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Collagen Hydrogel as Bio Interactive dressing for wound healing

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Abstract

In the present study, wound healing efficacy of the hydrogel prepared from fish skin collagen was evaluated in experimental full thickness wounds using albino rat model. The study demonstrated that within 2 weeks, the wound covered with gel were completely filled with new epithelium without any significant adverse reactions. The results clearly suggest that the hydrogel enhances re-epithelialization rather than a repair which was clear from the histopathology and biochemical analysis. 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, indicating the tissue regeneration potential of fish collagen.

Keywords: Collagen hydrogel, wound healing, epithelialization, histopathology, Hexosamine

Introduction

Wound healing is a complex process requiring coordination of a 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 in an inflammatory response, formation of new tissue and wound closure (Barrientos et al., 2008)

Hydrogels have been frequently utilized as scaffolds for soft tissue due to their excellent biocompatibility, biomimic microstructure and mechanical properties (Drury & 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 & Khare, 1993). 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 & Anseth, 2001). Some of the examples of hydrogel forming polymers of natural origin are collagen (Wallace & Rosenblatt, 2003), gelatin (Kim et al., 2004) and chitosan (Francis Suh & Matthew, 2000). Collagen is a natural substrate for cellular attachment, growth and differentiation, and promotes cellular proliferation and differentiation (Ho et al., 2001).

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 contains a number of biological functional groups and has been clinically used as a wound dressing. Its potential as artificial skin, bone grafts and pharmaceutics has been intensively investigated (Kuberka et al., 2002).

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

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Megaheal. The percentage of re-epithelialization was examined by histopathological studies and biochemical analysis of reformed skin.

Materials and Methods

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 the ratios of collagen to CM-chitosan at 10/0, 8/2 respectively. The gels were filled into tubes (inner diameter 10 mm) and subjected for ã 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.

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

Before making the wound, rats were anesthetized with 2.5% isoflurane. The mouse was strapped to a surgical board, the surgical area was shaved with an electric razor and additional anesthesia was provided via a nose cone. 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 down.

Collagen hydrogel and Megaheal ointment were applied topically, twice daily from day zero to day of complete healing or the 15th day postoperative whichever occurring earlier. There were 4 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 (Sample 1) Group IV: Collagen chitosan hydrogel (Sample 2). Wound contraction was noted by following the progressive changes in wound area, excluding the day of wounding. The progressive changes in excision wound area were measured in mm^2 by tracing the wound boundaries on transparent paper at 2 days interval until complete wound healing was achieved. Wound areas in all groups were recorded on graph paper. Wound contraction was expressed as reduction in percentage of the original wound formula

$$\% \text{ wound contraction} = \frac{(Ao - At)}{Ao} \times 100 \quad \text{.....(1)}$$

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

Wound area was measured and healed area calculated by subtracting from the original area.

Epithelialization time was noted as the number of days post-wounding required for the scar to fall off leaving no raw wound behind. From the healed wound, a specimen sample of tissue was collected 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 percent re-epithelialization. The average of all six sections from each wound site was calculated which was considered to be the average percent re-epithelialization for that wound.

$$\% \text{ re-epithelialization} = \frac{(Do - Db)}{Do} \times 100 \quad \text{.....(2)}$$

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

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

Circular wound area was excised and evaluated for various biochemical parameters at the end of the study. 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):

Collagen (μg) =

Hydroxyproline (μg) × dilution factor × 7.57

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 min. The hydrolysate was cooled and 2 mL of Ehrlich's reagent was added to it and mixed well. Absorbance was measured at 535 nm. Glucosamine standards of concentrations 20 to 80 mg were similarly processed and absorbance values were recorded. From the standard graph, concentration of hexosamine in the test sample was calculated.

The results are expressed as Mean ± 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)

Result and Discussion

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 (Group I). The changes in the wound area in the course of experimental period are shown in Fig. 1. Fig. 2 and 3 show the results for 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 sample 1 and sample 2 had significantly more re-epithelialization ($p<0.001$) than the control group. At the end of experiment, the percentage of re-epithelialization for sample treated group was 85±0.65% whereas for control it was 77.08±0.83%. Wound healing profile of control group and hydrogel treated group are shown in Fig. 4 and 5. Topical administration of collagen hydrogel renders moist environment to facilitate the smoothness essentially required for fast healing process.

