The Effect of Collagenase on Ischemic Wound Healing: Results of an In Vivo Study

Shengxian Jia, MD, PhD; Yanan Zhao, MD; Michael Law, PhD; Robert D. Galiano, MD; and Thomas A. Mustoe, MD

Abstract
Many chronic wounds have a limited blood supply and contain necrotic tissue that must be debrided. The effect of collagenase, an enzymatic debriding agent, has been studied in acute but not in chronic wounds. The purpose of this in vivo study is to evaluate the effect of collagenase on wound healing in ischemic wounds. The ears of eight young New Zealand White rabbits were rendered ischemic by ligation of caudal and central arteries and dermal circulation circumferentially so both ears were perfused only by the rostral artery, preserving the caudal, central, and rostral veins. Three 6-mm, full-thickness dermal punches were made on the inner surface of both ears down to perichondrium. One ear on each rabbit was treated with either collagenase or petrolatum ointment covered with a semi-occlusive dressing; wounds on the other ear of the same rabbit were covered with a semi-occlusive dressing only (control). On post-wounding day 8, wound samples were collected and processed for histological analysis of reepithelialization (epithelial gap, percentage healed, epithelial height, and epidermal area) and granulation tissue formation (peak height, granulation tissue distance, and area). Within-animal comparison showed no significant differences between the petrolatum and control wounds but epithelial height, epidermal area, wound peak height, and granulation tissue distance and area were significantly different between the collagenase and control-treated wounds. Between-animal comparison of petrolatum- and collagenase-covered wounds showed statistically significant ($P < 0.05$) differences for the following outcomes: epithelial gap, percentage healed, epithelial height, epidermal area, wound peak height, and granulation tissue distance and area. In this ischemic wound model, outcomes for most of the variables associated with healing were significantly better in wounds covered with collagenase and a semi-occlusive dressing than in the control or petrolatum group. Additional in vivo studies are warranted to confirm these results.

Keywords: in vivo study, wound healing, ischemic rabbit ear model, collagenase, petrolatum

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Wounds that have failed to return to functional and anatomical integrity in a timely fashion are defined as chronic.$^{1,2}$ Pressure ulcers, diabetic ulcers, and venous ulcers comprise the overwhelming majority of chronic wounds.$^2$ These ulcer types share common causative features: the cellular and systemic changes of aging, repeated ischemia-reperfusion injury, and bacterial colonization with a resulting inflammatory host response.$^3$ Although the etiologies of all chronic wounds are multifactorial, ischemia—ie, a decrease in blood flow—and/or ischemia-reperfusion injury appears to be a primary causality. One of the major reasons for impaired wound healing is an attenuated blood supply, which results in a decrease in the concentration of growth factors within the wound bed.$^2$ This is particularly true for lower extremity wounds and/or pressure ulcers.

Collagenases have been used as an enzyme-debriding agent for chronic dermal ulcers and severe burn wounds. Although collagenases act by degrading native helical collagen fibrils,$^4$ preliminary results from animal studies show that collagenase-treated, partial-thickness wounds in pigs healed...
quicker than control-treated wounds; furthermore, significant pro-angiogenic activity was observed in mice with basement membrane matrix implants (Matrigel®, BD, Franklin Lakes, NJ) (unpublished data). The effects of collagenase in acute wound healing has been studied in a swine back, acute, full-thickness wound model, but not for chronic wounds. Although many animal models are available, only a small number of these models actually mimic chronic wounds; one of these is the unique impaired wound healing model caused by ischemia in a rabbit ear. Because information about the potential effect of collagenase on chronic wound healing is limited, the purpose of this in vivo study was to evaluate the effect of collagenase on granulation tissue formation and reepithelialization under ischemic conditions.

