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Evaluation of the Efficacy of Bacterial Collagenase in Wound Healing

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Abstract

Introduction: The peptides resulting from the collagen degradation by collagenases have wide range of applications including wound healing. However, most of the wound healing studies involve collagenase obtained from anaerobic and pathogenic microorganisms thus increasing the production cost of the same.

Methodology: Earlier in our laboratory, collagenase was isolated and purified from aerobic, non-pathogenic Bacillus altitudinis and it has shown excellent activity in vitro. Thus, the present study was carried out to evaluate the wound healing potential of collagenase isolated and purified from Bacillus altitudinis in murine model. For this, male mice were divided in to 2 groups viz. test group (n=6) and control group (n=6) and burn wound was created on the dorsal side of each mouse with the help of a heated brass bar after exposing the skin. In case of test group purified collagenase (10µg/100 µl) was applied on the burn wound while phosphate buffered saline (100 µl) was applied in case of control group for 5 consecutive days. On day 6th the mice (n =3) were sacrificed from each group and wound biopsies were collected for histopathological examinations

Results: Histopathological examinations showed the absence of epithelium in control group whereas in test group initial signs of regeneration of epidermis were observed. The wound recovery when examined visually and by calculating wound area by using calliper the result showed that the wound was fully recovered. Conclusion: Purified collagenase can be used as therapeutic measure in burn wound healing.

Keywords: Collegenase; Bacillus altitudinis; Burn wound; Mouse model; Histopathological examination.

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INTRODUCTION

Collagenases are unique proteolytic enzymes that are capable of specifically breaking the peptide bond in the triple helical domains of native and denatured collagen.[1] Mostly the collagenases are found in animals and microorganisms however, the existence of collagenases from plant sources has also been described.[2,3] Out of all, microbial collagenases have mainly been used industrially due to their ability to cleave the collagen at several

sites and thus produce numerous active peptides.[4]

Now a days, the importance of collagenases has been extended from industry and has found widespread applications in medical industry as collagen forms one-third of the human body proteins.[5] For medical purposes, the most commonly used microorganism for obtaining collagenase is *Clostridium histolyticum*. Clostridium collagenase has been significantly used for treating Dupuytren's and Peyronie's disease in men[6,7] and uterine fibroids, capsular contracture around the breast implants and removal of human retained placentae in women.[5]

Burn wounds are a necessary public health hassle in the world.[8] According to trustworthy statistics, 2,65.000 deaths happen per year due to burns alone. Usually silver impregnated products such as silver sulfadiazine (SSD) are used for treatment of burn wounds due to its antimicrobial properties. Drawbacks of SSD consist of delayed wound recovery and eschar separation causing a pseudoeschar formation. The delays in wound healing is due to inhibition of nearby keratinocyte and fibroblast development, and suspension in eschar separation is because of the antimicrobial activities of SSD which help to stop the growth and release of bacterial collagenases and proteases.[9] The methods used for debridement are either surgical or mechanical and these methods are less specific and stressful. Clostridium collagenase has been used to dissolve burn scars as a replacement for traumatic surgical debridement.[10] The necrotic dead tissues are perceptively and painlessly damaged by the collagenase enzyme that yield collagen derived peptides leading to enhanced macrophage chemotaxis, increased cytokine secretion and thus enhance wound healing.

While most of the studies of Collagenase production have been reported from anaerobic pathogenic microorganisms, researchers are now looking for non-pathogenic microbial sources to reduce the collagenase production cost. Previously in our laboratory, collagenase had been isolated and purified by gel permeation chromatography (Sephadex G-200) and ion exchange chromatography[11,12] from an aerobic and non-pathogenic microorganism, *Bacillus altitudinis*. Therefore, the current study was further undertaken with an aim to evaluate the wound healing potential of bacterial collagenase isolated from *Bacillus altitudinis*.

MATERIALS AND METHODS

2.1 Microorganism

Bacillus altitudinis used in the present study was already available in the laborartory.[11]

2.2 Isolation and purification of collagenase from *Bacillus altitudinis*

Collagenase was extracted and purified from 72 h old cell culture of Bacillus altitudinis by the method previously standardized in the laboratory.[11,12] Briefly, the cell culture of Bacillus altitudinis was grown in tryptic soy broth, at 37◦C for 72h at 220 rpm. The culture was centrifuged at 10,000 rpm for 15 mins at 4. Further, the collagenase was purified from the supernatant by ammonium sulphate precipitation followed by gel permeation chromatography followed by ion exchange chromatography. To evaluate the purification status, sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) was carried out.

