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DOSE-DEPENDENT EFFECT OF INHIBITORS OF ANTIOXIDANT ENZYMES ON WOUND CHRONICITY

By

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A capstone project submitted for
Graduation with University Honors

August 28, 2018

University Honors
University of California, Riverside

APPROVED

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Abstract

Chronic wounds have become an increasingly serious and costly problem in healthcare settings. Chronic wounds, such as diabetic foot ulcers and pressure ulcers, fail to proceed through timely wound-healing processes because they have excessive oxidative stress levels and biofilm-producing bacteria at the wound site. While a balance of oxidative stress and antioxidant enzyme activity is essential for proper progression of wound healing, an excess of oxidative stress can contribute to wound chronicity. In diabetic mice, normal wounds proceed to close by around day 20; however, when we treat these mice with inhibitors of antioxidant enzymes (IAEs), wound closure is delayed by as much as 100 days after wounding and we observe the formation of mature biofilm by day 20.

To observe the effect oxidative stress has on wound healing and wound microbiome composition, we surgically wounded diabetic mice and treated them with various doses of IAEs. Then, we recorded wound percent closure over time and collected the skin for histology. Additionally, we collected bacteria from the wound over time and sequenced the DNA to create bacteria profiles. As we increased the dose of IAEs, which theoretically increased oxidative stress levels, we saw that healing was delayed, the wounds remained open longer, there was less diversity of bacteria species in the wound microbiome, and there was poor dermal-epidermal adhesion even after the wound had healed.

We found that as IAE dose increases, the wounds remain open longer, wound area increases, microbiome diversity decreases, and skin integrity decreases. From our results, we can conclude that antioxidant enzymes are critical for combatting the excessive oxidative stress that induces wound chronicity. Additional research about chronic wounds using animal models is needed because they allow us to observe critical, time-sensitive wound healing processes that occur early in the development of chronic wounds that we otherwise would not be able to study in humans. From this research, we can develop better treatments for chronic wounds or invent screening procedures to identify individuals that are at risk for developing chronic wounds.

Acknowledgments

First, I would like to thank our principle investigator, Dr. Manuela Martins-Green, for her mentorship and support for this project. I would also like to thank graduate student, Jane Kim, for helping me write scholarship proposals, teaching me new research techniques, and providing a unique and valuable experience in the laboratory. She has shown great patience with me as she often had to explain certain concepts or procedures, all the while she was working on her own experiments. She is not only the person I turn to for wisdom about science and academia, she is also a trustworthy mentor and a close friend of mine. Jane completed the DNA sequencing and created the bacteria profiles, and was there to supervise and offer advice to me throughout each step of this project. I would also like to thank previous undergraduate and laboratory manager, Anthony Castro, for his company and assistance with troubleshooting the sectioning of samples using the cryostat, and for teaching me how to use ImageJ to trace the wound areas.

Additionally, I would like to express gratitude to all of the members of the lab for sharing their knowledge with me at weekly meetings and in the laboratory. Each member was always so kind and willing to teach me what they were working on and they have taught me many valuable skills I will continue to use throughout my career. I am also grateful for the financial support provided by the Barry Goldwater Scholarship and Excellence in Education Foundation, and the Chancellor's Research Fellowship, as well as the social support from my friends and family. Finally, I would be remiss not to acknowledge the University of California, Riverside and the University's Honors Program for providing the opportunity to conduct research and share our findings with the academic community.

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Introduction and Background

Chronic wounds, wounds that fail to proceed through the normal regulated repair process in a timely manner, are increasingly significant clinical issues in the United States as the diagnosis rate of diabetes rapidly increases and extends to younger populations, and as a large portion of the population ages [1]. In the US alone, over 5 million people develop chronic wounds and over $20 billion is spent annually on chronic wound care [1]. Chronic wounds, can be classified into four categories according to the Wound Healing Society: diabetic ulcers, pressure ulcers, venous ulcers, and arterial insufficiency ulcers [2].

Elderly and diabetic patients have an increased risk of developing these wounds because they have reduced antioxidant activity, increased oxidative stress, and biofilm-producing bacteria in the wound [3]. While oxidative stress is needed for proper functioning of cells and normal wound-healing, an imbalance of oxidative stress can result in wound chronicity and other diseases [3]. Similar to our model's diabetic mice, diabetic patients have sustained hyperglycemia that induces excessive production of reactive oxygen species (ROS) [4]. Excessive levels of ROS cause cellular damage by oxidizing lipids and proteins [4, 5]. The imbalance of oxidative stress can lead to wound complications that are difficult and costly for clinicians to manage so they often must resort to amputation to prevent the spread of tissue necrosis and infection, imposing not only an economic burden on the health care system, but also a reduction in productivity on the individual [1, 4]. In addition to inducing cellular damage, oxidative stress has also been shown to activate collagenase, an enzyme involved with collagen remodeling, and decrease fibrillar collagen synthesis in fibroblasts [4]. This means that in the presence of high levels of oxidative stress, collagen deposition and normal wound healing are impaired; consequently, wound healing is delayed and the wound can become chronic. Diabetics

are prone to developing chronic wounds not only because of decreased collagen deposition, but also because they have an abnormal inflammatory response, impaired angiogenic response, reduced quality of granular tissue and impaired vascular endothelial function [4]. These impairments are similar to those associated with aging and this is why both diabetics and the elderly are predisposed to developing chronic wounds [6]. Understanding how to prevent or reverse wound chronicity is therefore an important task for improving the quality of lives of people suffering from diabetes or other ailments that confer an increased risk for wound complications.

