5-1-2021

COMPARISON OF VARIOUS COMPLEMENTARY AND ALTERNATIVE MEDICINE FOR THEIR WOUND HEALING PROPERTIES IN VITRO

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COMPARISON OF VARIOUS COMPLEMENTARY AND ALTERNATIVE MEDICINE FOR THEIR WOUND HEALING PROPERTIES *IN VITRO*

by

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Presented to the Faculty of the Honors College of

The University of Texas at Arlington in Partial Fulfillment

of the Requirements

for the Degree of

HONORS BACHELOR OF SCIENCE IN BIOMEDICAL ENGINEERING

THE UNIVERSITY OF TEXAS AT ARLINGTON

May 2021

ACKNOWLEDGMENTS

I would like to thank Dr. Kytai T. Nguyen for working in her lab for the past two years. She was one of the best mentors that I ever worked with. I would also like to thank Nanomedicine and Drug Delivery Lab members for their support throughout my years. I would also thank Uday Chintapula, Deepsundar Sahoo, Vy Nguyen, and Quincy Harper for their guidance and assistance during this project.

This work was partly supported by the National Institute of Health (NIH) R15 HL156076 (Dr. Kytai T. Nguyen's NIH award).

May 04, 2021

ABSTRACT

COMPARISON OF VARIOUS COMPLEMENTARY AND ALTERNATIVE MEDICINE FOR THEIR WOUND HEALING PROPERTIES *IN VITRO*

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The University of Texas at Arlington, 2021

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Currently, healthcare providers face significant challenges within the field of wound treatment. In the last decade, United Kingdom reported £2.3-3.1 billion expenditure on chronic wound treatments, whereas the United States spent over $25 billion on wound management. This indicates that unresolved wounds can place a significant burden on public health resources. A comprehensive review of global chronic wound pervasiveness found that the pooled prevalence of chronic wounds to be approximately 2.21 per 1000 individuals. Prevalence across the globe highlights the urgent need for more effective treatment strategies. One niche within the realm of wound treatment involves complementary and alternative medicines (CAMs), or treatments that are not involved in standard medical practice. This research aims to investigate the comparative impacts of

CAMs including, boric acid (BA) and curcumin, with respect to wound healing *in vitro*. Wound infliction plays a major role in determining the effects of CAMs. In this research, we optimized the mode of wound infliction using various methods and found 1mL pipette stamp is highly effective in treating wounds *in vitro* and improves the feasibility of assessing wound closure more accurately. In our study, BA at a concentration of 10μM and curcumin with 1 μM-10 μM showed effective wound closure in mouse dermal fibroblasts. With respect to the cell proliferation, BA at a concentration of 1mM and curcumin from 0.5 μM-10 μM showed an increase in cell proliferation rates compared to the untreated control. These compounds offer alternative wound healing treatments on improving the migration and proliferation stage of the wound healing process in dermal fibroblasts. CAMs like these have the potential to expand the available repertoire of compounds for wound healing treatment methods along with improved cost efficiency.

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CHAPTER 1

INTRODUCTION

1.1 Wound Healing

Currently, healthcare providers face significant challenges within the field of wound treatment. In the United States, it is reported that about $25 billion were spent annually for the treatment of chronic wounds and affect 6.5 million patients (Sen, et al, 2009). In 2008, it was estimated that the United Kingdom spent approximately between £2.3-3.1 billion on chronic wound treatment (Posnett & Franks, et al, 2008). This indicates that unresolved wounds can place a significant burden on public health resources and the economic ramifications to the society. Echoing these findings, a 2018 comprehensive review of global chronic wound pervasiveness found that the pooled prevalence of chronic wounds to be approximately 2.21 per 1000 individuals. While actual prevalence can vary across regions and populations, with significant differences stemming from environmental, economic, and cultural factors, the review highlights the global need for improved wound treatment methods and procedures (Martinengo et al, 2019). One particular niche within the realm of wound treatment involves complementary and alternative medicines (CAMs), or treatments that are not involved in standard medical practice. While CAMs generally do not possess bodies of evidence similar in the magnitude of standard medical treatments (Sointu et al, 2013), some compounds do show promising results in preliminary research. Some examples of CAMs include healing touch, acupuncture, essential oils, or even certain foods with healing properties like honey and ginger (Hall et al. 2020).