**Materials and Methods**

Eight (8) young adult New Zealand White rabbits (3 to 6 months, ~3 kg; Covance Research Products, Inc., Cumberland, VA) were acclimated, housed, and given access to food and water ad libitum under the conditions set forth in the Public Health Service Guide for the Care and Use of Laboratory Animals and the US Department of Agriculture Title 9–Animal Welfare Act and its revisions. The study was conducted under an approved experimental protocol from the Northwestern University Animal Care and Use Committee. Both ears in each of the eight rabbits were rendered ischemic by the method described by Ahn et al. Briefly, the rabbit was anesthetized with an intramuscular (IM) injection of ketamine (45 mg/kg) and xylazine (7 mg/kg) and prophylactically treated with penicillin (50,000 units/kg, IM) before surgery. The surgical sites and the dorsal surface of the rabbit ears were shaved with an electric clipper and depilated with Nair (Church & Dwight Co., Inc., Lakewood, NJ). The surgical sites then were painted with a betadine solution and an incision was made to the level of bare cartilage at the base of the ear. Under a dissecting microscope, the caudal and central arteries were ligated so the entire ear was perfused only by the rostral artery with preservation of the caudal, central, and rostral veins to render the rabbit ear ischemic (an important reason for delayed healing). This technique results in ischemia for 7 to 10 days. In aged rabbits with sustained ischemia, no healing has been known to occur up to 26 days. No truly chronic wounds have been noted in animals, but this model has been extensively characterized and used to test therapeutic options for clinical use. The incision was closed with a 5-0 polypropylene suture. Three 6-mm, full-thickness dermal punches then were made on the inner surface of both ears down to bare cartilage by removing the epidermis, dermis, and perichondrium. One ear of the rabbit served as a nontreated control ear covered by a transparent semi-occlusive dressing consisting of a thin polyurethane membrane coated with a layer of an acrylic adhesive (Tegaderm® dressing, 3M, St. Paul, MN). The wounds in the other ear of the same rabbit were treated with 0.05 mL of either collagenase ointment (Santhyl® Ointment, Healthpoint Biotherapeutics, Ltd, Forth Worth, TX—approximately 1.23 g collagenase in a petrolatum-based ointment) or petrolatum alone, and covered by the same semi-occlusive dressing. The same agent (either petrolatum or collagenase) was used on all three wounds (1 cm to 1.5 cm apart) on the treated ear. Each rabbit received one treatment in one ear and no treatment in the other ear. All animals were sacrificed at post-wounding day 8.

Tissue from all the wounds was collected and analyzed. The tissue of each wound was equally split into two halves. One half was processed for histological analysis and the other half was stored at -80°C or colder for future western blot analysis or RNA extraction. The collagenase and petrolatum test products were provided by Healthpoint Biotherapeutics, Ltd (Fort Worth, TX) in individually sealed, coded, and labeled aluminum tubes. The tubes were stored at room temperature. Histological analysis. The tissue from half of the circular wound taken for histological analysis was subject to routine paraffin embedding and sectioning. A 4-μm cross-section was obtained through the center of each circular wound. Tissues were stained with hematoxylin and eosin (H&E) for microscopic examination and measurement. Histological analyses included reepithelialization in terms of the epidermal gap (EG) and the percentage healed (PH); keratinocyte proliferation in terms of the epithelial height (EH) and the epidermal area (EA); and the formation of granulation tissue in terms of the peak height, granulation tissue distance, granulation tissue area, and the granulation tissue area per μm wound (see Table 1). All measurements were performed using a Nikon microscope and accompanying imaging software at 2X and 10X magnification.

**Key Points**

- Researchers compared the effect of collagenase ointment to untreated control and petrolatum ointment in an ischemic rabbit ear model.
- Within-animal comparisons showed no difference in the variables assessed between petrolatum-treated and control wounds, but some outcomes in the collagenase treated wounds were better than their respective control.
- Collagenase-covered wounds exhibited some better outcomes than wounds in the animals treated with petrolatum.
- Additional studies are needed to confirm these in vivo results.

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Because the measured wound size among slides was not identical due to differences in cutting sections or slightly off-center in some samples, the ratio of 6 mm (the original wound size) to the measured wound size was used to adjust the raw data, with the exception of PH and epidermal area per μm wound and granulation tissue area per μm wound measurements, which already were adjusted.

Data and statistical analysis. There were 12 wounds for each of the collagenase- and petrolatum-treated groups and their respective controls. To detect a difference between the collagenase or petrolatum treatment and its own control groups, the data were compared using two-tailed paired t-tests (SAS® Institute Inc., Cary, NC). Analysis of variance (ANOVA) with Tukey’s test was used to compare outcomes of the collagenase or petrolatum treatment groups from different animals while controlling for differences between animals. P < .05 was considered significant in all analyses.

