EFFECT OF CELL CULTURE CONDITION OR BISPHENOL A (BPA) EXPOSURE ON THE WOUND HEALING RESPONSE *IN VITRO*

_____

A Thesis

Presented to

The Faculty of the Department of Biological Sciences

Sam Houston State University

_____

In Partial Fulfillment

of the Requirements for the Degree of

Master of Science

_____

by

Emily E. Hays

December, 2023

EFFECT OF CELL CULTURE CONDITION OR BISPHENOL A (BPA) EXPOSURE ON THE WOUND HEALING RESPONSE *IN VITRO*

by

Emily E. Hays

_____

APPROVED:

James M. Harper, PhD
Committee Director

Aaron M. Lynne, PhD
Committee Member

Amber J. Ulseth, PhD
Committee Member

Melinda Holt, PhD
Dean, College of Science and Engineering Technologies

DEDICATION

I would like to dedicate this thesis to my parents, Garry and Erin Hays.

ABSTRACT

Hays, Emily E., *Effect of cell culture condition or bisphenol a (bpa) on the wound healing response in vitro*. Master of Science (Biology), December, 2023, Sam Houston State University, Huntsville, Texas.

Wound healing is a process comprised of overlapping phases: inflammation, tissue formation, reepithelization, and remodeling; all of which can be influenced by exogenous factors such as environmental pollutants. Bisphenol A (BPA), a monomer used in the manufacture of many plastics, is an endocrine-disrupting chemical that may influence cell proliferation and/or cellular migration during wound repair. Here I used an *in vitro* model of wound healing to investigate the effect of varying the composition of the cell culture media in conjunction with BPA exposure to determine how each of these factors may influence the kinetics of the wound healing response. Specifically, the degree of closure over 24 hours was measured in the presence or absence of bovine serum in the cell culture media in cells pre-exposed to varied concentrations of BPA. Using an algorithm developed for ImageJ to autonomously estimate the rate of wound closure, we found that the presence of serum in the culture media significantly increased this rate in ($p \leq 0.001$). Prior exposure to BPA had no effect on the rate of wound closure, regardless of dose or cell culture condition ($p \geq 0.05$). Collectively, these data suggest that serum growth factors are essential mediators of wound closure *in vitro*, whereas BPA exposure has no significant effect.

KEY WORDS: Wound closure, Culture conditions, Scratch assay, Bisphenol-A, *In-vitro*.

ACKNOWLEDGEMENTS

I would first like to acknowledge my academic advisor, Dr. Harper. Thank you for your help along every step of my graduate career. You taught me how to be a true biologist. I hope to one day be the mentor you are.

Dr. Lynne and Dr. Ulseth, thank you both for being vital to my career and research. Each of you has helped me in ways I could never fully thank you for.

Finally, I want to thank my family and friends for their constant support these last two years. I would not have been able to finish this degree without their love. 138.

TABLE OF CONTENTS

Page

DEDICATION iii

ABSTRACT iv

ACKNOWLEDGEMENTS v

TABLE OF CONTENTS vi

LIST OF TABLES viii

LIST OF FIGURES ix

CHAPTER I: INTRODUCTION 1

Stages of Wound Healing 1

Bisphenol A 4

In Vitro Scratch Assays 6

CHAPTER II: METHODS 7

Fibroblast Cell Line 7

In Vitro Scratch Assay Protocol 7

Microscopic and Image Analysis 9

Statistical Analyses 10

CHAPTER III: RESULTS 11

Experiment 1 11

Experiment 2 13

Experiment 3 14

CHAPTER IV: DISCUSSION 15

REFERENCES 22

APPENDIX A 28

APPENDIX B 29

APPENDIX C 30

VITA 31

LIST OF TABLES

Table **Page**

1 Culture Conditions and BPA Exposure Combinations 8

LIST OF FIGURES

Figure **Page**

1 Wound Healing Phases: Hemostasis, Inflammation, and Proliferation 1

2 Representative Images of a Wound for 24 Hours 10

3 BPA Treatment and Culture Conditions on the Area of Wound Closure 12

4 Area of Wound Closure Between Culture Conditions 13

CHAPTER I

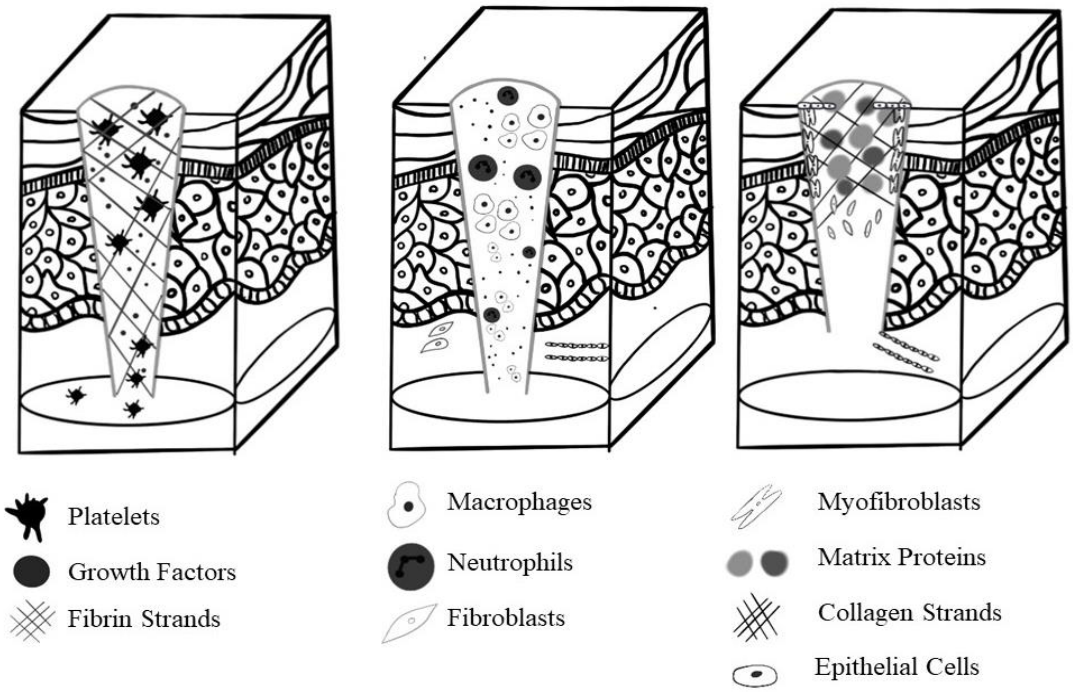
Introduction

Stages of Wound Healing

Within mammals, tissue damage results in the initiation of a wounding healing response consisting of four distinct, but overlapping, phases: (1) hemostasis, (2) inflammation, (3) remodeling, and (4) maturation (Figure 1).

Figure 1

Wound Healing Phases: Hemostasis, Inflammation, and Proliferation



Note. Pictorial rendering of wound healing using the integumentary system as a representative. (A) Hemostasis (B) Inflammation (C) Proliferation.