This paper investigates the comparative impacts of curcumin and boric acid (BA), two proposed alternative wound healing treatments, on the proliferation of skin cells and migration stage of the wound healing process. While earlier studies have investigated the individual efficacy of each compound, no comparison between the two has been made within the context of wound healing. By comparing the compounds, this study will perform the necessary job of narrowing the CAM niche for wound healing treatment by determining therapeutic efficacy *in vitro*. This can expand the available body of treatment methods available to both physicians and patients seeking less invasive or non-costly therapy.

Wound healing is a complex biological series of events that can last even years after an injury occurs (Wild et al, 2010). The process of wound healing can be divided into four overlapping stages, which are hemostasis, inflammation, migration-proliferation, and remodeling (Lindley, et al, 2016). The process of wound healing is not linear, and sometimes it can progress forward and backward through different phases depending on various intrinsic and extrinsic factors (Wild et al. 2010). Two prominent mechanisms implicated in this process include cell migration and cell proliferation, which along with angiogenesis and extracellular matrix formation, play an important role in the ultimate contraction of a wound (Falanga 2005). Cell migration, which can present as either single-cellular movement or collective migration (Grada et al, 2017), allows for the lateral closure of wounds as healthy cells move to regenerate physical structures (Friedl & Gilmour et al, 2009). Collective cell migration is particularly essential to the wound healing process as it allows for multicellular organ and tissue patterns to be replaced (Friedl & Gilmour 2009). Additionally, within the large context of skin regeneration and healing, cell types, including fibroblasts and melanocytes, also participate in collective migration (Grada et al, 2017).

Supplementing cell migration, cell proliferation generates new healthy cells for structure formation. This process becomes especially vital in cases where large wounds cannot be addressed sufficiently solely through cell migration (Falanga 2005). Ultimately, because of the essential processes of both cell migration and cell proliferation, the potential wound healing benefits of compounds can be determined through investigating their positive impacts on these vital processes.

1.2 Boric Acid

Boric acid (BA) is a compound with a wide range of researched and reported health benefits. Today, BA is known for its antiviral and antifungal properties and is utilized as a treatment for minor illnesses such as yeast infections and cold sores (NCI Thesaurus, n.d.). However, studies have indicated that its potential uses may extend much further. In 2009, it was found that BA possesses the ability to inhibit Ca^{2+} signaling in prostate cancer cells leading to a decreased risk of developing clinical cancer (Henderson et al, 2009). Further, BA has been found to impede certain proteases and peptidases such as prostate specific antigen (PSA), suggesting the existence of chemo-preventive properties (Tepedelen et al. 2016). Additionally, BA has been shown to cause cancer cells to become more sensitive to damaging agents, such as chemotherapy drugs, while simultaneously protecting normal cells from the same damage (Tepedelen et al, 2016).

Beyond potential significance for cancer treatment, BA has also shown itself to be beneficial during wound healing. In 2015, it was reported that when orally administered, BA improved the healing process for damaged Achilles' tendon tissue in rats (Kaymaz et al, 2015). In this study, it was found that the compound allowed for decreased vascularity, proper collagen orientation, as well as normalized distribution of tenocytes within the

injured tissues (Kaymaz et al, 2015). BA has also been found to accelerate cellular migration, leading to faster wound closure in HS-2 human epithelial cells (Tepedelen et al. 2016). These properties of BA may have influenced topical treatments and supplements for injuries such as facial wounds (Nzietchueng et al. 2002), with some studies suggesting it may one day be used to address chemotherapy induced inflammation (Tepedelen et al. 2016) and wound healing.

1.3 Curcumin

Alongside boric acid, the compound curcumin has also demonstrated potential within the context of wound healing. Historically used as a natural medicine in China and India (Topman et al, 2012), curcumin has been touted for its demonstrated antioxidant and anti-inflammatory properties (Maheshwari et al, 2006) as well as potential anti-cancer applications. One of the primary interests regarding curcumin concerns its apparent ability to negatively regulate a variety of oncogenic molecules, including growth factors, transcription factors, and inflammatory cytokines (Allegra et al, 2017). Curcumin has been shown to inhibit NF-κB, a family of transcription factors associated with carcinogenesis and proliferation activity, by preventing the phosphorylation and degradation of its inhibitor IκBα (Allegra et al. 2017, Singh & Aggarwal 1995). However, curcumin appears to behave differently when interacting with non-cancerous cells (Allegra et al, 2017), demonstrating promotional rather than inhibitory effects.

