

**COLLAGEN HYDROGEL AS BIO INTERACTIVE
DRESSING FOR WOUND HEALING**

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3.1. Introduction

A wound may be defined as disruption of the cellular or anatomical continuity of the normal organ structure. Wound healing is a complex process requiring coordination of cascade of cellular responses to injury including inflammation, epithelialization, proliferation, angiogenesis and remodeling. Healing involves migration, infiltration, proliferation, and differentiation of several cell types like keratinocytes, fibroblasts, endothelial cells, macrophages, and platelets which culminate an inflammatory response, the formation of new tissue and wound closure (Barrientos *et al.*, 2008)

It is widely accepted that a warm, moist environment encourages rapid healing and most modern wound care products are designed to provide these conditions. Fluid balance in burn injury is very important since heavy loss of water from the body by exudation and evaporation may lead to a fall in body temperature and increase in the metabolic rate. Besides this, dressing should have certain other properties like ease of application and removal, and proper adherence so that there will not be any area of non-adherence left to create fluid-filled pockets for the proliferation of bacteria (Conti *et al.*, 2000)

The wound-healing process consists of four highly integrated and overlapping phases: hemostasis, inflammation, proliferation, and tissue remodeling or resolution (Gosain and DiPietro, 2004). These phases and their biophysiological functions must occur in the proper sequence, at a specific time, and continue for a specific duration at an optimal intensity. There are many factors that can affect wound healing which interfere with one or more phases in this process, thus causing improper or impaired tissue repair. The factors include oxygenation, infection, age and sex hormones, stress, diabetes, obesity, medications, alcoholism, smoking, and nutrition (Mathieu *et al.*, 2006).

Since the early 1980s, numerous wound dressings have been developed to promote wound healing (Balakrishnan *et al.*, 2005). The ideal dressing needs to ensure that the wound remains moist with exudates, but not macerated, and free of infection, while fulfilling prerequisites concerning structure and biocompatibility (Purna and Babu, 2000). Furthermore, they should be non-cytotoxic and non-antigenic, guarantee uniform cell distribution, maintain cell viability and phenotype, and should induce migration and proliferation of epithelial cells, fibroblasts and endothelial cells, as well as the synthesis of extracellular matrix components required for wound repair. In addition, wound dressings should exhibit ease of application and removal, and proper adherence, in order to ensure that there will be no areas of non-adherence left to create fluid-filled pockets for bacterial proliferation (Paddle-Ledinek *et al.*, 2006)

The most significant advancement in wound care came with Winter's (1962) study in 60's, which showed that occluded wounds healed much faster than dry wounds and moist wound healing environment optimized the healing rates. He demonstrated that when wounds on pigs are kept moist, epithelialisation is twice as rapid as on wounds allowed to dry by exposure to air. Later Hinman and Maibach (1963) confirmed Winter's work on human beings in 1963. An open wound, which is directly exposed to air, will dehydrate and a scab or eschar is formed. This forms a mechanical barrier to

migrating epidermal cells and is then forced to move in a deeper level of tissue, which prolongs the healing process. Moist healing prevents the formation of scab as the dressing absorbs wound exudate secreted from the ulcer (Winter and Scales, 1963).

Hydrogels have been frequently utilized as scaffolds for soft tissue due to their excellent biocompatibility, biomimic microstructure and mechanical properties (Drury and Mooney, 2003; Jeon *et al.*, 2007). Natural polymers have similar components with native extra cellular matrix and are widely used for biomedical applications. Collagen and chitosan derivatives are among the most frequently used biomaterials due to their biocompatibility.

Hydrogels are attractive as biomaterials; they are highly permeable to water, ions, and small molecules (Peppas and Khare, 1993). Hydrogels comprised of naturally derived macromolecules have potential advantages of biocompatibility, cell-controlled degradability, and intrinsic cellular interaction. Hydrogels have structural similarity to the macromolecular based components in the body and are considered biocompatible (Jhon and Andrade, 1973).

