OVER THE COUNTER LIQUID BANDAGE APPLICATION RESULTS IN PRECOCIOUS WOUND CLOSURE FOLLOWING DISTAL AMPUTATION OF THE TERMINAL PHALANGEAL DIGIT IN MICE

An Undergraduate Research Scholars Thesis

by

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ABSTRACT

Over the Counter Liquid Bandage Application Results in Precocious Wound Closure Following Distal Amputation of the Terminal Phalangeal Digit in Mice

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CVS Liquid Bandage -- a “value brand” version of wound closure devices such as Dermabond -- following a distal, P3 amputation in mice initiates precocious wound closure; inducing a hypoxic microenvironment, and/or leading to reduced regeneration of the P3 digit.

Previous research has shown that epidermal closure and a hypoxic environment are crucial in mammalian regeneration; this study looks to confirm and expand this knowledge and potentially lead to new discoveries through the use of different immunohistochemical assays: Hif1α, and Hypoxyprobe for hypoxia and EdU as a marker for cell proliferation.

It is expected that the CVS Liquid Bandage will induce similar results to those produced by application of Dermabond: premature wound closure, creation of a hypoxic microenvironment, reduced degeneration and regeneration, and a similar expression pattern from the immunohistochemical assays.

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We would like to thank our PI, Dr. Muneoka, our mentor, Osama Qureshi, and the rest of our wonderful lab family for their support and assistance throughout this glorious research endeavor.

NOMENCLATURE

DPA	Days Post Amputation
CVS-LB	CVS Liquid Bandage
P3	Terminal Phalanx

CHAPTER I

INTRODUCTION

Currently one out of 200 Americans is an amputee. In total, more than 1.7 million people are living with limb loss in the United States and this number is projected to double by 2050, reaching an upwards of 3.6 million. Vascular diseases, cancer, and trauma are among the leading causes of death of both lower and upper extremity amputations (Ziegler-Graham 2008). Mortality rates for amputees suffering from vascular diseases have increased to 43-55%, now higher than the five-year rates for breast, colon, and prostate cancer (Robbins *et al.* 2008).

Although prosthetic technology has advanced significantly, amputees report their prostheses are often underutilized (Østlie *et al.* 2012). Despite the limitations of prosthetics, few other treatment options remain for those living with limb loss. Thus, studies in mammalian regeneration seek to discover a more viable and long term solution to help amputees restore motility and normalcy in their lives.

A Brief Survey of Vertebrate Regenerative Capabilities

While regenerative medicine will fill an important niche in the medical industry, it is clear that more research needs to be conducted before it becomes a viable option. Current research extensively uses animal models, with model organisms specifically chosen to cater to regenerative potential. For example, amphibians exhibit extensive regenerative capabilities, with many species able to successfully regenerate entire limbs (Goss 1969; Wallace 1981). However, neither humans nor any other mammalian species have shown the capacity for such regenerative ability; mammalian regeneration is limited to distal amputations of the third phalange (P3) that leave the nail organ intact (Borgens 1982; Neufeld & Zhao 1995; Han et al. 2008; Fernando et al. 2011, Chamberlain *et al.* 2017). Due to the differences in regenerative scope, amphibian

regeneration is not currently a high quality model to use for studying human regeneration. However, mice experience analogous regeneration in comparison to humans, particularly in regards to distal P3 amputations (Han *et al.* 2008). Accordingly, mice provide an excellent model for pre-clinical studies and were used in this study.

Overview of P3 Regeneration in Mice

The regenerative process following a distal P3 amputation in mice can be summarized into several phases: inflammation/histolysis, wound closure, blastema formation, regeneration, and finally redifferentiation (Simkin *et al*. 2013). In non-experimental cases, the inflammation/histolysis stage seems to be initiated by the remaining epidermis attaching to the bone stump created by the amputation. Large, multinucleated osteoclasts then form and degrade the bone stump for several days, creating a secondary amputation plane. Epithelial wound closure occurs and blastema formation begins at the distal end of the secondary amputation plane. The wound closure event-which generally occurs between 8 and 12 DPA - seems to demarcate the end of the degenerative aspect of the wound healing response and the beginning of the regenerative phases (Fernando *et al.* 2011). The regenerative phase is characterized by the formation of a blastema, a mass of undifferentiated, lineage-dependent cells and is responsible for the proliferative aspect of the regeneration response which will ultimately differentiate into a complete regenerate.

Although these phases have been relatively well characterized, the factors that delineate and trigger one phase leading to another have yet to be fully elucidated. It is in this light that we are investigating the effects of the wound closure product CVS brand liquid bandage (CVS-LB) on mammalian digit tip regeneration and comparing it to the effects of the previously-tested wound closure product Dermabond. Both CVS-LB and Dermabond are antiseptic wound closure

agents but have different methods of action: Dermabond employs the monomeric 2-octyl cyanoacrylate, which polymerizes within minutes of application, whereas CVS-LB employs eight active and inactive ingredients; of note, the ingredient nitrocellulose has previously been associated with accelerated wound closure and enhanced wound re-epithelialization (Xiafeng *et al.* 2016). Specifically, the influence of CVS-LB on epidermal wound closure and oxygen levels within the tissue was examined, as it has been thought that wound closure marks the end of the degradational phase and the advent of the regenerative phase in amputated digits. Normally, epidermal closure is completed between 8 and 12 DPA (Fernando *et al.* 2011), with a significant spike in hypoxia in the bone marrow at 7 DPA and in the blastema at 12 DPA (Sammarco *et al.* 2014). However, it is highly likely that oxygen levels regulate the level of distal bone degradation in the P3 digit and associated with a reduction in subsequent regeneration (Simkin *et al.* 2015). We seek to confirm, and potentially expand on, the findings of that project by also using the wound closure agent Dermabond (2-octyl cyanoacrylate), which was utilized by Simkin et al. in her previous study, as a positive control for premature wound closure.