In 2011, it was demonstrated that curcumin promotes the wound healing process at low doses in early passage human skin fibroblasts (Demirovic & Rattan et al. 2011). While the study found that curcumin dosages within the range of 5-20μM produced inhibitory and cytotoxic effects, it was shown that doses around 0.25μM stimulated wound closure

(Demirovic & Rattan et al. 2011). Further, curcumin treated wound biopsies have been shown to contain larger quantities of infiltrating macrophages, neutrophils, and fibroblasts when compared to untreated wounds (Maheshwari et al. 2006). This indicates that curcumin stimulates both the inflammation and migration-proliferation stages of wound healing which involve elevated quantities of these cell types (Falanga et al. 2005). However, while increased fibroblast appearance suggests enhanced migration/proliferation, additional studies have demonstrated that curcumin most likely fails to influence collective fibroblast migration *in vitro* (Topman et al, 2012). Ultimately, curcumin demonstrates potential wound healing applications that require further exploration.

CHAPTER 2

METHODOLOGY

This study consisted of three stages: optimization, cell migration quantification via wound healing assays, and cell proliferation quantification via MTS assays. Additionally, the optimization studies were further divided into the optimization of wound healing assays and optimal dosage determination. This ensured the methodology appropriately answered the research question through an experimental process of inquiry.

2.1 Cell Culture

Mouse fibroblasts cells (3T3) ware selected for their ability to demonstrate collective cell migration (Bindschadler & McGrath, 2006) and were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, and 1% Penicillin-Streptomycin antibiotic cocktail. Cells were seeded at confluency into 48-and 96 well plates for cell migration and proliferation assays.

2.2 Wound Infliction Optimization

To quantify collective cell migration *in vitro,* a wound healing assay was utilized. For the assay, a wound is inflicted onto a confluent cell monolayer and then tracked for gradual closure over a given period of time, typically 24 hours post treatment. By monitoring closure over time, it becomes possible to determine the rate at which cells migrate when injured. While wound healing assays are commonly used within a research setting, there is no standardized approach to wound infliction. To determine the most effective method for

wound infliction, several methods were tested in this study for consistency of the created wound. Two variations of a stamp assay, a method which had been utilized a reusable stamp to create reproducible and geometrically defined wounds (Riahi et al. 2012), were tested alongside a variety of chemical wound infliction and the most frequently used scratch assay, which typically implements a linear scratch induced via a micropipette tip, razor, or other tools (Grada et al. 2017).

To test the consistency and repeatability of each wound infliction method, wounds were created on confluent cells cultured in 48 well plates in replicates of four. Images were taken immediately after infliction and analyzed by using ImageJ (NIH. Bethesda, Maryland). Wounds whose size could not effectively be determined using the MRI Wound Healing Tool were analyzed manually using ImageJ's freehand selection and measurement tools. Repeatability of wound creation was quantified using coefficients of variation (CV) (Eq. 1,) which finds the ratio of the standard deviation to the mean for a set of wound areas. As each method of wound infliction creates wounds of different scales, CV values were employed as they are unitless and allow for variation to be compared regardless of relative size. Lower CV values indicate greater consistency of area, while larger CV values indicate high variability between samples in a set.

$$CV = \frac{\sigma\ wound\ area\ (\mu m\text{^}2)}{\mu \text{ wound area } (\mu\text{m}\text{^}2)}$$

2.2.1 Stamp Assays

For the two variations of the stamp assay, common household and laboratory materials were used to create easily sterilizable and durable reusable stamps. One stamp was created using a 1 mL serological pipette trimmed to approximately 2 inches (Fig. 2.1). Once trimmed, the pipette was sealed at either end using hot melt adhesive to create one solid piece that could be easily sterilized using ethanol. Both capped ends were then sanded down and polished using fine grit sandpaper to create smooth and even surfaces. For the second stamp, a 1 mm steel drill bit was modified so that its blunt end was capped with hot melt adhesive. Similar to the first stamp, the adhesive was again polished using fine grit sandpaper to create a smooth surface. Early trials, where an adhesive cap was not used, found that the blunt end of a drill bit would scratch the bottom of a cell culture plate, impeding the imaging of wounds.