Biocompatible hydrogels are currently used in cartilage wound healing, bone regeneration, wound dress, and as carriers for drug delivery. Hydrogels are often favorable for promoting cell migration, angiogenesis, high water content, and rapid nutrient diffusion (Bryant and Anseth, 2001). Some of the examples of hydrogel forming polymers of natural origin are collagen (Wallace and Rosenblatt, 2003), gelatin (Kim and Park, 2004) and chitosan (Francis Suh and Matthew, 2000)

Both artificial and natural polymers have been used to constitute hydrogels. Collagen is a natural substrate for cellular attachment, growth and differentiation, and promotes cellular proliferation and differentiation. Natural polymers such as fibrin (Keiser *et al.*, 1994; Siedler and Schuller-Petrovic, 2000), hyaluronic acid (King *et al.*, 1991; Murashita *et al.*, 1996), fibrinogen (Vacanti and Langer, 1998) and collagen (Ruszczak, 2000; Hansen *et al.*,

2001; Froget *et al.*, 2003; Gomathi *et al.*, 2003; Ruszczak, 2003) have been recently tested in different matrix systems for local drug delivery and wound healing. Collagen is unique in possessing different levels of structural order: primary, secondary, tertiary and quaternary (Ho *et al.*, 2001). In vivo, collagen molecules form fibers having a specific internal and structural orientation and are strengthened together by two types of covalent crosslinking: intramolecular and intermolecular. Intermolecular cross-linking is essential to form macromolecular fibers and consequently, for its mechanical stability and other physical properties.

Collagen acts as a natural substrate for cellular attachment, growth and differentiation in its native state. In addition to its desirable structural properties, collagen has functional properties. Certain sequences of the collagen fibrils are chemotactic and promote cellular proliferation and differentiation. Collagen provides considerable strength in its natural polymeric state. The source of collagen either purified from animal sources or as an integral component of a more complex extracellular matrix, and its treatment prior to use are important variables in the design of tissue-engineered devices.

Biomaterials made of collagen offers several different advantages: They are biocompatible and nontoxic to tissues (including neural and brain tissue) and have well-documented structural, physical, chemical, biological and immunological properties. Additionally, mechanical and immunologic properties of collagen scaffolds can be influenced by modification of matrix properties (porosity, density) or by different chemical treatment affecting its degradation rate (Schoof *et al.*, 2001). Collagen contains a number of biological functional groups and has been clinically used as a wound dressing. Its potential as artificial skin, bone grafts and pharmaceuticals has been intensively investigated (Kuberka *et al.*, 2002).

Chitin and chitosan are regarded as appropriate biomaterials due to their physicochemical and biological properties. However,

acetic acid or organic solvents should be applied for material preparation, which would impart certain cytotoxicity to the final product (Shanmuga sundaram *et al.*, 2001). Carboxymethyl chitosan (CM chitosan), a water-soluble derivative of chitosan, has the merits of chitosan and has improved biocompatibility over chitosan (Zhu & Fang, 2005). Therefore, CM-chitosan has been extensively utilized in biomedical materials including moisture-retention agents, bactericides, wound dressings, artificial skin, blood anticoagulants and so on (Zhang *et al.*, 2000). Furthermore, CM-chitosan was capable of stimulating the extracellular lysozyme activity of fibroblasts, promoting the proliferation of normal skin fibroblasts and inhibiting the proliferation of keloid fibroblasts (Chen *et al.*, 2002).

An ideal wound dressing should have several key attributes. The dressing should protect the wound from bacterial infection, control evaporative water loss and prevent dehydration, control permeability of oxygen and carbon dioxide, absorb wound exudate, and enhance the healing. Additionally, it should be composed of materials that are non-toxic, non-immunogenic, flexible, durable, and comfortable when worn. Synthetic materials such as poly(urethane) (Hinrich *et al.*, 1963; Wright *et al.*, 1998) poly(vinyl alcohol) (Suzuki *et al.*, 1997), poly hydroxy ethyl methacrylate (Dressler *et al.*, 1980), and copolymers (Kim *et al.*, 2000), as well as biological materials like bovine collagen (Kim *et al.*, 2000; Boyce *et al.*, 1988b; Yannas and Burke, 1980 and Yannas *et al.*, 1982), chitin (Conti *et al.*, 2000 and Su *et al.*, 1997), and alginate (Choi *et al.*, 1999 and Choi *et al.*, 2001) have been investigated. However, until now there have been few reports of fish collagen based materials for wound dressings.

The present study aims to examine the wound healing activity of hydrogel prepared from queen fish skin collagen by conducting *in vivo* studies in albino rats. Circular incision wound model was used to screen the wound healing activity. Percentage closure of original wound area was calculated on various days and results indicated that

the percentage wound closure and re-epithelialization for the gel formulation treated group was comparable with those of standard group treated with megaheal. The percentage of re-epithelialization was examined by histopathological studies and biochemical analysis of reformed skin.