Hemostasis

Upon injury, the constriction of damaged blood vessels rapidly occurs to redirect blood flow around the damaged tissue as well as limiting the degree of hemorrhaging (Fitridge & Thompson, 2011). In particular, endothelial disruption exposes circulating platelets to Type I Collagen. This results in platelet activation and the subsequent release

of a number of bioactive compounds including glycoproteins (e.g., fibrinogen, fibronectin, and von Willebrand factor) which aggregate to form a platelet plug after thrombin converts fibrinogen to fibrin (Harmening, 1996). The resultant fibrin strands create a network around the platelet plug to provide stabilization while creating a barrier and scaffolding for migrating immune cells (Bryant & Nix, 2007; Harmening, 1996). Additionally, activated platelets will release platelet-derived growth factor (PDGF), transforming growth factor beta (TGF-β), transforming growth factor alpha (TGF-α), fibroblast-derived growth factors (FGF), and vascular endothelial growth factor (VEGF) that are important during later phases of the wound healing response (Barrientos et al, 2008). Simultaneously, clotting factors (V & VIII) are produced in the liver resulting in coagulation to stem the degree of hemorrhaging further (Harmening, 1996). Finally, both platelet and immune cell-derived growth factors as well as damage-associated molecular patterns (DAMPs) recruit neutrophils and macrophages to the wound site to initiate an inflammatory response (Bryant & Nix, 2007).

Inflammation

The innate immune system is responsible for mounting an inflammatory response at the site of injury that is characterized by heat, swelling, pain, and redness around the injury. The loss of cell membrane integrity leads to an immediate release of various DAMPs, such as ATP and DNA fragments, which bind to pattern recognition receptors (PPRs) on innate immune cells to activate nuclear factor kappa beta (NF-kB) and mitogen-activated protein kinase (MAPK) signaling pathways (Rodrigues et al., 2019; Johnson et al., 2020). Ultimately, activated neutrophils and macrophages secrete cytokines such as interleukin-1 (IL-1), interleukin-6 (IL-6), and tumor necrosis factor-

alpha (TNF-α) which act as the primary inflammatory mediators (Johnson et al., 2020). In addition to pro-inflammatory cytokines, both neutrophils and macrophages secrete a suite of growth factors to serve as a chemoattractant for fibroblasts, to activate macrophages, stimulate re-epithelialization and induce an angiogenic response (Barrientos et al., 2008).

Remodeling

Fibroblasts utilize cell adhesion molecules such as integrins to migrate to the site of injury via binding to fibronectin and fibrin fibers present at the wound site (Fitridge & Thompson, 2011). In addition, PDGF secreted by platelets and macrophages stimulate fibroblast activation/differentiation into proto-myofibroblasts while producing collagenase (Barrientos et al., 2008). At the same time, TGF-β increases the production of collagen and elastin to aid with the formation of a new extracellular matrix (Barrientos et. al., 2008). Together, these factors shift the wound microenvironment to support cell proliferation (Fitridge & Thompson, 2011).

Eventually, fibrinolysis downstream of plasmin activity dissolves the fibrin clot which will ultimately be replaced by collagen strands at the wound edges. Due to tensile forces, the proto-myofibroblasts at the wound edges further differentiate to myofibroblasts which express α-smooth muscle actin (α-SMA). Contraction of these actin fibers then aid with the mechanical closure of the gap (Fitridge & Thompson, 2011). Meanwhile, VEGF stimulates the production of matrix proteins to fill the gaps between the individual collagen strands to form granulation tissue while stimulating nearby capillaries to bring additional oxygen and nutrients into the wound site (Barrientos et al., 2008; Bryant & Nix, 2007).

Lastly, fibroblast-derived growth factors induce the proliferation and migration of epithelial cells at the wound edges toward the center to create a monolayer (Bryant & Nix, 2007). This is followed by their differentiation into a proliferative state (Fitridge & Thompson, 2011). The final step of remodeling is to restore barrier function by rebuilding the stratified layers of the epidermis if it is a surface wound via the action of keratinocytes (Fitridge & Thompson, 2011). Interestingly, the wound healing response is essentially the same regardless of wound site except for the involvement of keratinocytes in surface wounds.

With time, tissue homeostasis is restored due to the constant synthesis, deposition, and reorganization of the ECM along with a decrease in capillary density and thickening of collagen fibers to increase the tensile strength of the tissue (Rodrigues et al., 2019). Unneeded fibroblasts and immune cells will either migrate away from the wound site or undergo apoptosis (Rodrigues et al., 2019).

Bisphenol A

Bisphenol A (BPA) is a plastic monomer used in the production of polycarbonate and epoxy resins to increase flexibility and durability (Vandenberg et al., 2007). It is commonly found in food containers, dental fillings, medical equipment, and water pipes. Importantly, BPA can leach from the final products into the environment due to changes in temperature and/or pH (Vandenberg et al., 2007). It is estimated that the daily exposure of humans to BPA is approximately 30.76 and 60.08 ng/kg body weight for adults and children, respectively (Vandenberg et al., 2007). Detectable levels of BPA have been found in serum, blood, placenta, fetal plasma, and breast milk (Vandenberg et al., 2007).

BPA is classified as an endocrine-disrupting chemical which are classified based on their ability to hinder physiological function downstream by mimicking or antagonizing normal endocrine production and/or signaling pathways (Diamanti-Kandarakis et al., 2009). In the case of BPA, it binds to the estrogen receptor (ER) to act as either an antagonist or an agonist (Shanle & Xu, 2011). In actuality, the estrogen receptor exists as one of two isoforms known as ER-α and ER-β which differ in the structure of the C-terminal binding domain (Matthews and Gustafsson, 2003). ER-α is expressed in the mammary glands, liver, and the uterus; whereas ER-β is typically expressed in bladder, colon, and the immune system (Paterni et al., 2014). As is the case with all intracellular steroid receptors, ER-α and ER-β act as nuclear transcription factors to regulate gene expression in response to estrogenic signaling (Shanle & Xu, 2011).

Estrogen is implicated in every phase of the wound healing response (Cooper et al., 2015; Koh et al., 2015; Kovats et al., 1997; Zhou et al., 2016), playing a prominent role in fibroblasts where it affects collagen production by increasing TGF-β secretion (Ashcroft et al., 1997; Verdier-Sévrain et al., 2006). Therefore, I hypothesized the wound healing response *in vitro* would be modulated by BPA (Le-Magueresse-Battistoni et al., 2018 & Liu et al., 2021). Interestingly, BPA exposure can alter the wound healing response *in vivo,* but it could either be stimulated or delayed dependent on the route of exposure (Wen et al., 2022). Specifically, local application induced ER-β expression to promote repair, whereas dietary exposure suppressed ovarian secretion and delayed wound repair (Wen et al., 2022).

In Vitro Scratch Assays

A common and cost-effective model system to study cell migration or proliferation is the *in vitro* scratch assay. In brief, a monolayer of cells is disrupted using different experimental techniques (e.g., pipette scratch) to mimic or create an injury in this model system. Commonly, cells are monitored over 24-hours to assess cellular proliferation or migration within the wound to determine the rate of wound closure.