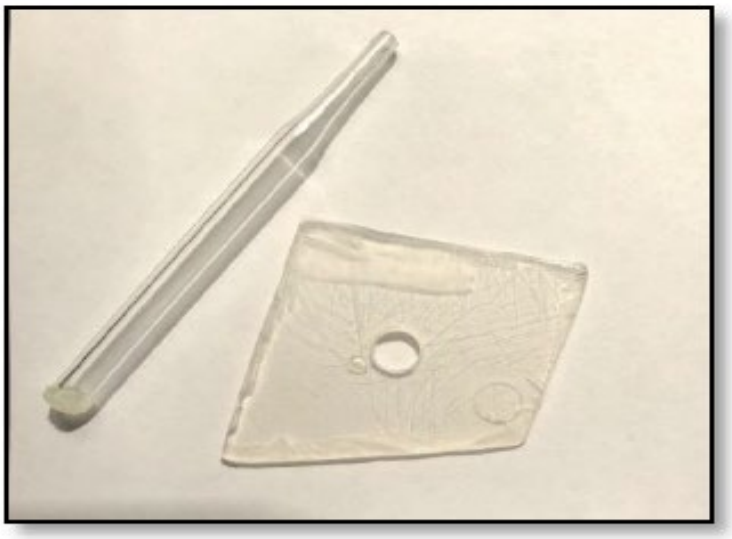

Figure 2.1: Modified 1 mL serological pipette stamp and 1 inch sterilizable plastic guide

2.2.2 Chemical Assay

For the chemical variation of wound infliction, the study opted to use NaOH because of its demonstrated usage in other studies, including a paper that racked wound healing in human bronchial epithelial cells *in vitro* (Legrand et al. 1999), as well as its ready availability in the lab. To create wounds, 0.5 µL of 1 M NaOH was deposited onto a confluent cell monolayer using a micropipette aimed at the center of each well. The NaOH droplets were then rapidly neutralized using approximately 1 mL of phosphate buffered saline (PBS) to prevent total cell destruction (Fig. 2.2).

Figure 2.2: Wound created using chemical assay method

2.2.3 Scratch Assay

This study opted to test the micropipette variation of the scratch assay because of the availability of inexpensive micropipette tips within a lab setting. For the assay, a straight line was drawn using a 200 µL micropipette tip across each cell containing well in a single vertical motion. This method created long geometrically defined wounds with two directions for cell migration to occur from (Fig. 2.3).

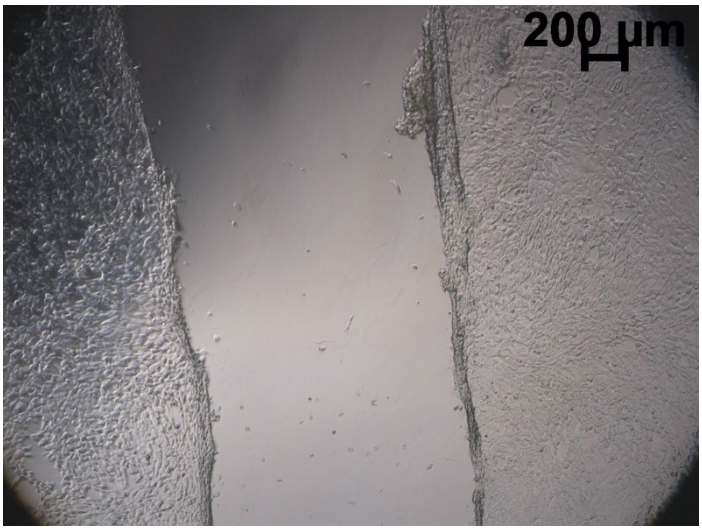

Figure 2.3: Wound created using scratch assay method

2.3 Quantification of Cell Migration

To quantify collective cell migration, this study utilized the optimized stamp wound healing assay from section 2.2. Cells were cultured in 48 well plates until confluency was achieved. Each plate contained 20 replicates treated with the concentrations of either BA (10um-1mM) or curcumin (1um-25um), 4 replicates treated with the FGF positive control at a concentration of 100 ng/mL, and 4 untreated replicates. Wounds were treated directly following infliction, and images were taken at 0 hours and 24 hours to determine the average rate of closure (µm/hour) and average percent wound closure (μm^2).

2.4 MTS Assay

In order to gain a quantitative estimate of the impacts of BA and curcumin on cell proliferation, an MTS tetrazolium assay was utilized. While studies of similar scope have elected to use an MTT tetrazolium assay (Tepedelen et al, 2016), the MTS assay offers a more streamlined and efficient process as the reagent does not require the use of DMSO as a solvent (Riss et al, 2013). By allowing cells to reduce the MTS tetrazolium salt reagent

into a colored formazan product, cell metabolic and proliferative activity can be directly quantified by measuring absorbance of light at 490 nm.