3.2 Materials and methods

3.2.1 Preparation of the hydrogels

Collagen gel and CM-chitosan powder, and distilled water at different ratios were mixed by a hybrid mixer for 10 min to form a homogenous gel. The total polymer concentration was fixed to 50% by weight with collagen to CM-chitosan at the ratio 1:0 and 4:1 respectively. The gels were filled into tubes (inner diameter 10mm) and subjected for γ irradiation using a ^{60}Co radiation facility, which was performed at room temperature with a dose rate of 20 Gy/min at a desired absorbed dose.

3.2.2 In vivo wound healing

The wound healing characteristics of the collagen hydrogel was evaluated in subcutaneous circular incision wound model on albino rats. The study was conducted with the approval from the Institutional Animal Ethical Committee (IAEC) of CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animals). Total 24 adult Wistar male rats (150-200gm) were divided into four groups with 6 animals in each group. Animals were housed under standard environmental conditions of temperature and 12 hours light and dark cycle. All the animals were provided with food and water *ad libitum*.

Before making a wound, the rats were anesthetized with 2.5% isoflurane. The surgical area was shaved with an electric razor, the mouse was strapped to a surgical board, and additional anesthesia was provided via a nose cone. After a deep surgical plane of general anesthesia had been reached, a wound, approximately 1cm in diameter, was created on the dorsal side of the mouse using curved

blade surgical scissors. Both the epidermal and dermal layers were removed.

3.2.3 Study design and Dosing schedule

Collagen hydrogel and megaheal ointment were applied topically, twice daily from day zero to day of complete healing or the 15th postoperative day whichever occurred earlier. There were four groups in the study viz. control, standard (Megaheal cream) and test groups.

Treatment Protocols: The animals were numbered, weighed and then divided into four groups with six animals in each as follows:

Group I: control group without any treatment.

Group II: standard ointment (Megaheal) applied.

Group III: Collagen hydrogel (test 1)

Group IV: Collagen chitosan hydrogel (test 2)

3.2.4 Wound contraction measurement

Wound contraction was noted by following the progressive changes in wound area planimetrically, excluding the day of wounding. The progressive changes in excision wound area were measured in mm² by tracing the wound boundaries on transparent paper on each 2 days interval until complete wound healing was achieved. The wound areas in all groups were recorded on graph paper. Wound contraction was expressed as reduction in percentage of the original wound formula

$$\% \text{ wound contraction} = \frac{(A_o - A_t)}{A_o} \times 100 \dots\dots\dots (1)$$

Where A_o is the original wound area and A_t , the area of wound at end of treatment.

Wound area was measured by tracing the wound margin using a transparent paper in each 2 days interval and healed area calculated by subtracting from the original area.

Figure 3.1 showing Mega heal ointment and the hydrogel prepared



Figure 3.2(A) Photographic representation of measurement of wound area in excised rat(B)Collagen application in wounded area.

(A)



(B)



3.2.5 Epithelialization period

Epithelialization time was noted as the number of days after wounding required for the scar to fall off leaving no raw wound behind. From the healed wound, a specimen sample of tissue was isolated from each group of rats for histopathological examination.

The un-epithelialized wound diameter was measured using an eyepiece micrometer. This measurement, together with the original wound diameter, was used in Eq. (2) to determine the percent re-epithelialization. The average of all six sections from each wound site was calculated and determined to be the average percent re-epithelialization for that wound.

$$\% \text{re-epithelialization} = \frac{(D_o - D_b)}{D_o} \times 100 \dots\dots\dots (2)$$

Where D_o is the original wound diameter, D_b is the length of un-epithelialized tissues at the times of biopsy.

Histopathological examination and biochemical parameters were carried out by using tissue specimen isolated from the healed skin of each groups of rat.

3.2.6 Histopathology

Formalin (10%) was used to fix the tissue and was embedded in paraffin wax. Serial sections of paraffin embedded tissues of 4µm were made. Sections were stained with hematoxyline eosin (H&E). All sections were analyzed using light microscopy (Olympus BX 45, Olympus, Hamburg Germany) by two pathologists in a blinded manner. The microscopic slides were photographed. Congestion, edema, PMNL, mononuclear cells, fibroblasts and vascularization were qualitatively evaluated as well as ulceration, necrosis and epithelialization were examined in the skin tissues.