Effects of Hypoxia and Wound Closure on Regeneration in Mice

Oxygen tensions vary throughout the reparative process. Although normoxia is ultimately required for proper wound healing, a brief hypoxic microenvironment can initiate necessary processes such as angiogenesis and fibroblast growth through activation of *HIF-1α,* a master regulator of the adaptive responses responsible for normalizing oxygen levels (Hong *et al.* 2014). During inflammation/histolysis, the wound site becomes increasingly hypoxic due to lesioned vasculature and the arrival of metabolically demanding inflammatory cells, limiting oxygen availability and delivery. A hypoxic microenvironment can be observed within the marrow throughout the degradative phase. In the concluding stages of digit regeneration, regions of

hypoxia are only visible in the vasculature along the regenerated structure as well as in the trabecular bone (Sammarco *et al.* 2014; Simkin *et al.* 2015).

The timing of wound closure has a major impact on the overall degree of regeneration. When wound closure is immediate - suturing of the skin flaps over the wound immediately following amputation - there is a complete lack of a regenerative response (Douglas, 1972; Mescher, 1976). Simkin *et al.* 2015 demonstrated that normal wound closure is regulated, at least partially, by the degree of oxygen saturation in the microenvironment of the wound site. Application of a wound closure agent, such as Dermabond, causes this microenvironment to become hypoxic earlier than normal which leads to accelerated wound closure, and the premature wound closure results in an abbreviated histolytic phase which alters the overall regenerative process (Simkin *et al*. 2015). Similarly, the presence of a hyperoxic microenvironment causes delayed wound closure with a corresponding extended histolytic phase and increased level of wound closure (Sammarco *et al.* 2014).

Figure 1 displays the bone volume progression after a distal, P3 amputation of untreated (control) mice vs Dermabond-treated mice, obtained from Simkin *et al.* 2015. The application of Dermabond to the wound site clearly reduced the level of degeneration (loss of bone volume) and regeneration (gain of bone volume).

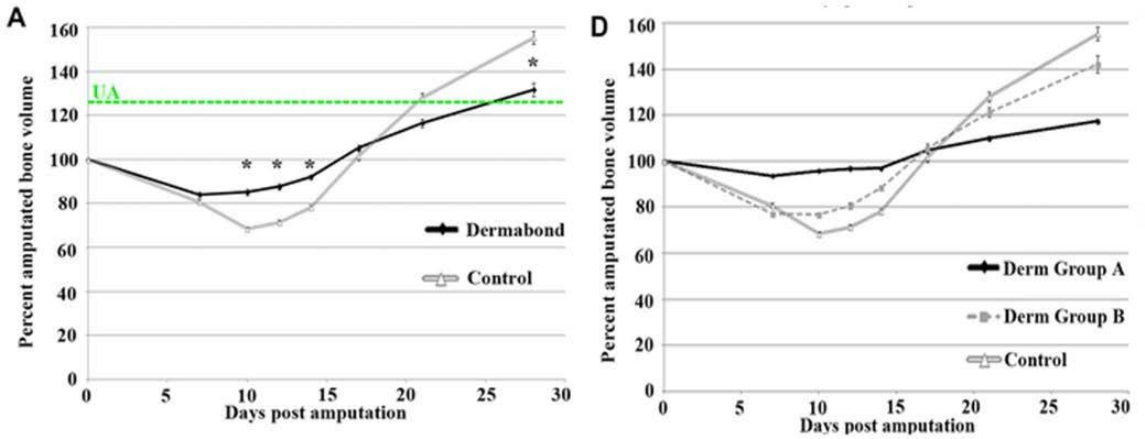

Figure 1. Results from Simkin *et al.* 2015, which were the basis for the use of Dermabond treated digits as the positive control for this experiment. These results display the average normalized (to the 0 DPA) bone volume of digits at 7, 10, 12, 14, 17, 21 and 28 DPA. The control sample shows how bone volume normally changes in an untreated sample. In A, it is apparent that the samples treated with Dermabond experienced significantly less degeneration and regeneration than the control samples. In D, the Dermabond treated digits were divided into Group A and Group B. Group A digits were chosen by a visual analysis of a phenotypic difference from the control samples, while Group B digits were those that underwent a regenerative process much more similar to that of the control.

CHAPTER II

METHODS

A selection of female mice will be exposed to the anesthetic agent isoflurane in a controlled environment until unconscious. Following amputation at the distal tip of the third phalangeal digit of the second, third and fourth toes on both hind limbs, 10 microliters of CVS liquid bandage will be applied to the wound site and then be re-applied each of the next two days. Digits will be collected 2,3,4,5, and 7 DPA. Amputated digits will be fixed, processed, and serially sectioned. One slide from each digit will be stained with Mallory trichrome to observe the overall morphology. The second slide will be subject to a dual immunohistochemical assay of Edu, which highlights proliferating cells, and Hif1, which is an indicator of a hypoxic microenvironment. Micro CT (μCT) scans of all subjects are taken at designated time points and edited using BoneJ, an ImageJ program plugin. Images will then be viewed using ImageJ's 3D viewer plugin to visualize morphological changes in a three dimensional perspective. Bone volume is computed using the BoneJ volume fraction plugin and documenting differences in both bone volume and length throughout the period of regeneration are integral to understanding the extent of regeneration/degeneration elicited by the application of both treatments (Doube *et al.* 2010).