For the assay, 3T3 fibroblasts were cultured in 96-well cell culture plates with a seeding density of $3x10^4$ cells per well at 37° C with ~5% CO_2 for 24 hours. Once cells had been allowed to adhere following the initial incubation period, cells were exposed to treatment group same as the migration assay for both BA (10um-1mM) or curcumin (1um-25um), and then incubated for an additional 4 hours. Longer periods of time allowed the 3T3 cells to reach confluency, causing contact inhibition and carrying capacity to inhibit any further proliferative activity. After 4 hours post incubation, 20 µL of the MTS assay reagent were pipetted into each well before the plate was returned to the incubator to allow the reagent to metabolize. After one and four hours, absorbance at 490 nm was recorded using a 96-well plate reader. The same procedure was done for the 24- and 48-hour groups as well. Recording absorbance at three separate time points allowed for initial and change in proliferative activity over time to be determined, ensuring the data was comparable across the BA and curcumin samples.

CHAPTER 3

RESULTS

3.1 Wound Infliction Optimization Results

It was determined that the first iteration of the stamp assay, which utilized a modified 1 mL serological pipette tip stamp, produced the most consistent wounds with the lowest CV value of 11.7% in comparison to other methods. While the linear scratch method had a similar CV value of 15.4%, the wounds produced by the method possess boundaries that extended beyond the scope of the microscope's camera to track. As a result, it is difficult to determine whether the wound is being imaged in the same place across multiple time points. This can skew results and prevent accurate area determination over time. The version of the chemical assay tested in this study yielded wounds with the extremely variable areas, as illustrated by a high CV value of 41.1% (Fig. 3.1). This is likely because it is difficult to create geometrically defined and consistent wounds using a liquid media. Further, 1 M NaOH, even in quantities as small as 0.5 µL, is highly corrosive and rapidly kills cells. Additionally, chemically induced wounds were significantly larger than those inflicted using other methods with a mean area of 985343.3 μm^2. This produced imaging limitations similar to those posed by the linear scratch assay. Ultimately, the study opted to use the 1 mL serological pipette stamp for the wound healing assay because of its demonstrated consistency and ability to be easily sterilized.

Samples with NaOH inflicted wounds saw significantly lower cell viability, including some samples with total cell death in an earlier trial. Additionally, chemically induced wounds were significantly larger than those inflicted using other methods with a mean area of 985343.3 μm². This produced imaging limitations similar to those posed by the linear scratch assay. Ultimately, the study opted to use the 1 mL serological pipette stamp for the wound healing assay because of its demonstrated consistency and ability to be easily sterilized.

A

Type of Wound	Standard Deviation (μm²) at 0 hrs	Mean at 0 hrs (μm²)	Coefficient of Variance (%)
Linear Scratch	372034.519	2420845	15.368%
Drill Bit	84169.284	449976.5	18.705%
1 mLSerological Pipet Tip	49057.488	419782.5	11.686%
NaOH Chemical	404542.873	985343.3	41.056%

B

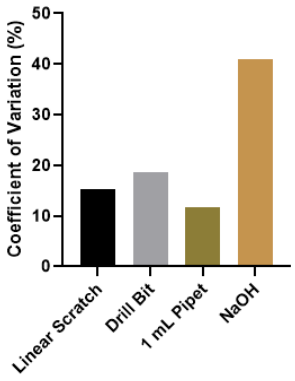

Figure 3.1: Optimizing wound infliction method A: Standard deviation (μm²), mean area (μm²), and coefficient of variation values for each type of wound. Magnitude of area varies across assays, resulting in significantly different standard deviation values. B: Coefficients of variation for the linear scratch assay, drill bit stamp assay, 1 mL modified serological pipette stamp assay, and NaOH chemical assay shown as a vertical bar chart.

3.2 Dose dependent study of CAM compounds in wound healing

3.2.1 Boric Acid

To determine the optimal concentration of BA for wound healing applications, various concentrations were tested using the 1 mL serological pipette stamp wound healing assay. Following wound infliction, cells were treated to BA at various concentrations of (10 μM-10 mM). (Tepedelen et al, 2016). Fibroblast growth factor (FGF) was used as a

positive control at a concentration of 30 ng/mL alongside a set of untreated sample control. Images were taken of the samples at 0 and 24 hours and then analyzed to determine average percent wound closure (μm²) and average rate of closure (μm²/hour). It was determined that the optimal concentration of BA within the context of wound healing is 10 μM (Fig. 3.2).