3.2.7 Biochemical parameters

Circular wound area was excised and evaluated for various biochemical parameters at the end of the study. Especially collagen content, hydroxyl proline and hexosamine was estimated for evaluating the healing properties of collagen.

3.2.7.1 Estimation of hydroxyproline & collagen from reformed wound tissue

Hydroxyproline content was determined by Ehrlich's hydroxyproline assay (Reddy *et al*, 1996). Repaired wound skin tissues were dried in a hot air oven at 60–70°C to constant weight and were hydrolyzed in 6 N HCl at 130°C for 4 h in sealed tubes. The hydrolysate was neutralized to pH 7.0 and was subjected to Chloramine-T oxidation for 20 min. The reaction was terminated by addition of 0.4 M perchloric acid, colour was developed with the help of Ehrlich reagent at 60°C and the absorbance was measured at 557 nm using spectrophotometer.

Hydroxyproline content was converted to collagen content using the following equation (Ignateva *et al*, 2007):

$$\text{Collagen } (\mu\text{g}) = \text{Hydroxyproline } (\mu\text{g}) \times \text{dilution factor} \times 7.57$$

3.2.7.2 Estimation of hexosamine from reformed wound tissue

The hexosamine content was determined by the method of Wagner (Wagner *et al*, 1979). An aliquot of de-fatted sample was hydrolyzed with 3N HCl in a boiling water bath for four hours and neutralized. To 0.8 mL of neutral hydrolysate added 0.6 mL of acetyl acetone reagent and heated in a boiling water bath for 30 minutes. The hydrolysate was cooled and 2 mL of Ehrlich's reagent was added to it and mixed well. Absorbance was measured at 535nm. Glucosamine standards of concentrations 20mg to 80mg were similarly processed and absorbance values were recorded. From the standard graph, concentration of hexosamine in the test sample was calculated.

3.2.8 Statistical analysis

The results are expressed as Mean \pm SE from n=6 observations. The findings were also analyzed for determining significance of difference by ANOVA test followed by pair-wise comparison of various group by LSD. The differences among groups were considered to be significant at $p < 0.001$. The analysis was carried out by using SAS system version 9.3 (SAS Institute Inc., Cary, NC, USA)

3.3 Result and Discussion

3.3.1 Changes in wound area

Wound healing was assessed by monitoring wound contraction and re-epithelialization. Wounds supplemented with the hydrogel, had improved wound healing results compared to those wounds without any treatment (control group). The changes in the wound area in the course of experimental period are shown in table 3.1. Fig. 3.4 and 3.5 shows the results for both wound contraction and re-epithelialization, respectively. No significant difference in wound contraction was observed between any of the four experimental groups after 15 days of experimental period.

On the other hand, by day 15, wounds treated with test 1 and test 2 had significantly more re-epithelialization ($p < 0.001$) than the controls group. At the end of experiment, the percentage of re-epithelialization was found to be 85 ± 0.65 % for test treated group; whereas for control wounds this was 77.08 ± 0.83 %.

Table 3.1 Changes in wound area (in mm^2) for in vivo wound healing experiments

| Group | 0 th day | 2 nd day | 5 th day | 8 th day | 10 th day | 12 th day | 15 th day |
|----------|---------------------|---------------------|---------------------|---------------------|----------------------|----------------------|------------------------------|
| Control | 452.6 \pm 05.6 | 314.5 \pm 9.8 | 267.5 \pm 11.5 | 181.0 \pm 18.0 | 53.3 \pm 23.6 | 26.67 \pm 12.6 | 23.3 \pm 09.3 ^b |
| Std drug | 455.5 \pm 14.3 | 336.1 \pm 12.4 | 213.6 \pm 8.5 | 141.8 \pm 6.4 | 38.5 \pm 8.7 | 12.6 \pm 2.04 | 11.8 \pm 03.8 ^a |
| Test 1 | 453.8 \pm 13.5 | 319.7 \pm 25.2 | 263.3 \pm 15.9 | 165.2 \pm 17.5 | 53.5 \pm 16.2 | 28.6 \pm 12.6 | 12.2 \pm 17.9 ^a |
| Test 2 | 458.3 \pm 13.6 | 320.6 \pm 22.6 | 256.12 \pm 11 | 177.66 \pm 13.9 | 55.8 \pm 8.0 | 26.3 \pm 15.8 | 11.6 \pm 14.2 ^a |