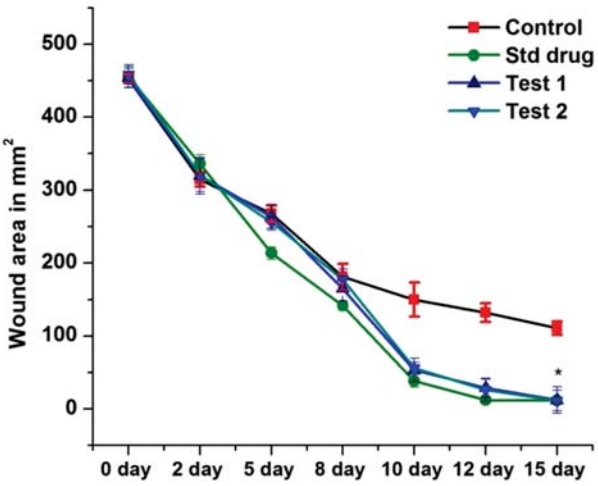

Fig. 1. Changes in wound area during the course of experimental period

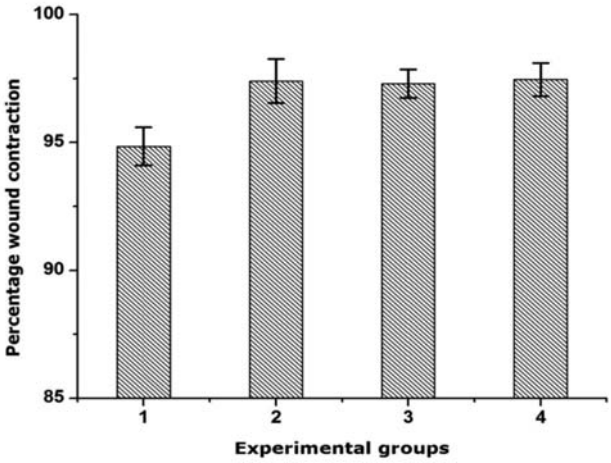

Fig. 2. Percent wound contraction for the in vivo wound healing experiments. Results are shown for the four experimental groups, treated with megaheal (group 2), sample1 (group 3) and sample 2 (group 4) and compared against control group (mean±SD, n = 6)

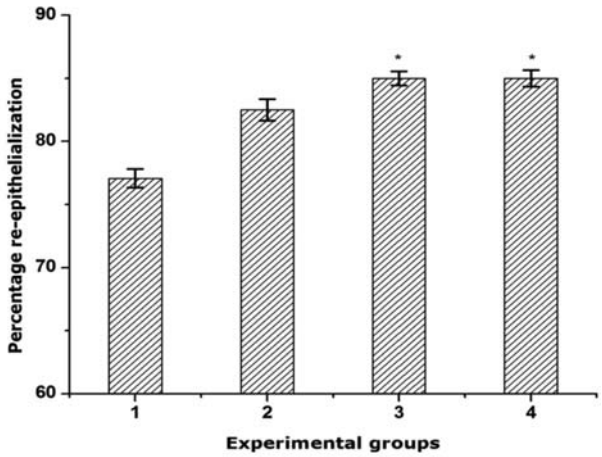

Fig. 3. Percent re-epithelialization for the in vivo wound healing experiments. Results are shown for the four experimental groups, treated with megaheal (group 2), sample1 (group 3) and sample 2 (group 4) and compared against control group. (mean±SD, n = 6)(* shows significance at $p<0.001$)

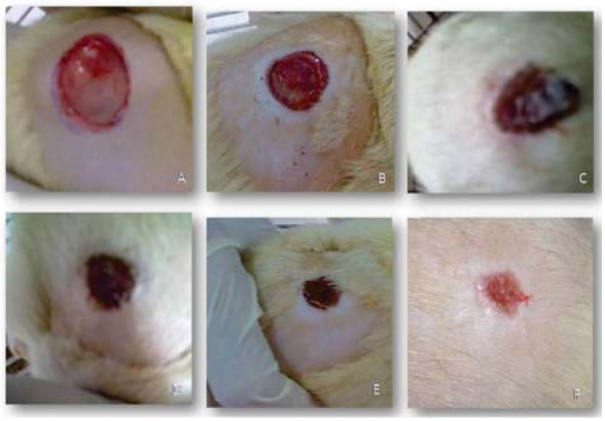

Fig. 4. Wound healing profile of control group without any treatment. Changes in the wound on 1st day, 3rd day, 5th day, 8th day, 12th day & 14th day shown in picture A- F respectively

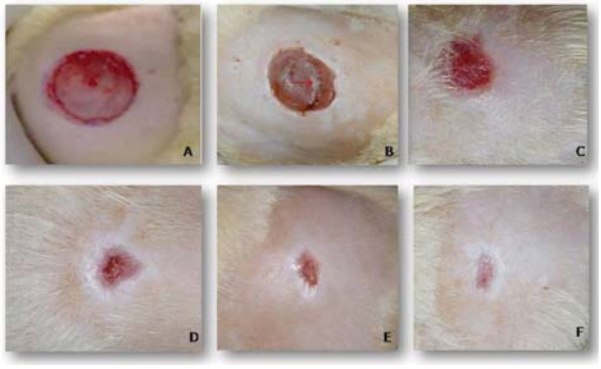

Fig. 5. Wound healing profile of collagen hydrogel treated group. Treatment effects in the wound on 1st day, 3rd day, 5th day, 8th day, 12th day & 14th day shown in picture A- F respectively

Histopathological studies show that treatment of wounds with hydrogel and standard drug led to reduced macrophages, oedema, necrosis and increased collagen fibril and blood vessel formation (Fig. 6). It can be seen that wounds treated with hydrogel were fully re-epithelialized with a well-structured layer of epidermis and also mature 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. Increased collagen deposition within the wound bed has effectively potentiated vascularization process in wound healing. Also the angiogenesis in granulation tissues is possibly improved blood circulation to the wound site, thus providing nutrients and oxygen essential for the healing process.