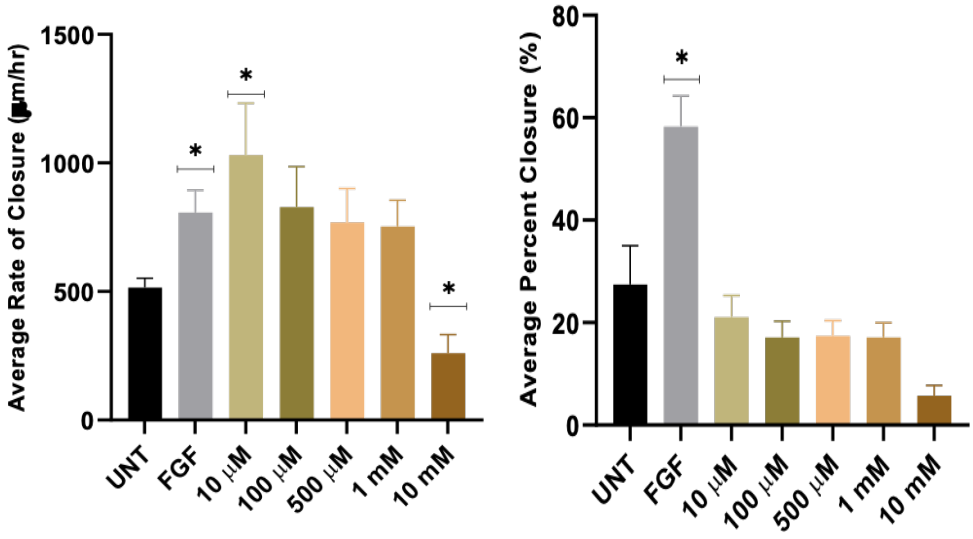

Figure 3.2: Average rates of closure (μm/hr) and average percent closure (%) for tested concentrations over 24 hours via wound healing assays for BA. Fibroblast growth factor (FGF) at 100ng/ml (positive control) and untreated (negative control). *showing significance compared against the untreated group (UNT).

3.2.2 Curcumin

The optimal concentration of curcumin was also found using the 1 mL serological pipet stamp wound healing assays. Wounds were exposed to curcumin at concentrations of (0.25-10μM). Fibroblast growth factor (FGF) was used as a positive control at a concentration of 100 ng/mL alongside a set of untreated sample control. Images were taken of the samples at 0 and 24 hours and then analyzed to the determine average percent wound

closure (μm²) and average rate of closure (μm/hour). It was determined that the optimal concentration of curcumin within the context of wound healing is 1 μM (Fig. 3.3).

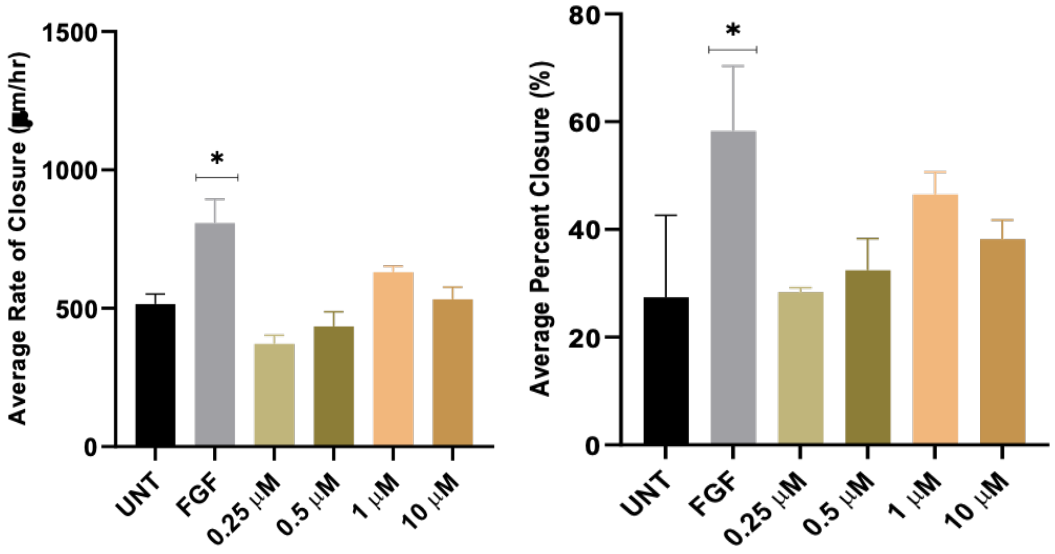

Figure 3.3: Average rates of closure (μm/hr) and average percent closure (%) for tested concentrations over 24 hours via wound healing assays for curcumin Fibroblast growth factor (FGF) at 100ng/ml (positive control) and untreated (negative control). *showing significance compared against the untreated