Cell culture media formulations typically include serum, usually from cattle, to serve as a source of growth factors. These growth factors aid with cell growth and proliferation and ensure cell viability. Scratch assay protocols often advise supplementing the cell culture media with 10 – 20% bovine serum to prevent cell detachment while minimizing excessive cell growth (Liang et al., 2007). Nevertheless, there is a lack of a consensus on whether the presence (or absence) of serum in the cell culture media will yield similar results among experiments, including when the serum is present. That is, does the media contain serum during the entire protocol? Or only during a select period (e.g., pre-scratch versus post-scratch)?

This study aims to explore using a spontaneously immortalized primary fibroblast cell line. This line was originally isolated from the skin of a mouse with an uncharacterized genetic background purchased from a retail pet store.

CHAPTER II

Methods

Fibroblast Cell Line

A spontaneously immortalized dermal fibroblast cell line that was generated from an abdominal skin biopsy collected from a mouse with an uncharacterized genetic background purchased at a pet supply chain was used for this study. This cell line will be referred to as Sam Houston State University Fibroblast A (SHSU FA) and was used at varying passage numbers over the course of the study. Prior to experimentation, cells were maintained in Complete Dulbecco Modified Eagle Medium (DMEM) high glucose (4.5 g/L) without glutamine and sodium pyruvate supplemented with 10% Fetal Bovine Serum and 1% Penicillin Streptomycin.

In Vitro Scratch Assay Protocol

To generate the wound, a 1000 µL pipette tip was used to draw a line through a confluent monolayer of SHSU FA cells under each of the conditions below. Specifically, cells were maintained in each of two media formulations during the pre-wound or post-wound period in the presence of varying amounts of BPA. Overall, there were 16 different culture condition x BPA treatments (Table 1). In particular, the individual culture conditions consisted of cells grown in the presence of either complete or serum-free media during the pre-wound and/or post-wound period. Complete media consisted of DMEM supplemented with 10% Fetal bovine serum (FBS) and antibiotics. The serum-free media was comprised of DMEM with antibiotics and 2% Bovine Serum Albumin (BSA). There was no FBS added to the media. For the BPA treatments, a 10 mM stock solution of BPA that was dissolved in 100% ethanol was generated, then serially diluted

with complete DMEM to achieve the final concentrations of 1 µM, 10 µM, and 100 µM to be used in this study.

Table 1

Culture Conditions and BPA Exposure Combinations

BPA Treatment	Name	Pre-Scratch Media	Post-Scratch Media
0µM			
	Condition A	Complete	Complete
	Condition B	Complete	Serum-free
	Condition C	Serum-free	Complete
	Condition D	Serum-free	Serum-free
1µM			
	Condition A	Complete	Complete
	Condition B	Complete	Serum-free
	Condition C	Serum-free	Complete
	Condition D	Serum-free	Serum-free
10µM			
	Condition A	Complete	Complete
	Condition B	Complete	Serum-free
	Condition C	Serum-free	Complete
	Condition D	Serum-free	Serum-free
100µM			
	Condition A	Complete	Complete
	Condition B	Complete	Serum-free
	Condition C	Serum-free	Complete
	Condition D	Serum-free	Serum-free

Note. Culture conditions and BPA exposure combinations were used in this study. Each BPA dosage had four different culture conditions. (A) Complete to Complete (B) Complete to Serum-free (C) Serum-free to Complete (D) Serum-free to Serum-free

In total, three different experiments were conducted using each of the 16 culture x BPA combinations. In two cases, scratch assays were performed in a 24-well plate with cells grown to confluency then seeded at 60,000 cells per well in complete media overnight to ensure attachment. The first scratch assay was performed by another graduate student in 2017; the protocol and raw images were used in this analysis (Breuer, 2017). The third scratch assay was performed in a 25 cm^2 flask seeded with 500,000 cells

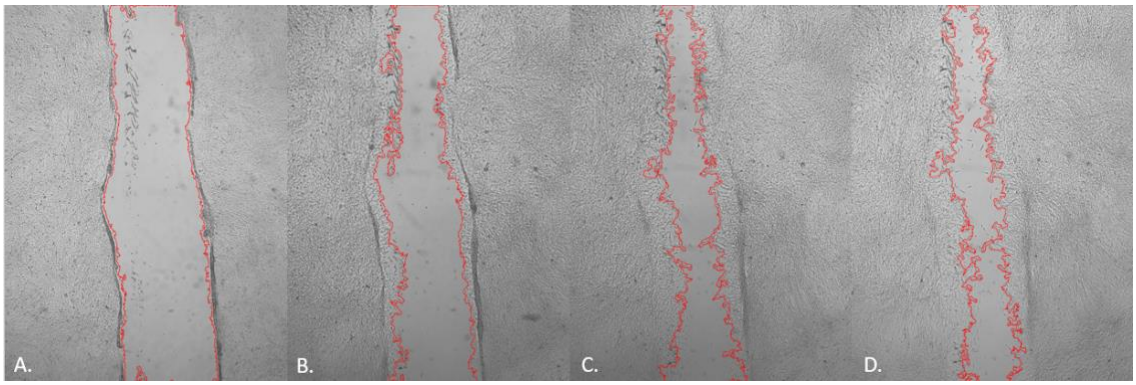
and grown to confluency. In each case, the confluent monolayer was washed using the appropriate media formulation for the subsequent experiment then maintained in either complete or serum-free media for 24 hours. On day 3, cells were washed with the media containing BPA (control, 1 µM, 10 µM, or100 µM) then maintained in the presence of the same BPA dose for an additional 24 hours. Prior to scratching, cells were washed with complete or serum-free medium. After scratching, the medium was removed, then replaced with one of the 16 media x BPA combinations.

Microscopic and Image Analysis

Wound closure for the first experiment using a 24-well plate were imaged at 0-, 8-, 16-, and 24-hours post-scratching at 4x magnification using a condenser on an inverted microscope. During the second experiment, imaging protocol was the same except no images were taken at 16- hours. For the third experiment, scratches were imaged using the Echo Revolve at 4x magnification for 0-, 8-, 16-, and 24- hours post- scratching. To ensure the wound was imaged in the same spot, a line was drawn to use as a guide. Images were exported into Adobe Lightroom, and a B&W preset was applied. Wound closure analysis was conducted in ImageJ using an automated script (Breuer, 2017). In brief, the images were flattened and cropped (Appendix 1), then the outline of the wound was detected and the area inside was measured (Appendix 2). To ensure non-scratch areas were not included in the final analysis, each individual image was manually checked to remove any detected non-scratch areas (Appendix 3). See Figure 2.

Figure 2

Representative Images of a Wound for 24 Hours



Note. Images of a wound over 24 hours. (A) Hour 0 (B) Hour 8 (C) Hour 16 (D) Hour 24.

Statistical Analyses

A multiple linear regression model was performed to explore the relationship between culture condition and BPA treatment on the degree of wound closure with hour as a covariate. Initially, the full model was run and if there were significant interactions within the model, then the model was simplified further. To investigate the best fit of the model, Akaike Information Criterion (AIC) was performed. Finally, any negative numbers were excluded from these analyses. Statistical analyses were run using R version 4.3.1 (R Code Team, 2022).

