An In Vivo Biocompatibility Analysis of A Novel Tissue Regeneration Matrix Using A Pig Model

Shamar Thomas
shamart@g.clemson.edu

Follow this and additional works at: https://tigerprints.clemson.edu/all_theses

Part of the Biomedical Engineering and Bioengineering Commons, Diseases Commons, Materials Science and Engineering Commons, and the Medical Specialties Commons

Recommended Citation
https://tigerprints.clemson.edu/all_theses/4027
AN IN VIVO BIOCOMPATIBILITY ANALYSIS OF A NOVEL TISSUE REGENERATION MATRIX USING A PIG MODEL

A Thesis
Presented to
the Graduate School of
Clemson University

In Partial Fulfillment
of the Requirements for the Degree
Master of Science
Bioengineering

by
Shamar Thomas
May 2023

Accepted by:
Dr. Brian Booth, Committee Chair
Dr. Dan Simionescu
Dr. Angela Alexander-Bryant
ABSTRACT

The goal of this project is to develop an injectable bead scaffold to promote tissue regeneration in the void created by lumpectomy and to alleviate post lumpectomy problems by preventing local recurrence and minimizing surgical-related infections. Microbeads were synthesized from collagen type I and crosslinked with tannic acid to form the basis for this injectable therapeutic. Tannic acid acts as a therapeutic anticancer agent. The action mechanisms of tannins in breast cancer cells have been studied with studies showing tannins to be cytotoxic to cancer cells in a dose-dependent manner. Tannic acid induces apoptosis in breast cancer cells via caspase pathways. Tannic acid has also demonstrated antimicrobial properties. Collagen type I is a biomaterial routinely used in reconstructive surgeries.

To test the viability of tissue regeneration aspect of this potential therapy, an in vivo study was conducted using a pig model. The collagen/tannic acid beads were seeded with pig adipocytes to attach and grow on the matrix. The cells remodel the collagen as they grow thus releasing the tannic acid into the surrounding environment. Beads were injected orthotopically into the mammary glands, then excised at two time points for histological analysis. Tissue samples were fixed, embedded in paraffin, sectioned, and stained to determine cell types present. Additionally, samples of the kidneys and livers were collected for analysis. Our results indicate that the collagen beads were successful at promoting tissue regeneration, specifically by increasing expression of VEGFR, presence of adipocytes, intermediate filaments, and fibroblast.
ACKNOWLEDGMENTS

First, I would like to thank God for the opportunities He has provided me with and for the people He has placed in my life to help me achieve success. I would like to thank my family and friends for their constant encouragement, love, and support. Next, I would like to thank my advisor, Dr. Brian Booth for his guidance and support. I am so appreciative for your mentorship and for your direction and patience with me throughout my thesis completion. I would also like to thank my committee members, Dr. Dan Simionescu and Dr. Angela Alexander-Bryant for their time and insight during this process. I would also like to Dr. Heather Dunn as well for her help along the way. Thank you to Dr. Luigi Strizzi for your helpful analysis on the kidney and liver samples. I would like to thank my fellow lab members for their assistance on this project. Thank you to the Clemson University Department of Bioengineering for accepting me and providing me with the foundation and tools necessary for a career in bioengineering. To all my professors and to the departmental staff that helped me along the way, thank you. Lastly, I would like the CURF-Maturation fund for the funding that made this research possible.
TABLE OF CONTENTS

Page

TITLE PAGE.................................................................................................................................................. 1

ABSTRACT ....................................................................................................................................................... 2

ACKNOWLEDGMENTS..................................................................................................................................... 3

LIST OF FIGURES.......................................................................................................................................... 6

LIST OF TABLES............................................................................................................................................. 7

CHAPTER

I. INTRODUCTION AND BACKGROUND................................................................. 8

1.1 Breast cancer ......................................................................................................................... 8
1.2 Tannic acid ........................................................................................................................... 10
1.3 Collagen type I ...................................................................................................................... 11
1.4 TA crossed linked with collagen type 1 for tissue regeneration ...................................... 13
1.5 Tannic Acid (TA) Applications........................................................................................... 14
1.6 Tannic acid to prevent cancer recurrence ........................................................................ 15
1.7 Effects of tannic acid on various cancer types ................................................................... 16

II. AN IN VIVO BIOCOMPATIBILITY ANALYSIS OF A NOVEL TISSUE REGENERATION MATRIX USING A PIG MODEL.................................................... 22

2.1 Introduction ............................................................................................................................ 22
2.2 Material and Methods.......................................................................................................... 23
2.3 Collagen Bead Preparation.................................................................................................... 24
2.4 In Vivo Study ......................................................................................................................... 29
2.5 Tissue Sectioning................................................................................................................... 31
2.6 Mammary Tissue Histological Staining ............................................................................. 32
2.7 Kidney and Liver staining .................................................................................................... 32

III. RESULTS.................................................................................................................................................. 34

3.1 Kidney and Liver Analysis..................................................................................................... 34
3.2 Mammary Tissue Analysis..................................................................................................... 35
3.3 Immunohistochemistry Staining (IHC) ............................................................................. 37
IV. CONCLUSION ........................................................................................................ 48

4.1 Conclusions ........................................................................................................ 48
4.2 Future Research ................................................................................................... 49

