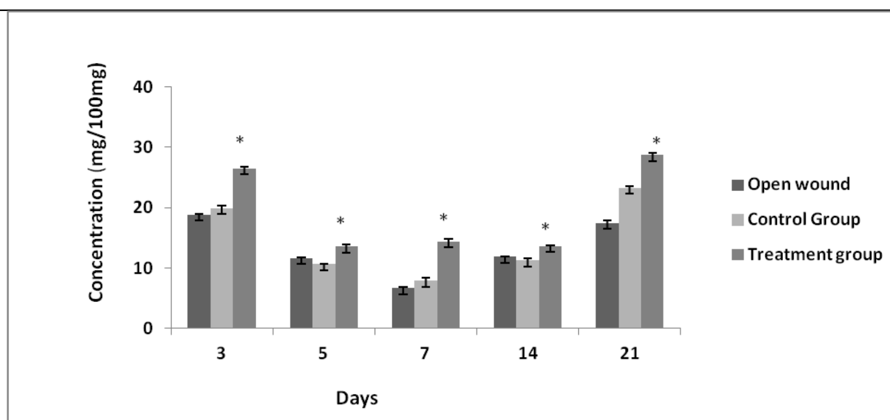


Figure 6: Hydroxyproline content in the granulation tissues of experimental groups at different time intervals (n = 6). Results are presented as mean ± SE. *Statistically significant data, P <0.05.

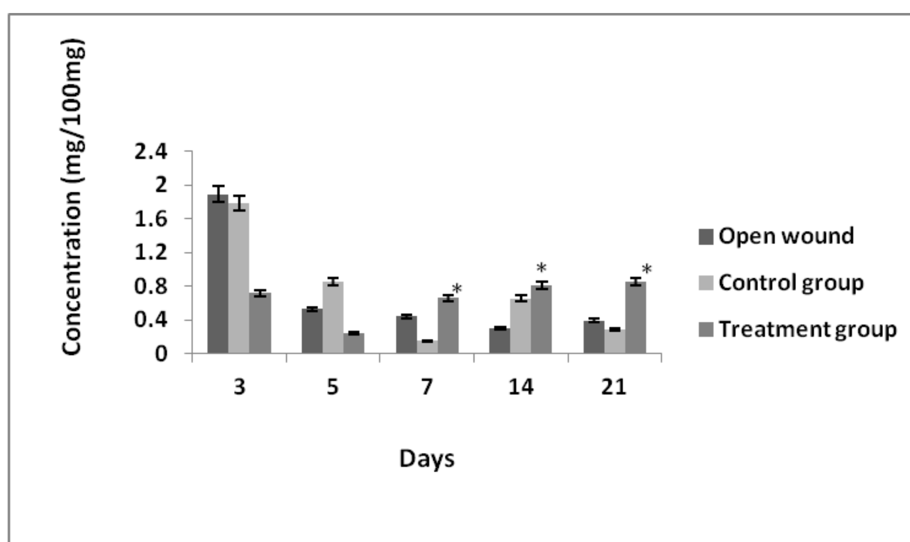


Figure 7: Uronic acid content in the granulation tissues of experimental groups at different time intervals (n = 6).Results are presented as mean ± SE. *Statistically significant data, P <0.05.

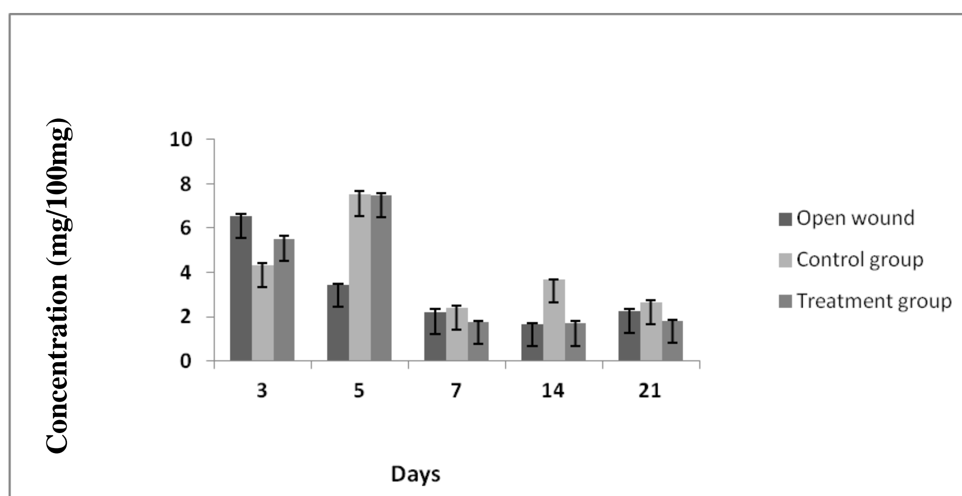


Figure 8: Hexosamine content in the granulation tissues of experimental groups at different time intervals (n = 6). Results are presented as mean ± SE. *Statistically significant data, P <0.05.