Bacteria are also necessary for survival of humans and other organisms; however, complex interactions among bacteria in the wound microenvironment can lead to biofilm development that impedes healing [7]. Biofilms are aggregates of sessile microorganisms embedded in a self-secreted extracellular polymeric substance that provides structural foundation and resources for harbored bacteria to thrive. Biofilms cause difficult problems in several sectors such as medical facilities, water systems, and food industries [8]. In addition to diabetic foot ulcers and pressure ulcers, biofilms are commonly found in the lungs of cystic fibrosis patients, on implant devices during infection, catheters, central lines, ventilators, and on teeth and oral surfaces [8, 9, 10]. Biofilm protects the bacteria within from external physiochemical aggressions, host immune responses, and antibiotic treatments, leading to slower wound closure and antibiotic-tolerant bacteria [11]. It is possible that an imbalance of oxidative stress at the wound site causes or contributes to the change of interactions among bacteria species, thus leading to biofilm development. If this is the case, then oxidative stress levels could be considered the most important aspect in wound chronicity and is a critical aspect to target when developing treatments for chronic wounds. Understanding how oxidative stress and bacteria at

the wound site lead to wound chronicity will help guide future research towards creating better treatments for chronic wounds or inventing preventative screening methods to identify individuals that are susceptible to developing chronic wounds.

Our chronic wound model allows us to observe critical, time-sensitive wound healing processes that occur early in the development of chronic wounds and that cannot be studied in healthcare settings. Previously, our lab has found that we can generate chronic wounds in diabetic mice by treating diabetic mice with two inhibitors of antioxidant enzymes (IAEs): mercaptosuccinic acid (MSA) and 3-amino-1, 2, 4-triazole (ATZ). MSA and ATZ inhibit catalase and glutathione peroxidase, respectively, both of which are important enzymes for scavenging free radicals and ROS that generate oxidative stress. We saw that the wounds of diabetic mice not treated with IAEs closed by day 20 while wound-healing of the mice treated with IAEs was delayed up to 100 days after wounding [12]. In a separate preliminary experiment, we have found that transplanting biofilm from the chronic wound of one mouse treated with IAEs onto the clean wound of another mouse not treated with IAEs is not sufficient to induce wound chronicity, suggesting oxidative stress is necessary for wounds to become chronic. Additionally, we found that the wound of a mouse treated with IAEs does not become chronic after cleaning it of bacteria, suggesting that oxidative stress is not sufficient on it's own to induce wound chronicity and that the bacteria are also needed.

To understand the influence of oxidative stress on chronic wound development in an *in vivo* system, we treated diabetic mice with various doses of ATZ and MSA and observed the effect on biofilm composition, skin integrity, and wound closure. We hypothesized that increasing the dose of IAEs further exacerbates levels of oxidative stress at the wound site and this induces wound chronicity by influencing bacterial interactions and reducing skin integrity.

To test this hypothesis, we conducted two separate experiments:

Experiment 1 After wounding the mice, we administered either a half, quarter, or eighth dose of IAEs and placed tegaderm over the wound to prevent contamination. To monitor wound progression, we photographed the mice every day for 5 days after surgery, then every 5 days thereafter until wound closure. To observe the effect of oxidative stress on bacteria composition in the wound over time, we removed the tegaderm and used a sterile cotton Q-tip to swab for bacteria at the same time points as when we took pictures of the wounds. We sequenced the bacterial DNA collected from these swabs to generate a bacteria profile at several time points. Once the wounds had closed, we sacrificed the animals and collected the skin for histology in order to observe the effect of oxidative stress on skin integrity.

Experiment 2 We wounded the mice and administered either a half, quarter, or eighth dose of IAEs just as we had done in Experiment 1; however, for this experiment we did not collect bacteria from the wound site and, consequently, we did not remove the tegaderm. We photographed the mice every day for 5 days after surgery, then every 5 days thereafter until the first wound closed completely by day 20. At day 20, we sacrificed all of the animals and collected the tissue for histology.

In conclusion, we found that as we increased the dose of IAEs, wound healing was delayed, wound area increased, diversity in the wound microbiome decreased, and skin integrity decreased.

Methodology

Chronic Wound Model

We utilized our chronic wound mouse model as described previously by our lab [12]. The diabetic (*db/db*) mice were housed in the UCR vivarium and were used in accordance with protocols approved by the Institutional Animal Care and Use Committee (IACUC). We removed the hair on the backs of 6-7 month-old mice the day prior to surgery by shaving and using Nair. On the day of surgery, the mice were given two intraperitoneal injections of buprenex (0.05 mg/kg) for pain management; once 30 minutes prior to surgery and once 6 hours after surgery. The mice were also treated once intraperitoneally with either a half, quarter, or eighth dose of ATZ (TCI America; Portland, Oregon) 30 minutes prior to surgery. (Note: the full dose concentration of ATZ is 1 g/kg body weight for our model.)

