

# ACTIVATED COLLAGEN ACCELERATES WOUND REPAIR AND MODULATES CYTOKINE PRODUCTION IN WHOLE BLOOD AND PBMC CULTURES.

Gregory B. Pott<sup>1,2</sup>, K. Scott Beard<sup>1,2</sup>, Matthew Regulski<sup>3</sup>, and Leland Shapiro<sup>1,2</sup>.

<sup>1</sup>Department of Medicine, Denver Veterans Affairs Medical Center, Denver, CO, USA, <sup>2</sup>Department of Medicine, University of Colorado Denver, Aurora, CO, <sup>3</sup>Wound Care Center of Ocean County, NJ.

## Abstract

Several reports have shown that exogenous collagen fragments enhance wound repair through poorly understood mechanisms. CellerateRX is an activated (fragmented) product derived from Type I Collagen that is applied topically as a gel or powder on open wounds that are often infected or colonized with bacteria. In three case series, topical CellerateRX in conjunction with standard care healed infected surgical wounds and diabetic ulcers more rapidly than standard therapy alone. We conducted an interim analysis of 16 patients with lower extremity diabetic ulcers. Eight patients received CellerateRX in addition to standard treatment, and 8 received standard treatment alone. A quantitative analysis of wound healing at 14 weeks showed that CellerateRX-treated wounds were 100±0% healed, compared to 59±13.5% healing in control subject wounds. To examine possible mechanisms by which CellerateRX enhances wound healing, cytokine production was assessed in 24 hr cultures of whole blood and peripheral blood mononuclear cells (PBMC) from 10 healthy subjects. These cultures were conducted in the absence or presence of stimulation with heat-killed *Staphylococcus epidermidis* (S.epi) or lipopolysaccharide (LPS). In whole blood, CellerateRX significantly increased spontaneous production of Interleukin (IL)-8, IL-6, IL-1 $\beta$ , tumor necrosis factor  $\alpha$  (TNF $\alpha$ ), and IL-10. In whole blood stimulated with S.epi, cytokine synthesis was augmented by CellerateRX, except for TNF $\alpha$ , which was suppressed. In PBMC cultures, CellerateRX significantly induced spontaneous production of IL-8, IL-6, IL-1 $\beta$ , TNF $\alpha$  and IL-10. In PBMC stimulated with LPS, CellerateRX significantly augmented IL-8, IL-6, and IL-10; TNF $\alpha$  was suppressed. CellerateRX inhibition of stimulated TNF $\alpha$  in whole blood and PBMC suggests selective suppression of detrimental TNF $\alpha$  inflammatory and cell death effects *in vivo*. These results suggest that CellerateRX enhances wound healing, and a possible mechanism involves specific modulation of the cytokine response in bacteria-containing wounds.

## Background

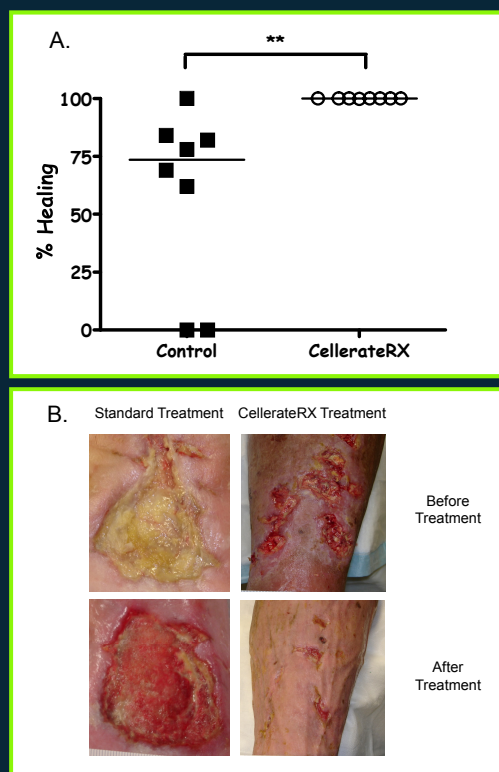
The innate immune response is activated in wounds and includes cytokine biological effects, which are rapid and highly regulated. Cytokine activities important for physiological wound healing include effects on coagulation, inflammation, epithelialization, angiogenesis, matrix and tissue remodeling, and pathogen control. CellerateRX, composed primarily of proteolytically cleaved collagen, is thought to accelerate wound healing by incompletely understood mechanisms. We investigated the effect of CellerateRX in a clinical investigation of wound healing, and we assessed CellerateRX effects on production of pro- and anti-inflammatory cytokines thought to be relevant in wound repair and pathogen control.