CHAPTER III

Results

Experiment 1

Kinetics

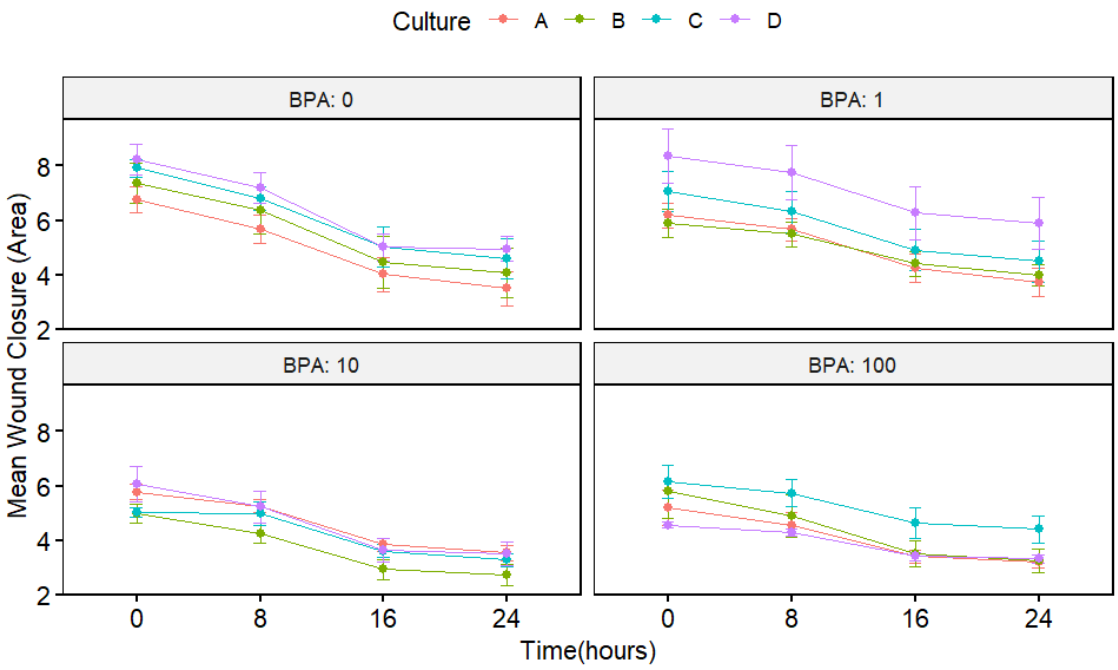
The regression of culture condition and BPA treatment with hour as covariate on the area of wound closure was significant (R^2 = 0.570, df =19, 356, F = 27.11, p = 0.001). However, BPA did not significantly predict the degree of wound closure over time at any dosage; 1 µM (β = -0.100, t = -0.280, df = 19, 356, F = 27.11, p = 0.780), 10 µM (β = 0.42172, t = 1.120, df =19, 356, F = 27.11, p = 0.263) or 100 µM (β = -0.193, t = -0.513, df = 19, 356, F = 27.11, p = 0.609, Figure 3). Moreover, there was a significant predictive relationship on wound closure for conditions C (β = -1.319, t = -3.031, df = 19, 356, F = 27.11, p = 0.003) and D (β = -2.646, t = -6.080, df = 19, 356, F = 27.11, p = 0.001), whereas condition B had no predictive effect on the degree of closure (β = -0.550, t = -1.264, df =19, 356, F = 27.11, p = 0.207). Compared to the slope for condition A, the slope for condition B was 6.8% smaller, condition C was 22.5 % smaller, and condition D was 54.46% smaller (Figure 4). Furthermore, there was a significant interaction between condition B and the 10 µM dose of BPA as a prediction of wound closure (β = 1.700, t = 3.26, df = 19, 356, F = 27.11, p = 0.001). None of the remaining interaction terms between BPA and culture condition were significant predictors of the degree of wound closure (p > 0.05).

Hour, the covariate regressed against area of wound closure was also statistically significantly (β = -0.178, df = 19, 356, F = 27.11, t = -11.987, p = 0.001). Condition B and hour had a significant predictive influence on the degree of wound closure (β =

0.091, df = 19, 356, F = 27.11, t = 4.222, p = 0.001). Likewise, the interaction between hour and condition C predicted a significant effect on the area of wound closure (β = 0.048, df = 19, 356, t = 2.326, p = 0.001). Hour and condition D significantly predicted degree of wound closure (β = 0.123, df = 19, 356, F = 5.972, p = 0.001).

Figure 3

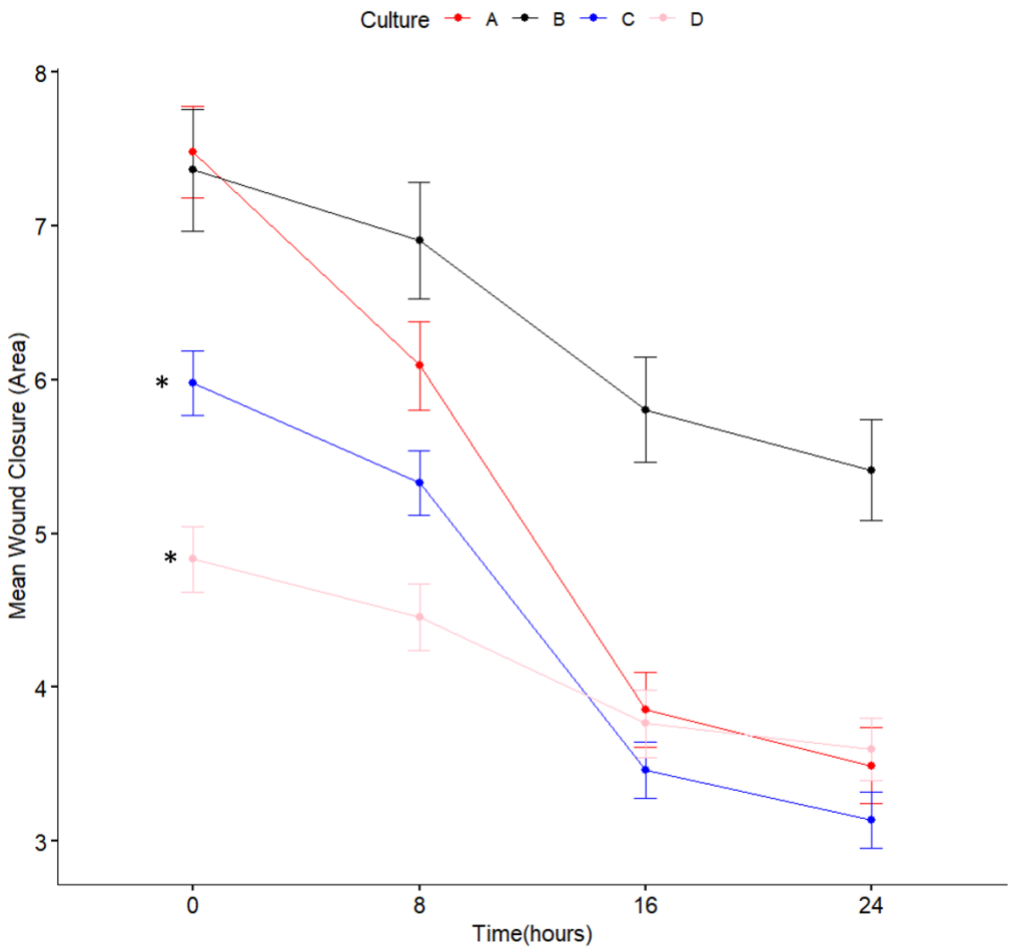
BPA Treatment and Culture Conditions on the Area of Wound Closure



Note. Area of wound closure over time with different doses of BPA treatment and four culture conditions. (A) Complete to Complete (B) Complete to Serum-free (C) Serum-free to Complete (D) Serum-free to Serum-free. Bars represent ± 1SE. Overall regression analysis is significant (R^2 = 0.570, df = 19, 356 F = 27.11, p = 0.001). BPA had no significant effect (R^2 = 0.570, p > 0.05 for all dosages).