Three trials were performed in this study, each differing in number of applications. In the first round, digits were treated with three applications of CVS-LB and evaluated histologically. Digits that were subject to μCT analysis were treated with only one application. A second iteration was done, this time including Dermabond. Digits were treated with three applications of either CVS-LB or Dermabond and stained with Hypoxyprobe to track regions of hypoxia. μCT

analysis was done for only the CVS-LB samples. The first two iterations of this project served to guide studies for the third trial involving the wound closure devices and are not the focus of the paper; thus, the results of those experiments are located in the appendix. To confirm Simkin's results, the third round of this study, which forms the basis of this paper, included volumetric analysis of both CVS-LB and Dermabond digits and number of applications remained the same.

CHAPTER III

RESULTS

Degradation and Regeneration

To analyze bone degradation/regeneration, scans were taken at designated time points between -1 DPA to 28 DPA (Figure 2). Original digit morphology prior to amputation is shown in the first column of Figure 2, labeled as -1 DPA. Once amputated at a distal level, the digit normally undergoes degradation between 8-12 DPA and this is characterized by loss in bone volume. This is observed in the untreated (control) digits. Conversely, both CVS-LB and Dermabond samples experienced reduced degradation and regeneration within this time frame (4-12 DPA), although this reduction was more significant for Dermabond-treated samples.

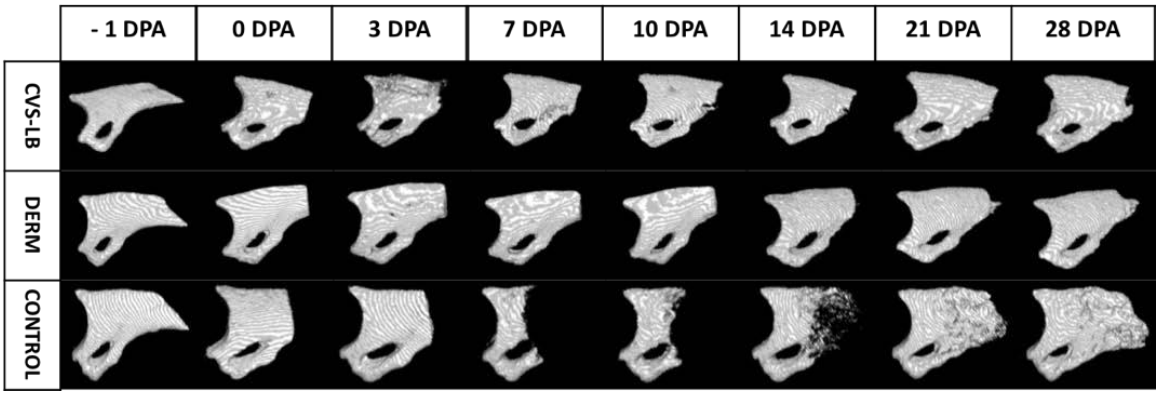

Figure 2. μCT Scans of Treated and Control Digits. These scans range from pre-amputation (-1 DPA) to 28 DPA and show the progression of degeneration/regeneration in 3 separate digits that have been treated (CVS-LB or Dermabond) or left untreated to serve as controls.

The next stage of analysis was a volumetric analysis of the data obtained through μCT scans. Each individual digit was tracked from 0-28 DPA, which enabled normalization of the data by dividing each obtained digit volumes by that particular digit's volume at 0 DPA. Figure 3 displays the basic data obtained from this study. Graph A reveals that digits treated with CVS-

LB experienced more degradation than digits treated with Dermabond, while both experienced less degradation than the control digits. The CVS-LB average volumes were highly comparable to the Dermabond volumes obtained in Simkin's experiment, and the Dermabond samples experienced even less degeneration than Simkin's Dermabond treated digits. Graph B displays the control data and the Group A data for both Dermabond treated samples and CVS-LB treated samples. Group A consists of digits that stayed within 20% of their 0 DPA volume for the entire 28 day period - they experienced reduced degeneration and regeneration. One of eight digits treated with CVS-LB and two of eight digits treated with Dermabond qualified as Group A digits. All of the digits that failed to qualify for Group A were designated as Group B.

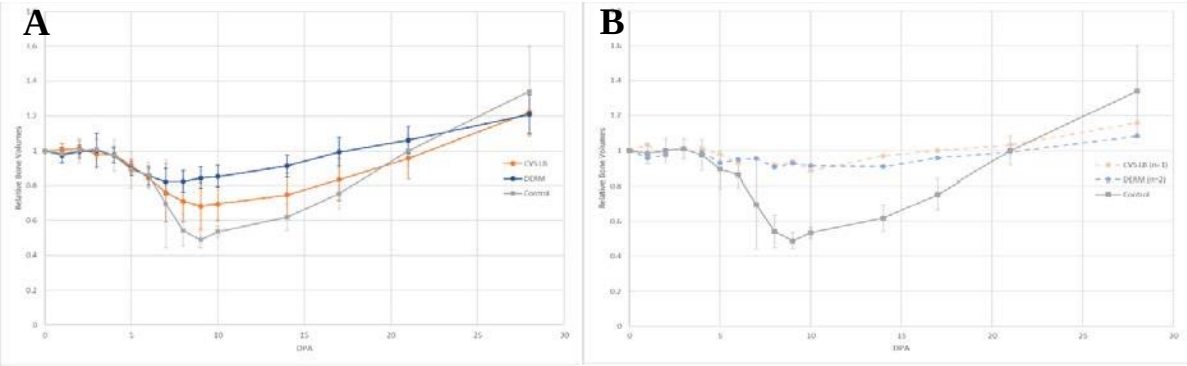

Figure 3. Overall Volumetric Results. A) This graph displays the overall average normalized bone volumes (with 95% confidence intervals shown by the vertical bars) for the CVS-LB, Dermabond, and control samples. B) This graph displays the Group A digits from both the CVS-LB and Dermabond samples; Group A digits are those that stayed within 20% of their 0 DPA bone volume for the entire 28 days.

