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The Effects of *Staphylococcus aureus* Secreted Products on Fibroblast Lattice Compaction and Correlated Tension Generation.

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BY:

Pratiksha Kshetri

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The Effects of *Staphylococcus aureus* Secreted Products on Fibroblast Lattice Compaction and Correlated Tension Generation.

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By _____

Name Committee Chairperson

_____

Name Committee Member

_____

Name Committee Member

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ABSTRACT OF THESIS

University of Central Oklahoma

Edmond, Oklahoma

NAME: PRATIKSHA KSHETRI

TITLE OF THESIS: The effects of *Staphylococcus aureus* secreted products on fibroblast lattice compaction and correlated tension generation.

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Abstract: Chronic wounds such as diabetic foot ulcers, pressure ulcers, venous leg ulcers have become a worldwide problem, as they fail to heal in a regular period due to the presence of an extended inflammatory stage. Bacterial infection is one contributing factor that impairs the wound healing process. The ability of the dermal fibroblast to migrate, proliferate and differentiate is the critical factor for the normal progression of wound healing. Studies have shown that chronic wound biofilms colonize the dermal tissue which makes it important to investigate dermal fibroblast behavior in the presence of bacterial secreted products. The objective of the study was to determine if products secreted by *Staphylococcus aureus* in planktonic or biofilm phenotypic states would play a role in wound chronicity by affecting fibroblast functions.

A 3-dimensional collagen lattice model was used to study fibroblast tension generation day by day with the presence or absence of the bacterial secretion products. Using this model, we conducted our observation by measuring height and fibroblast tension generation of collagen lattices and followed by immunostaining the lattices to identify structural changes.

On each day of 5-day height measurements of fibroblast lattices, I observed that planktonic conditioned medium (PCM)-treated lattices had the most significant effect on compaction compared to biofilm conditioned medium (BCM), control and TGF-beta treatments. Similarly, when lattices were released PCM inhibited the lattice contraction followed by BCM. Lattices treated with TGF- beta were found to have the highest anchored compaction and were very contractile when released. Compaction and contraction were associated with the number of differentiated myofibroblasts and total cell number with respect to the treatment. Fibroblast lattices exposed to PCM showed no presence of myofibroblasts. Our findings are highly relevant to the medical field curing chronic wounds and should help the scientific community understand chronic wound healing at the cellular level.

Introduction

Wound healing is the process of repairing the injured skin by itself (Li and Wang, 2011). After an injury, to maintain the integrity of skin, it involves normal interconnected and overlapping phases of inflammation, proliferation, contraction, and tissue remodeling. Wound healing involves various cell types such as keratinocytes, fibroblasts, endothelial cells, macrophages, and platelets. To maintain skin integrity, the migration, infiltration, proliferation, and differentiation of these cells are necessary (Barrientos et al., 2008). Skin wound healing is categorized as either acute and or chronic. Acute wounds heal within a predictable time and involve all phases of healing whereas chronic wounds fail to follow the typical phases of wound healing and do not heal or require an extended period, which takes more than three months to heal.

In acute healing, to restore the skin, the damaged capillaries trigger the clotting of blood. While clotting blood, the wound tissue releases multiple pro-inflammatory cytokines such as fibroblast growth factor (FGF), transforming growth factor-beta (TGF-β) and epidermal growth factor (EGF) (S. Guo and L.A. DiPietro., 2010). Tissue repair starts with inflammation where the infiltration of neutrophils, monocytes, lymphocytes, and differentiation of macrophages occur, and these cells migrate toward the wound site (Ian A Darby et al., 2014). Neutrophils phagocytose the invading microbes. Macrophages play critical roles in healing. Macrophages recruit lymphocytes, induce apoptosis and clear apoptotic cells. The phenotypic state of a macrophage changes to a reparative state to promote tissue regeneration that stimulates keratinocytes, fibroblasts, and angiogenesis (S. Guo and L.A. DiPietro, 2010). The reparative state of macrophages is identified by the expression of anti-inflammatory mediators such as Il-R antagonist, decoy IL-1 receptor

type II and IL-10 and production of growth factors such as transforming growth factor (TGF-β1), vascular endothelial growth factors (VEGF), and insulin-like growth factor (IGF)-1. The transition of macrophage to reparative state is the signal that switch the inflammation to proliferation during wound healing (Koh and DiPietro, 2011).

The proliferative phase follows and overlaps with the inflammatory phase. Fibroblasts and endothelial cells are considered as the most important cell types that support capillary growth, the formation of collagen and granulation tissue at the wound site. TGF-β and other growth factors produced in this phase stimulate the fibroblast. Stimulated fibroblasts produce the collagen within the wound site which is the main protein component of the extracellular matrix (ECM). Collagen and fibronectin provide the structural integrity to granulation tissue that enables the fibroblast to change its phenotype into a contractile form called the myofibroblast (Darby and Hewitson, 2007). The myofibroblast present in wounds plays a role in wound closure through its contractile nature. The robust proliferation and ECM synthesis take wound healing to its final phase, i.e., the remodeling phase (Gosain and Dipietro, 2004).

In the remodeling phase, the maximum tensile strength is achieved through reorganization of tissue, in the extracellular matrix. To achieve a normal tissue, the granulation tissue finally gets remodeled, forming scar tissue that is less cellular and vascular (Stunova & Vistejnova, 2018). Type I collagen replaces the type III collagen secreted by fibroblasts in the previous phase. The high contraction and excessive collagen deposition form hypertrophic scarring during wound healing. (Menon et al., 2012).

Chronic wounds fail to follow the orderly process of wound healing and take a more extended period to heal or may not heal at all. It is a common and major health issue.

Chronic wounds are common in people who suffer from leg ulcers, pressure ulcers, and diabetic foot ulcers as well as venous and arterial ulcers. In the United States, around three million people are suffering from pressure ulcers and venous ulcers (Soliman et al., 2018). The total cost to treat chronic wounds in the USA each year is over US$25 billion, with the growing number of patients prevalence of diabetes and other chronic diseases from 6.5 million (Han and Ceilley, 2017). A chronic wound is the result of prolonged inflammation containing proinflammatory cytokines, high protease levels, and neutrophils failing to go through the normal healing process (Wolcott RD et al., 2008). In a chronic wound, the matrix supports inflammatory cells, an d macrophages while fibroblast functions are impaired; together these delay the healing process (Stadelmann, 1998). Further consequences of a continuing chronic wound include septicemia, chronic pain, amputations, and death (Jeffery et al., 2015). Therefore, it is essential to understand the cellular and molecular level of wound healing which will improve the medical approach treating chronic wounds.

Multiple factors affect one or more phases of wound healing. Bacterial infection is one of the significant challenges that impedes wound healing and is responsible for delayed tissue repair in chronic wounds (Jeffery et al., 2015). Most chronic wounds are colonized by bacteria and progress to infection when their host has a weak immune response (Edwards and Harding., 2004). The chronic wound bacteria exist in two states: planktonic and biofilm. A free-living state of bacteria in a liquid is considered planktonic (Percival et al., 2012) whereas a cluster of bacteria attached to a surface and surrounded by a protective extracellular polymeric matrix is considered biofilm (Percival et al., 2015). Both states of bacteria might play a role in the pathogenicity and delay of wound healing in a chronic

wound (Perez Diaz et al., 2016). The two-different phenotypic states of bacteria have different gene regulation and expression. Around 80 % of bacterially infected wounds prevail in the form of biofilms. Biofilms show more resistance to immune response and antibiotics than planktonic (Resch et al., 2005). The biofilm develops a protective microenvironment and is impervious to antibiotics. The wound bed under the bacterial biofilms remains in a persistent inflammatory state (Jeffery et al., 2015). Chronic wounds provide a favorable environment for a bacterial infection which leads to biofilm formation that can delay the healing process (Zhao et al., 2010). *Staphylococcus aureus* and *Pseudomonas aeruginosa* are the predominant bacteria associates common in chronic wounds. These bacteria play an essential role in wound pathogenesis (Guo and DiPietro., 2010). The bacteria association attached to the surface as biofilm in chronic wound pathogenesis is not very well understood (Werthen et al., 2010). Usually, there are more than one bacterial species associated with a wound infection. The most prevalent species in chronic wounds are *Staphylococcus, Pseudomonas aeruginosa, Enterobacter, peptoniphilus, Enterobacter, Stenotrophomonas, Finegoldia and Serratia spp* (Kirker and Garth., 2017; Xu and Z., 2018). We used *S. aureus*, a single strain bacterium for our study to better understand wound pathogenicity. It is one the most prevalent bacteria in chronic wounds that infects and colonizes the wound bed. *S. aureus* causes a wide variety of skin diseases and chronic wound infections. Various studies have been carried out to understand the pathogenicity of *S. aureus* in related chronic wound areas, but its pathogenicity is still unclear. Therefore, it is essential to investigate on how *S. aureus* may be impairing the healing process.