The mice were anesthetized with aerosolized isoflurane at a flow rate of 5% for 1.5-2 minutes and then the flow rate was reduced to 2% for the remainder of the surgery. After wiping non-sterile surgical scissors and the skin of the mice with 95% ethanol, full thickness 7 mm punch wounds (excision of the skin and the underlying panniculus carnosus) were made on the backs of each mouse. After wounding, tegaderm was placed over the wounds to prevent contamination and we injected MSA (Aldrich Chemistry; St. Louis, MO) with the same corresponding dose (either half, quarter, or eighth) underneath the tegaderm. (Note: the full dose concentration of MSA is 150 mg/kg body weight for our model.)

Wound Area Measurement

For Experiment 1, we photographed the mice every day for 5 days after surgery, then every 5 days thereafter until wound closure. For Experiment 2, we photographed the mice every day for 5 days after surgery, then every 5 days thereafter until the first wound completely closed (day 20). We used Lightroom computer software to crop the images, ImageJ to measure wound areas, and Microsoft Excel to graph wound area over time and percent wound closure as a function of IAE dose, and also to perform statistical analyses including one-way ANOVA, two-way ANOVA, and Student T-test.

Bacteria Collection and Analysis

For Experiment 1, we used sterile cotton swabs to collect bacteria from the wound bed while minimizing disruption to the wound microenvironment. After collecting the bacteria, new tegaderm was placed over the wound. The content of each swab was suspended in 1.0% w/v protease peptone and 20.0% v/v glycerol solution and stored at 20ºC. DNA extractions were performed on the thawed swabs using the MOBio PowerSoil DNA Isolation Kit as described by the manufacturer, with a 90-second bead-beating step. For the construction of the library, performed by graduate student Jane Kim, polymerase chain reaction (PCR) amplified the ITS region using bacterial ITS rRNA primers (ITS-1507F GGTGAAGTCGTAACAAGGTA, ITS-23SR GGTTBCCCCATTCRG) with specific barcode sequences for each sample. The thermos cycles are 94°C for 5 minutes; 40 cycles of 94°C for 20 seconds, 56°C for 20 seconds, and 72°C for 40 seconds; followed by 2°C for 10 minutes and 26°C for 20 minutes. The PCR product was cleaned using a MinElute 96 UF PCR Purification Kit and measured using a NanoDrop to obtain the final concentration of the product. A normalized amount of DNA from each sample was then sent to the MiSeq sequencer. The sequence reads were processed by removing barcodes and

identifying the sequences to known bacteria. An OTU table was generated separating read counts for specific bacteria across different samples via Qiime (1.9.1). Qiime was utilized to obtain relative percentages of bacteria on the same day over time on the same mouse and and ggplot2 (2.2.1) in R (3.4.0) was used to graph the mean% with more different colors than the graphics tool in Qiime can provide. Creation of these bacteria profiles were also done by Jane Kim.

Histology

After we euthanized the animals with carbon-dioxide, we collected, washed, and embedded part of the wound tissue in OCT and stored it at -80ºC. We used a cryostat microtome to section the tissues, then we stained them with Hematoxylin and Eosin and visualized them under a light microscope.

Results and Discussion

Wound Area Over Time

For experiment 1, we see that wound healing was delayed in all of the mice, regardless of IAE dose. We also observed a ring of damaged tissue surrounding the initial wound. We thought this could have been due to the acidity of the MSA applied topically; however, when we applied neutralized MSA, we still observed this ring. Wounds of mice given the eighth dose of IAEs begin to show wound closure at day 10 and continue to heal into day 25, whereas the wounds of mice given the half and quarter doses of IAEs are still open and chronic, showing no sign of wound closure at day 10 (Figure 1A). The images shown in Figure 1 are representative of each of the IAE doses.

For experiment 2, we see a ring of damaged tissue surrounding the initial wound, just as we had seen in experiment 1, and the ring size appears to decrease as IAE dose decreases. We also observe that biofilm can be found on the wounds of mice given the quarter dose of IAEs by day 3 and eighth dose by day 10. When the regrowth of hair prevented the tegaderm from covering the wound, the wound dried and appeared to heal faster. For the wounds of mice given the half and quarter doses of IAEs that remained covered by tegaderm, we saw the wounds increased or remained constant in size until day 15, with the exception of one mouse. The wounds of mice given the eighth dose of IAEs begin to show wound closure at day 10, with the exception of one wound that became infected around day 10 (Figure 1B).

In our statistical analyses we did not include mice that died prior to day 20. We also did not include wounds that had dried out due to a regrowth of hair that prevented the tegaderm from covering the wound. The wound of one mouse in experiment 2 appeared to have become infected

between days 10 and 15, so we also did not include it in our statistical analyses. To assess wound closure over time for each mouse, we graphed the percent wound closure between day 0 and day 20 (Graph 1). Taking the average of the half, quarter, and eighth dose IAE percent wound closures, we can see that wounds of mice treated with the half dose IAEs had increased in area, the wounds of mice treated with the quarter dose IAEs had slightly increased in area, and the wounds of mice treated with the eighth dose IAEs had decreased in area by day 20 (Graph 2).