As Figure 4 depicts, the Group B for the CVS-LB was much more similar to the control than the Group B of Dermabond. However, it is important to note that even the Group B data for both treatment groups experienced statistically significantly less degeneration than that of the control. Additionally, the Group A data for both treatment groups was highly similar to Simkin's Group A data, and the entire CVS-LB-treated Group A vs Group B graph looked was similar to the data obtained by Simkin *et al*. for Dermabond-treated digits.

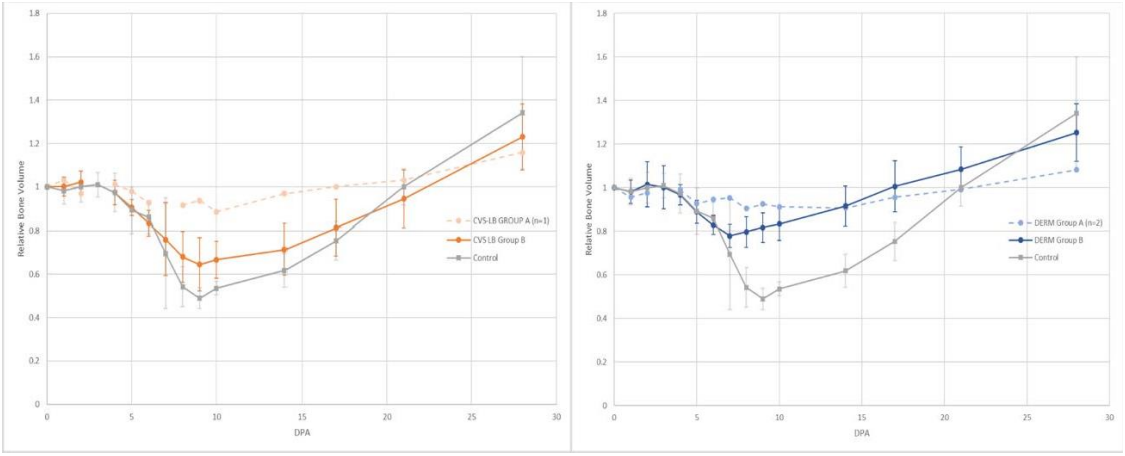

Figure 4. CVS-LB-Specific and Dermabond-Specific Volumetric Results. A) The graph of the average, normalized bone volumes of Group A and Group B samples that were treated with CVS-LB. B) The graph of the average, normalized bone volumes of Group A and Group B samples that were treated with Dermabond. Digits that fell into Group A stayed within 20% of their 0 DPA volume for the entirety of the 28 days, and all other digits fell into Group B.

Hypoxia and Wound Closure

Images of digits that were collected at 2, 3, 4, 5, or 7 DPA and then stained with the Mallory Trichrome procedure or a Hif1-α/EdU immunohistochemical assay. All images were oriented so that the wound site is located on the right. The images that underwent a Mallory Trichrome stain are useful for identifying histology, while the images that underwent a Hif1-α/EdU immunohistochemical assay are used to identify areas of hypoxia and/or cellular proliferation. The structural characteristics to note in the Mallory stained images are the dark blue - bone; strong pink/red - scab and outer layer of skin; and light pink - epidermis. Mallory stains revealed if wound closure had occurred, defined by the dorsal and ventral wound epidermises connecting at the distal tip (Figure 5).

