In Vitro Testing of the Protease Modulating Activity of a Nitric Oxide Generating Foam

Daniel J. Gibson, Ph.D.
University of Alabama Capstone College of Nursing

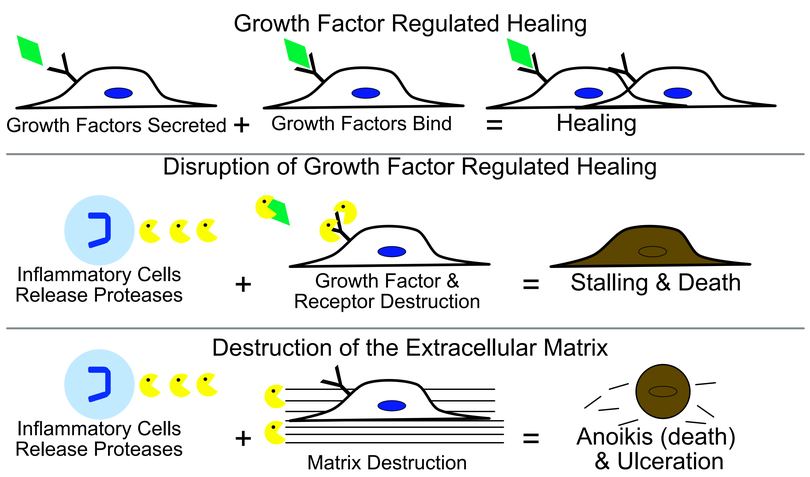
The data presented herein were funded in whole via a sponsored research agreement with NOxy Health Products

Purpose

The purpose of this study was to test a two-component foaming liquid's effects on a variety of proteases.

Introduction

One of the molecular hypotheses for wound chronicity is that there is a disruption of healing due to the activities of proteases. The protein destroying activities hypothetically lead to a lack of pro-healing growth factors, growth factor receptors being stripped from the surface of cells, and extracellular matrix destruction. Host-derived elastases have been found to be associated with growth factor destruction and receptor striping, whereas host-derived matrix metalloproteases have been associated with matrix destruction. Bacterial proteases have a mixture of activities, though *P. aeruginosa*'s acid protease is sufficient to generate a corneal ulcer.

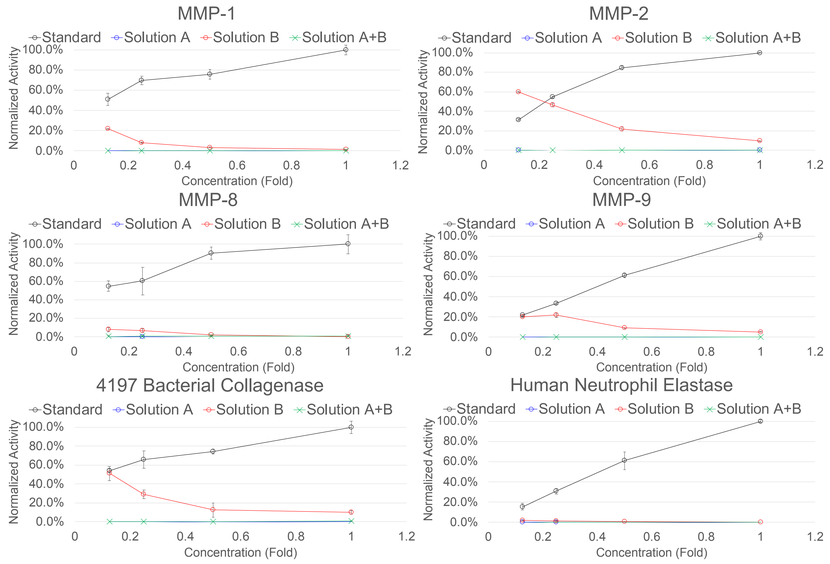


A novel 2-part nitric oxide generating foam is in pre-clinical testing to determine the effects on known barriers to healing. Canonically, nitric oxide is a known vasodilator, and the increased blood flow is anticipated to improve healing. A non-exhaustive list of activities of nitric oxide include regulation of apoptosis(1,2,7), neuroprotection(3), antibacterial(4), and antiviral(5). These activities are commonly considered to be "double edged" in that they seem to affect both the pro- and con- side of biological activities. The same can be said in the case of proteases: where there is evidence of activation(6) and of inhibition(7).

Here, the impact on *in vitro* protease activity using wound-relevant proteases is assessed for the two individual parts and the 1:1 combined final mixture.