The dermis of the skin is composed of dense irregular connective tissue. The fibroblast serves as the primary resident cell and is responsible for forming new granulation tissue, regulating angiogenesis, supporting re-epithelization, creating new extracellular matrix molecules and synthesizing collagen to provide support for other cells to enable effective wound healing (Stunova, A., and Vistejnova 2018). The fibroblast is mainly responsible for remodeling of tissue, which gives a better understanding of wound healing and other fibrotic disease progressions. A fibroblast synthesizes type I collagen and is most abundantly found in the dermis. During dermal wound healing, ECM signaling has different effects on fibroblast proliferation, migration, collagen production and differentiation (Tracy et al., 2016).

After an injury, the fibroblast starts migrating towards the wound site while it reorganizes the ECM along the lines of stress providing the mechanical tissue tension for the cell (Tomasek et al., 2002). With this tension, the fibroblast starts differentiating its phenotype to the myofibroblast, a cell that generates high contractile force and synthesizes ECM to facilitate wound closure(Li & Wang, 2011). It is characterized by having stress fibers that express cytoplasmic α-smooth muscle actin fibers (Tomasek, Gabbiani, Hinz, Chaponnier, & Brown, 2002) and demonstrating high contractile force (Hinz et al., 2007) through the interaction of actin with non-muscle myosin II. Tension generation is essential to enable the myofibroblasts phenotype (Tomasek et al., 2002) and during wound closure the presence of myofibroblasts in granulation tissue is essential. The activation of Rho promotes the assembly of stress fibers and fibroblasts contraction. The activity of myosin light chain kinase (MLCK) and myosin light chain phosphatase (MLCPPase) enzymes regulates fibroblast contraction (Parizi and et al., 2000). Growth factors produced by

platelets and cells present in the blood clot trigger further differentiation into the myofibroblast (Darby and Hewitson., 2007). TGF-β, ECM proteins and mechanical stiffness activate the differentiation of fibroblast (Van De Water et al., 2013). Alpha smooth muscle actin incorporation into stress fibers provides structural elements and focal adhesions containing proteins such as vinculin (Vaughan et al., 2000). In chronic wounds, the hypoxic environment decreases myofibroblast formation (Van De Water et al., 2013). Matrix remodeling and fibroblast proliferation change the mechanical properties of the tissue. These mechanical changes are important to study to provide knowledge of wound healing.

Wound biofilms and chronicity can be studied using appropriate yet simplified *in vitro* models. Collagen matrices provide a well-established in vitro model that mimics wound-healing granulation tissue and wound contraction (Tomasek et al., 1992; Grinnell, 1994; Carlson and Longaker, 2004; Herwig and Vaughan, 2014). The lattice model simulates wound healing mechanisms and offers numerous opportunities for reasonable investigation of the fibroblast role during wound contraction. Collagen lattices are the combination of fibroblasts and type I collagen. The fibroblast-populated collagen lattice simulates granulation tissue that acts similarly to the processes of proliferation, apoptosis, protein synthesis, and contraction (Carlson and Longaker, 2004). In this model, the fibroblasts start spreading, migrating and reorganizing the collagen matrix using tractional force. The mechanical tension produced in the collagen lattice is vital for fibroblast differentiation. Myofibroblasts need a tension-maintaining environment which is satisfied by an anchored collagen lattice model. The lattices were treated with TGF-β as a positive control for increased migration and contraction as expected due to its presence in normal wound

healing. It is appropriate to use TGF-β because in lattices the treated fibroblasts are activated. When the lattices are physically released, tension is relieved, and the lattice will contract; subsequently, the cells may undergo apoptosis (Grinnell., 1999) or revert to a fibroblast phenotype. These things suggest that the model we use is entirely appropriate for studying at least two phases of wound healing, i.e. granulation and contraction. By recognizing the need to study tension regeneration and its ability to help increase the rate of chronic wound healing, our understanding of wound biofilms and wound chronicity can improve. This study will help improve the knowledge of biofilms within the scientific community, which may foster future research to improve chronic wound.

Objective of the study

The purpose of the research was to investigate the *Staphylococcus aureus* biofilm and planktonic secreted products' effects on tension generation by normal dermal fibroblasts in collagen lattices, and whether these effects are related to proliferation and/or myofibroblast differentiation. This study helps to bridge the knowledge gap between wound contraction and extended inflammation, where myofibroblast differentiation that could lead to wound closure is prohibited. Since the pathogenicity of these bacterial biofilms on dermal tissue is unknown, it is important to investigate fibroblast behavior with the presence of bacterial biofilms' secreted products (Kirker et al. 2012). Fibroblasts play a critical role in exerting force, first by using migration mechanisms and then by differentiating into myofibroblasts and generating tension. We hypothesized that the secreted bacterial products of *S. aureus* inhibit the fibroblast contraction of the lattices which is correlated to the proliferation and differentiation of myofibroblasts. We proposed to use a simplified in *vitro* model to better understand wound biofilms and chronicity. It is

important to investigate the effects of bacterial biofilm on dermal fibroblasts using an anchored 3-D collagen matrix since it closely resembles *in vivo* tissue-like attributes, including morphology, cell division, and protein expression compared to conventional 2-D models (Grinnell, 1994) (Grinnell et al., 2006). Also, we observed the collagen lattice compaction over time which helped us monitor the tissue tension increase in a matrix to its maximum and afterward.

Materials and Methods

Fibroblast culture: Normal human dermal fibroblasts were cultured in a T-75 tissue culture flask using Dulbecco's Modified Eagle Medium (DMEM) supplemented with 5% fetal bovine serum, 2mM glutamine, and 1% antibiotic – anti-mycotic and incubated at 37°C in a CO_2 incubator. We carried out all experiments when cells were actively dividing and in log-phase growth. The UCO Institutional Review Board approved our studies.

HaCaT culture: HaCaT an immortalized human epidermal keratinocyte, (Boukamp et al.,1988) were cultured in T-25 tissue culture flask maintained with DMEM containing 10% Fetal bovine serum and Penicillin/ Streptomycin in a CO_2 incubator at 37°C. In a 24-well plate, the cell concentration of $3x10^4$ per well were prepared to run an experiment.

Biofilm Conditioned media (BCM): *Staphylococcus aureus* ATCC 6538 strain is one of the prevalent biofilm forming bacteria, so we prepared its biofilm in tissue culture inserts. Briefly, tryptic soy broth (TSB) was used to grow bacteria and adhere to on the surface of tissue culture inserts. An overnight culture of *S. aureus* was inoculated in 5 ml of TSB. The inoculated bacteria of 10 µl was placed into tissue culture inserts. Every 24 hrs. for a total of 72 hrs. The broth was replaced, and inserts were then moved to new wells of 24-well

plates. After 72 hrs., the inserts with bacteria were washed with phosphate buffer solutions to remove excess TSB. Finally, DMEM without serum was supplied for 72 hrs. and every 24 hrs. Then exposed media was collected and tissue culture inserts were transferred to new wells. The secretions produced by *S. aureus* biofilm were collected and filtered using a 0.45μm syringe and were stored at -20°C. (Tankersley et al. 2014).

Planktonic Conditioned Media (PCM): PCM was prepared by overnight culture of *S. aureus* bacteria that was inoculated in 5ml of TSB and was placed on a rotary shaker for agitation at 150 rpm at 37°C. After 24 hrs. of incubation the tube of bacteria was centrifuged at 1500 rpm for 7 minutes and the TSB was removed. Then 5ml of PBS with pH 7.4 was used to wash the pellet. 5ml of DMEM without serum was then supplied for another 24 hrs., if necessary 48 hrs. in agitation at 150 rpm. Finally, the culture was centrifuged and the supernatant DMEM was collected, filtered and stored at -20°C (Tankersley et al., 2014).