Figure 4

Area of Wound Closure Between Culture Conditions



Note. Culture conditions influence the degree of wound closure. Due to the lack of an effect of BPA, data from all four treatment conditions were combined. (A) Complete to Complete Condition (B) Complete to Serum-free Condition (C) Serum-free to Complete Condition (D) Serum-free to Serum-free Condition. Error bars represent the mean ± 1SE. Culture C and D had significant association on the area of wound closure ($R^2 = 0.570$, df = 19, 356, F = 27.11, $p < 0.001$). Culture B has no significant association ($p = 0.207$).

Experiment 2

Kinetics

Initially, a multiple linear regression model was planned to be performed. However, this experiment did not pass the assumption of the homogeneity of the regression slope. Therefore, there were no significant effect of the culture condition (df =

3,196, F = 2.349, p = 0.074) or BPA treatment (df = 1, 196, F= 0.405, p = 0.525) on wound closure. The interaction of culture condition and BPA treatment was also non-significant (df = 3, 196 F = 0.687, p = 0.053). Hour, the covariate, was significant in this model (df = 1, 196 F = 117.789, p = 0.001), but there was no significant interaction with BPA (df = 1, 196, F = 0.260, p = 0.611), culture condition (df = 3,196, F = 0.796, p = 0.497), or BPA and culture condition (df = 3, 196, F = 1.321, p = 0.269).

Experiment 3

Kinetics

The proposed plan was to conduct a multiple linear regression model, but the homogeneity of the regression slope assumption was not met. Thus, it was found that there was no significance for the two factors: Culture condition (df = 3, 164, F = 2.209, p = 0.090.) or BPA treatment (df =1, 164, F= 2.565, p = 0.111) on the degree of wound closure. Also, the interactions were found not to be significant between the factors (df = 3, 164, F = 1.276, p = 0.284). The covariate, hour, was significant (df = 1, 164, F = 263.325, p = 0.001). However, in this model, hour was not significant in the interaction with BPA (df = 1, 164, F = 0.029, p = 0.864), culture (df = 3, 164, F = 2.112, p = 0.101), or BPA and culture (df = 3, 164, F = 0.297, p = 0.828) on the degree of wound closure.

CHAPTER IV

Discussion

During the wound healing process, cells can either proliferate or migrate during wound closure. Each of these processes is strongly influenced by the presence of growth factors (Islam et al., 2016; Iyengar et al., 2009; Rose, 2012). Therefore, it was not surprising that the presence of serum in the cell culture medium during the post-wound period significantly increased the degree of wound closure relative to serum-free conditions (Figure 3). In addition, the difference in the slopes among the four culture conditions indicated that the rate of wound healing differed among the conditions. Specifically, the presence of serum increased the rate of wound closure, most likely due to the presence of various metabolites, growth factors, other hormones, trace elements and proteins.

In the case of the serum-free conditions, the slowed rate of wound closure is most likely due to a need to synthesize key bioactive compounds such as growth factors prior to the onset of wound closure by the cells. Overall, these data clearly indicate that the presence/absence of serum is a key modulator when assessing the wound healing response *in vitro*. Whether this is due to differences in the degree of cell proliferation and/or cell migration is unknown.

BPA is a plasticizing agent commonly used in the manufacture of many household products. It is a well-known endocrine disruptor with negative effects on the reproductive and metabolic health of humans and other species. Despite its suppressive effect on the inflammatory response (Loffredo et al., 2020; Malaise et al., 2020; Zia,

2020), I found that BPA had no significant main effect on the degree of wound closure even at a relatively high dose (100 uM) under any culture condition (Figure 3).

Interestingly, this is in contrast with other studies. Specifically, Wen et al. (2022) found that BPA actually enhanced the rate of migration during wound healing using primary mouse fibroblasts in conjunction with an increased expression of ER-β, collagen, and α-SMA (Wen et al, 2022). In addition, Hu et al. (2016) also found that BPA increased the rate of cell migration, but in human cardiac fibroblasts rather than murine dermal fibroblasts (Hu et al., 2016). In each case, these differences could be a reflection of differences in the degree of estrogen responsiveness and/or variable expression of growth factor receptors in these cells versus the SHSU FA cell line.

Furthermore, BPA has been shown to modulate the degree of wound closure *in vivo* as well (Wen et al., 2022). In this case, wound closure could be delayed or stimulated dependent of on the route of delivery in that topical treatment of the wound with BPA promoted healing, while ingesting BPA delayed it. Meanwhile, BPA delayed the rate of wound closure in ovariectomized mice, while estradiol replacement in conjunction with BPA promoted wound closure (Wen et al., 2022). This suggests there is a complex interplay between estrogen signaling, BPA and the wound healing response in intact animals. Although it is a useful surrogate for many processes, a cell culture model cannot replicate the complexity of an *in vivo* response. For example, immune cells such as neutrophils and macrophages are important modulators of the wound healing response but are missing from the monoculture condition typically used for *in vitro* scratch assays.

Interestingly, there was a lack of an effect of BPA treatment on the induction of stress-induced senescence in murine 3T3 cells (Alper, 2016). These cells are often used

as a model for studies of basic fibroblast biochemistry and physiology, but they do not express the estrogen receptor (Gaben and Mester, 1991). Estrogen also has no significant effect on the wound healing response in human primary dermal fibroblasts (Carnesecchi et al., 2015). Together, these data suggest that estrogenic activity may not have a direct influence on aspects of fibroblast biology *in vitro*, perhaps as a consequence of differential estrogen-dependent signaling. It is unknown whether the SHSU FA cell lines expressed a functional estrogen receptor.

Although we used a clonal cell line, the individual experiments were conducted months apart and were separated by multiple passages, or generations of cells. Consequently, some of the heterogeneity between experiments are likely the result of chance mutations and the subsequent fixture of specific alleles that could have altered the dynamics of the response. For example, differences in the degree of growth factor receptor expression, the rate of DNA replication and subsequent cell doubling times, or the production and secretion of various bioactive compounds that are central to the wound healing response (e.g., collagen). In a study using 13 individual cell lines that were cultured in parallel, but in different laboratories, demonstrated marked differences in the genetics, biochemistry and physiology of the individual clone maintained in each lab (Ben-David et al., 2018). This could be due to chance mutation as noted above, or it could be due to inadvertent selective pressures that were introduced from maintaining individual clones in different environments. For example, the microenvironment of the individual laboratories (e.g., the rate of airflow, the degree of room air turnover and/or differences in the ambient temperature within the individual cell culture rooms). For this

study, Experiment 1 was conducted in 2017 in a different building than Experiments 2 and 3.