Bacteria Profiles

After I collected the bacteria from these mice and extracted the DNA, graduate student Jane Kim performed DNA sequencing and created the representative bacteria profiles over time. From these bacteria profiles, we see in general that as the concentration of IAEs increases, the alpha diversity index decreases, meaning there is less diversity of bacteria species present at the wound site (Appendix figure 1-5). Thus, increasing the dose of IAEs allows opportunistic pathogens, such as *Pseudomonas aeruginosa*, to dominate the wound microbiome and outcompete other bacterial communities for nutrients and resources. How this is accomplished could be attributed to the effect of oxidative stress on bacterial interactions early in wound healing, possibly via altering quorum sensing molecules or pathways. Another possibility is that the IAEs could be affecting the availability of resource for the bacteria or they could be affecting the ability of the bacteria to utilize available resources. It has been speculated that bacteria reduce their metabolism in an effort to reduce their own ROS production when faced with excessive oxidative stress in their environment [13]. Slowing bacteria metabolism is important for switching between the mobile, planktonic stage and the stagnant, biofilm form of growth. Yet another possible way in which increasing IAEs reduces diversity is by causing an increase in the

expression of genes that promote growth and survival in biofilm-producing bacteria or by causing an increase in the expression of genes related to death and dormancy in nonbiofilm-producing bacteria. Further research is necessary to elucidate the mechanism behind our observations.

Histology

In normal skin, the dermal-epidermal junction serves as mechanical support for the epidermis and gives the skin it's barrier function. While we could not include photographs of the sections due to technological issues, we saw that the skin of the mice given a half dose of IAEs appeared abnormal with poor epidermal-dermal adherence, the skin of the mice given an eighth dose of IAEs appeared similar to normal skin, and the skin of the mice given a quarter dose of IAEs varied between the half and eighth dose. These findings are consistent with the observations we made of the wound areas (Graph 1) and with previous observations in chronic wound studies in pigs [14]. It has been speculated that while the skin of biofilm-infected chronic wounds may appear visually closed, they may actually behave as an open wound due to a compromise in function. This theory is supported by our findings that as IAE dose increases, wounds become more chronic, biofilm burden increases, and dermal-epidermal adherence decreases, leading to poor barrier function and functionally impaired skin.