Figure 3.3 Changes in wound area during the course of experimental period

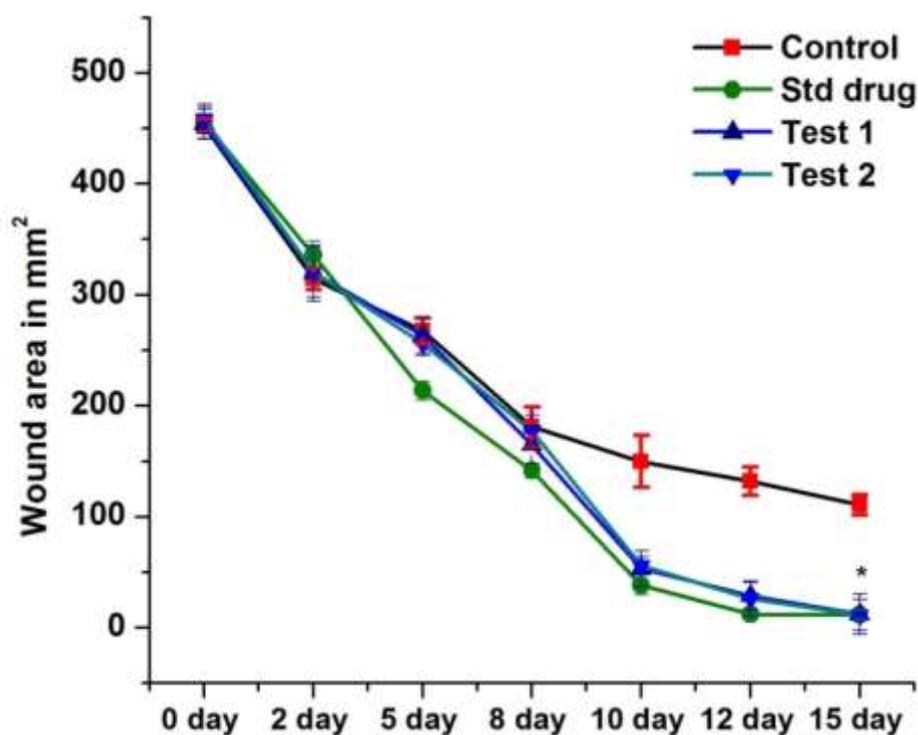


Figure 3.4 Percent wound contraction for the *in vivo* wound healing experiments. Results are shown for the four experimental groups, treated with megaheal (group 2), test 1 (group 3) and test 2 (group 4) and compared against control group. (mean \pm SD, n = 6).

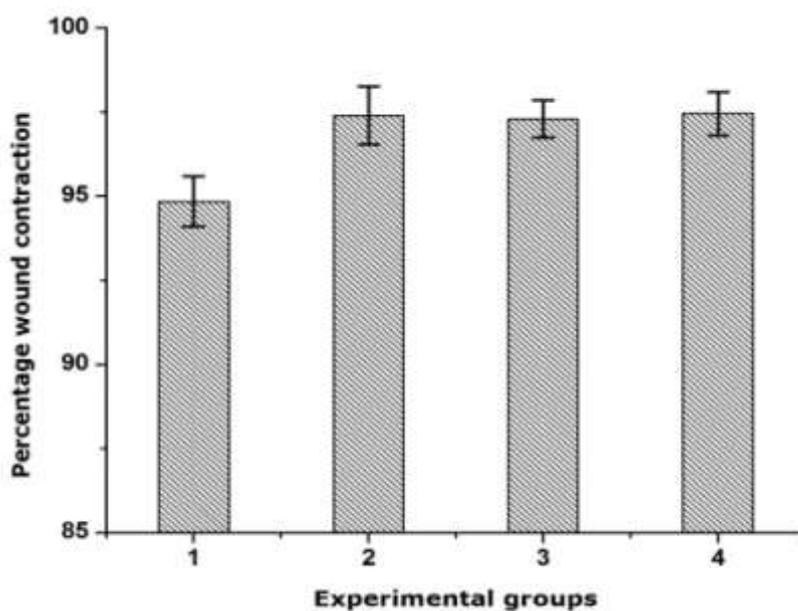
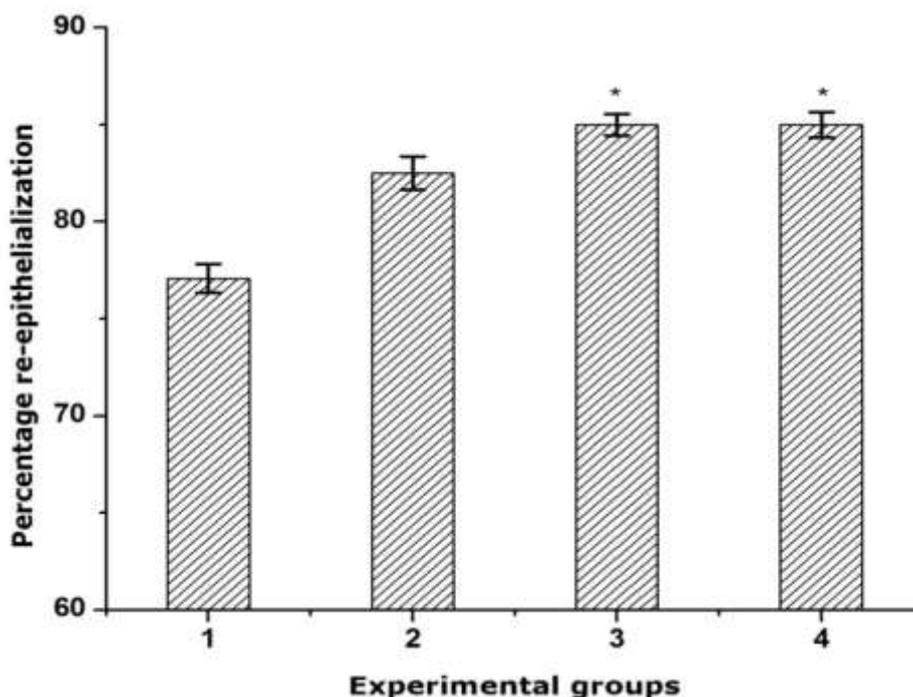