Another source of variation could be the use of different plasticware among the individual experiments. More specifically, experiments 1 and 2 were conducted in 24-well plates while Experiment 3 was conducted in a T-25 flask. Due to differences in the design and specifications of 24-well cell culture plates versus T-25 flasks, there are differences in the rate and degree of gas exchange subsequent to variations in airflow, the available surface area over which gas exchange can occur and/or the depth of the media within the individual plates/flasks. For example, CO_2 and other gases flows over the individual wells in a cell culture plate, whereas in a cell culture flask the gases flow into and fill the airspace within the flask, leading to pockets of stagnant air. Likewise, the relatively larger surface area and the greater depth of the cell culture media in a flask versus cell culture plate will alter the rate of diffusion through the media to the cell layer below.

Each well/flask was marked with a lab marker to provide a point of reference so that the same area of the wound could be imaged post-scratching. This is essential to be able to accurately monitor the wound area over time. Nevertheless, there were instances where the wound area increased with time suggesting that the same area had not been imaged. A difference of even a millimeter of two could have a significant impact at the cellular level since this would shift the image by tens of microns. Consequently, individual measures that suggested an increase in wound size over time were not included in the final models.

Whether the SHSU FA cell line expresses a function estrogen receptor is unknown. A simple first step would be Western blotting for both ER-α and ER-β to determine whether the protein is expressed. If the receptor is absent, this suggests the lack of an effect of BPA is due to an inability to alter estrogen-dependent signaling within the cell. If the receptor is present, however, this still does not guarantee it is functional and/or that can bind BPA. This could be assessed by looking for a change in the expression of known estrogen-dependent genes after estradiol treatment in the presence and absence of estrogen receptor antagonists and ligand binding assays, respectively. Although it is immortal, whether the SHSU FA cell line has undergone transformation is also unknown. Because cell transformation results in the loss of normal homeostatic control this could have a significant impact on the response to wounding, as well as BPA. Various protocols, such as a Soft-Agar assay, can be used to assess whether these cells have undergone cell transformation.

Nevertheless, future studies would benefit from using established cell lines with known doubling times and patterns of gene expression, especially genes that may influence the wound healing response. The 3T3 cell line is commonly used for the investigations of basic fibroblast biology, but it does not express the estrogen receptor (Gaben and Mester, 1991). This makes it a useful model to investigate the influence of estrogen signaling on wound healing in the presence or absence of both estradiol and/or BPA. Specifically, 3T3 cells could be transfected to express either ER-α, ER-β or both ER-α and ER-β followed by estradiol, BPA and estradiol plus BPA after wounding to monitor what effect, if any, estrogen signaling has on the wound healing response.

Alternatively, an *in vivo* study using a laboratory strain of mice such as C57BL/6J could be conducted to investigate the effect of BPA on wound healing in an intact individual. Others have shown that the effect of BPA on wound healing can vary with the route of exposure. Using a full-thickness skin wound Wen et al., (2022), found that dietary exposure delayed the wound closure, whereas topical exposure accelerated wound closure. In another study using ovariectomized mice, Wen et al. (2020) demonstrated the importance of estrogen receptor signaling and the inflammatory response in mediating wound closure.

Finally, wound closure after a scratch assay is the result of either cell proliferation from the wound edges, cell migration into the wound site, or combination of these factors. Cell migration and proliferation are key processes in every phases of wound healing. To what degree either, or both, of these processes contributed to wound healing in the SHSU FA model is unknown. One approach to address this question would be to use Antigen Kiel 67 (Ki-67) staining after scratching under each of the culture condition x BPA treatments; Ki-67 is a well-characterized marker of cell proliferation both *in vivo* and *in vitro*. Meanwhile, cell labeling using fluorescent markers is a reliable means to track individual migrating cells.

In conclusion, it was found that the presence of serum during the post-wounding period was an important modulator on the rate of wound closure in an uncharacterized, spontaneously immortalized fibroblast cell line derived from an abdominal skin biopsy collected from a mouse of an unknown genetic background using a scratch assay. The scratch assay is a widely used *in vitro* model of wounding and the wound healing

response. This is most likely due to the presence of growth factors and essential nutrients that are lacking in serum-free conditions.

In addition, it was found that bisphenol A (BPA) had no effect on the rate of wound closure, despite its ability to impair inflammation at the cellular level. Prior work had shown that BPA impairs the release of pro-inflammatory cytokines from a mouse macrophage cell line in response to LPS. However, because the SHSU FA cell line is uncharacterized, it is unknown whether it is estrogen responsive and/or can maintain normal homeostatic control. Future studies aimed toward characterization of the SHSU FA cell line are warranted, as well as using established, commercially available cell lines to investigate the potential role of BPA as a mediator of wound healing in vitro.

REFERENCES

Alper, S. J. (2016) *Effect on bisphenol a on stress-induced senescence* [Master's thesis, Sam Houston State University]. Scholarly Works @ SHSU.

Ashcroft, G.S., Dodsworth, J., Van Boxtel, E., Tarnuzzer, R.W., Horan, M.A., Schultz, G.S., & Ferguson, M.W.J. (1997). Estrogen accelerates cutaneous wound healing associated with an increase in TGF-β1 levels. *Nature Medicine*, 3, 1209–1215. https://doi.org/10.1038/nm1197-1209

Barrientos S, Stojadinovic O, Golinko M, Brem H, & Tomic-Canic M. (2008). Growth factors and cytokines in wound healing. *Wound Repair and Regeneration*. *16*(5):585-601. https://doi.org/10.1111/j.1524-475x.2008.00410.x

Ben-David, U., Siranosian, B., Ha, G., Tang, H., Oren, Y., Hinohara, K., Strathdee, C. A., Dempster, J., Lyons, N. J., Burns, R., Nag, A., Kugener, G., Cimini, B., Tsvetkov, P., Maruvka, Y. E., O'Rourke, R., Garrity, A., Tubelli, A. A., Bandopadhayay, P., Tsherniak, A., Vazquez, F., Wong, B., Birger, C., Ghandi, M., & Golub, T. R. (2018). Genetic and transcriptional evolution alters cancer cell line drug response. *Nature*, *560*(7718), 325–330. https://doi.org/10.1038/s41586-018-0409-3

Breuer, M. (2018). *Scratch assay digital research journal.* Sam Houston State University.

Bryant, R., & Nix, D. (2007). *Acute & chronic wounds: Current management concepts*. Elsevier.

Carnesecchi, J., Malbouyres, M., de Mets, R., Balland, M., Beauchef, G., Vié, K., Chamot, C., Lionnet, C., Ruggiero, F., & Vanacker, J. M. (2015). Estrogens induce rapid cytoskeleton re-organization in human dermal fibroblasts via the

non-classical receptor GPR30. *PloS one*, *10*(3), e0120672. https://doi.org/10.1371/journal.pone.0120672

Cooper R. L., Segal R. A., Diegelmann R. F., & Reynolds A. M. (2015). Modeling the effects of systemic mediators on the inflammatory phase of wound healing. *Journal of Theoretical Biology*, 367, 86–99. https://doi.org/10.1016/j.jtbi.2014.11.008

Diamanti-Kandarakis, E., Bourguignon, J. P., Giudice, L. C., Hauser, R., Prins, G. S., Soto, A. M., Zoeller, R. T., & Gore, A. C. (2009). Endocrine-disrupting chemicals: An Endocrine Society scientific statement. *Endocrine Reviews*, *30*(4), 293–342. https://doi.org/10.1210/er.2009-0002

Fitridge, R. & Thompson, M. (2011). *Mechanism of vascular disease: Principles of wound healing*. Adelaide.