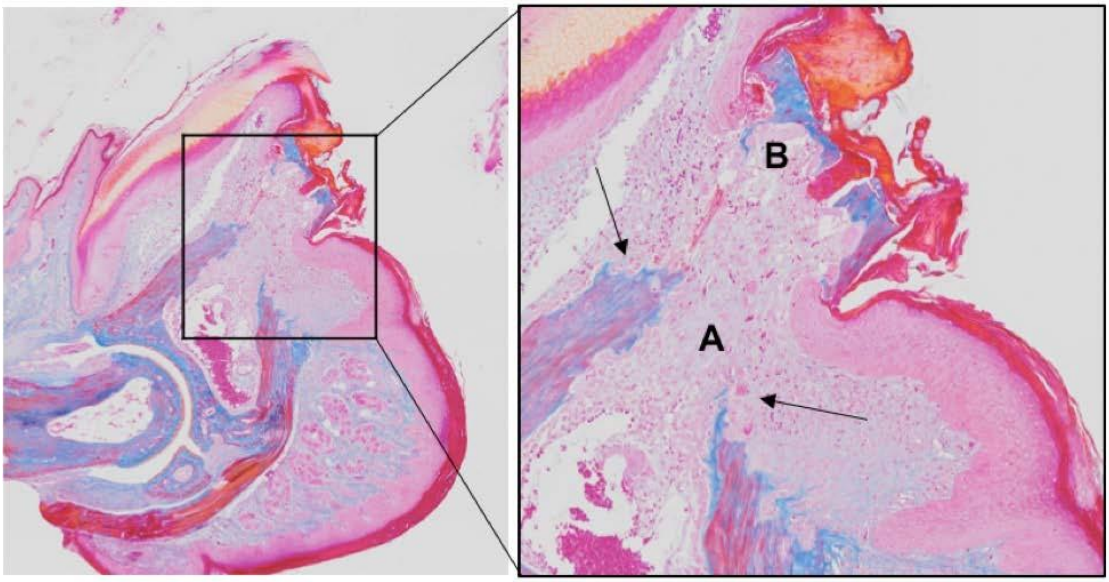

Figure 5. Secondary Amputation May Precede Wound Closure to Reveal A Secondary Amputation Plane. A) Wound closure can be observed when the ventral and dorsal edges of the epidermis (light pink) migrate across the amputated bone stump (as marked by the arrows). B) A secondary amputation occurs during the degradative phase, resulting in the cleavage and expulsion of the distal bone fragment.

In each set of images in Figure 6 there is a row of images on the top (Panel A) with a corresponding row of images below it (Figure 6B).The images from Panel A were used to determine if wound closure had occurred. The images in each Panel B were used to identify areas of hypoxia - increased expression of "green" - and cell proliferation - expression of "red". The nail (top), red blood cells (inside the center of the bone), and the fat pad (bottom) would auto fluoresce and were not taken into consideration. Due to the role of hypoxia in epidermal migration, Hif1-α was expressed at the distal portion of the epidermis prior to wound closure, as expected. Expression would spike at and shortly after wound closure, then slowly decrease (as seen in the lack of expression at 7 DPA in the Dermabond images of Figure 6). Cell proliferation appeared to generally only occur in 7 DPA images, if at all. This proliferative event occurs after 7 DPA which presumably corresponds with the development of the blastema. Due to our lack of data post-7 DPA, it was hard to draw any connections between cellular proliferation and wound

closure/hypoxia, but it did appear that wound closure had to occur before any significant level of cell proliferation could occur.