Conditioned medium

Biofilm conditioned media was produced by growing *S. aureus* ATCC 6538 biofilms on a tissue culture inserts and the planktonic *S. aureus* was grown in a cell culture medium with agitation to produce the planktonic conditioned medium. In some cases, the pH of both BCM and PCM in the media was too acidic. The pH indicator in the media (phenol red) indicated acidic conditions (turned yellow) in both PCM and BCM under submerged condition (Tankersley et al., 2014). Subsequent emerging of the bacteria in the media as suggested by Kirker Kelly (personal communication) reduced the pH variability.

Detection of Inflammatory cytokines:

This experiment was carried out to observe if bacterial secretion products cause any inflammation. HaCaT cells were plated in a 24-well tissue culture plate at a concentration of 10^5 cells/well. After the cells reached 80-90 % confluency, they were treated with BCM, PCM and the vehicle control (DMEM lacking serum and antibiotics) for 24 hrs at 37°C. After 24 hours of exposure the BCM, PCM, and control media were collected and stored at -20°C. Some of the BCM, PCM, and control from each specific group was used as a treatment for a collagen lattice to study effects on myofibroblast and the remainder were stored until the ELISA was performed. An ELISA kit (R&D Systems) was used as per instructions to test for IL8/CXCL8 after the supernatants were collected from the treatment and control group (Tankersley et al. 2014).

Optimization of the Collagen Lattice Assay: The preparation of collagen lattices is a critical step which requires an optimal time for mixing of cell and collagen components and then plating in a pre-warmed dish. We used three different collagen distributing companies' products: Millipore, Corning, and Invitrogen. Three different vendors collagen products were mixed and plated at different time periods. The number of cells falling through to the plate underneath the collagen lattice and the texture of collagen was observed, to determine the effective plating time required to achieve homogeneous collagen composition and uniform cell distribution. Image J texture plugin was used to analyze the flocculence of the collagen.

Anchored collagen lattice preparation: The group of anchored collagen lattice assay was prepared with a mixture of dermal fibroblasts and type I collagen (Vaughan et al., 2000). To equilibrate the acidic and hypotonic collagen, the components 0.1N NaOH, 10X

EMEM, 10X $NaHCO_3$ were mixed to bovine collagen (final concentration 1mg/ml) to make an isotonic collagen solution of pH 7.4. Then, cells and collagen solution were mixed with the collagen solution on ice for the optimized time (4 minutes) and 80 µl drops were plated on 40 mm tissue culture plates (TPP; Midwest Scientific) then allowed to polymerize at 37°C for one hour. Subsequently, 2 ml of growth media in the presence or absence of bacterial biofilm extracts were added to each plate. TGF-β (1µg/ml) was used as a positive control to stimulate the fibroblast differentiation to myofibroblast. Then, lattices were incubated in a 5% CO_2 incubator for specific times, ranging from 24 hours to 5 days to allow us to find the peak of tension in the presence or absence of extracts or HaCaT-exposed bacterial secretions. During the 5-day incubation, the lattices' height was measured every day (as a measure of compaction). On the final day, the anchored lattices were released from the bottom of the dish, and contraction (diameter reduction over time) was measured. A replicate anchored lattice was used for immunostaining to quantify cell proliferation and differentiation as described below.

Lattice height Measurement: Human fibroblast collagen lattice height was measured every 24 hrs to predict the associated fibroblast contraction (manuscript in preparation). The collagen lattice was placed on an inverted microscope stage (Leica Primovert). The matrix was observed under the highest magnification (200x total magnification) at the light source maximum. The lattice apex was moved to the middle of the light circle and focused through the collagen fibers which were on the upper surface of the lattice keeping the fine and coarse focus knob at the marked zero line. Using the fine focus, we visually focused through the collagen lattice to its bottom. The presence of a flattened cell in focus in the collagen was identified as the bottom of the matrix. We counted the number-line units on

each turn of fine focus knob while focusing through the entire depth of the lattice (from top to bottom). We measured the height of the matrix every day until day 5. The collected height values were imported into a spreadsheet for further analysis (Microsoft Excel). The height values were changed into micrometers using formula (= ((cell value/640) *1000)). The calibration of the depth measurement and preparation of the microscope were already performed in our lab, where we found that 640 units corresponded to one millimeter, the thickness of a microscope slide. Everyday measurement of lattice height helped us to predict the correlated contraction until day 5.

Stress-relaxed collagen lattice assay: A 3-D stress-relaxed collagen lattice model was used to indirectly assess tension generated by normal dermal fibroblasts with the presence of *Staphylococcus aureus* BCM and PCM. It was performed on day 5 using a dissecting microscope (Olympus SZ-61) and a camera with software (SPOT Diagnostics) to collect images (Herwig and Vaughan, 2014). Lattices were first imaged and photographed while anchored. Some of the lattices were released from the bottom of the dish and pictures were taken at 1 minute, 2 minutes, 10 minutes, 30 minutes, and 60 minutes after release. They were released to observe the speed and intensity of contraction as an indirect method for studying tension generation (Tomasek et al., 1992). Collected images were then analyzed using a software program (ImageJ; Herwig and Vaughan, 2014). The mean lattice diameter change over time for each treatment was graphed with the standard deviation represented by error bars.

Immunostaining to determine proliferation and myofibroblasts differentiation: An immunofluorescence technique was used to visualize labeled nucleotide incorporated during mitosis, and alpha-smooth muscle actin, the hallmark protein of the myofibroblasts

(Hinz et al., 2007). Ethynyl-deoxyuridine (EdU) is a modified nucleotide that binds DNA that is being replicated before cell proliferation. On day 5, two to three numbers of lattices from control and extract-treated groups were pulsed for one hour with an EdU nucleotide for proliferation analysis (Vaughan et al., 2014). EdU-pulsed lattices were fixed with paraformaldehyde, permeabilized with ice-cold methanol, and stained with mouse anti-human alpha-smooth muscle actin (to identify myofibroblasts differentiation), followed by secondary antibody goat anti-mouse Alexa488 to visualize the actin antibody. A click-chemical reaction then linked a fluorescent probe (Alexa 594) to the incorporated EdU nucleotides (Vaughan et al.,2014). EdU staining facilitated the observation of fibroblast and myofibroblast proliferation within the lattice section. DAPI staining was used as a blue nuclear counterstain. Lattices stains were photographed with an inverted fluorescence microscope (Olympus IX-71). A camera (DP72) photographed multiple images from the same field of view, and a software program (CellSens) was used to combine the blue (DAPI), red (EdU) and green (a-sm actin) photographs into a single image (figure 7) to enable quantification of 4 cell types: Non-proliferative fibroblasts (NPF), Proliferative fibroblasts (PF), Non- proliferative myofibroblasts (NPM) and proliferative myofibroblasts (PM).

Statistical analysis:

To determine if there were significant differences in treatments with respect to mean change in contraction and mean height a repeated measure analysis of covariance (ANCOVA) was performed. For contraction the response variable was the change in contraction from baseline (time 0) at times 1, 2, 10, 30 and 60 minutes, and the factors were

treatment (control, biofilm, planktonic and TGF-β) and ln(time) with baseline contraction as the covariate. For compaction, the response variable was the change in height from baseline (day 0) at days 1, 2, 3, 4, and 5, and the factors were the treatment (control, biofilm, planktonic, TGF-β) and ln (days) with baseline height as the covariate. To achieve linearity, the time and days factors were log-transformed. In both cases, an unstructured covariance matrix for the repeated measurements was assumed. The ANCOVA was followed by Tukey's multiple comparisons and was done using proc mixed in SAS. V.9.4. The correlation between the mean total number of cells and the mean height at day 5 was computed using SAS v.9.4. ANOVA test was performed to analyze the significant difference in the cytokine production between the treatment.

Results

Optimization generated reproducible results

Cell distribution changed with mixing time. We observed that when lattices were plated early with little or no mixing, cells would sink to lower regions of the collagen lattices and onto the plate below as shown in figure 1A and B. Graphed results showed different times of mixing were necessary to achieve even distribution (figure 2).