Results
Wound reepithelialization. No differences in epithelial gap and percentage healed were observed between the collagenase-treated ischemic and nontreated control wounds (EG: 1,123 ± 389 μm versus 1,779 ± 497 μm, P = .4; PH: .81 ± .06 versus .70 ± .08, P = .4). No significant difference in reepithelialization measures was found between petrolatum-treated and nontreated controls (EG: 3,858 ± 754 μm versus 2,707 ± 583 μm, P = .3; PH: .36 ± .13 versus .55 ± .10, P = .3). However, between-animal comparison of collagenase- and petrolatum-treated wounds showed significant differences (EG collagenase 1,123 ± 389 μm versus 3,858 ± 754 μm petrolatum group, P = .004 and PH collagenase 81% ± .06 versus 36% ± .13 petrolatum group, P = .004) (see Figures 1 and 2). Keratinocyte proliferation, as measured by epithelial thickness and area, was significantly higher in collagenase-treated wounds than either of the respective control (untreated ischemic ear) or the petrolatum-treated wounds (EH: 165 ± 11 μm versus 117 ± 8 μm or 79 ± 18 μm, P = .004 or <.0001; EA: 152 ± 6 μm² versus 98 ± 8 μm² or 66 ± 16 μm², P < .0001 or <.0001). However, no significant difference was noted in keratinocyte proliferation between petrolatum-treated wounds and their own nontreated controls (EH: 79 ± 18 μm versus 124 ± 15 μm, P = .10; EA: 66 ± 16 μm² versus 100 ± 10 μm², P = .10) (see Figures 3 and 4).

<table>
<thead>
<tr>
<th>Term and Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>Epithelial gap (EG)</td>
<td>Distance between the tip of the new epidermal tongue on the left side and that on the right side of the wound or the length of unepithelialized wound area when harvested at post-wounding day 8</td>
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<tr>
<td>Percentage healed (PH)</td>
<td>The ratio of EG over the distance (diameter of the wound) between nick (or the edge of left side granulation tissue if no nick is visible) and nick (or the edge of right side granulation tissue if no nick is visible) of the wound</td>
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<tr>
<td>Epithelial height (EH)</td>
<td>Average epithelial height of both the left and right sides of the wound. Because the thickness of newly formed epidermis varies throughout, the height of newly formed epidermis was measured at two points and averaged</td>
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<tr>
<td>Peak height</td>
<td>Thickness of the wound tissue at its thickest point, including granulation tissue and overlying epidermis. Two measurements are made—one on the left and one on the right side of wound—and average calculated as peak height of the whole wound</td>
</tr>
<tr>
<td>Epidermal area (EA)</td>
<td>Quantification of epidermal area over the 1-mm wide wound area</td>
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<tr>
<td>Granulation tissue distance</td>
<td>Distance between the nick (or edge of the wound on day 0) to the most advanced tip of the granulation tissue ingrowth (tongue) in the wound. Two measurements are made—one on the left and one on the right side of wound—and average calculated as granulation tissue distance of the whole wound</td>
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<tr>
<td>Granulation tissue area</td>
<td>Sum of the area of granulation tissue measured from the left and right sides of the wound using Nikon imaging software (NIS-Elements BR 2.30)</td>
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<tr>
<td>Granulation tissue area per μm wound</td>
<td>Average amount of granulation tissue area per μm wound</td>
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**Granulation tissue formation.** Average tissue peak height, granulation tissue distance, and tissue area were higher in the collagenase than in the collagenase-control and petrolatum-treated wounds. Peak height measured 684 ± 28 μm versus 535 ± 42 μm or 442 ± 56 μm, P = .002 or .0006. Granulation tissue distance measured 1,451 ± 110 μm versus 989 ± 111 μm or 713 ± 170 μm, P = .02 or .0002. Granulation tissue area was 790,907 ± 118,010 μm² versus 407,288 ± 85,867 μm² or 285,596 ± 127,739 μm², P = .007 or .003. Granulation tissue area per μm wound was 132 ± 20 μm² versus 68 ± 14 μm² or 48 ± 21 μm², P = .007 or .003). Yet no significant difference was noted regarding granulation tissue formation between petrolatum-treated wounds and their own nontreated controls (peak height: 442 ± 56 μm versus 451 ± 40 μm, P = .90; granulation tissue distance: 713 ±170 μm versus 852 ± 60 μm, P = .50; granulation tissue area: 285,596 ± 127,739 μm² versus 317,127 ± 62,178 μm², P = .80; and granulation tissue area per μm wound: 48 ± 21 μm² versus 53 ± 10 μm², P = .80) (see Figures 5 through 8).