2.3 Experimental Animals

Adult BALB/c Mice, 6-8 weeks old, 20-25 gram obtained from Central Animal House, Panjab University, Chandigarh were used in the present study. These animals were housed in polypropylene cages and were randomly divided into 2 treatment groups i.e. test and control group with 6 mice in each group. Mice were kept under standard laboratory conditions with a photoperiod of 12 hour of light & 12 hour of darkness. All the mice were given access to standard pellet diet consisting of 20-21% crude protein, 4% fat, 5.0- 7.5% crude fibre, 8-9% ash, 1.0-1.5% calcium, 0.6-0.8% phosphorus and 50% nitrogen free extract (M/s Ashirwad Industries Pvt. Ltd) and water *ad libitum*. To avoid unwanted variation in results, the animals were acclimatized in the new environment for 5-6 days before performing any experiment.

2.4 Murine burn wound model

2.4.1 Burn wound establishment

Mice were anaesthetized with ether fumes and the hair was shaved from the dorsal side of the mice to expose the skin with the help of a commercially available hair removal cream. The skin was cleaned with a solution of povidine iodine and burn was produced with the help of a heated brass bar (10x10x100mm) for 45 seconds. Immediately after the burn, all the mice were injected intraperitoneally with 0.5ml of sterile physiological saline for fluid replacement to prevent overt shock and analgesic (0.25mg/ml) was given as post burn analgesic in drinking water.[13] The burn injury was confirmed with histopathological examinations.

2.4.2 Collagenase treatment

10µg/100 µl of purified collagenase was applied dermally at the site of burn for consecutive 5 days in case of test group (n=6). While 100 µl of phosphate-buffered saline (PBS) (pH-7.2) was applied in case of control group (n=6) and observed for healing.[14] After that a transparent occlusive dressing such as opsite was done to cover the wound.

2.4.3 Calculation of wound area

The wound area was measured using caliper on day 0, 6, 10, 14, 18 and 22. The wound area was calculated by measuring the horizontal (A) and mid-line (B) diameters of the wound and applying formula: (radius A) × (radius B) × π, wound area of burn wound can be calculated.[15]

2.4.4 Histopathological examination

The regeneration of skin cells was assessed on the basis of histopathological examination. On day 6th, mice were sacrificed from test group (n=3) and control group (n=3) and wound biopsies were collected by excising skin tissue of the burn wound. The tissues were preserved in 10% formalin and then dehydrated with different concentrations of alcohol (70% - 100%). The tissues were embedded, sectioned and stained with hematoxylin and eosin.[16]

RESULTS

Collagenase was isolated and purified from Bacillus altitudinis, with already standardized procedure in our laboratory.

3.1 Collagenase treatment

To determine thewound healing potential of purified collagenase in murine burn wound model, it was applied (10µg/100 µl) for 5 days consecutively on the wound in case of test group whereas in case of control group PBS (100 µl) was applied. Wound recovery was examined visually and by calculating wound area until the wound was fully recovered. The actual wound area of control and test group was 78.5 mm2 square on day 0. After 5 days of treatment, on day 6, the burn wounds treated with PBS (control) showed an average increase in wound area and became circular as compared to original burn wound. However, in collagenase treated burn wound (test), there was no significant increase in wound area, nevertheless granulation tissues appeared at the margins indicating initiation of wound healing. On day 10 and 14, the wound area of control group remained the same while in case of test group the wound area was found to be70.84 mm2 and 50.24 mm^2, respectivelywith contraction in the wound edges. On day 18 no changes were observed in the wound area in case of control group as compared to the test group where the wound area was reduced to 12.56 mm^2 and formation of new tissues started around the burn wound making wound healing more evident. On day 22 the wound gets fully recovered from the burn injury in test group which indicates complete reephithelization however no significant recovery was observed in case of control group (Fig. 1).

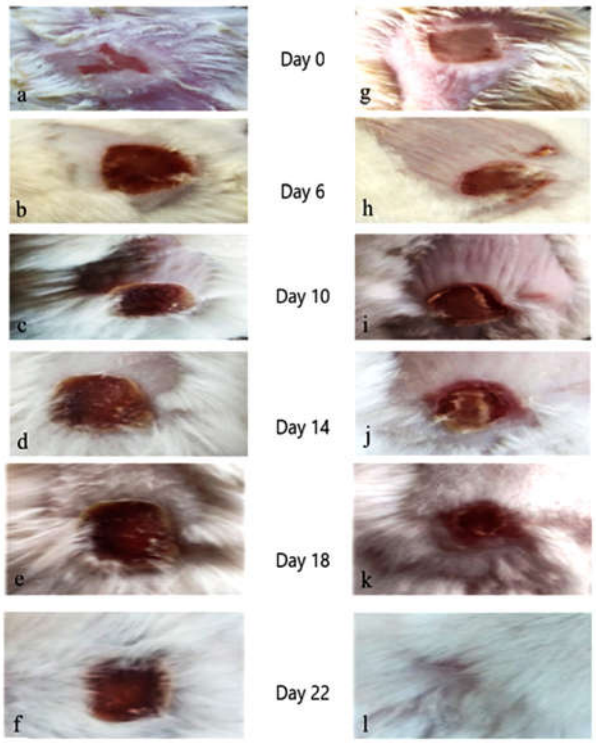

Fig. 1: Photomicrograph showing the wound area of control (a, b, c, d, e, f) and test group (g, h, i, j, k, l)on different days of the study.