Collagen lattice organization changed with mixing time. When collagen is brought to isotonic conditions (37°C, and pH 7.4), it begins to polymerize or gel. After too much mixing the collagen texture became flocculent (resembling cotton) (Elsdale and Bard.,1972) (figure 3A), likely because collagen pre-polymerized during the mixing process. We used Image J texture plugin to identify when flocculence became prevalent for each collagen type (figure 3B). In our study for each collagen, there was an optimum mixing time to allow best distribution of cells and lowest flocculence. Bovine collagen of concentration at 1mg/ml was optimized by mixing within a minute and plating by 4 minutes (figure 4). The optimum time for even distribution and homogenous collagen consistency is shown in figure 4. All subsequent experiments were set up during this optimum time.

PCM inhibited lattice height compaction to a greater extent than BCM

Anchored lattices in control conditions are height-compliant (susceptible to compaction in the z-axis) and the compaction by fibroblasts in control lattices was greater than compaction in the presence of bacterial secreted products. The control height compaction (figure 5, blue line) agrees with a previous study (Guidry and Grinnell,1985) where the lattice height decreased in the presence of growth media. The interaction between

treatments and ln(days) was significant (F=14.5, p = 0.001). The differences in the treatment means were analyzed each day separately. The mean height for all three treatments were all significantly different ($p < 0.001$). Each day of lattice height measurement (figure5), the PCM-treatment inhibited collagen lattice compaction to a greater extent than did the BCM treatment. Lattices treated with TGF-β were found to be at least inhibited, resulting in the smallest mean lattice height (greatest compaction).

PCM inhibited collagen lattice contraction:

The contraction of a lattice (i.e., reduction in diameter over time) provides an indirect measurement of tension generation (Tomasek, Haaksma, Eddy, & Vaughan, 1992). We predicted from our result of inhibited lattice compaction by the PCM treatment that subsequent lattice contraction would also be inhibited. The interaction between treatment and ln (time) was significant (F=10.5, p= 0.001). Also, at each time, the differences in the treatment means were analyzed separately at each time. There was no significant difference between control and biofilm at the 1-minute time point (t=-2.8, p=0.073) but at all other time point 2, 10, 30 and 60 minutes there were significant differences between all other treatments ($p< 0.001$) (Figure 6). PCM-treated lattices were significantly different ($p< 0.001$) from TGF-β, control, and BCM at every released time point. The PCM treated lattices contracted the least, and TGF-β-treated lattices contracted the most with the smallest resultant lattice diameter (Figure 6).

Ratios of the 4-measured cell types changed with treatments

The inhibition of lattice compaction and contraction was correlated with cell number as previously published (Tomasek et al., 1992) and myofibroblast differentiation as

previously published (Vaughan et al., 2000). The total number of cells for each type of cell and treatment are displayed in the Table 1. There was a significant association between type of cell and treatment ($\chi 2 = 14.1$, $p = 0.003$) for NPF and PF. In the PCM-treated lattices there were significantly more NPF cells, and in the TGF-β treatment, there were fewer NPF cells than expected. The proliferative fibroblasts were significantly fewer in the planktonic treated lattices whereas the TGF-β treated had significantly more PF cells. The values of myofibroblast differentiation and cell number are indicated in Table 1. There were little or no proliferating myofibroblasts in PCM-treated lattices.

Inflammatory cytokines response in HaCaT cells exposed to secretion products

ELISA detected the production of inflammatory cytokine IL-8 /CXCL8, a pro-inflammatory cytokine. It is important to detect IL-8 because of a pro-fibrogenic chemokine involved in fibrotic skin disease (Sahin and Wasmuth, 2013). The presence of IL-8 was detected highest in BCM than PCM or control (figure 8) as a result suggested previously (Tankersley and Brennan 2014). PCM exposed to HaCaT cells did not produce a significant amount of IL-8. A significant difference ($p < 0.05$) was observed between all the groups.

HaCaT exposed PCM inhibited the collagen lattice height

Bacterial secretion products were exposed to HaCaT cells and were collected to treat collagen lattices. There was significant difference ($F = 400.0$, $p < 0.001$) between treatment and ln(days). The differences in the treatment each day were separately analyzed. At day 1, there was a significant difference between control and planktonic ($t = -12.9$, $p < 0.001$) and biofilm and planktonic ($t = -8.1$, $p < 0.001$), but control and biofilm were not

significantly different (t = -2.2, p = 0.143). Planktonic treatment resulted in the highest mean lattice height (least compaction) and control with the smallest mean lattice height (greatest compaction) (Figure 10). The mean heights of the three treatments at all other days were significantly different ($p < 0.05$) from each other.

HaCaT exposed PCM inhibited the contraction

The tension generation with the presence or absence of HaCaT-exposed bacterial secretion was analyzed via stress relaxed contraction. The interaction between treatment and ln(time) was not significant (F = 1.9, p = 0.205), but the main effect for treatment was (F = 146.0, $p < 0.001$). Tukey's multiple comparisons for treatment indicate there is a significant difference in the mean contraction of all three treatments ($p < 0.05$), with planktonic resulting in the least contraction and control affected to most contraction (figure 11).

HaCaT- exposed secretion affected proliferation and differentiation

Four different phenotypes of cells were quantified after the treatment with HaCaT exposed BCM and PCM. The total number of cells for each type of cell and treatment are displayed in Table 2. There was no significant association between NPMF and PMF of different treatment ($\chi 2 = 4.2$, $p = 0.124$). For NPF and PMF, there was also no significant association between type of treatments ($\chi 2 = 2.8$, $p = 0.244$). Whereas visually we noticed number of cells were fewer in PCM treated lattices compared to control. Also, there were fewer myofibroblasts, whether proliferating or not in the presence of PCM compared to BCM and control.

Discussion

This research was focused on addressing the effect of bacterial conditioned media exposed to human fibroblasts and evaluating its function on compaction, contraction, proliferation and differentiation using the appropriate model. We used the anchored 3-D collagen lattice model to assess fibroblast functions accurately. We optimized the collagen matrix model for proper study because it has much variability. Therefore, optimization was necessary to avoid variability and achieve reproducible results. The soluble products produced by planktonic *S. aureus* inhibited compaction and contraction in a fibroblast collagen lattice, and it was correlated with cell number and myofibroblast. Also, the fibroblast response to bacterial secretion products was examined by quantifying inflammatory cytokine IL8.

To set up the experiment, finding the optimum mixing and plating time was extremely important. Our previous results with BCM and PCM varied with each experiment. We observed that this variability was correlated with uneven distribution of cells or heterogeneous mixture of collagen (M. B. Vaughan, personal observations). These properties change with mixing and plating time. To increase reproducibility, we dedicated a set of lattices to finding the appropriate mixing and plating time. We found that for each different collagen tested it was necessary to find the peak time for mixing and plating. The optimized lattice allowed us to avoid uneven cell distribution and collagen pre-polymerization. By optimizing, we could generate reproducible results for lattices exposed to bacterial conditioned media.

In our study, the lattice height measurement gave us the prediction of how much lattice tension had been generated and how much the lattices would contract upon release. The reduction of collagen lattice height depends on the ability of cell migration (tractional force

migration; Dallon and Elrich, 2008) and reorganization of the collagen matrix. After injury fibroblasts migrate into the wound (Tomasek et al., 2002) and reorganize granulation tissue using migratory properties (Ehrlich & Hunt, 2012). Cells in the collagen lattice can produce tractional force in the matrix. If there is tractional force present in the collagen matrix, then the height of lattice is reduced, but if no tractional force is within the matrix, then the lattice height remains unchanged. With our study, it has been clear that PCM is capable of inhibiting lattice height reduction. The cells in the PCM-treated lattice could not spread and reorganize the collagen matrix which inhibited lattice compaction more than any other treatment. The lattice height measurement is directly correlated with the lattice contraction. The contraction of collagen lattices is produced by the contractile force generated by myofibroblasts under stressed conditions rather than by migratory properties (Jiang et al., 2008). In vivo, fibroblasts during the wound healing process promote contraction of granulation tissue that provides the permissive environment for the myofibroblast phenotype (Tomasek et al., 2002). In our study, PCM treatment inhibited the permissive environment for the myofibroblast phenotype. Therefore, it caused the inhibition in contraction of a lattice that might be related with the chronic wound presence of bacteria inhibition in closing the wound. The TGF-β treated collagen lattices contracted the most and the results resembled to the finding of Vaughan et al. (2000) where they showed that TGF-beta promoting the formation of myofibroblasts resulted in increased contractile force generation. Also, in our study collagen lattices treated with TGF-β increased compaction by a mechanism like floating matrix contraction as previously shown (Montesano and Orci 1988). Our collagen assay with TGF-β contracting most resembled previous findings (Grinnell and Ho, 2003). Our findings with bacterial conditioned media determined that

PCM significantly inhibited the contraction of fibroblast collagen lattices compared to the BCM and control.