Figure 3.5 Percent re-epithelialization for the *in vivo* wound healing experiments. Results are shown for the four experimental groups, treated with megaheal (group 2), test1 (group 3) and test 2 (group 4) and compared against control group. (mean±SD, n = 6). (* shows significance at $p < 0.001$)



3.3.2 Histopathological observations

Treatment of rat wounds with hydrogel and standard drug treated animals led to reduced macrophages, oedema, necrosis and increased collagen fibril and blood vessel formation. It can be seen that wounds treated with hydrogel were fully re-epithelialized with a well-structured layer of epidermis. Collagen was present in dermis. On the contrary, in control group increased number of macrophages, oedema, necrosis and less collagen fibril formation were observed. For some control wounds, moderate number of inflammatory cells was still present in the upper dermis. And the surface of the defect was not completely covered with new epithelium.

Figure 3.6 Wound healing profile of control group without any treatment. Changes in the wound on 1st day, 3rd day, 5th day, 8th day, 12th day & 14th day shown in picture A- F respectively



Figure 3.7 Wound healing profile of collagen hydrogel treated group (test 2). Treatment effects in the wound on 1st day, 3rd day, 5th day, 8th day, 12th day & 14th day shown in picture A- F respectively



3.3.3 Biochemical evaluation of reformed skin

Biochemical parameters of wound healing was evaluated and presented in figure 3.9 (A) and (B). There was a significant increase in the hydroxyproline content that is 74.93 ± 2.214 and 74.00 ± 2.729 $\mu\text{g}/\text{gm}$ in test 1 and test 2 treated group respectively which was much more higher than control and standard drug treated group which showed the values of 46.13 ± 0.675 and 62.15 ± 3.935 $\mu\text{g}/\text{gm}$. Increased hydroxyproline content is a reflection of increased cellular proliferation and therefore increased collagen synthesis (Ignateva *et al.*, 2007). Generally an increase in hydroxyproline content is ultimately responsible for increase in collagen levels. In the present study control and standard drug treated animals showed much lesser collagen content which was 369.066 ± 5.401 and 497.173 ± 31.481 $\mu\text{g}/\text{gm}$ as compared to test 1 and test 2 groups which showed 599.466 ± 16.99 and 592.00 ± 21.839 $\mu\text{g}/\text{gm}$ concentration of collagen respectively.

For assessing wound healing property, the hexosamine content was evaluated in the reformed animal tissues. The hexosamine content was 22.91 ± 10.55 and 25.15 ± 7.96 mg/gm in the test 1 and 2 treated groups and the values for control and standard drug treated group were 6.15 ± 13.25 and 16.31 ± 12.50 mg/gm respectively. The values were statistically significant at $P < 0.001$ when compared to control group. Hexosamine content increases in the early stages of wound healing and that the fibroblasts actively synthesize the ground substances on which the collagen can be laid on (Chitra *et al.*, 1998).