3.3 Cell proliferation assays

In order to gain a quantitative estimate of the impacts of BA and curcumin on cell proliferation, an MTS tetrazolium assay was utilized. By allowing cells to reduce the MTS tetrazolium salt reagent into a colored formazan product, cell metabolic and proliferative activity can be directly quantified by measuring the absorbance of light at 490 nm. The reading was taken 0-hour, 24-hour, and 48-hour post treatment to determine cell proliferation rate.

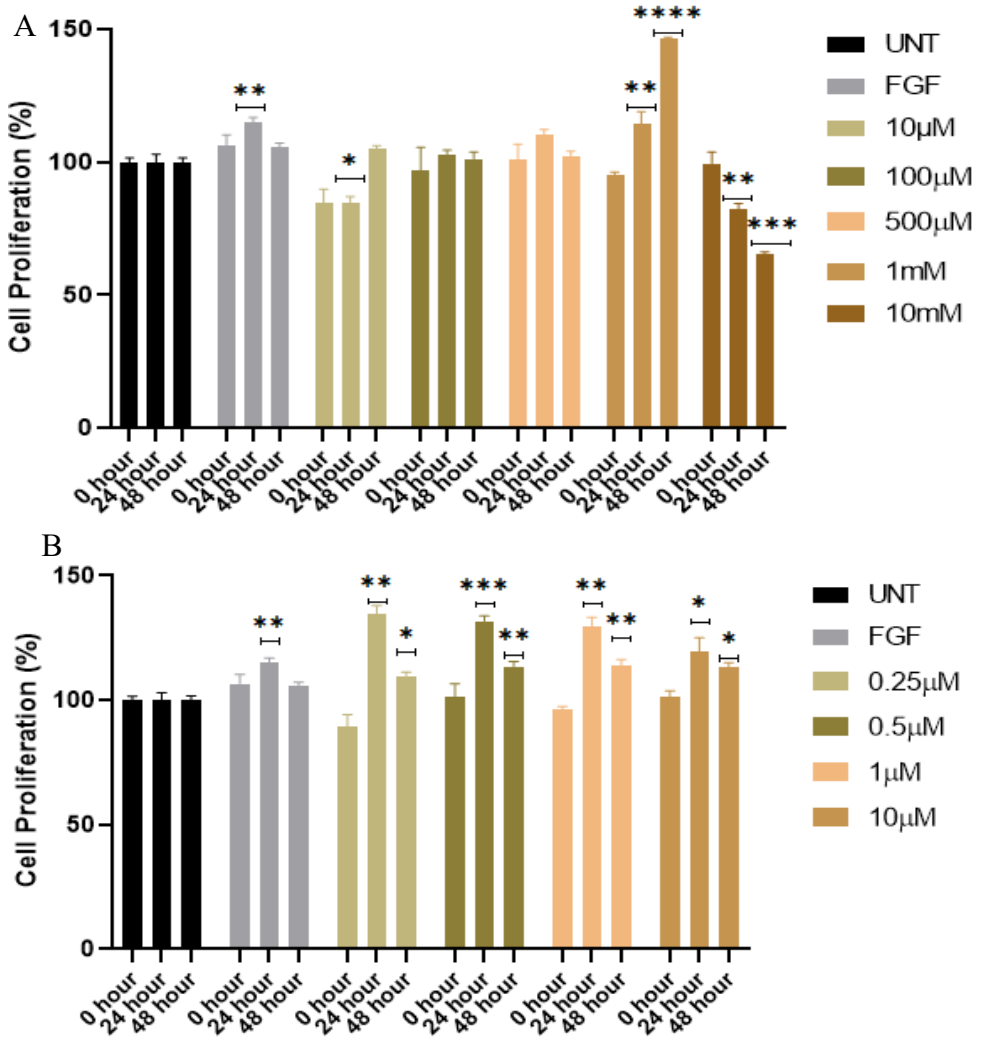

Figure 3.4: Cell proliferation after exposure to A: Boric Acid and B: Curcumin using MTS assays. *P<0.05, **P<0.005, ***P<0.0005, ****P<0.0001; significantly differences compared against the untreated group (100%) for different hours. n=3.