Because contraction of collagen lattice is affected by the number of cells present (Tomasek et al.,1992) and number or percentage of myofibroblasts (Tomasek et al.,2002), our next goal was to study these properties. To investigate, we stained our lattices to determine proliferation and myofibroblast differentiation. Visual inspection of PCM treated lattices showed a reduced number of cells; because cells in the anchored lattice can persist, proliferate, or undergo apoptosis (Grinnell et al., 1999), any of these properties can affect the number of cells, which by itself affects compaction (Grinnell et., 1999) or contraction (Tomasek et al. 1992, Vaughan et al., 2000). While we did not monitor apoptosis, we can confirm the cell death in the future by using a TUNEL assay or staining to demonstrate caspase-3 upregulation (Archana et al., 2013). Similar but much lesser effects were seen in BCM-treated lattices. We found that the PCM-treated lattices had little or no myofibroblasts present whereas the TGF-β-treated lattices had the highest number of myofibroblasts. Our results agreed with the study carried out in a coverslip experiment (Hall et al., 2016) where cells are growing under maximum tension. Fibroblasts treated in the presence or absence of BCM or PCM on a coverslip, then immunostained showed PCM-treated coverslips had decreased myofibroblast differentiation compared to BCM, similar to lattices, which suggests that PCM is not inhibiting permissive environment but something else that leads to myofibroblast formation. This can be tested by treating lattices under maximum tension with PCM.

The inflammatory response of the host plays a critical role in healing wounds. The production of inflammatory cytokines is one of the inflammatory response. In our study,

because of the number of myofibroblasts and the total number of cells in the presence of PCM were reduced, we wanted to know whether signaling molecules were responsible. Therefore, we measured the production of IL-8 in bacterial secreted products. The BCM had the highest percentage of IL-8/CXCL8 than PCM and control resembling the findings with Tankersley et al. 2014. IL8 is one of the fibrogenic cytokines that is responsible for myofibroblast activation and proliferation (Bagalad, Mohan Kumar, & Puneeth, 2017). CXCL8 stimulates the differentiation of fibroblasts into myofibroblasts (Rees et al., 2015). Debreva, I et al., 2006 discovered that Il-8 secreted by fibroblasts in response to low density lipoprotein in a P38 MAPK-dependent manner resulted in cell spreading and allowed wound closure. In our study, we think that IL-8 production is the reason why BCM is less inhibitory. The prostate stromal fibroblast undergoes phenoconversion to the myofibroblast easily when exposed to a combination of CXCL8 and TGF-β1 (Gharaee-Kermani et al., 2012). Also, IL8- has ability to promote angiogenesis and cell proliferation in some cancer types. If we could inhibit IL-8, BCM might have mimicked the PCM results. In association with inflammation specific genes that are overexpressed in the PCM (IL1F9, IL28RA, and CXCR7; Tankersley et al., 2014) they have a biased expression in skin. These overexpressed genes might be some of the reasons that PCM is actively inhibiting fibroblast functions.

For further evaluation of inflammation, we exposed HaCaT cells to the bacterial secreted products and then used the media from the exposed HaCaT cells to treat the fibroblast collagen lattice. We observed that the PCM exposed HaCaT media tended to inhibit the lattice compaction followed by BCM. The lattice compaction was directly correlated with the contraction followed by BCM. Visually the staining results suggested that the number

of cells and myofibroblast were comparatively less than control. Whereas statistically due to less number of about four replicate samples the number of cells was not significantly different in each treatment. In HaCaT exposed treatments we observed, an immediate contraction of the collagen matrix after release than compare to the regular treatment group. This signifies that the mechanical tension is responsible for sharp contraction rather than the cellular activity (Vaughan et al., manuscript in preparation). It suggests that the cellular activity could be achieve by letting cell in collagen to mature for more than 5 days. Therefore, our cell phenotype ratio data was not significant difference between HaCaT exposed treatments because of unmatured cells where the proliferation and differentiation might be late than for the regular treatment. The differentiation of fibroblasts to myofibroblasts depends on the tension environment, and for collagen, the tension is generated when fibroblasts use tractional forces to reorganize the collagen (Grinnell, 1994).

In this study, we have seen that PCM has a more detrimental effect on fibroblast functions than does BCM. The collagen lattices treated with biofilm secretion were also able to inhibit compaction and contraction (correlated with the number of cells and myofibroblasts) compared to control and TGF-Beta. We think one reason might be the effectiveness of biofilm is greater when it is present in the wound rather than just in the secreted products. The study by Izano et al., 2008, where they degraded the biofilm by enzymes dispersin B and lysostaphin, and we predicted that biofilm secretion effect is more within its polysaccharide sheath and when a protective sheath is disrupted the biofilm secretion might be effective on inhibiting the fibroblast function. This study is currently underway in our lab to see if the biofilm secretion after disruption releases compaction/ contraction inhibitors.

Conclusion and Future studies

In our study, the investigation of the effects of bacterial secretion products on fibroblast tension generation, migration, proliferation, and differentiation enhance the understanding of the role of *S. aureus* in chronic wound pathogenesis. We found that PCM has a more significant effect on the fibroblast collagen lattice height and force generation by contraction. PCM created a permissive environment of inhibition (reduced compaction and reduced number of cells) resulting in reduced myofibroblast differentiation and contraction (Figure 9). The fibroblast migration, proliferation, differentiation and tension generation are critical aspects of wound healing and tissue remodeling. This study will help improve the knowledge of bacterial association in the wound as both planktonic and biofilm state and might help to reduce infection on a chronic wound. We can support the healing regeneration which restores normal wound healing that might contribute a better understanding to the scientific community. Further understanding of molecular properties and finding the pathway of inhibition by *S. aureus* PCM on fibroblast is in progress. This study will help narrow the role of planktonic and biofilm bacterial role in pathogenicity towards chronic wound healing. The breaking down of biofilm by biofilm disturbing enzymes (Izano et al., 2008) and treating the fibroblast collagen lattice is already in progress. For further biofilm investigations, we can work with co-culture model where biofilm tissue culture will be sitting on top of the collagen lattice. This way biofilm effect on fibroblast cell is direct, where we can assess fibroblast function more closely like in chronic wound. These studies will further advance the knowledge and foster future research to improve chronic wound healing.

Figures

A **B**

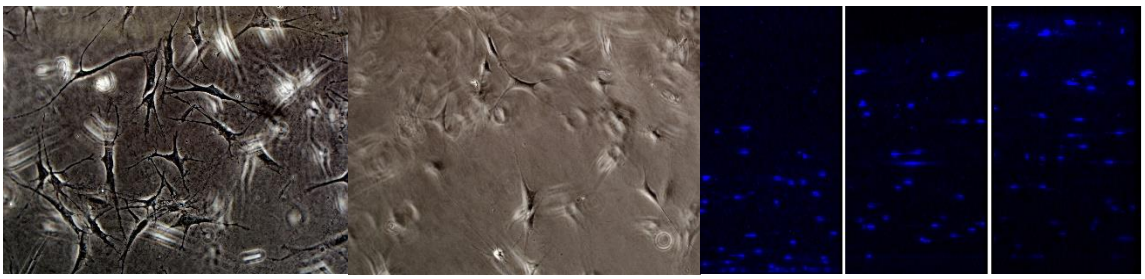

Figure 1. Distribution of cells is affected by mixing time. After lattices had polymerized and media was added, images were taken of the plate beneath the lattices. When lattices were plated early, more cells appeared on the plate (A, left); after a sufficient amount of mixing time, the number of cells remained constantly low (A, right). Lattices that were DAPI-stained and imaged with confocal laser microscopy showed the effect of mixing time; lattices plated immediately showed the majority of cells in the lower half of the lattice (B, left). After an appropriate amount of mixing time appeared more evenly distributed (B, right).