Figure 3.8 Histopathological examination of newly formed 15th day wound tissue.

A: Control group B: Ointment (Mega heal) C: test 1 D: test 2 (x) Area of ulceration and (y) mixed type inflammatory cells.

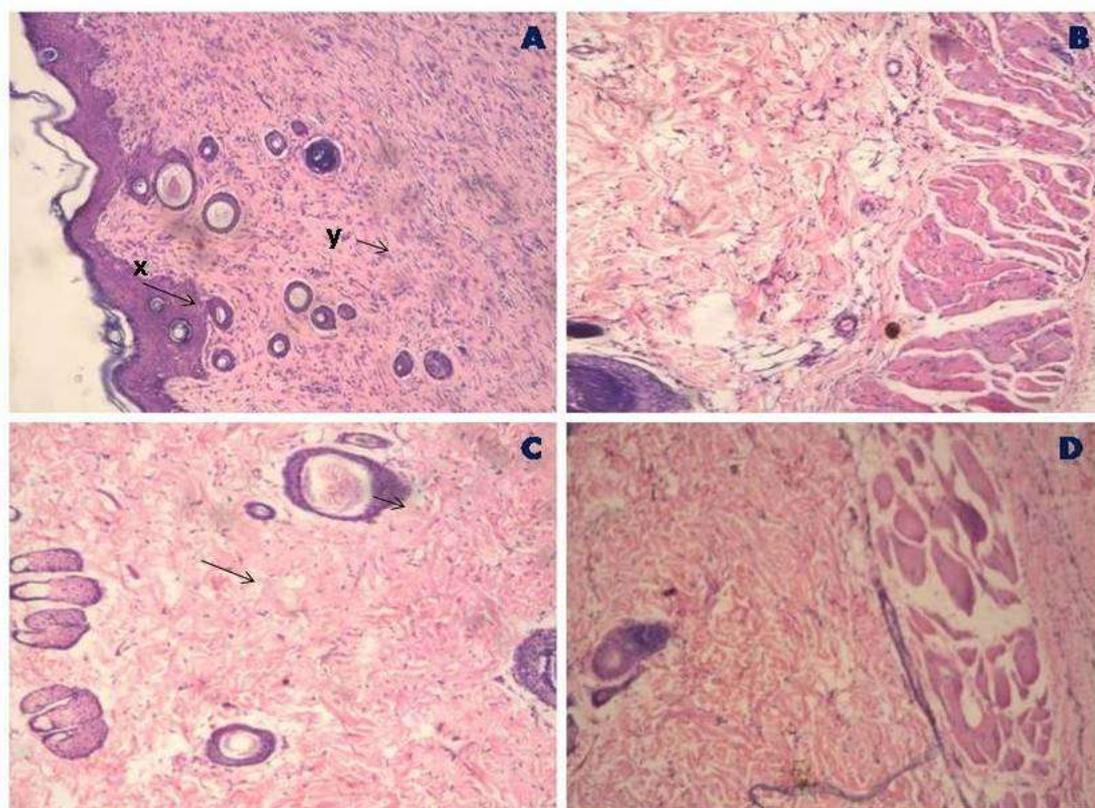


Figure 3.9 (A) *Hydroxyproline and Hexosamine content in different experimental groups. The graph shows that the increase in hexosamine content is obvious in the test rats when compared to control. (mean \pm SD, n = 6) ($p < 0.001$). This indicates that the fibroblasts are actively synthesized, the ground substance on which the collagen can be laid on.*