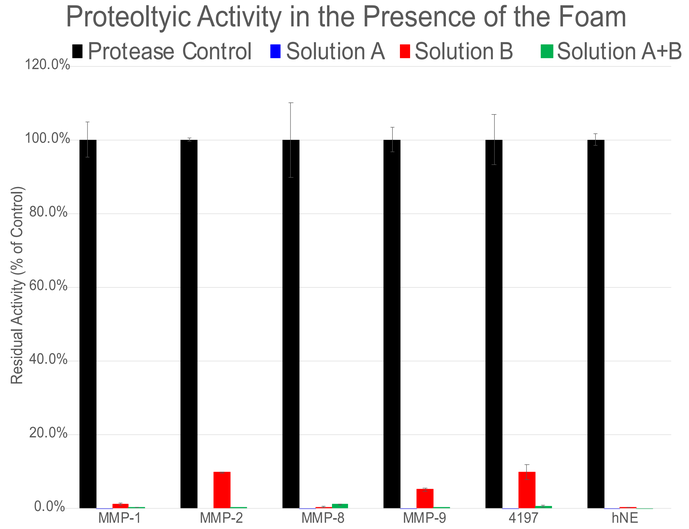
Materials & Methods

A mix-and-read fluorescent substrate-based assay was used to measure the protease activity of matrix metalloprotease (MMP) -1, -2, -8, -9, human neutrophil elastase (hNE), and a type-I collagenase mixture from *Clostridium histolyticum*. The assay was run in a 96-well plate format using a phosphate buffered saline buffer augmented with 1mM $CaCl_2$ and 5μM $ZnCl_2$ + 0.05% polysorbate 20. The enzymes were tested at levels associated with extremely high proteolytic activity in human chronic wounds (5-10 μg/ml). A standard curve was generated for each enzyme to ensure a proportional response. The test product comes as a two part unmixed pair of solutions (Part A and Part B); when applied the two solutions as foams are mixed 1:1 prior to application to the wound and periphery. Three test groups were utilized consisting of Part A, Part B, and a 1:1 mixture of Part A:B. Dilutions of each agent with diH_2O resulting in 100%, 50%, 25%, and 12.5% active agent were tested. The reaction was monitored for fluorescence generation with 485nm excitation and 528nm emission, 1 reading per minute for 10 minutes. The relative fluorescence unit (RFU) per minute slope was calculated for each sample while the reaction was still in the linear phase. The residual activity was reported as the RFU/min observed divided by the RFU/min of the non-treated control.



An effect was found among the control, Part A, Part B, and 1:1 mixture (multiple p-values, all $p \leq 1.69 \times 10^{-8}$). All components and the final mixture reduced or eliminated detectable protease activity. Dilution had no apparent effect on activity reduction for Part A, and there was a dose-dependent loss of inhibition with Part B. The dilution of the 1:1 mixture had a slight reduction of residual protease activity; with the most pronounced effect being with the *C. histolyticum* collagenase (0.7% residual activity in the 100%, down to 0.1% in the 12.5% diluted sample).

Results



Protease	p-value
MMP-1	4.12×10^{-11}
MMP-2	2.59×10^{-19}
MMP-8	1.69×10^{-8}
MMP-9	3.07×10^{-12}
Bacterial Collagenase (4197)	1.30×10^{-9}
Human Neutrophil Elastase	4.06×10^{-15}

Conclusions

These initial results indicate a definite broad-spectrum anti-protease activity when tested in vitro. The anti-protease activity appears to be stable up to at least a 12.5% dilution. Additional testing in wound fluids or wound fluid surrogates is necessary to determine if the effects will be diminished in the presence of additional biomolecules.

References

1. Dimmeler S, Zeiher AM. Nitric Oxide and Apoptosis: Another Paradigm for the Double-Edged Role of Nitric Oxide. Nitric Oxide. 1(4). 1997 275-281. DOI: 10.1006/niox.1997.0133
2. Kim YM, Bombeck CA, Billiar TR. Nitric Oxide as a Bifunctional Regulator of Apoptosis. Circulation Research. 1999. 84:253-256. DOI: 10.1161/01.RES.84.3.253
3. Chieuh, CC. Neuroprotective Properties of Nitric Oxide. Annals of the New York Academy of Sciences. 890: 1999 301-311. https://doi.org/10.1111/j.1749-6632.1999.tb08007.x
4. Miller C, McMullin B, Ghaffari A, Stenzler A, Pick N, Roscoe D, Ghahary A, Road J, Av-Gay Y. Gaseous nitric oxide bactericidal activity retained during intermittent high-dose short duration exposure. Nitric Oxide. 20(1) 2009 16-23 DOI: 10.1016/j.niox.2008.08.002.
5. Saura M, Zaragoza C, McMillan A, Quick RA, Hohenadl C, Lowenstein JM, Lowenstein CJ. An Antiviral Mechanism of Nitric Oxide: Inhibition of a Viral Protease. Immunity. 10(1) 1999. 21-28 DOI: 10.1016/S1074-7613(00)80003-5.
6. Murrell GAC, Jang D, Williams RJ. Nitric Oxide Activates Metalloprotease Enzymes in Articular Cartilage. Biochemical and Biophysical Research Communications. 20(1) 1995 15-21 DOI: 10.1006/bbrc.1995.1003.
7. Dimmeler S, Haendeler J, Nehls M, Zeiher AM; Suppression of Apoptosis by Nitric Oxide via Inhibition of Interleukin-1β–converting Enzyme (ICE)-like and Cysteine Protease Protein (CPP)-32–like Proteases. J Exp Med 1997; 185 (4): 601–608. doi: https://doi.org/10.1084/jem.185.4.601