Gaben, A. & Mester, J. (1991). Balbc mouse 3T3 fibroblasts expressing human estrogen receptors: effect of estradiol on cell growth. *Biochemical and Biophysical Research Communications*, *176*(3), 1473-1481. https://doi.org/10.1016/0006-291X(91)90453-E

Harmening, D. (1996). *Clinical hematology and fundamentals of hemostasis: Introduction to hemostasis*. FA Davis Co.

Hu, Y., Zhang, L., Wu, X., Hou, L., Li, Z., Ju, J., Li, Q., Qin, W., Li, J., Zhang, Q., Zhou, T., Zhang, L., Xu, C., Fang, Z., & Zhang, Y. (2016). Bisphenol A, an environmental estrogen-like toxic chemical, induces cardiac fibrosis by activating the ERK1/2 pathway. *Toxicology Letters*, 250-251, 1–9. https://doi.org/10.1016/j.toxlet.2016.03.008

Islam, M. R., Yamagami, K., Yoshii, Y., & Yamauchi, N. (2016). Growth factor induced proliferation, migration, and lumen formation of rat endometrial epithelial cells in vitro. *The Journal of Reproduction and Development*, *62*(3), 271-278. https://doi.org/10.1262/jrd.2015-158

Iyengar, L., Patkunanathan, B., McAvoy, J. W., & Lovicu, F. J. (2009). Growth factors involved in aqueous humour-induced lens cell proliferation. *Growth Factors*, *27*(1), 50–62. https://doi.org/10.1080/08977190802610916

Johnson. B.Z., Stevenson, A.W., Prele, C.M., Fear, M.W., & Wood, F.M. (2020). The role of IL-6 in skin fibrosis and cutaneous wound healing. *Biomedicines*, *8*(5), 101. https://doi.org/10.3390%2Fbiomedicines8050101

Koh, K.K., Mincemoyer, R., Bui, M.N., Csako, G., Pucino, F., Guetta, V., Waclawiw, M., & Cannon, R.O. (1997). Effects of hormone-replacement therapy on fibrinolysis in postmenopausal women. *New England Journal of Medicine, 336*, 683–691. https://doi.org/10.1056/NEJM199703063361002

Kovats S. (2015). Estrogen receptors regulate innate immune cells and signaling pathways. *Cellular Immunology*, *294*(2), 63–69. https://doi.org/10.1016/j.cellimm.2015.01.018

Le Magueresse-Battistoni B., Multigner L., Beausoleil C., & Rousselle C. (2018). Effects of bisphenol a on metabolism and evidences of a mode of action mediated through endocrine disruption. *Molecular and Cellular Endocrinology*, *475*, 74-91. https://doi.org/10.1016/j.mce.2018.02.009

Liang C, Park A, Guan J. (2007). *In vitro* scratch assay: a convenient and inexpensive method for analysis of cell migration *in vitro*. *Nature Protocols, 2*, 329-333. https://doi.org/10.1038/nprot.2007.30

Liu X, Wang Z, Liu F. (2021). Chronic exposure of BPA impairs male germ cell proliferation and induces lower sperm quality in male mice. *Chemosphere, 262*, 127880. https://doi.org/10.1016/j.chemosphere.2020.127880

Loffredo, L. F., Coden, M. E., & Berdnikovs, S. (2020). Endocrine Disruptor Bisphenol A (BPA) Triggers Systemic Para-Inflammation and is Sufficient to Induce Airway Allergic Sensitization in Mice. *Nutrients*, *12*(2), 343. https://doi.org/10.3390/nu12020343

Malaise, Y., Lencina, C., Cartier, C., Olier, M., Menard, S., & Guzylack-Piriou, L. (2020). Perinatal oral exposure to low doses of bisphenol A, S, or F impairs immune functions at intestinal and systemic levels in female offspring mice. *Environmental Health*, *19*(1), 93. https://doi.org/10.1186%2Fs12940-020-00614-w

Matthews, J., & Gustafsson, J. A. (2003). Estrogen signaling: a subtle balance between ER alpha and ER beta. *Molecular interventions*, *3*(5), 281–292. https://doi.org/10.1124/mi.3.5.281

Melnick, R., Lucier, G., Wolfe, M., Hall, R., Stancel, G., Prins, G., Gallo, M., Reuhl, K., Ho, S. M., Brown, T., Moore, J., Leakey, J., Haseman, J., & Kohn, M. (2002). Summary of the National Toxicology Program's report of the endocrine disruptors low-dose peer review. *Environmental Health Perspectives*, *110*(4), 427–431. https://doi.org/10.1289/ehp.02110427

Paterni, I., Granchi, C., Katzenellenbogen, J. A., & Minutolo, F. (2014). Estrogen receptors alpha (ERα) and beta (ERβ): subtype-selective ligands and clinical potential. *Steroids*, *90*, 13–29. https://doi.org/10.1016/j.steroids.2014.06.012

R Code Team. (2022). *R: A Language and Environment for Statistical Computing*. R Foundation for Statistical Computing.

Rodrigues, M., Kosaric, N., Bonham, C. A., & Gurtner, G. C. (2019). Wound Healing: A Cellular Perspective. *Physiological reviews*, *99*(1), 665–706. https://doi.org/10.1152/physrev.00067.2017

Rose, M.T. (2012). Effect of growth factors on the migration of equine oral and limb fibroblasts using an in vitro scratch assay. *Veterinary Journal*, *193*(2), 539-544. https://doi.org/10.1016/j.tvjl.2012.01.010

Shanle, E. K., & Xu, W. (2011). Endocrine disrupting chemicals targeting estrogen receptor signaling: identification and mechanisms of action. *Chemical Research in Toxicology*, *24*(1), 6–19. https://doi.org/10.1021/tx100231n

Vandenberg, L, Hauser, R, Marcus, M, Olea, N, & Welshons, W.V. (2007). Human exposure to bisphenol a (BPA). *Reproductive Toxicology*, *24*(2), 139-177. https://doi.org/10.1016/j.reprotox.2007.07.010

Verdier-Sévrain, S., Bonté, F., & Gilchrest, B. (2006). Biology of estrogens in skin: implications for skin aging. *Experimental Dermatology*, *15*(2), 83–94. https://doi.org/10.1111/j.1600-0625.2005.00377.x

Wen, X., Zhu, M., Li, Z., Li, T., & Xu, X. (2022). Dual effects of bisphenol A on wound healing, involvement of estrogen receptor β. *Ecotoxicology and Environmental Safety*, *231*, 113207. https://doi.org/10.1016/j.ecoenv.2022.113207

Zia, Irma. (2020). *The effect of plasticizer treatment of inflammation and wound healing* [Master's thesis, Sam Houston State University]. Scholarly Works @ SHSU.