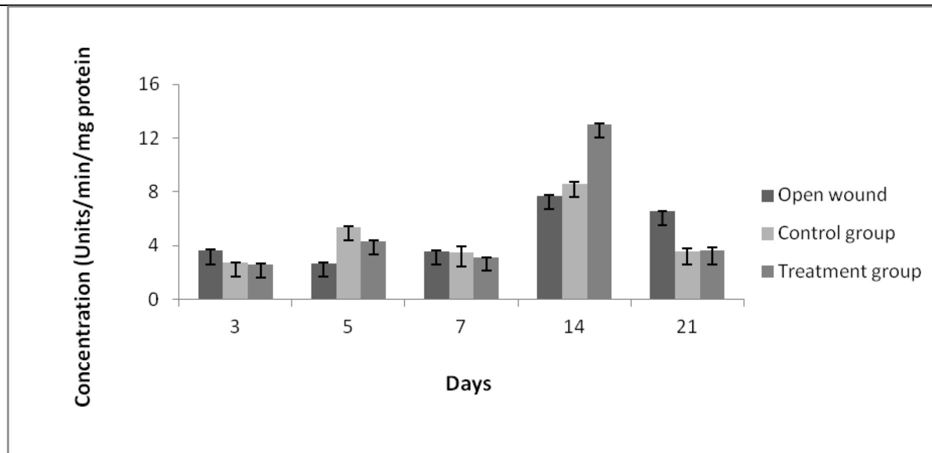


Figure 9: SOD content in the granulation tissues of experimental groups at different time intervals (n = 6). Results are presented as mean ± SE. *Statistically significant data, P <0.05.

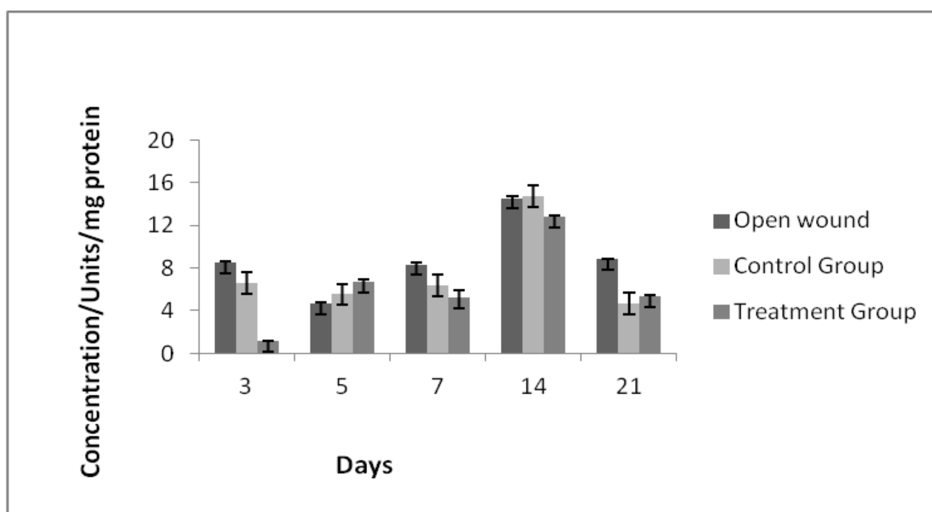


Figure 10: Catalase content in the granulation tissues of experimental groups at different time intervals (n = 6). Results are presented as mean ± SE. *Statistically significant data, P <0.05.