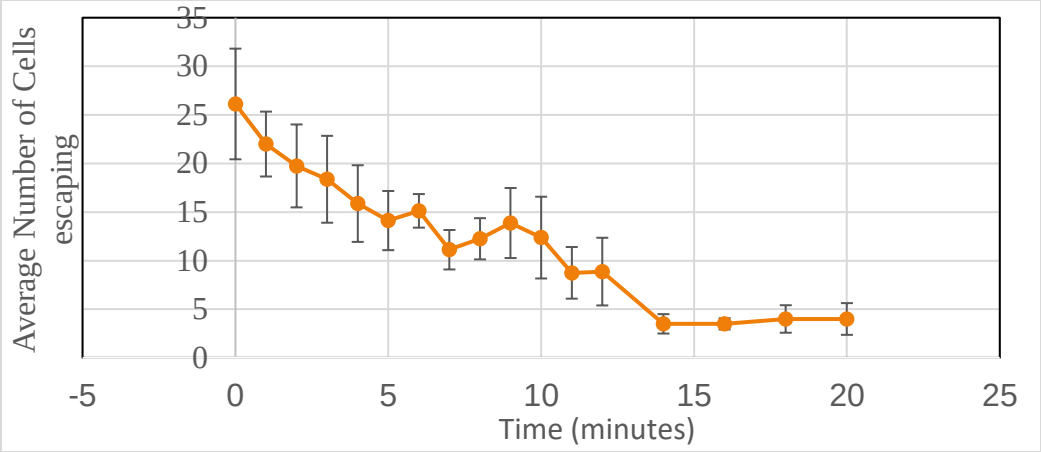

Figure 2: Cell appearance below the lattices was reduced over time. Graphed results of replicate images similar to figure 1 A were averaged. For this experiment, the cell distribution appears to have equilibrated after 14 minutes of mixing time**.**

Collagen Texture

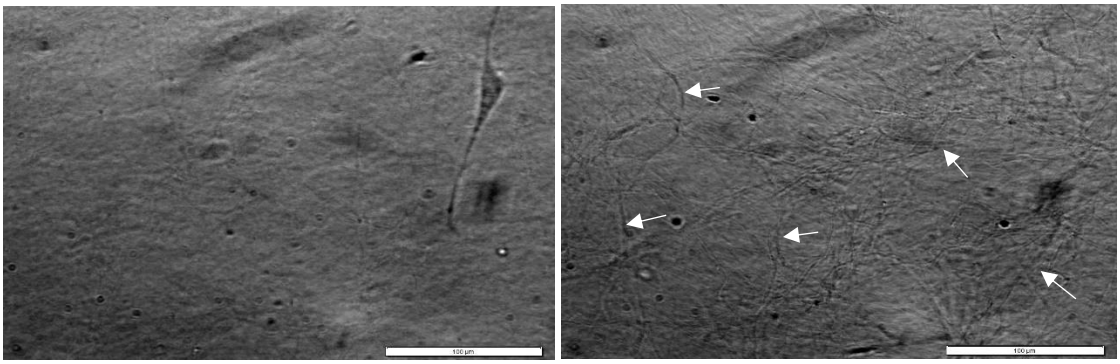

Figure 3 A: Collagen consistency changes with mixing. When the collagen/ cell mixture is plated early, the collagen appears homogenous, without fibers showing (left image); after a longer mixing time, fibers begin to appear in the collagen (arrow in the right image).

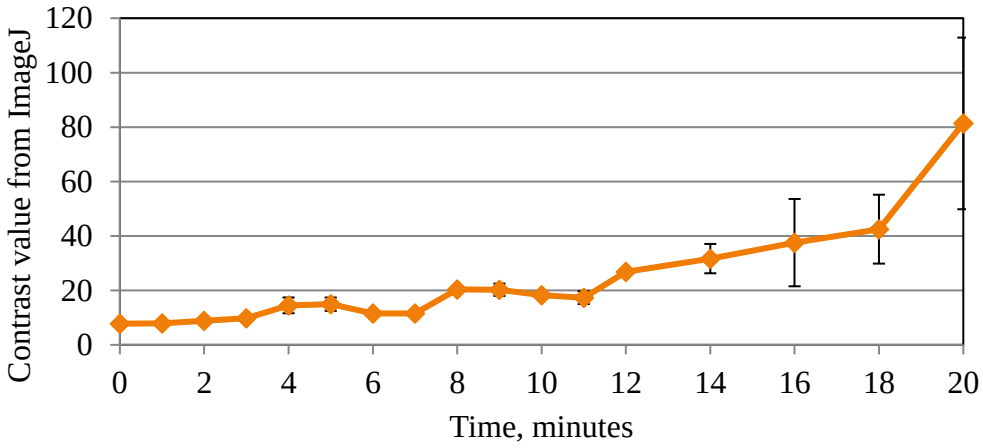

Figure 3 B: Collagen texture contrast increases with mixing time. Numerous images were analyzed using the Image J GLCM plugin, and the contrast values were combined and averaged. Contrast values, and therefore flocculence, began to increase after 7 minutes, suggesting that the optimum plating time for collagen is within 7 minutes for this experiment.

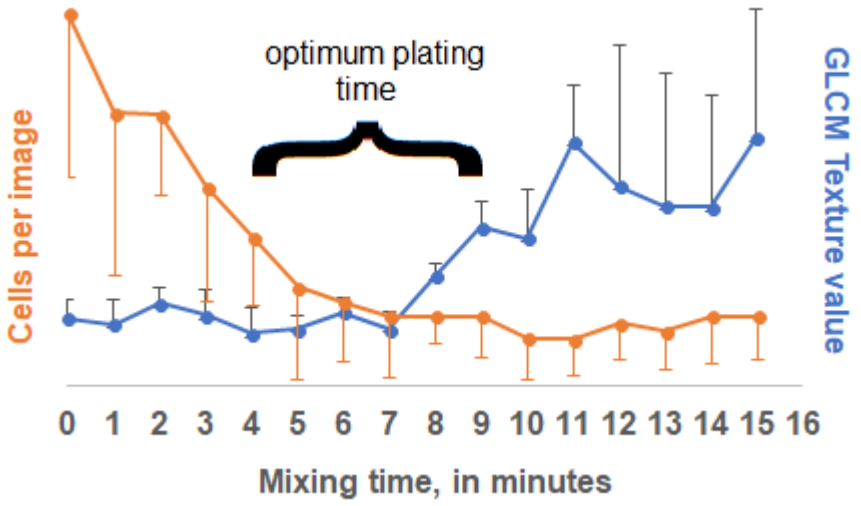

Figure 4: Graph overlay for cell escape and flocculence provides the optimum plating time to reduce both effects. Based on this set of data, the collagen lattices should be plated between 4 and 8 minutes of mixing time.

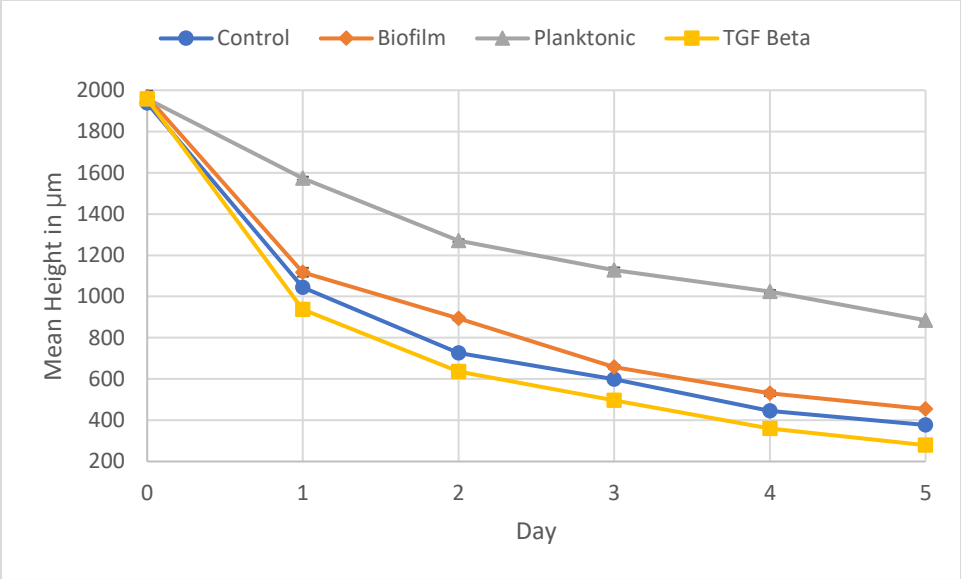

Figure 5: Raw mean height over time for the fibroblast collagen lattices treated with PCM tends to have the highest height throughout day 5. PCM inhibited the fibroblast collagen lattice compaction significantly compared to BCM, Control and TGF-Beta. Our result represents the replicates of four sample for each treatment. The mean height of all treatments was significantly different ($p < 0.001$) from each other resulting the PCM to have the greatest inhibition.

