Novel method to determine MMP-9/TIMP-1 ratio for predicting wound healing ability using an Enzyme Linked Immunosorbent Assay (ELISA)

David Liou, DPM, Gar Yee Koh, MSc, PhD, Adam Landsman, DPM, PhD, FACFAS

Cambridge Health Alliance, Department of Surgery, Division of Podiatry

Introduction

Matrix metalloproteinases (MMPs) are endopeptidases that degrade extracellular matrix proteins inside ulcerations to assist with initial phase of wound healing. Over abundance of MMPs can cause persistent inflammation of the ulceration site and prevent healing of ulceration. Tissue inhibitor of metalloproteinases (TIMPs) are enzymes that inhibit specific MMP activity to control the persistent degradation process. The ratio of MMP-9/TIMP-1 is a predictor of ulcerations' ability to heal, with higher ratio being predictive of slower healing. Past methods to obtain sample for ratio analysis are complex, invasive, and often involved unnecessary quantification of protein. In this study, **we aimed to develop, optimize, and evaluate a noninvasive method to determine ratio of MMP-9/TIMP-1 with single swab method via an enzyme linked immunosorbent assay (ELISA).**

Methodology

8 samples using the standard aerobic wound cultures were obtained from 4 diabetic ulcerations from 3 patients. To assess the intra-ulceration variability, 3 separate swabs from different quadrants of the ulcerations were obtained from 2 ulcerations (Patient G & R). Samples were processed according to manufacturer's instruction from commercially available MMP-9 and TIMP-1 ELISA kits. Sample dilutions were also performed at 0X, 50X and 200X dilutions. Each sample was performed in triplicates to ensure consistency and color intensity (representative of protein concentration) was detected at 450nm.

ELISA

Enzyme-linked immunosorbent assay (ELISA) is an analytical biochemistry assay. The assay utilizes solid phase antibodies to detect presence of a protein in a liquid solution. The commercially available ELISA plates are pre-coated with specific antibodies for the protein of interest. Sample is added to the plate, and the protein binds to the antibodies. Secondary antibody is then introduced which bind to the protein. Other chemicals are added to convert the enzyme into a measurable signal.

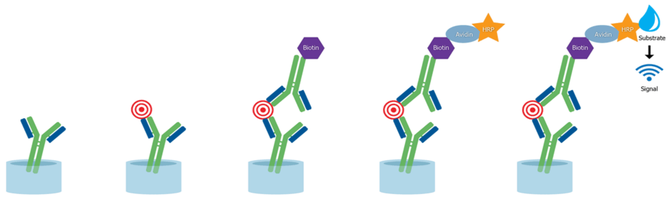

Fig. 1. Solid phase sandwich type ELISA with protein binding, and subsequent conversion into measurable signal.

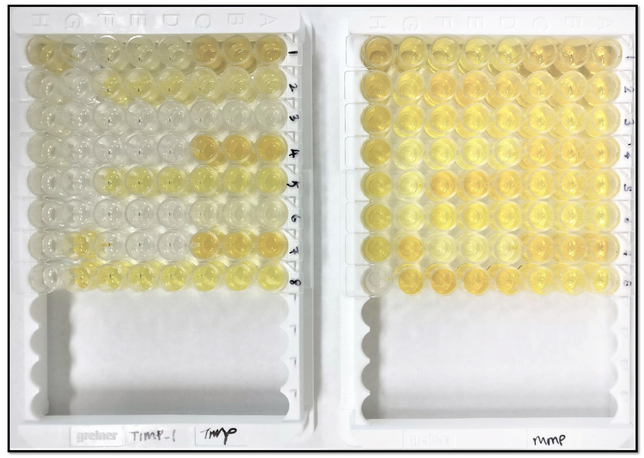

Fig. 2. Commercially available MMP-9 and TIMP-1 ELISA plates with florescent signal in individual wells indicative of different concentrations of protein after antibody hybridization.

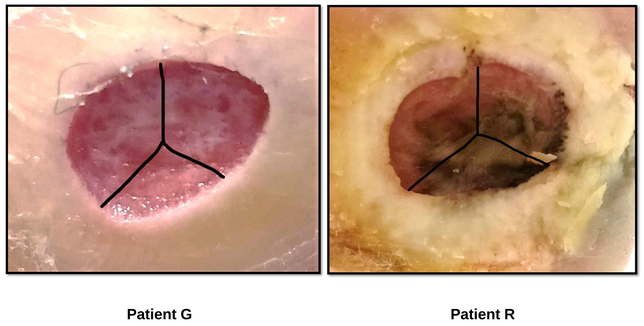

Fig. 3. Ulcerations from Patient G and Patient R, with 3 different quadrants where 3 separate swabs were obtained to test for intra-ulceration variability.