CHAPTER 4

DISCUSSION

This study sought to investigate and compare the impacts that boric acid and curcumin have on wound healing properties in vitro, specifically within the context of collective cell migration and cell proliferation. It was initially hypothesized that BA would have a more pronounced effect on these processes, however, the optimal concentration determination step suggests differently.

For BA, 10 mM BA samples had a faster average rate of closure and greater average percent closure when compared to the other samples with greater BA concentrations (Fig. 6). Yet, when the set is compared to the untreated samples, it does not appear that the BA is producing any stimulatory effects except that 10 mM, which shows a significancy on the average rate of wound closure. This is surprising, considering earlier studies have suggested BA concentrations between 1 and 2.5 mM significantly accelerates these two stages of the wound healing process (Tepedelen et al. 2016). This study was not able to affirm those findings; however, the statistical significance of the optimal concentration determination step is severely limited. In a future iteration of this study, optimal concentration determination should be repeated with additional trials to yield greater certainty. In contrast to the determined optimal concentration of BA, 1 μM curcumin did appear to produce stimulatory effects when compared to untreated and positive control samples.

In an ideal trial, the FGF treated samples would have significantly faster closure rates and greater percent closures than the untreated negative control samples. Nonetheless, 1 μM curcumin performed well compared to its cohort of samples with differing concentrations. When compared the treatment samples against the untreated group, there was no statistical significance.

The MTS assay for BA showed different than the migration assay study, the 1 mM showed to have a higher significance compared against the untreated group for both of the 24 and 48 hours. For curcumin, the study shows 0.25, 0.5 and 1 μM have a higher statistical significance when compared against the untreated group. More repetitions of these studies will be done in mice and human dermal fibroblasts for reproducibility and determining effective concentrations of wound healing potential in BA and curcumin.

CHAPTER 5

CONCLUSION

This study sought to compare the effects of two proposed alternative wound healing treatments, BA and curcumin, specifically within the context of collective cell migration and cell proliferation. By determining which compound has a more significant impact on the wound healing process at these stages, it would become possible to narrow the complementary and alternative medicine (CAM) niche of wound healing treatments. This would provide both medical practitioners and patients a treatment option less invasive and costly than current methods, potentially lessening the burden posed by chronic wounds.

1ml pipette stamp showed the least coefficient of variance, indicating a more robust method to inflict wounds than conventional scratch assays to study various treatment responses in wound healing. 1µM curcumin and 1mM BA concentrations showed better average percent wound closure compared to untreated groups. Boric acid at higher concentrations was needed as compared to the lower concentration of curcumin to aid in *in vitro* wound closure study. Cell proliferation is a significant factor for the assessment of the wound healing potential of drugs.

In our study, over 48 hours, curcumin at lower concentrations (0.5µM -10µM) showed significant cell proliferation, while BA showed significant improvement at 1mM over a positive control. Combinedly, BA and curcumin were well tolerable by cells at their significant concentrations of wound closure compared to a positive control. Further

investigation of CAMs compounds including BA, curcumin, neem, and holy basil will be performed in human primary fibroblasts to assess their wound healing potential.

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BIOGRAPHICAL INFORMATION

Ahmed's personal journey has been full of hope, and has shaped the person he has become, and has helped him to define the legacy he wants to leave in this world. He was born and raised in Somalia, a country in East Africa. His family migrated to the United States of America to seek new opportunities, leaving him back home. After he finished high school in his home country, he moved to the United States to reunite with his family. Having been raised in Somalia, he always had a deep curiosity about the cutting-edge science in developed countries and dreamt about how he could contribute to the scientific community. The motivation to improve his knowledge and skills and his eagerness to be part of the scientific community were the elements that propelled him to pursue his undergraduate education at the University of Texas Arlington (UTA) and study to earn an Honors Bachelor of Science in Biomedical Engineering. He has been working in Dr. Kytai T. Nguyen's lab to develop a nanoparticle-based water filtration device which was funded by the Dean of Engineering at UTA. He also participated in a summer research experience program at the University of Delaware during the summer of 2019 while he participated in the McNair program in the summer of 2020. After graduation, he will be joining the Biomedical Engineering Ph.D. program at Cornell University.