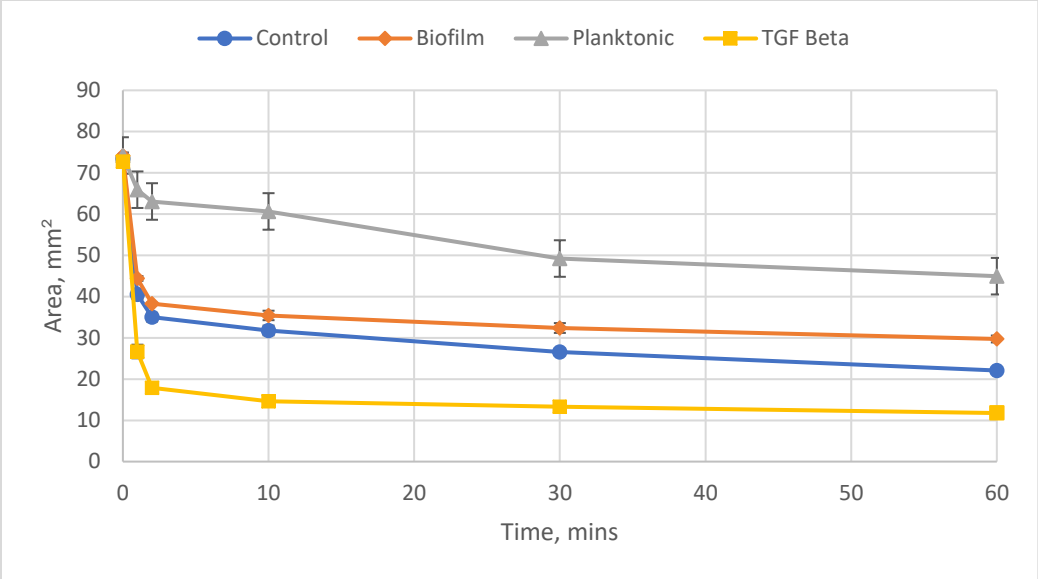

Figure 6: PCM inhibited the contraction of fibroblast collagen lattices when released at day 5. PCM-treated lattices significantly reduced contraction of the lattice diameter at each time point 1, 2, 10, 30 and 60 minutes from other treatments BCM, Control and TGF-β. We also observed that BCM-treated lattices were significantly different ($p < 0.001$) from control lattices after the time point of 1min.

Table 1. Total number of cells phenotype with respect to their treatments.

Cell Type	Control	BCM	PCM	TGF-β
Non-proliferating fibroblasts (NPF)	160	187	*78*	*162*
Proliferating fibroblasts (PF)	40	31	*9*	*47*
Non-proliferating myofibroblasts (NPMF)	52	41	18	100
Proliferating myofibroblasts (PMF)	19	16	1	39

	Control	BCM	PCM	TGF-β
Total number of cells:	271	275	106	348
Myofibroblast percentage:	26.19 %	20.72 %	17.92 %	39.94%

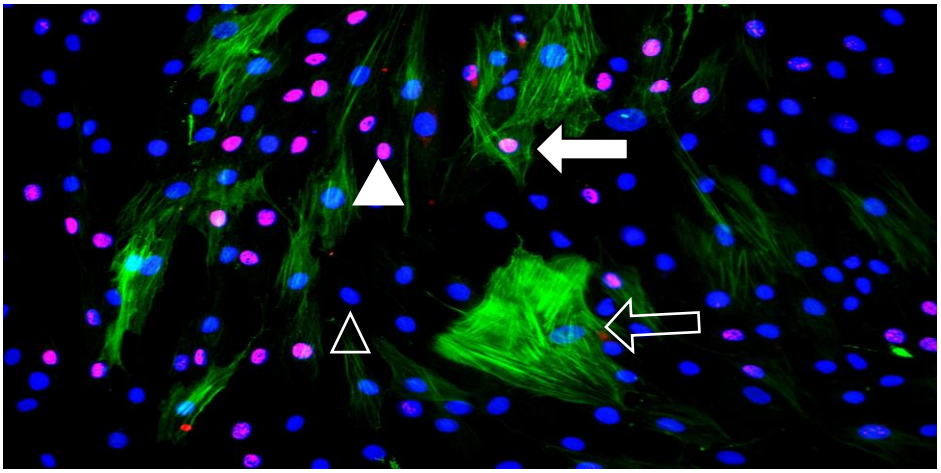

Figure 7: Four different cell phenotypes were observed. Each field of view images were collected using the blue (dapi-nuclei counter stain)**,** red (EdU-proliferation) and the green (α-sma- differentiation) filter. The images were combined that identified for four different cell phenotypes: non-proliferative fibroblasts (open arrowhead), proliferative fibroblasts (solid arrowhead), non-proliferative myofibroblasts (open arrow) and proliferative myofibroblast (solid arrow).

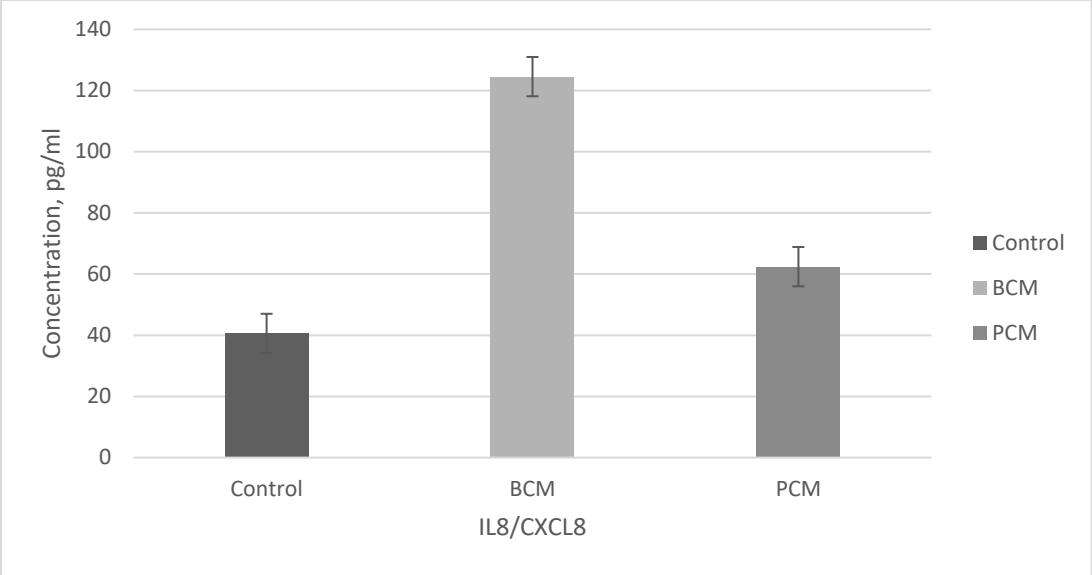

Figure 8: This is the ELISA results for PCM, BCM and control. BCM has high production of IL8/CXCL8 than compared to PCM and control. Analysis of variance (ANOVA) was performed to observe statistical difference. The values represent the mean of three independent experiments. There is a significant difference ($p<0.05$) between the groups. The measurements are reported in pg/ml concentration.