Figures

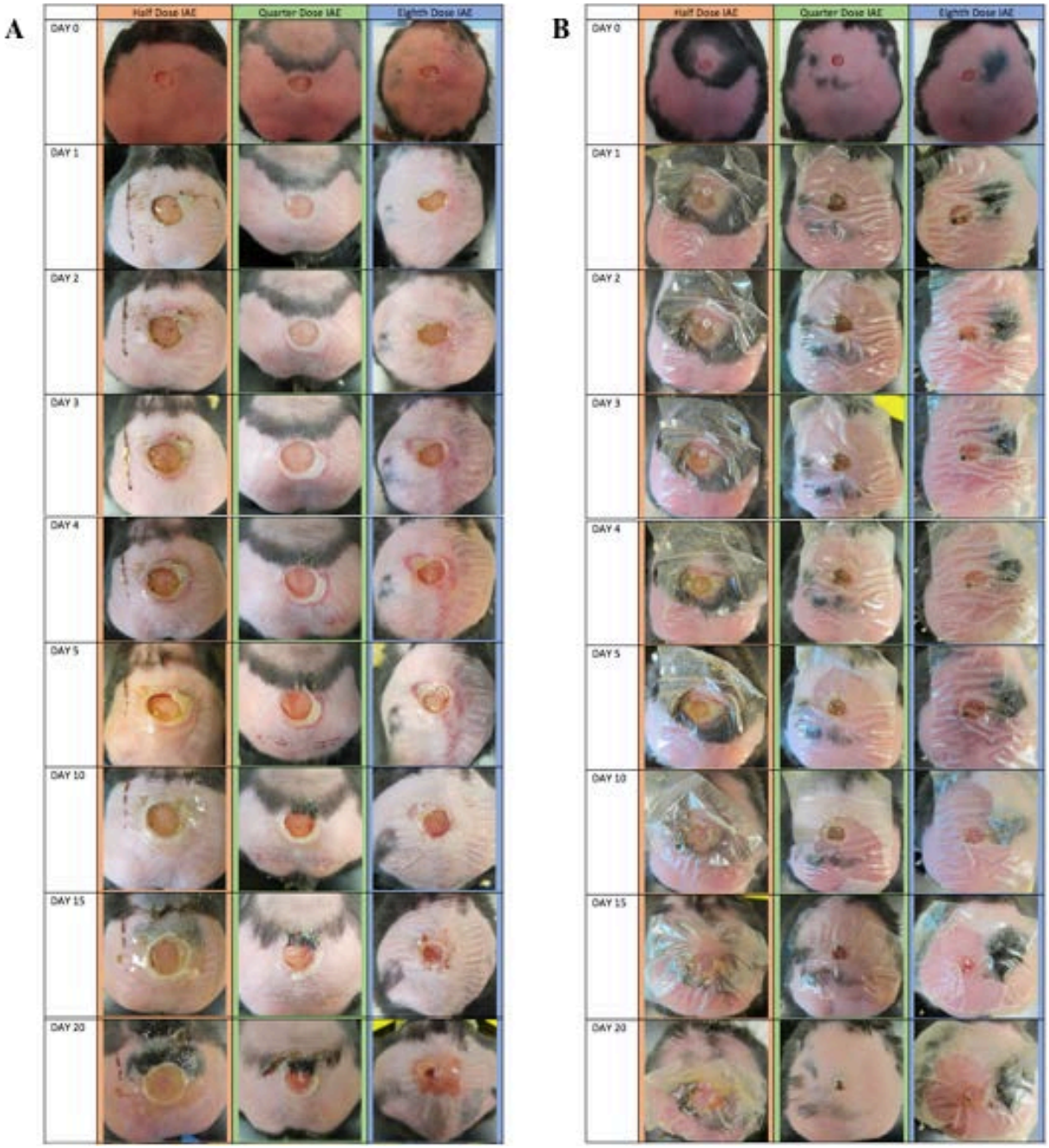
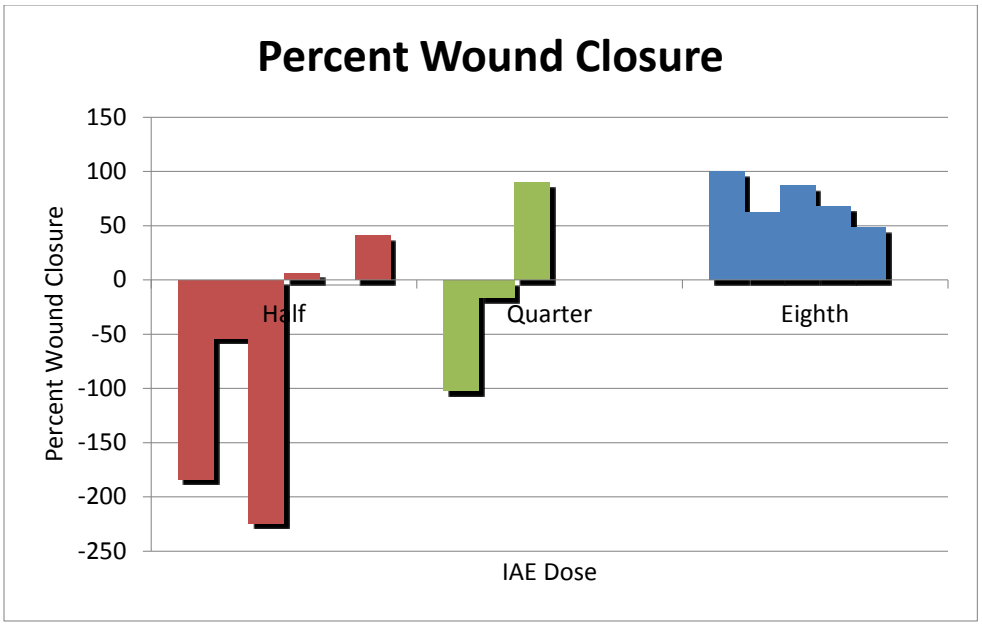
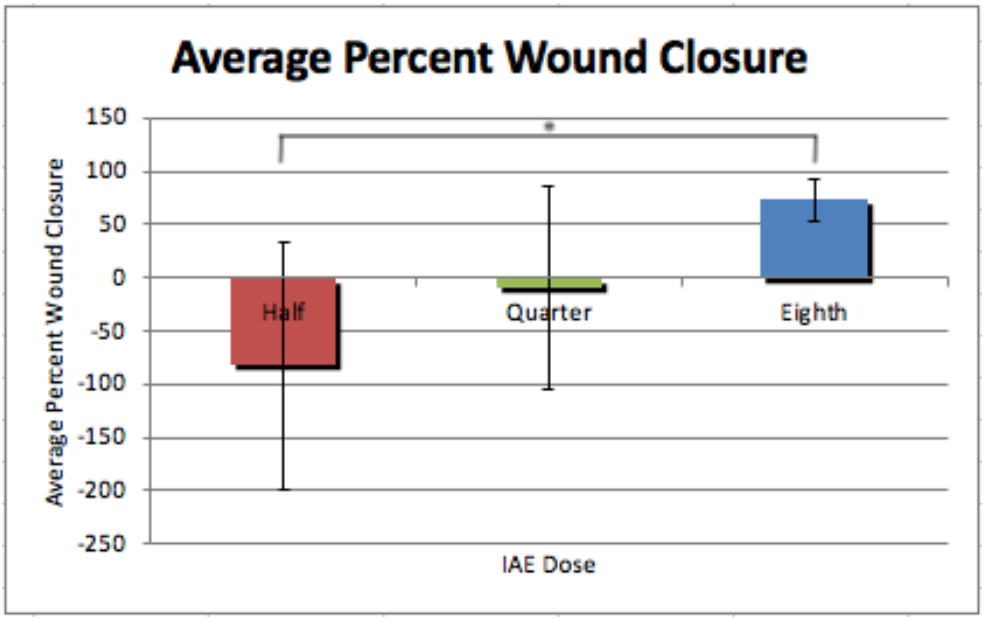