Zhou, T., Yang, Z., Chen, Y., Chen, Y., Huang, Z., You, B., Peng, Y., & Chen, J. (2016). Estrogen accelerates cutaneous wound healing by promoting proliferation of epidermal keratinocytes via Erk/Akt signaling pathway. *Cellular Physiology and Biochemistry*, *38*(3), 959–968. https://doi.org/10.1159/000443048

APPENDIX A

Flatten and Crop ImageJ Macro

```
showMessage("Specify the directories in the following order:\n\n1. Where the
tiffs converted from the raw files are stored\n\n2. Where to store the flattened
tiffs\n\n3. Where you would like to store the newly-cropped tiffs")

dir1 = getDirectory("Choose a Directory");
dir2 = getDirectory("Choose a Directory");
dir3 = getDirectory("Choose a Directory");
list = getFileList(dir1);

setBatchMode(true);

for (i=0; i<list.length; i++) {
   showProgress(i+1, list.length);
   open(dir1+list[i]);
run("Flatten");
getDimensions(width, height, channels, slices, frames);
if (height>width) {
run("Rotate 90 Degrees Right");
  }
saveAs("Tiff", dir2 + list[i]);
close();
}

list2 = getFileList(dir2);
setBatchMode(true);

for (j=0; j<list.length; j++) {
   showProgress(j+1, list.length);
   open(dir2+list[j]);

makeRectangle(1600,400,2100,2900);
run("Crop");
saveAs("Tiff", dir3 + list[j] + "_cropped");
close();
}
```

Note. ImageJ macro used to flatten and crop the image tiff to complete analysis.

APPENDIX B

Automatic Scratch Detection and Measurement ImageJ Macro

```
showMessage("Select the directories in the following order:\n\n1. Location of cropped
images to be analyzed\n\n2. Location to save output\n\n3. Location to move the
unedited cropped images that have already been analyzed");
dir1 = getDirectory("Choose a Directory");
dir2 = getDirectory("Choose a Directory");
dir3 = getDirectory("Choose a Directory");
list = getFileList(dir1);
setBatchMode(true);

for (i=0; i<list.length; i++) {
 showProgress(i+1, list.length);
  open(dir1+list[i]);
name = File.nameWithoutExtension;

run("Enhance Contrast...", "saturated=0.2");
run("Bandpass Filter...", "filter_large=40 filter_small=5 suppress=None tolerance=5");
run("Find Edges");
run("Find Edges");
run("8-bit");
setAutoThreshold("Huang");
run("Find Edges");
run("Convert to Mask");
run("Fill Holes");
run("Open");
setBatchMode(false);
run("Analyze Particles...", "size=5000-Infinity pixel include add");

run("Revert");

run("Scale to Fit");

selectWindow("ROI Manager");

roiManager("save", dir2 + name + "_roi.zip");

roiManager("reset");
selectWindow(list[i]);
run("Close");
setBatchMode(true);

File.rename(dir1 + list[i], dir3 + list[i]);
}
```

Note. ImageJ macro code

APPENDIX C

Non-Scratch Area Removable ImageJ Macro

```
showMessage("Select the directories in the following order:\n\n1. Location of cropped
images\n\n2. Location of ROI files from automated analysis\n\n3. Location to save the new
measurements \n\n4. Location to save the new overlaid images");
dir1 = getDirectory("Choose a Directory");
dir2 = getDirectory("Choose a Directory");
dir3 = getDirectory("Choose a Directory");
dir4 = getDirectory("Choose a Directory");
list = getFileList(dir1);

setBatchMode(true);
for (i=0; i<list.length; i++) {
  showProgress(i+1, list.length);
  open(dir1+list[i]);
name = File.nameWithoutExtension;

setBatchMode(false);
run("Scale to Fit");
roiManager("open", dir2 + name + "_roi.zip");
roiManager("Set Color", "red");
roiManager("Set Line Width", 3);

selectWindow("ROI Manager");
waitForUser("Remove non-scratch particles");

roiManager("deselect");
roiManager("multi measure append");
saveAs("Measurements", dir3 + "measurements_" + name + ".csv");

roiManager("Show All without labels");
run("Flatten");
saveAs("Tiff", dir4 + name + "_overlay");
roiManager("reset");
run("Close");

selectWindow("Results");
run("Close");
selectWindow(list[i]);
close();
setBatchMode(true);
}
```

Note. ImageJ Macro code

VITA

Emily E. Hays

Education

08/2021 – present: **Master of Science, Biology**
Sam Houston State University
Huntsville, TX

08/2018 – 05/2021: **Bachelor of Arts, Psychology**, *cum laude*
Minor, Microbiology
University of Arizona
Tucson, Az

Research Interests

- Effects of plasticizing agents and cell culture conditions on the wound healing response *in vitro*.
- Physiological functions with aging and longevity using model organisms.
- Understanding the stress of migration to the United States in Latinx Adolescent Children and their attachment to their caregiver.
- Phylogenies and or stress of tick-borne viruses and their effects on rodents and humans, specifically, Colorado Tick Fever Virus and Lone Star Virus.

Peer Reviewed Publications

- **Hays, E.E.** & Harper, J.M. *In revision.* Weight gain and longevity in speckled cockroaches (*Nauphoeta cinerea*): Effect of food availability during development. *Trends in Entomology*.

Posters and Presentations

- **Emily E. Hays** and James M. Harper. 2023. Do Cell Culture Conditions or Bisphenol A (BPA) Treatment Affects the Wound Healing Response *In Vitro*? Texas Academy of Science, 126th Meeting, San Angelo State University.
- **Emily E. Hays** and Jeremy Bechelli. 2022. Phylogenetic Analysis of Colorado Tick Fever Virus. American Society of Microbiology, Texas Branch Meeting, Online.

TEACHING AND ACADEMIC WORK EXPERIENCE

- Graduate Assistant, Department of Biological Sciences, Sam Houston State University, Huntsville, TX, Fall 2021-present.
- Academic Review Panel Graduate Student Member, College of Science Engineering Technology, Sam Houston State University, Huntsville, Tx, Spring 2022-present.
- Graduate/Undergraduate Instructor Academy, Sam Houston State University, Huntsville, TX, Fall 2021, Spring 2022, Fall 2022, Spring 2023.
- Bring Em' Back Kats Workshop, College of Education, Sam Houston State University, Huntsville, Tx, September 2022.

- Undergraduate Preceptor, Department of Microbiology, University of Arizona, Tucson, AZ, Spring 2021.

COURSES TAUGHT

- General Biology
- Introductory Applied Microbiology
- General Microbiology

HONORS & AWARDS

- COSET Student Excellence in Teaching Award, College of Science Engineering Technology, Sam Houston State University, Huntsville, TX, Summer 2023.
- Joey Harrison Biological Sciences Student Research Award, Department of Biological Sciences, Sam Houston State University, Huntsville, TX, Summer 2023. - **$1000**
- Graduate Travel Award, Sam Houston State University, Huntsville, TX, Spring 2023. **-$600**

MEMBERSHIPS IN PROFESSIONAL SOCIETIES

- Texas Academy of Science, 2021-present
- American Society of Microbiology, 2021-present
- Entomological Society of America, 2022-present