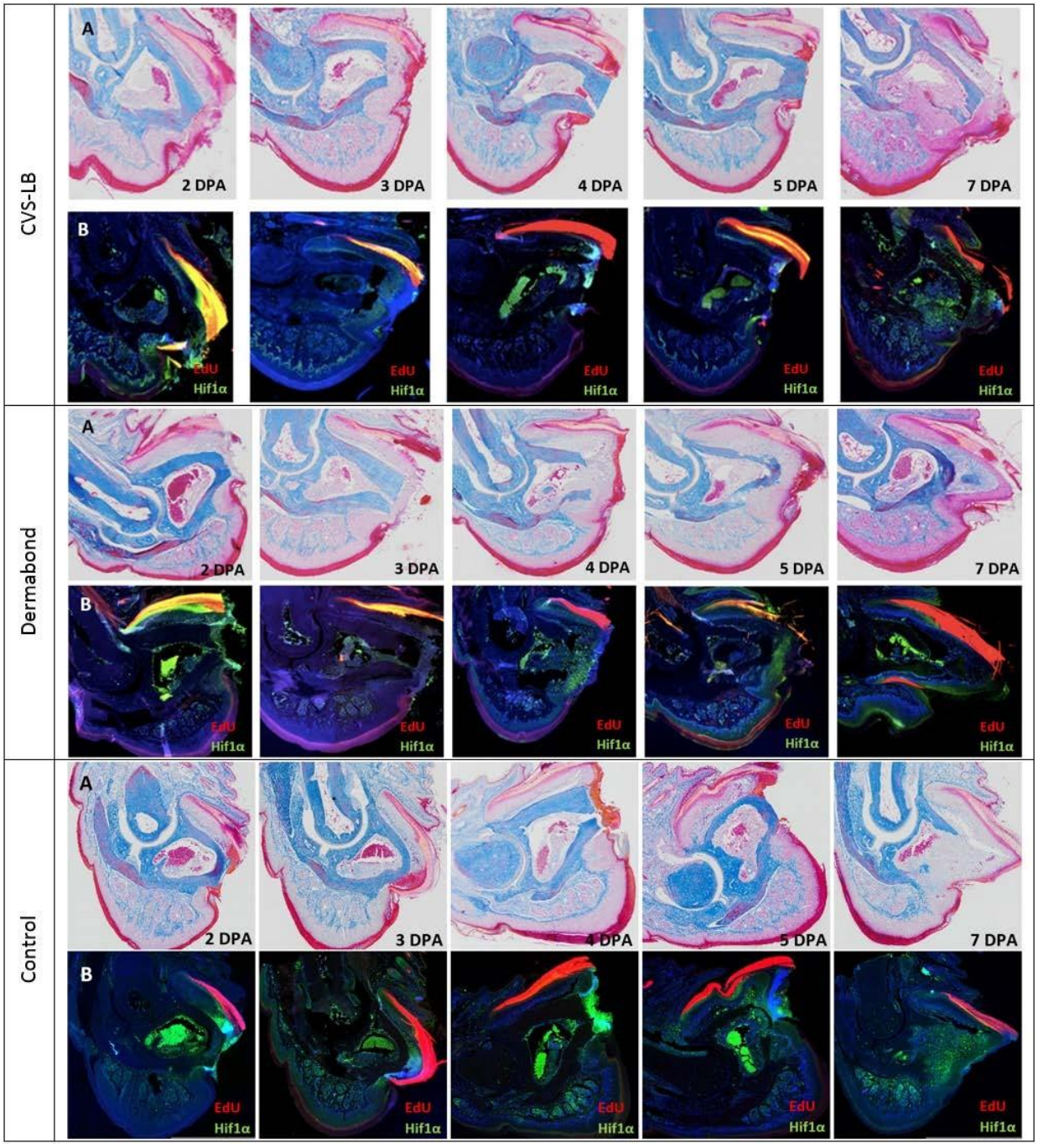

Figure 6. Images of Stained, Treated Digits. Each vertical pair is the same digit with a different staining procedure, but there is no association between digits horizontally other than that they underwent the same treatment. A) Images of samples stained with Mallory Trichrome, used to determine if wound closure had occurred. These samples showed positive wound closure at 3, 5 and 7 DPA for the CVS-LB images, 4, 5, and 7 DPA for the Dermabond images, and 7 DPA for

the control images. B) Images of samples-which correspond to the Mallory stained sample above them-that underwent an EdU/Hif1-α immunohistochemical assay. Hypoxia is found at the leading edge of the epidermis throughout, and has widespread expression after wound closure. The apparent extreme distal expression of Hif1-α in the 2 DPA (all) and 4 DPA (CVS-LB, Control) immunos is autofluorescence from the scab, not hypoxia in the tissue at the wound. There was only slight expression of EdU in the wound site for the 7 DPA Dermabond and CVS-LB samples.

The Mallory stained slide from every mouse digit was analyzed for wound closure, and the results were recorded in Table 1. It also contains the data from the previous two iterations of the CVS-LB/Dermabond experiment and those obtained from Simkin *et al.* 2015. In all three rounds, closure in both Dermabond and CVS-LB digits was identified as early as 3 DPA. Rates greatly varied from 9% (1/11) to 83% (5/6) for digits treated with CVS-LB and from 0% (0/5) to 50% (3/6) in Dermabond samples. Closure occurred by 7 DPA in every sample in both rounds I and II, but only 1/5 (20%) of CVS-LB treated digits closed in round III at the same time point. (Table 1).

Table 1. Rate of Wound Closure in Rounds I-III.

	ROUND I	**ROUND II**		**ROUND III**		**Simkin Data**	
	CVS-LB 1 application	**CVS-LB** 1 application	**DERM** 1 application	**CVS-LB** 3 applications	**DERM** 3 applications	**Control**	**DERM** 1 application
2 DPA	0/5 (0%)			0/6 (0%)	0/6 (0%)	0/3 (0%)	1/3 (33%)
3 DPA	5/6 (83%)	1/11 (9%)	3/6 (50%)	2/4 (50%)	0/5 (0%)	----	----
4 DPA				0/6 (0%)	2/4 (50%)	0/4 (0%)	2/4 (50%)
5 DPA	4/5 (80%)	0/12 (0%)	6/6 (100%)	1/5 (20%)	5/6 (83%)	----	----
6 DPA	----			----	----	0/5 (0%)	4/5 (80%)
7 DPA	3/3 (100%)	3/3 (100%)	3/3 (100%)	1/5 (20%)	5/5 (100%)	----	----
10 DPA	3/3 (100%)			**----**	----	----	----

CHAPTER IV

CONCLUSION AND DISCUSSION

The use of wound closure devices Dermabond and CVS-LB has shown to produce accelerated although variable levels of epidermal wound closure in all three rounds of this study (Table 1). With both treatments, wound closure was observed between 3-7 DPA; this is far faster than the average timeframe (8-12 DPA) for untreated digits. Timing of wound closure also coincided with expression of hypoxia, suggesting that it may be an indicator of wound closure. Only the results of samples treated with Dermabond have shown potential for a possible correlation with reduced degradation and regeneration. This could not be concluded with CVS-LB as volumetric analyses of all three rounds showed very little consistency.

Differences of Dermabond/CVS-LB and Microbial Ramifications

Although both CVS-LB and Dermabond are wound closure agents, they differ in composition. CVS-LB contains antiseptic/analgesic properties and establishes a microbial barrier to prevent infection. This may affect the microbial environment of the wound and contribute to the variability in responses to precocious wound closure of the Dermabond and CVS-LB treated samples. Disruption of the microflora can influence the histolytic immune response by affecting the differentiation of monocytes to macrophages rather than osteoclasts. Monocytes are leukocytes that are recruited by MCP-1 (among other things), a chemoattractant secreted by cells that regulate macrophage and monocytic activity at the site of inflammation or infection (Conti and DiGioacchino, 2001; Melgarejo 2008).

Further Studies

When looking through the scope of regenerative medicine, the primary effect of wound closure devices such as Dermabond and CVS-LB is the inhibition of bone degradation. The osteoclasts responsible for bone degradation are formed by osteoclastogenesis in the days-to-week after the wound event; however, this process is presumably disrupted by the early wound closure event caused by the application of a wound closure agent, which leads to the decreased degradation. The process of wound closure itself, however, is still not fully known. More specifically, it is not known exactly what events trigger the epidermal migration which leads to the actual wound closure. For example, previous and ongoing studies have demonstrated that several treatments negatively affect wound closure, such as exposure to hyperbaric oxygen (effectively inhibiting a normally hypoxic microenvironment in the wound), although this can be recovered to a control-like wound closure rate by the application of Dermabond (Simkin *et al*. 2015). Mice that had the nerves of the amputated toes removed did not experience wound closure at any point, even with the application of Dermabond (unpublished data). Similarly, hindlimb unloaded mice do not experience any acceleration to wound closure rates when Dermabond is applied post-amputation (unpublished data). Addition of the bisphosphonate clodronate severely inhibits the function of osteoclasts (Simkin 2017). If macrophages are eliminated from the wound site, wound closure and regeneration as a whole are entirely eliminated (Simkin 2017).