3.2 Histopathological results

Analysis of hematoxylin and eosin (H&E) stained sections of wound biopsies on day 6th of control group showed presence of ulcer at the wound and normal skin junction and absence of epithelium whereas the test group showed the initial signs of regeneration of epidermis with presence of inflammatory infiltrate (Fig. 2).

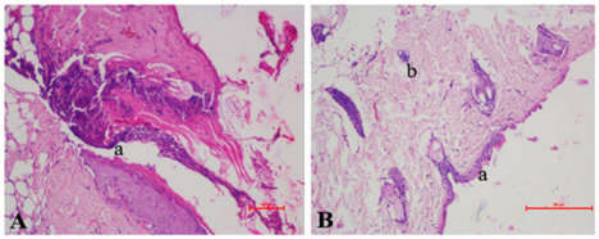

Fig. 2: Photomicrograph of burn wound on 6th day: A. Control group a)ulcer at burn wound and normal skin junction with no epithelium; B. Test group (a) regeneration of epidermis, (b) inflammatory infiltrate

DISCUSSION

Burn wounds are the major problem in medical maintenance worldwide. Over the past few decades, different techniques have been established for wound healing, which include debridement, irrigation, provision of giving of antibiotics, tissue grafts, and use of proteolytic enzymes for the treatment of chronic wounds.[17] In an injury when the wound becomes chronic, it causes a significant amount of load on the patient and affects the lifespan, wellness, and physical abilities.[18] Wound dressings play an important role in the treatment of different kinds of exposed wounds (e.g. traumatic, thermal, or chronic), except when the wound bed is provided with conditions like moisture, nutrition, and warmness, and then growth of microbes start to begin and the process of wound healing is halted by the colonization of bacteria and succeeding to cause an infection, which would eventually cause an extreme and prolonged inflammatory response.

Debridement is the elimination of irrelevant substances along with devitalized or contaminated tissue from a wound bed for improvement in wound healing. This method, can be done surgically, chemically, mechanically, or by autolytic removal of the tissue and is an important factor in wound bed preparation.[19] Major problem in medical practice is the care of non-healing wounds[20], and from last few years finding its cure has been the main goal for the researchers.[21] Enzymatic debridement using collagenase is the most commonly used method of debridement of wound healing.[22] However, enzymatic methods appear to be barely extra tremendous in treatment of wounds because clostridial collagenase used in treatment of wound debridement proved to be expensive and needs a good investment. Herein, we demonstrated the wound healing capacity of bacterial collagenase isolated from a non-pathogenic and aerobic strain Bacillus altitudinis using in vivo model.

The collagenase enzyme is responsible for proteolysis of collagen, is essential for several biological functions such as tissue remodeling, morphogenesis, and wound healing.[23,24] In the present study, we had done the dermal application of the purified collagenase daily for 5 days in murine model to burn wound site in test group and PBS in the control group. The purified collagenase promoted re-epithelization and accelerated wound healing in the test group as compared to control group which showed delay in the appearance of granulation tissue. An in vivo experiment performed by 14 observed that the purified collagenase could potentially aid in wound care and healing in rat model. Hence proving that collagenases have a countless potential for their therapeutic effect in wound care.

Histopathological examinations, on day 6th showed that the process of healing was better in the collagenase treated group that is test group than the control group with PBS treatment. It revealed that the purified collagenase in test group hastens the process of epithelial repair and formation of granulation tissue as compared to the control group however inflammatory infiltrate was present in the both groups. These results are in concordance with 25, wherein they showed that the healing of burned wound skin in rats was better in collagenase than the other groups treated with silver sulfadiazine (SSD) and cold cream.

The rate of wound healing was significantly different in both the groups on day 10 and 22. The development of epithelium was observed in the test group on 22nd day with fully healed wound whereas in the control group, epidermis was absent and no evident wound healing was observed. Moreover, the mean wound area was significantly reduced to 12.56mm^2 on day 18th from 70.84 mm 2 on day 10th in the test group treated with collagenase in comparison to 78.5 mm^2 in the control group treated with phosphate buffered saline.

Consequently, the data and observations collected in this study indicated that the purified collagenase can be used as dermal application to treat the burn wounds.

CONCLUSION

The rate of epithelization of wound was faster and wound area reduced rapidly, revealing that direct application of purified collagenase to burn site promotes the healing process and improves the wound health in experimental animals.

Conflict of interest

The authors declare that there is no conflict of interests.

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