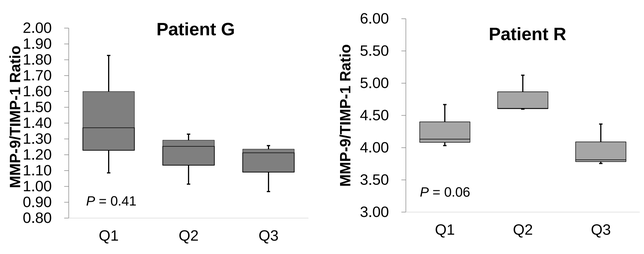

Fig. 4. Box-plot diagram demonstrating the intra-ulceration variation collected within the 3 swabs (Q1, Q2, Q3) collected from different quadrants of 2 independent ulcerations from Patient G and Patient R, respectively. Box = 25th and 75th percentiles; bars = min and max values. Statistical significance is achieved at $P < 0.05$.

Results

The ratio of MMP-9/TIMP-1 was successfully obtained from single swab of ulceration using the ELISA method with no sample dilutions required. The method is reproducible and consistent as confirmed by low standard error within sample. No intra-ulceration variation was observed within the 3 swabs collected from different quadrants of 2 independent ulcerations (Fig. 4), with an average ratio of MMP-9/TIMP-1 was **1.26 ± 0.09 (*P* = 0.41)** and **4.34 ± 0.23 (*P* = 0.06)**, respectively (Fig.5).

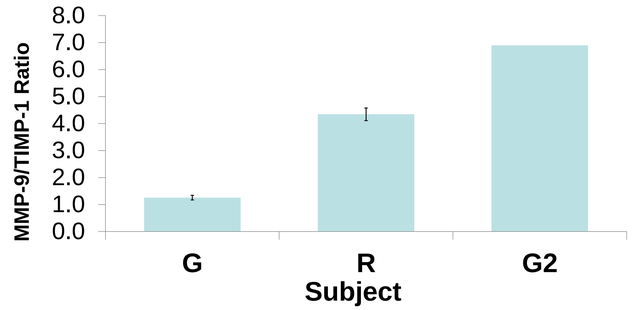

Fig. 5. Average ratio of MMP-9/TIMP-1 obtained from single swab of ulceration(s) from 3 individuals. Each sample was analyzed in duplicate on the same ELISA plate. Data are expressed as mean ± standard error (n = 1 – 3).

Discussion

This novel approach to obtain MMP-9/TIMP-1 ratio is **feasible, reproducible and consistent** as confirmed by low variability observed within samples. Intra-ulceration variability is minimal and statistically insignificant, suggesting the sufficiency of a single swab method via ELISA for calculation of MMP-9/TIMP-1 ratio.

Sample dilutions were performed at 0X, 50X and 200X factors to determine the optimal concentration for the assay. All 3 dilution factors fall within the standard curve generated from the commercial ELISA kit. However, through statistical analysis, dilution is noted to introduces variability in the triplicate samples. The variability is further compounded by the calculation of the MMP-9/TIMP-1 ratio factoring in the dilution factors. Therefore, dilution of the swab samples for analysis is not necessary and may introduce variability.

References

Muller, M., et al. "Matrix Metalloproteinases and Diabetic Foot Ulcers: the Ratio of MMP-1 to TIMP-1 Is a Predictor of Wound Healing." *Diabetic Medicine*, vol. 25, no. 4, 2008, pp. 419–426., doi:10.1111/j.1464-5491.2008.02414.x.

Liu, Y., et al. "Increased Matrix Metalloproteinase-9 Predicts Poor Wound Healing in Diabetic Foot Ulcers." *Diabetes Care*, vol. 32, no. 1, 2008, pp. 117–119., doi:10.2337/dc08-0763.

Rayment, E.a., et al. "Increased Matrix Metalloproteinase-9 (MMP-9) Activity Observed in Chronic Wound Fluid Is Related to the Clinical Severity of the Ulcer." *British Journal of Dermatology*, vol. 158, no. 5, 2008, pp. 951–961., doi:10.1111/j.1365-2133.2008.08462.x.

Rockland Antibodies & Assays. "AccuSignal ELISA Kits." *AccuSignal ELISA Kits*, rockland-inc.com/ELISA-Kits.aspx.