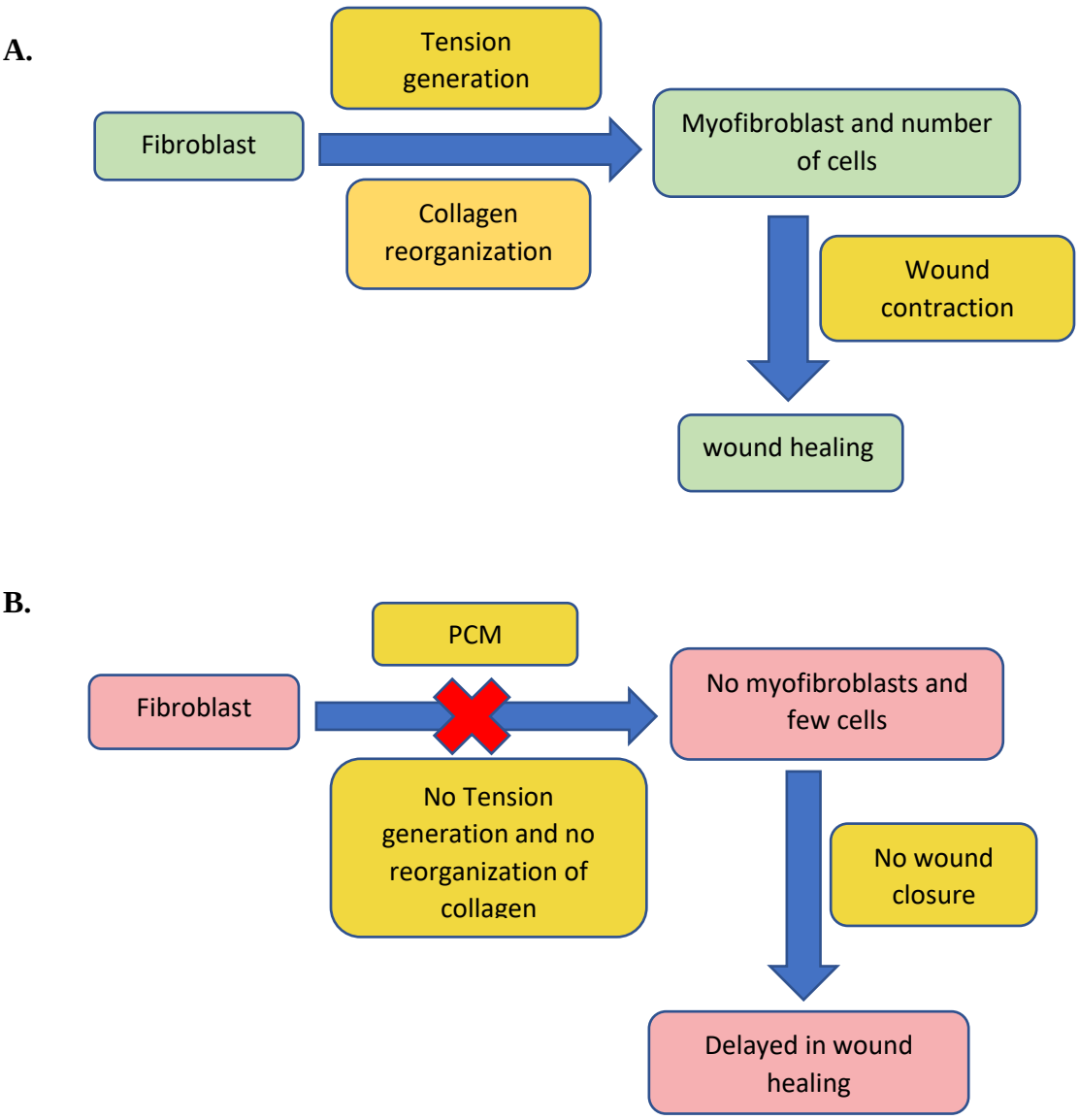

Figure 9: A diagram scheme for the fibroblast role in wound healing. **A:** Tension generation allows a fibroblast to differentiate into a myofibroblast and cell locomotion supports reorganization of collagen that results in wound closure. **B**. In our scenario where fibroblasts were exposed to planktonic secretion products (PCM,) no myofibroblasts were produced because of lack of permissive environment with no tension generation. Without cell locomotion or reorganization of collagen, would healing would be prolonged with no contraction of wound.

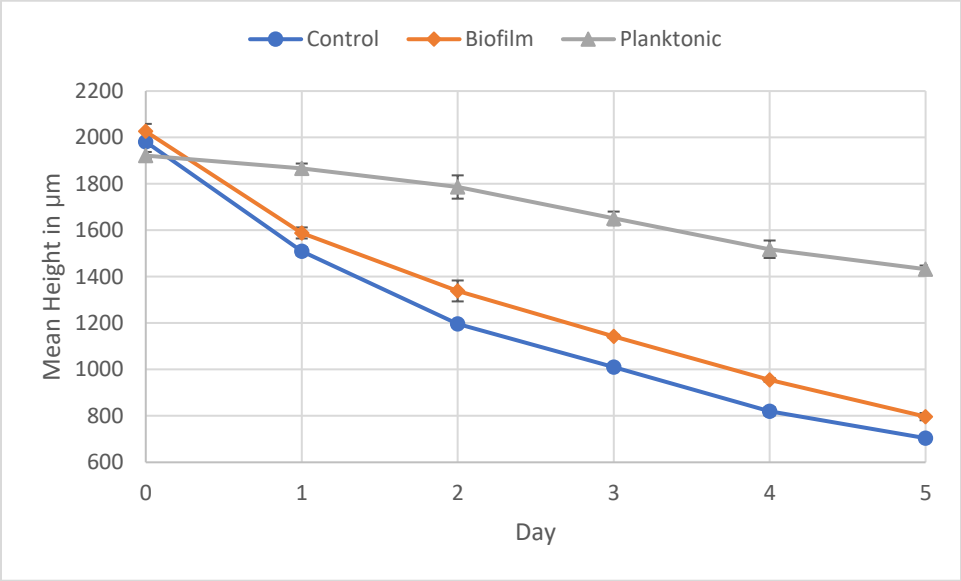

Figure 10: This graph demonstrates compaction effects due to HaCaT- conditioned medium. Under control conditions, compaction occurs in an asymptotic manner (blue line) over 5 days. Mean averages of four replicates for each treatment were used. PCM exposed to HaCaT-conditioning medium greatly reduced the compaction (grey line) while BCM only modestly reduced compaction (orange line).

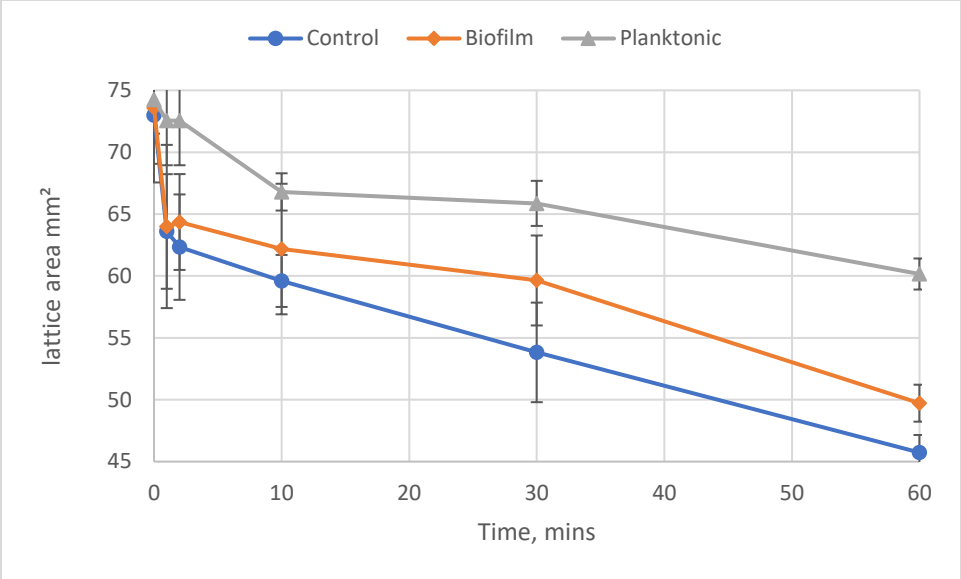

Figure 11: PCM greatly inhibited fibroblast populated collagen lattice contraction, indicating little or no force generation. BCM was slightly inhibitory while control lattices contracted the greatest.

Table 2. Total number of cells by type and treatment for HaCaT treatments

Cell Type measured	Control	BCM	PCM
Non-proliferating fibroblasts (NPF)	329	266	141
Proliferating fibroblasts (PF)	33	29	9
Non-proliferating myofibroblasts (NPMF)	53	35	12
Proliferating myofibroblasts (PMF)	13	8	0

	Control	BCM	PCM
Total number of cells:	428	338	162
Myofibroblast percentage:	15.42%	12.72%	7.4%

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