Figure 1 **A** Pictures of wound progression until all of the wounds closed

B Pictures of wound progression until the first wound completely closed



Graph 1 Percent wound closure for each mouse



Graph 2 Average percent wound closure

Conclusion

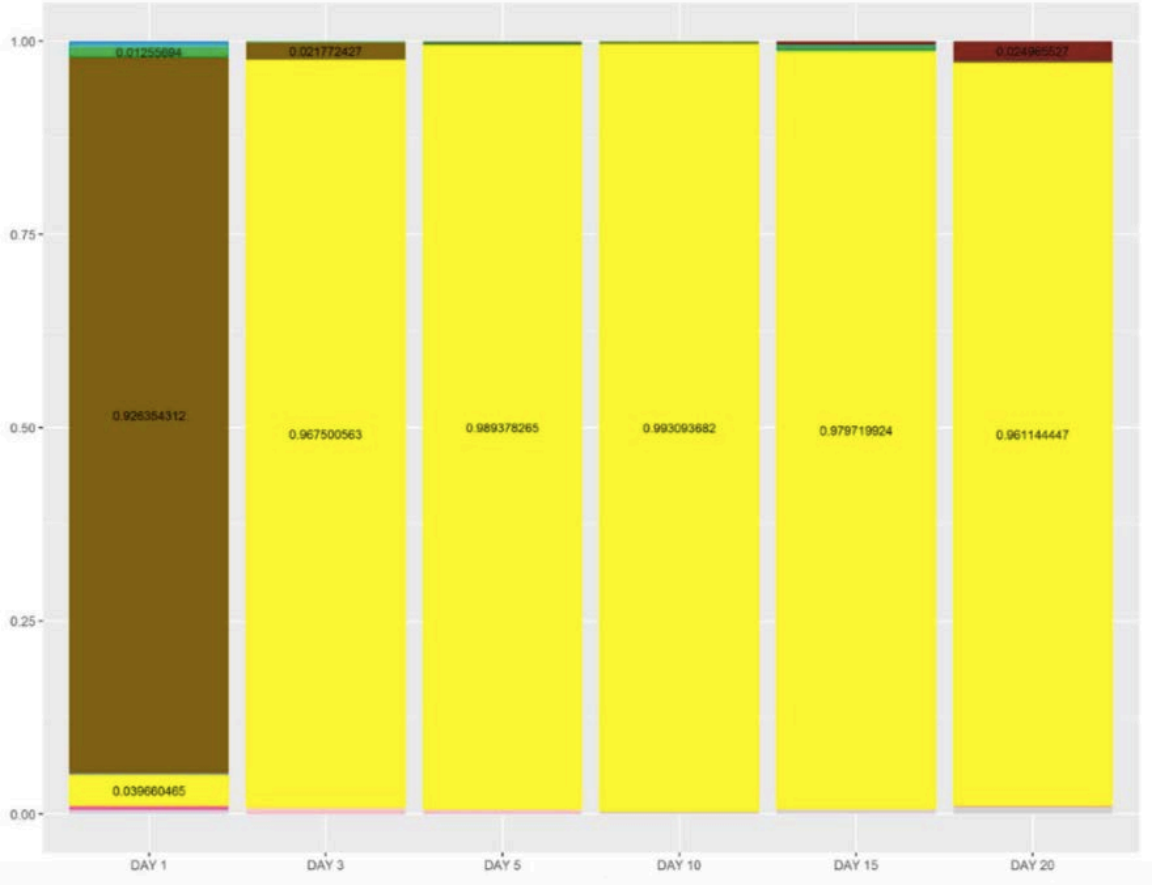
Chronic wounds are serious and costly problems in healthcare. Our results show that oxidative stress is a major player in the development of chronicity. As we increased the dose of IAEs, which theoretically increased oxidative stress levels, we saw that healing was delayed, the wounds remained open longer, there was less diversity of bacteria species in the wound microbiome, and there was poor dermal-epidermal adhesion even after the wound had healed. This work can potentially be used to help design better treatments for chronic wounds and in further research on the implications of oxidative stress on healing. While much of our findings may be translated to humans, we should take into consideration the differences in healing due to the panniculus carnosus muscle, a muscle found in mice but not in humans. Further studies extending from our work could include elucidating the mechanism by how oxidative stress levels influence bacteria interactions and metabolism, and how oxidative stress levels hinder the establishment of the dermal-epidermal junction at the molecular or cellular level.

Appendix

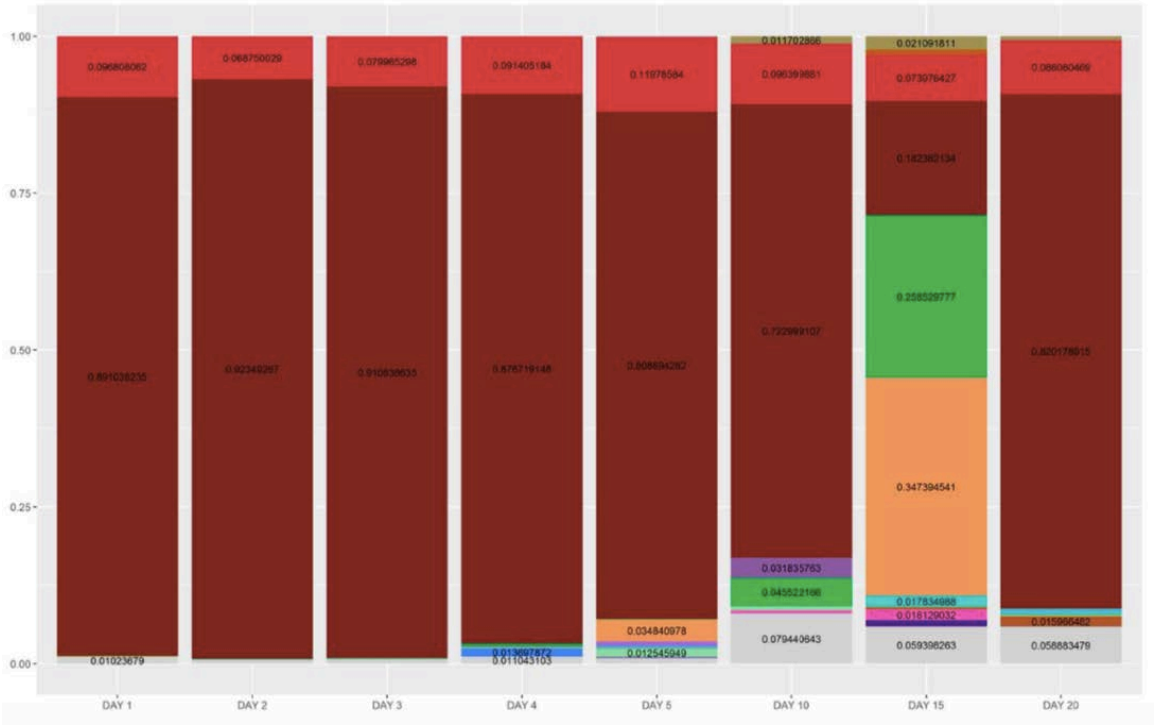
Formula for percent wound closure for each mouse