## Methods

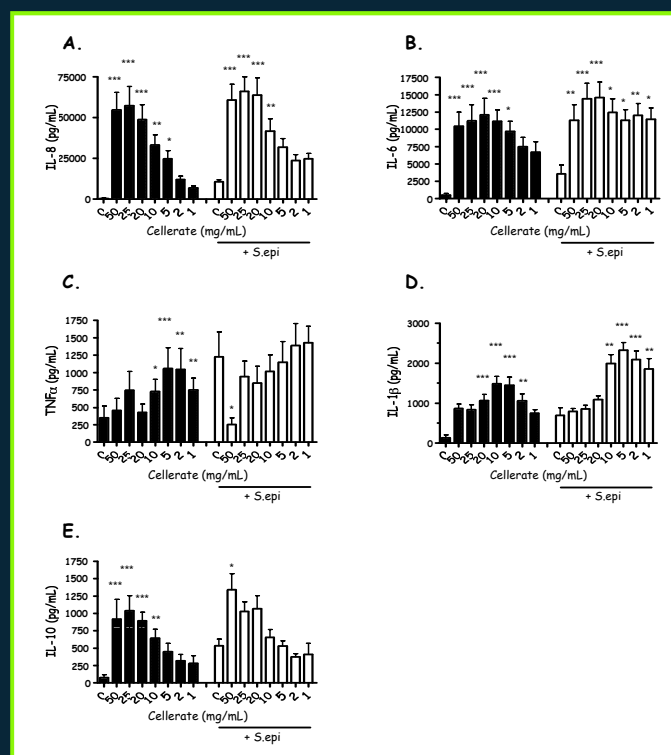
**Wound healing in diabetic ulcers.** Sixteen patients with below-knee diabetic ulcers were recruited. Eight patients (Controls) were treated with standard therapy, consisting of an absorbent occlusive dressing secured with an elastic wrap. Eight patients (CellerateRX), were treated with an application of 1.0-1.5 g CellerateRX powder (Wound Care Innovations, LLC, Florida) in addition to the standard therapy. Wounds were treated and photographed weekly, and wound areas were quantified using image analysis.

**Whole blood assays.** Heparinized human blood was obtained from 10 healthy volunteers and diluted 1:5 with RPMI tissue culture medium in the absence (CellerateRX=0) or presence of CellerateRX. After 1 hr of incubation at 37°C, cultures remained unstimulated, or were stimulated with 1.2  $\mu$ g/mL (protein) heat-killed *Staphylococcus epidermidis* (S.epi). All final volumes were 1.0 mL. After 18 hrs of incubation, supernatants were assayed for cytokine concentrations using ELISAs. Cytotoxicity in each culture was determined from cell-free supernatants using the CytoTox 96 Non-Radioactive Cytotoxicity Assay (no cytotoxic effects were observed).

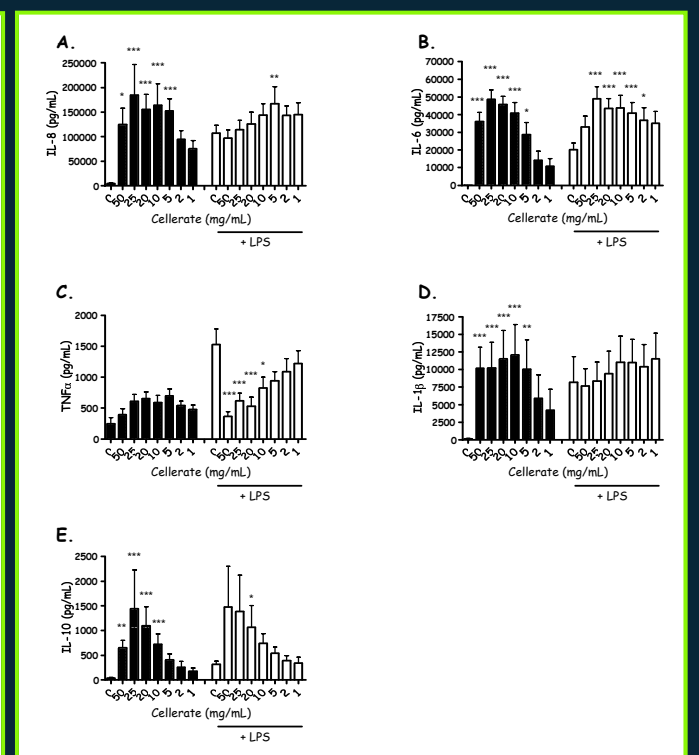
**PBMC assays.** Heparinized human blood was obtained from 10 healthy volunteers, centrifuged over a cushion of ficoll-hypaque, and the peripheral blood mononuclear cells (PBMC) were isolated and cultured in tissue culture medium in the absence (CellerateRX=0) or presence of CellerateRX. After 1 hr of incubation at 37°C, cultures remained unstimulated, or were stimulated with 10 ng/mL lipopolysaccharide (LPS). After 18 hrs of incubation, cell lysates were assayed for cytokine concentrations using ELISAs. Cytotoxicity in each culture was determined from cell-free supernatants using the CytoTox 96 Non-Radioactive Cytotoxicity Assay (no cytotoxic effects were observed).



**Figure 1. CellerateRX accelerates wound healing in diabetic patients.** A. 16 patients with lower extremity diabetic ulcers were treated with standard therapy alone (Control, N=8) or with a topical application of CellerateRX in conjunction with standard therapy (N=8). An interim analysis was performed at 14 weeks post-treatment by evaluating percent wound closure. For Control patients, the mean±SEM value for percent wound healing was 59.4±13.5%, and the median was 73.5%. CellerateRX-treated patients had a mean±SEM percent wound healing of 100±0% and a median value of 100%. \*\*p=0.002 by Mann-Whitney test. Horizontal bars indicate median values. B. Representative photographs of lower extremity ulcers on patients treated with standard therapy (left panels) or treated with standard therapy plus CellerateRX (right panels).