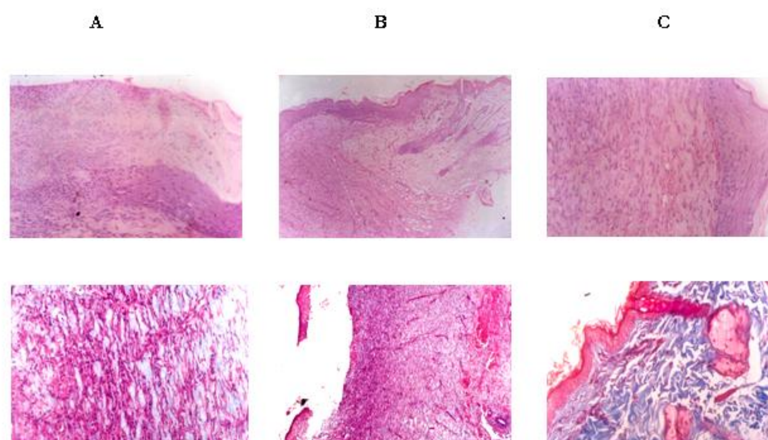
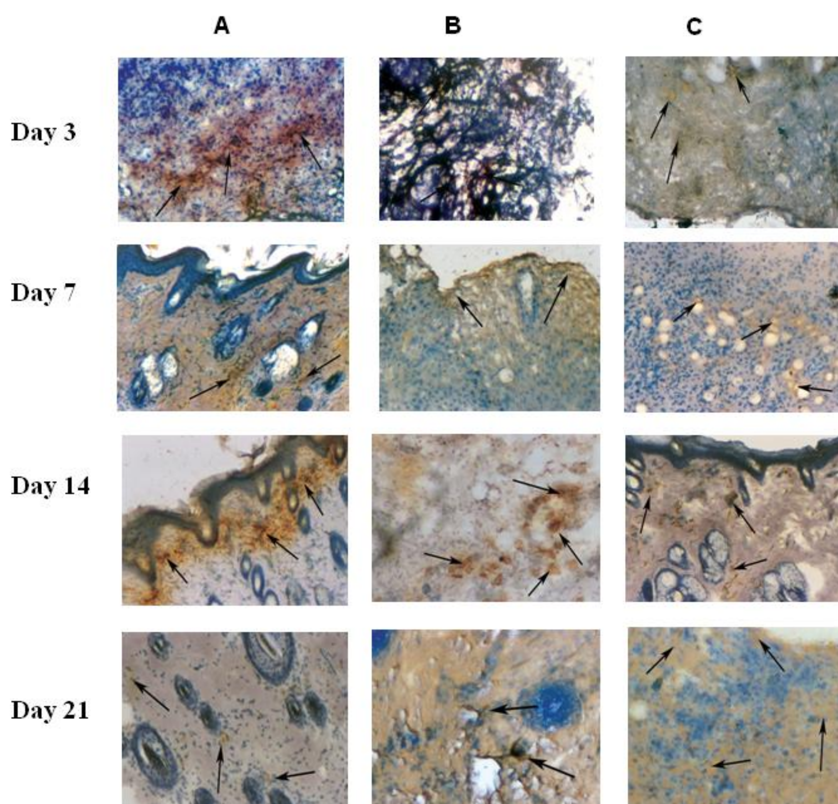


Figure 11: H&E & Masson's Trichrome stained histological sections of the granulation tissue on day 21 of wound creation of different experimental groups. A -open wound, B-Control, C-Treatment Group. Figures A-C are at similar magnifications (320 X). Note: In MTC stained sections blue color indicates staining of the deposited collagen bundles.



Figures 12: A-C: Photomicrograph showing immunohistochemical analysis of Integrin-αV on day 3-21 day of wound creation in the different groups. A- Open wound, B- Control collagen, C-Treatment 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).

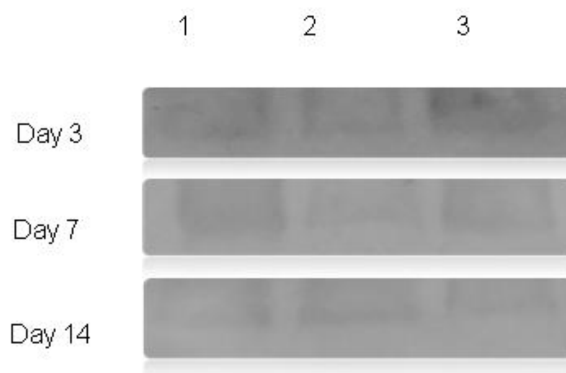


Figure 13: Western blot analysis of Integrin-αV expression in the regenerating wounds. Lanes 1-3 shows expression of tissue antigens in 1) Open wound 2) Control 3) Treated group.