**Discussion**

Pierce and Mustoe described the basic principles of optimal wound healing to include minimizing tissue damage, débriding nonviable tissue, maximizing tissue perfusion and oxygenation, ensuring proper nutrition, and providing a moist wound-healing environment. Most preclinical study designs control certain conditions, such as minimizing tissue damage (by exclusion from study) and proper nutrition.
Collagenase is an enzymatic debridement agent and although this study did not focus on maintaining a moist wound environment, all wounds (including control) were covered with a semi-occlusive dressing. The observed increased rate of reepithelialization in the collagenase-treated compared to the petrolatum-treated wounds is similar to that observed in another study, although the control in the latter study is dry gauze. However, following clinical and laboratory studies, Ghadially et al. found that applying petrolatum did not completely occlude the epidermis of acetone-treated skin in

**Figure 5.** Average peak height of wounds and standard error of the mean (n=12 for each group).
- a Own control comparison \( P = 0.002 \) for collagen/collagen control and \( P = 0.9 \) for petrolatum/petrolatum control
- b Between-animal comparison (collagenase/petrolatum) \( P = 0.0006 \)

**Figure 6.** Average granulation tissue distance and standard error the mean (n=12 in each group)
- a Own control comparison \( P = 0.02 \) for collagen/collagen control and \( P = 0.5 \) for petrolatum/petrolatum control
- b Between-animal comparison (collagenase/petrolatum) \( P = 0.0002 \)

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both humans and mice; the authors concluded that petrolatum permeates throughout the epidermis interstices to allow recovery of the skin barrier function. Hence, petrolatum affects wound healing despite being viewed as inert.\textsuperscript{20}

In the present study, collagenase ointment was significantly more effective compared to treatment with petrolatum for the following wound healing variables assessed: reduction in wound epithelial gap, percentage healed, epithelial height, epidermal area, peak tissue height, granulation tissue distance, and granulation tissue area. When compared with their own respective control wounds (an ischemic ear with no ointment applied), no significant differences were observed between the petrolatum and petrolatum control wounds; whereas, epithelial height, epidermal area, peak height, granulation tissue distance, granulation tissue area, and granulation tissue area per µm wound were significantly different between the collagenase and collagenase control wounds. Differences between collagenase and control wounds in epithelial gap and percentage healed were not significant. Additional \textit{in vivo} studies including direct petrolatum control or using other wound healing models are needed to confirm these results.

**Conclusion**

In this ischemic wound model, wounds treated with a topical debriding agent (collagenase) covered with a semi-occlusive dressing had better wound healing-related outcomes than wounds covered with a semi-occlusive dressing alone (untreated control) or covered with petrolatum. Additional preclinical studies are warranted and clinical studies to confirm the potential effects of collagenase ointment on healing chronic wounds are needed.

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**Figure 7.** Average granulation tissue area and standard error of the mean (n=12 in each group)

- \( a \) Own control comparison \( P = 0.007 \) for collagen/collagen control and \( P = 0.8 \) for petrolatum/petrolatum control
- \( b \) Between-animal comparison (collagenase/petrolatum) \( P = 0.003 \)

**Figure 8.** Average granulation tissue area per µm and standard error of the mean (n=12 in each group)

- \( a \) Own control comparison \( P = 0.007 \) for collagen/collagen control and \( P = 0.8 \) for petrolatum/petrolatum control
- \( b \) Between-animal comparison (collagenase/petrolatum) \( P = 0.003 \)

**References**