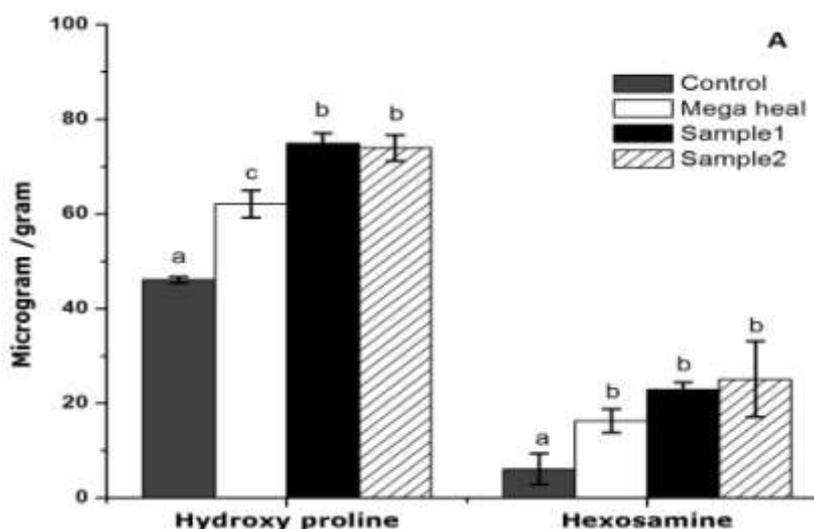
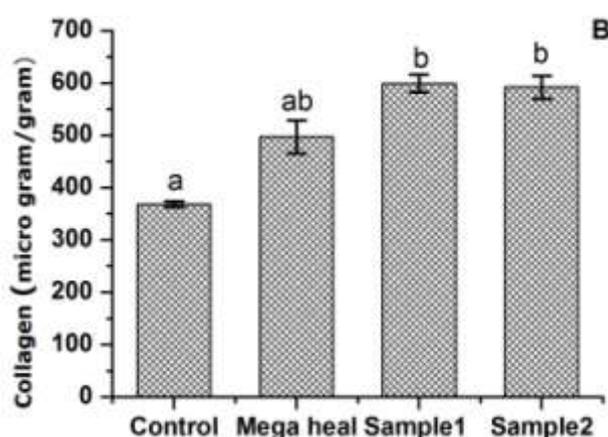


Figure 3.9 (B) Collagen content in different experimental groups. It is evident from the graph that the amount of collagen has been increased in the test rats compared to control. (mean \pm SD, n = 6)($p < 0.001$). It has been stated that collagen provides tensile strength to tissues especially of healing wounds.



3.5 Conclusion

In the present study, the wound healing efficacy of hydrogel was evaluated in experimental full thickness wounds using a rat model which demonstrated that within 2 weeks, the wound covered with gel was completely filled with new epithelium without any significant adverse reactions. There is significant increase in angiogenesis, collagen deposition, hexosamine content, epithelialization and wound contraction in hydrogel treated rats without inflammatory cells compared to the control group. Wound healing effects may be due to regulation of collagen expression and an increase in tensile strength of the wounds. Enhanced healing activity has been attributed to increased collagen formation and angiogenesis. Angiogenesis in granulation tissues improves blood supplementation to the wound site, thus providing nutrients and oxygen essential for the healing process.

Also the hydrogel provided moist environments which facilitate the smooth healing process. Winter showed that epithelialization can be accelerated if the wound is kept moist (Winter, 1962). Keratinocytes migrated more easily over a moist wound surface than underneath a scab (Winter and Scales, 1963). Epidermal cells can migrate at a speed of about 0.5 mm/day over a moist wound surface which is twice as fast as under a scab in dry wounds (Winter, 1972).

Wound contracture is a process that occurs throughout the healing process, commencing in the fibroblastic stage whereby the area of the wound undergoes shrinkage. It has 3 phases, inflammatory, proliferative, and maturational and is dependent upon the type and extent of damage, the general state of the host's health, and the ability of the tissue to repair. The inflammatory phase is characterized by hemostasis and inflammation, followed by epithelization, angiogenesis, and collagen deposition in the proliferative phase. In the maturational phase; the final phase of wound healing, the wound undergoes contraction resulting in a smaller amount of apparent scar tissue.

During wound healing, the edges of the wound pull inwards to reduce the overall wound area. Wound fibroblasts begin to assume a myofibroblast phenotype characterized by large bundles of actin-containing microfilaments and the establishment of cell-cell and cell-matrix linkages (Clark, 1996). The fibroblasts link to extracellular fibronectin and collagen and to each other through adherens junctions. Collagen bundles at the wound edge and the underlying dermis crosslink to form a collagen network. These cell-cell, cell-matrix, and matrix-matrix links provide a network through which the traction of the fibroblasts can be transmitted across the wound, leading to wound contraction.

Re-epithelialization is the process by which new cutaneous tissue covers the wound defect. This process requires the uninjured keratinocytes along the wound edges to migrate laterally to cover the wound bed. Both wound contraction (Noormohamed and Ray, 1998;