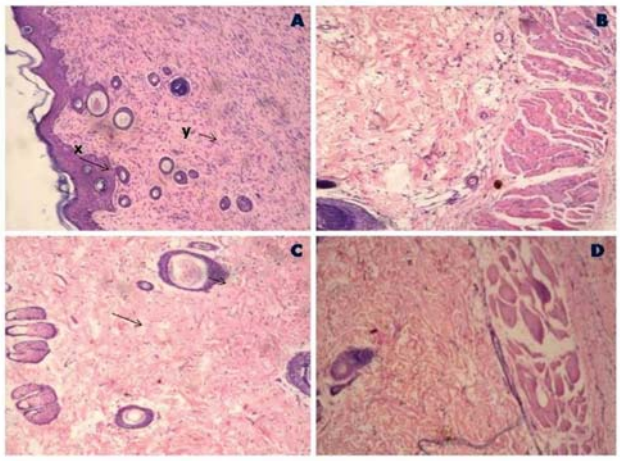

Fig. 6. Histopathological examination of newly formed wound tissue on 15th day

A: Control group B: Ointment (Mega heal) C: Sample1 D: Sample 2 (x) Area of ulceration and (y) mixed type inflammatory cells

Biochemical parameters of wound healing were evaluated and presented in Fig. 7(a) and 7(b). There was a significant increase in the hydroxyproline content in groups I II and IV with 74.93±2.21 and 74.00±2.73 $\mu g\ g^{-1}$ respectively which was higher than control and standard drug treated group that showed significantly lower values of 46.13±0.68 and 62.15±3.94 $\mu g\ g^{-1}$ respectively. 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 369.07± 5.40 and 497.17 ± 31.48 μg g^{-1} respectively as compared to Group III and IV which are having 599.47±16.99 and 592± 21.84 μg g^{-1} concentration of collagen respectively.

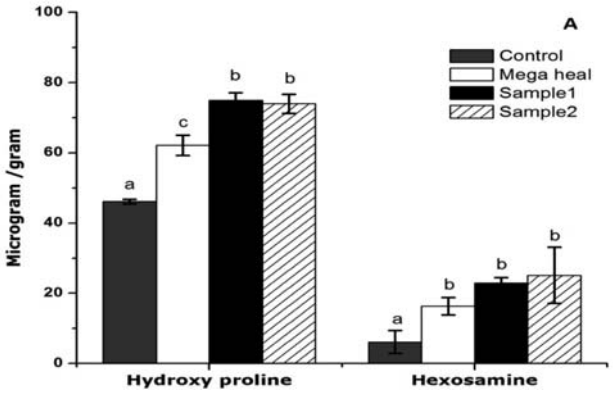

Fig. 7(a) Estimation of Hexosamine. The figure shows that the increase in hexosamine content is obvious in the test rats when compared to control. (mean± SD, n=6)($p<0.001$). This indicates that the fibroblasts are actively synthesized, the ground substance on which the collagen can be laid on.

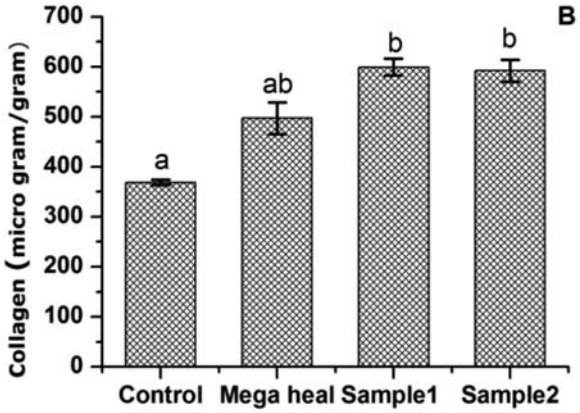

Fig. 7(b) Estimation of Collagen. It is evident from the graph that the amount of collagen has been increased in the test rats compared to control. (mean±SD, n = 6)($p<0.001$). It has been stated that collagen provides tensile strength to tissues especially of healing wounds

For assessing healing property, hexosamine content was evaluated in the reformed animal tissues. The hexosamine content was 22.91±10.55 and 25.15±7.96 mg gm^{-1} in Group III and IV and the values for control and standard drug treated group were 6.15±13.25 and 16.31±12.50 mg gm^{-1} 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 fibroblasts actively synthesize the ground substances on which the collagen can be laid (Nakamura et al., 2008).

Wound healing effects of fish collagen may be attributed to its capability of tissue remodeling process associated with collagen synthesis at the wound site, which is evident from increased tensile strength of skin. It is concluded from the study that the hydrogel formulation can be effectively used as an active ingredient in the formulation of wound healing ointments.

Acknowledgements

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