While there are many questions yet to be answered about the wound closure process, one potential starting point would be to use Keratin 17 as an immunohistochemical wound marker to attempt to find any differences in the "wound identifying" behaviors between control digits, CVS-LB treated digits, and Dermabond-treated digits. Furthermore, replicating this study with an addition of CVS-LB's active ingredients, benzethonium chloride (0.2%) and dyclonine

hydrochloride (0.75%) in addition to Dermabond treatment may help illustrate whether the two wound closure devices' differential response can be attributed to analgesic agents found only in CVS-LB.

REFERENCES

Borgens, R. B. “Mice Regrow the Tips of Their Foretoes.” *Science*, vol. 217, no. 4561, Aug. 1982, pp. 747–50. *science.sciencemag.org*, doi:10.1126/science.7100922.

Chamberlain, Connie S., et al. “Level-Specific Amputations and Resulting Regenerative Outcomes in the Mouse Distal Phalanx.” *Wound Repair and Regeneration*, vol. 25, no. 3, May 2017, pp. 443–53. *Wiley Online Library*, doi:10.1111/wrr.12544.

Conti, P., and M. DiGioacchino. “MCP-1 and RANTES Are Mediators of Acute and Chronic Inflammation.” *Allergy and Asthma Proceedings*, vol. 22, no. 3, June 2001, pp. 133–37.

Doube, Michael, et al. “BoneJ: Free and Extensible Bone Image Analysis in ImageJ.” *Bone*, vol. 47, no. 6, Dec. 2010, pp. 1076–79. *PubMed*, doi:10.1016/j.bone.2010.08.023.

Fernando, Warnakulasuriya Akash, et al. “Wound Healing and Blastema Formation in Regenerating Digit Tips of Adult Mice.” *Developmental Biology*, vol. 350, no. 2, Feb. 2011, pp. 301–10. *PubMed*, doi:10.1016/j.ydbio.2010.11.035.

Goss, Richard J. Principles of Regeneration. Academic Press, 1969.

Han, Manjong, et al. “Development and Regeneration of the Neonatal Digit Tip in Mice.” *Developmental Biology*, vol. 315, no. 1, Mar. 2008, pp. 125–35. *PubMed*, doi:10.1016/j.ydbio.2007.12.025.

Han, Manjong, et al. “Development and Regeneration of the Neonatal Digit Tip in Mice.” *Developmental Biology*, vol. 315, no. 1, Mar. 2008, pp. 125–35. *PubMed*, doi:10.1016/j.ydbio.2007.12.025.

Mu, Xiaofeng, et al. “Nano-Porous Nitrocellulose Liquid Bandage Modulates Cell and Cytokine Response and Accelerates Cutaneous Wound Healing in a Mouse Model.” *Carbohydrate Polymers*, vol. 136, Jan. 2016, pp. 618–29. *ScienceDirect*, doi:10.1016/j.carbpol.2015.08.070.

Melgarejo, Esther, et al. “Monocyte Chemoattractant Protein-1: A Key Mediator in Inflammatory Processes.” *The International Journal of Biochemistry & Cell Biology*, vol. 41, no. 5, May 2009, pp. 998–1001. *PubMed*, doi:10.1016/j.biocel.2008.07.018.

Mescher A.L. “Effects on adult newt limb regeneration of partial and complete skin flaps over the amputation surface.” *The Journal of Experimental Zoology*, vol. 195, Jan. 1976, pp. 117-128.

Mu, Xiaofeng, et al. “Nano-Porous Nitrocellulose Liquid Bandage Modulates Cell and Cytokine Response and Accelerates Cutaneous Wound Healing in a Mouse Model.” *Carbohydrate Polymers*, vol. 136, Jan. 2016, pp. 618–29. *ScienceDirect*, doi:10.1016/j.carbpol.2015.08.070.

Neufeld, Daniel A., and Weiguang Zhao. “Bone Regrowth after Digit Tip Amputation in Mice Is Equivalent in Adults and Neonates.” *Wound Repair and Regeneration*, vol. 3, no. 4, Oct. 1995, pp. 461–66. *Wiley Online Library*, doi:10.1046/j.1524-475X.1995.30410.x.

Østlie, Kristin, et al. “Prosthesis Use in Adult Acquired Major Upper-Limb Amputees: Patterns of Wear, Prosthetic Skills and the Actual Use of Prostheses in Activities of Daily Life.” *Disability and Rehabilitation. Assistive Technology*, vol. 7, no. 6, Nov. 2012, pp. 479–93. *PubMed*, doi:10.3109/17483107.2011.653296.

Robbins, Jeffrey M., et al. “Mortality Rates and Diabetic Foot Ulcers: Is It Time to Communicate Mortality Risk to Patients with Diabetic Foot Ulceration?” *Journal of the American Podiatric Medical Association*, vol. 98, no. 6, Dec. 2008, pp. 489–93.

Sammarco, Mimi C., et al. “Endogenous Bone Regeneration Is Dependent upon a Dynamic Oxygen Event.” *Journal of Bone and Mineral Research: The Official Journal of the American Society for Bone and Mineral Research*, vol. 29, no. 11, Nov. 2014, pp. 2336–45. *PubMed*, doi:10.1002/jbmr.2261.