**Figure 2. Effect of CellerateRX on cytokine production in whole blood.** Whole blood was incubated without (CellerateRX=0) or with CellerateRX in the absence (black bars) or presence (white bars) of S.epi stimulation for 18 hr. Supernatants were collected and cytokine production depicted as measured concentrations. Shown are mean±SEM concentrations of IL-8 (A), IL-6 (B), TNF $\alpha$  (C), IL-1 $\beta$  (D), and IL-10 (E). \*p<0.05, \*\*p<0.01, and \*\*\*p<0.001 compared to CellerateRX=0.



**Figure 3. Effect of CellerateRX on cytokine production in PBMC.** PBMC were incubated without (CellerateRX=0) or with CellerateRX in the absence (black bars) or presence (white bars) of LPS stimulation for 18 hr. Supernatants were collected and cytokine production depicted as measured concentrations. Shown are mean±SEM concentrations of IL-8 (A), IL-6 (B), TNF $\alpha$  (C), IL-1 $\beta$  (D), and IL-10 (E). \*p<0.05, \*\*p<0.01, and \*\*\*p<0.001 compared to CellerateRX=0.

## Results

- Clinical Wound Healing**
  - Treatment of diabetic ulcers with CellerateRX and standard therapy significantly accelerated wound healing compared to standard therapy alone.
- Whole Blood Culture Cytokines**
  - CellerateRX significantly increased spontaneous (unstimulated) levels of IL-8, IL-6, and IL-10. A biphasic CellerateRX effect on TNF $\alpha$  and IL-1 $\beta$  was observed, with stimulation at lower CellerateRX concentrations and no effect at higher concentrations.
  - CellerateRX significantly enhanced S.epi-stimulated levels of IL-8, IL-6, and IL-10. Low CellerateRX concentrations enhanced S.epi-induced IL-1 $\beta$  and did not affect TNF $\alpha$  production. High CellerateRX concentrations did not affect S.epi-induced IL-1 $\beta$  and significantly decreased TNF $\alpha$  production.
- PBMC Culture Cytokines**
  - CellerateRX significantly increased spontaneous (unstimulated) levels of IL-8, IL-6, IL-1 $\beta$ , and IL-10. Spontaneous TNF $\alpha$  synthesis was not significantly affected by any CellerateRX concentration tested.
  - CellerateRX significantly enhanced LPS-stimulated levels of IL-8, IL-6, and IL-10, but IL-8 and IL-10 levels were only marginally increased.
  - CellerateRX did not affect LPS-stimulated IL-1 $\beta$  production and significantly decreased LPS-stimulated levels of TNF $\alpha$ .

## Discussion

Diabetes-related lower extremity infections are a major clinical problem. In the U.S., 6-10% of all diabetic patients experience lower extremity ulcer infections, which result in over 50,000 amputations each year. Lower extremity diabetic ulcer care costs an estimated 1.2 billion dollars in the United States (U.S.) annually for treatment alone. Improved treatments for diabetic foot infections are urgently needed to prevent progression to bone infections (osteomyelitis) and amputations. These studies examined the effect of CellerateRX, a product derived from fragmented Type I Collagen, on wound healing. Patients treated with CellerateRX experienced significantly accelerated wound healing compared to patients treated with standard therapy (Figure 1). To examine possible mechanisms by which CellerateRX might achieve enhanced wound healing, we evaluated the effects of CellerateRX on cytokine synthesis in whole blood (Figure 2) and PBMC (Figure 3) cultures. We used S.epi and LPS as stimuli in these studies since these substances are likely pivotal as cytokine modulators in natural wounds. We found that CellerateRX alone was a potent inducer of IL-6 and the neutrophil chemo-attractant, IL-8, which are important for the acute response to injury and bacterial infection. CellerateRX appeared to induce spontaneous (unstimulated) IL-1 $\beta$  but had no notable effect on stimulated IL-1 $\beta$ . CellerateRX increased spontaneous and stimulated levels of IL-10 in whole blood and PBMC, and IL-10 possesses important anti-inflammatory properties that include inhibitory effects on TNF $\alpha$  production. Interestingly, CellerateRX inhibited stimulated TNF $\alpha$  production in both whole blood and PBMC cultures. While TNF $\alpha$  is important in activating the immune response to wounds, excess TNF $\alpha$  is potentially damaging to surrounding healthy tissues, and needs to be controlled. The specific suppressive effect of CellerateRX on TNF $\alpha$  production is therefore intriguing. CellerateRX appears to generate and fortify a specific innate immune/inflammatory response that may be beneficial for wound healing and pathogen control. Although it is reasonable to assume that CellerateRX concentration in wounds is large, the actual CellerateRX levels in wounds is unknown. Therefore, the biological relevance of our *in vitro* data requires further study and validation.