$$\left[\frac{(day\ 0\ wound\ area - day\ 20\ wound\ area)}{day\ 0\ wound\ area}\right] * 100\% = percent\ wound\ closure$$

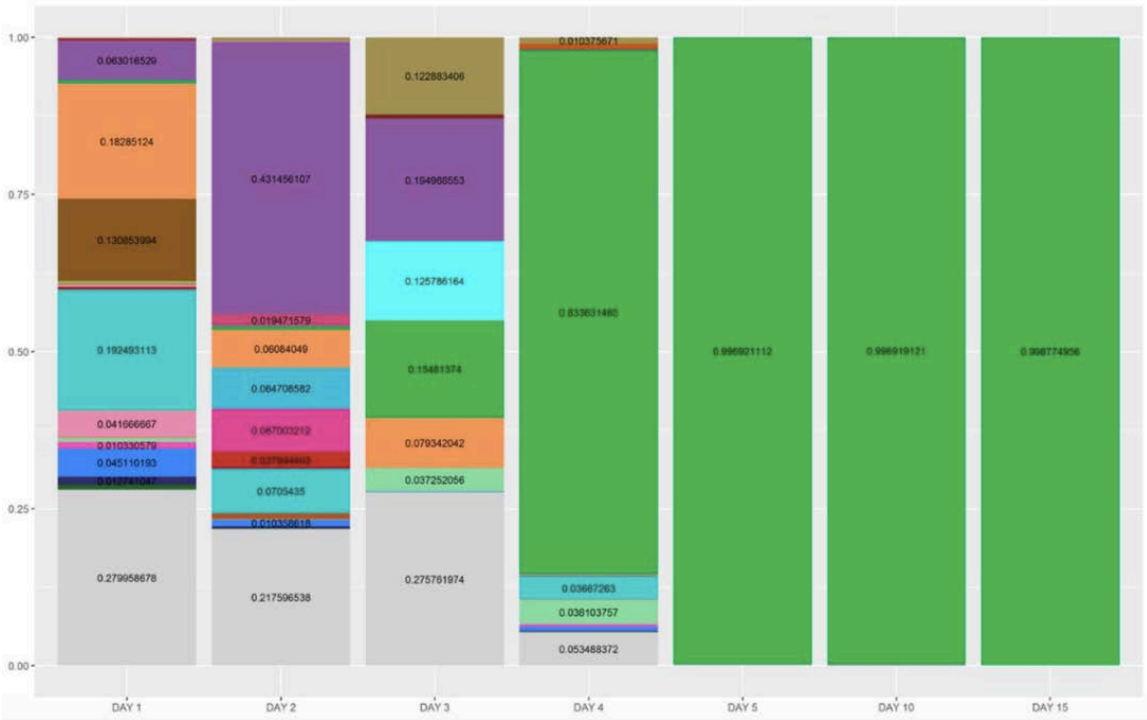
Bacteria profiles



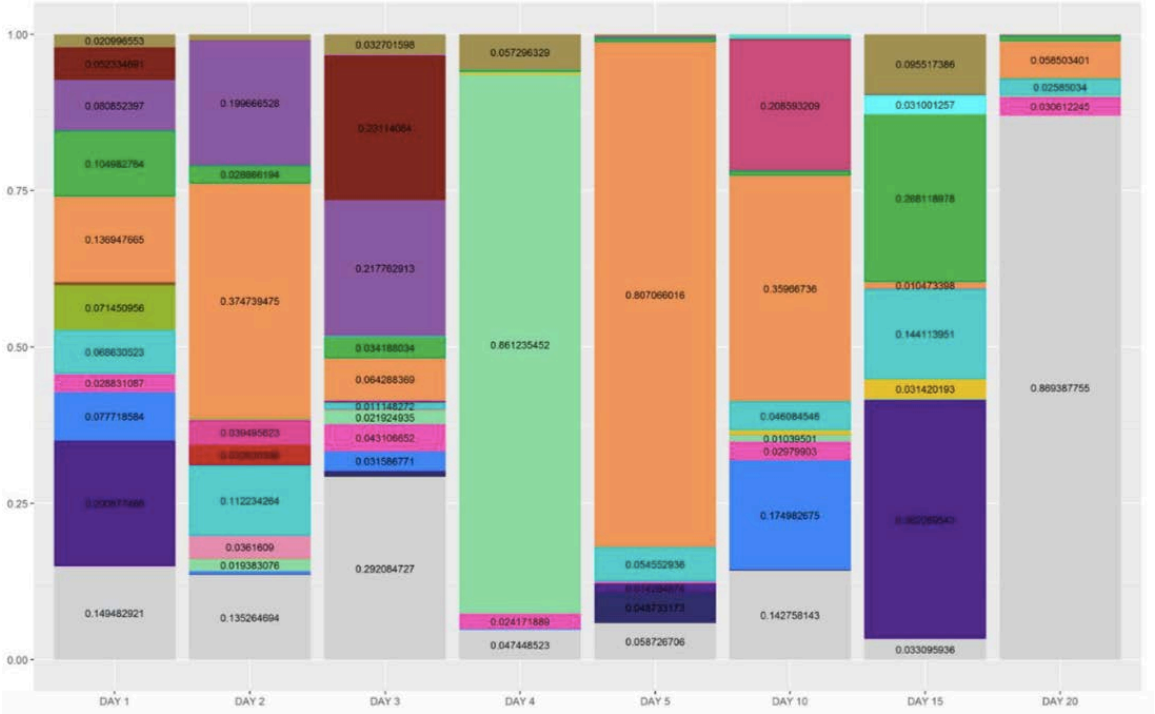
Appendix figure 1 Bacteria profile for full dose I



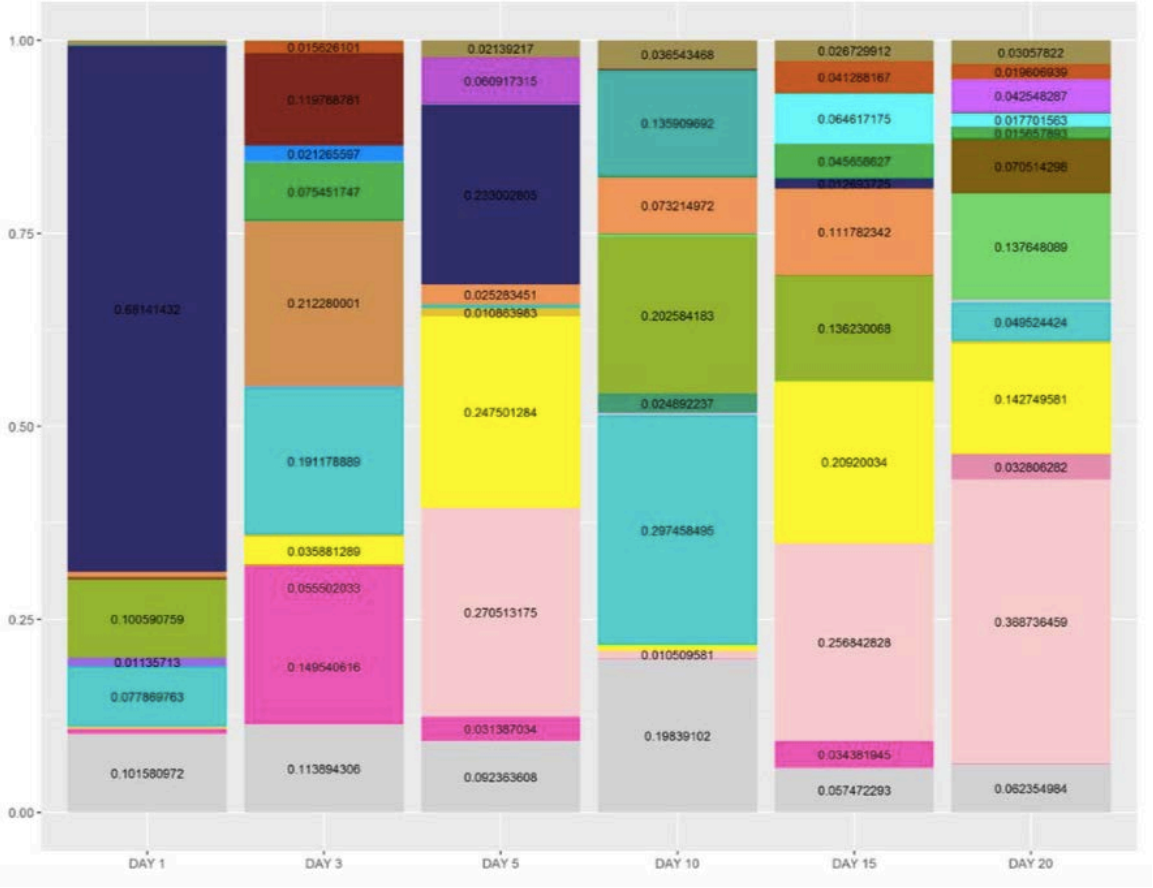
Appendix figure 2 Bacteria profile for half dose IAE



Appendix figure 3 Bacteria profile for quarter dose IAE



Appendix figure 4 Bacteria profile for eighth dose IAE



Appendix figure 5 Bacteria profile for a nonchronic wound

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