Simkin, Jennifer, Mimi C. Sammarco, et al. “Epidermal Closure Regulates Histolysis during Mammalian (Mus) Digit Regeneration.” *Regeneration*, vol. 2, no. 3, June 2015, pp. 106–19. *Wiley Online Library*, doi:10.1002/reg2.34.

Simkin, Jennifer, Manjong Han, et al. “The Mouse Digit Tip: From Wound Healing to Regeneration.” *Methods in Molecular Biology (Clifton, N.J.)*, vol. 1037, 2013, pp. 419–35. *PubMed*, doi:10.1007/978-1-62703-505-7_24.

Wallace, H., et al. “Regeneration of Subnormally Innervated Axolotl Arms.” *Journal of Embryology and Experimental Morphology*, vol. 62, Apr. 1981, pp. 1–11.

Ziegler-Graham, Kathryn, et al. “Estimating the Prevalence of Limb Loss in the United States: 2005 to 2050.” *Archives of Physical Medicine and Rehabilitation*, vol. 89, no. 3, Mar. 2008, pp. 422–29. *PubMed*, doi:10.1016/j.apmr.2007.11.005.

APPENDIX

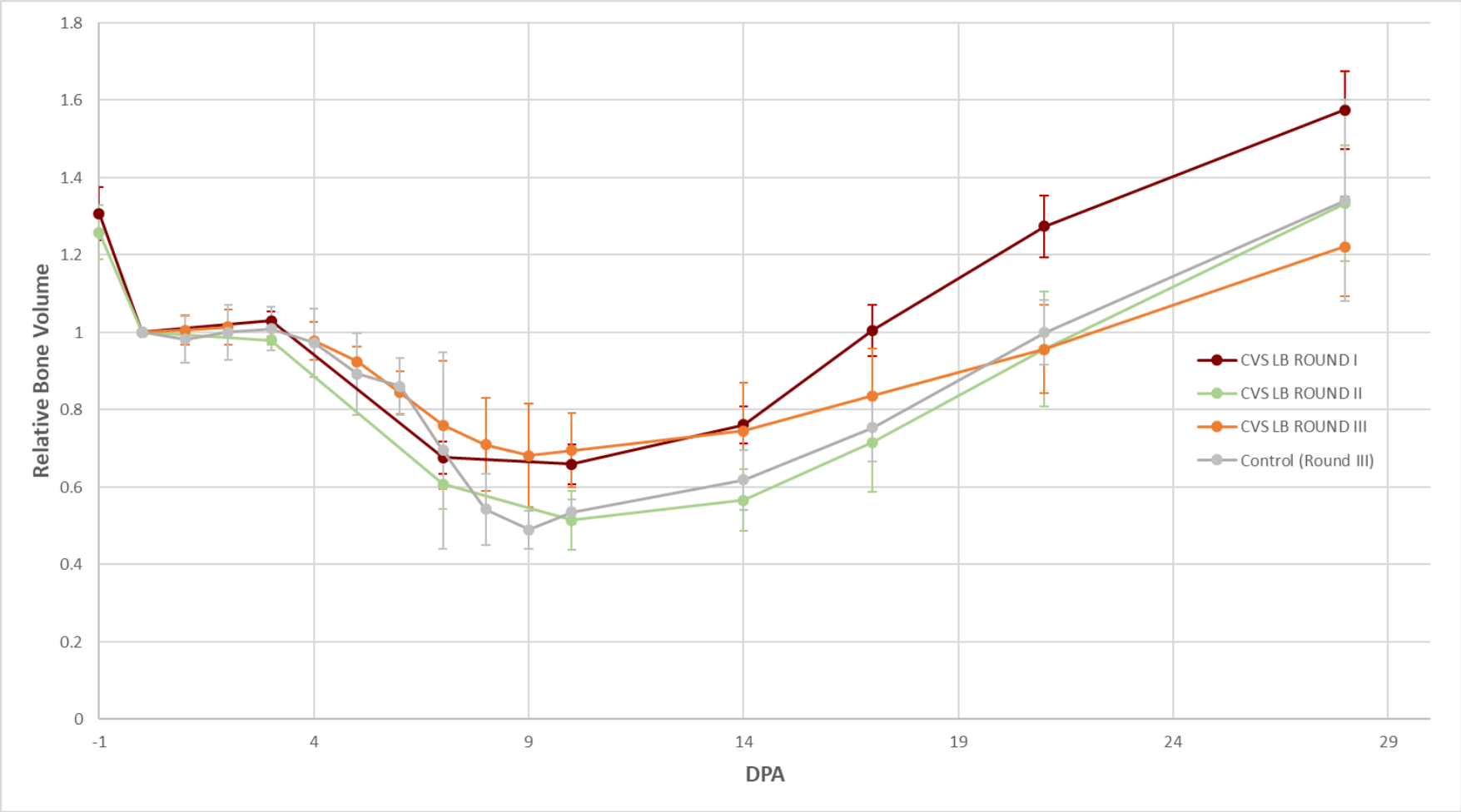

Figure S1. This graph compares the average relative bone volumes of CVS-LB treated digits for each iteration of the experiment. Round I saw reduced degeneration, but incredibly large levels of regeneration, Round II was very similar to the control volumes, and Round III saw slightly decreased levels of degeneration and regeneration. Overall, CVS-LB application led to decreased degeneration, but had wildly inconsistent results.