REFERENCES ........................................................................................................... 51
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figures</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1 Lumpectomy Procedure</td>
<td>9</td>
</tr>
<tr>
<td>1.2 Chemical Structure of Tannic Acid</td>
<td>10</td>
</tr>
<tr>
<td>1.3 Hydrogen bonding between collagen and tannic acid</td>
<td>12</td>
</tr>
<tr>
<td>2.3.1 Electrostatic Bead Generator Setup</td>
<td>28</td>
</tr>
<tr>
<td>2.3.2 Electrostatic Bead Generator Components</td>
<td>29</td>
</tr>
<tr>
<td>2.4 In Vivo study experimental design</td>
<td>31</td>
</tr>
<tr>
<td>3.1 H&amp;E Staining of Kidney Sections</td>
<td>34</td>
</tr>
<tr>
<td>3.2.1 H&amp;E Staining of Mammary Tissue Sections</td>
<td>36</td>
</tr>
<tr>
<td>3.2.3 Masson’s Trichrome Staining of Mammary Tissue Sections</td>
<td>38</td>
</tr>
<tr>
<td>3.3.1 Adiponectin Staining of Mammary Tissue Sections</td>
<td>40</td>
</tr>
<tr>
<td>3.3.3 Vimentin Staining of Mammary Tissue Sections</td>
<td>41</td>
</tr>
<tr>
<td>3.3.5 CD163 Staining of Mammary Tissue Sections</td>
<td>43</td>
</tr>
<tr>
<td>3.3.7 VEGF-R Staining of Mammary Tissue Sections</td>
<td>44</td>
</tr>
<tr>
<td>3.3.9 CD68 Staining of Mammary Tissue Sections</td>
<td>46</td>
</tr>
</tbody>
</table>
## LIST OF TABLES

<table>
<thead>
<tr>
<th>Tables</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.7 TA effects on various cancer cell lines</td>
<td>21</td>
</tr>
</tbody>
</table>
CHAPTER ONE
INTRODUCTION AND BACKGROUND

Tannic acid as a potential cancer therapeutic

This manuscript has been submitted to The International Journal of Molecular Sciences

1.1 Breast cancer

Breast cancer is currently the most common cancer globally, accounting for 12.5% of all new annual cancer cases worldwide, and breast cancer the second most common cancer in women after skin cancer in the United States. About 13% (about 1 in 8) of U.S. women will develop invasive breast cancer in the course of their lives. On average, every 2 minutes a woman is diagnosed with breast cancer in the United States. Breast cancer accounts for about 30% of all new cancer cases in women each year in the United States [1]. Almost 5% to 10% of breast cancers can be linked to known gene mutations inherited from one’s mother or father. Mutations in the BRCA1 and BRCA2 genes are the most common. In 2022, an estimated 287,850 new cases of invasive breast cancer were expected to be diagnosed in women in the U.S., along with 51,400 new cases of non-invasive breast cancer. This year an estimated 43,550 women will die from breast cancer in the U.S. Nearly 4 million women in the US alone have been diagnosed with breast cancer [1].

Current treatment options for breast cancer include surgery, chemotherapy, and radiation. Unfortunately, there are negative side effects associated with each of these treatment options, and they may not be appropriate for every patient. Surgical removal of breast tumors is an important step in treating breast cancer. In addition, local breast cancer recurrence presents a high risk, especially for patients undergoing procedures known as "Breast-conserving surgery" (BCS). BCS
removes cancer while leaving as much normal tissue as possible. Usually, some surrounding healthy tissue and lymph nodes also are removed to ensure all the cancer is out of the area. The main advantage is that a woman keeps most of her breast. One type of breast-conserving surgery is lumpectomy. A lumpectomy removes the least amount of breast tissue needed to remove the tumor. In many cases patients will also need radiation. Women who have mastectomy for early-stage cancers are less likely to need radiation. Radiation therapy uses high-energy particles or waves, such as x-rays, gamma rays, electron beams, or protons, to destroy or damage cancer cells. Cancer cells grow and divide faster than most normal cells. Radiation works by making small breaks in the DNA inside cells. These breaks keep cancer cells from growing and dividing and cause them to die. Nearby normal cells can also be affected by radiation [2].

![Lumpectomy procedure](image_url)

**Figure 1.1**: Lumpectomy procedure (Keck School of Medicine of USC, 2016).

### 1.2 Tannic acid (TA)
Polyphenol-based materials have attracted wide-spread interest from academic and industrial communities because of their unique structure and physicochemical properties [4]. Tannic acid (TA) is a form of tannin, a naturally occurring plant polyphenol that is taken into consideration for a variety of medical applications. TA has been approved as a safe compound by the US Food and Drug Administration (FDA), thereby it can be used as an excipient/active supplement in food, drink, and pharmaceutical formulations. TA is abundantly available, and is present in plant leaves, green tea, fruits, vegetables, nuts, red wine, coffee, and wood bark [5]. TA is one of the main examples of tannins that can be efficiently produced from natural sources with high efficiency, and thus, it attracts much scientific interest. TA interacts with various substances (proteins, polysaccharides, and metals) through several modes including hydrogen bonding, hydrophobic interactions, and electrostatic interactions. TA also possesses a variety of restorative, therapeutic and pharmacological properties, including anti-cancer, anti-inflammatory, antimicrobial, antiviral, antioxidant, wound healing, and homeostatic characteristics [3,4]. TA causes cell cycle arrest and apoptosis of a variety of cancer cells [4].

Tannins are divided into two classifications: hydrolysable or condensed [6,7]. The main structure of hydrolysable includes a polyhydric alcohol core and hydroxyl groups that are either partially or wholly esterified by hexahydroxy diphenic or gallic acids seen in Figure 1.2 Condensed tannins are a product of polymerized flavan-3-ols and flavan-3,4-diols [8].
1.3 Collagen type I

Collagen type I is the most abundant protein in animals and humans and is routinely used as a biomaterial in reconstructive surgeries [9]. In most soft tissues, collagen type I fibrils create a network that comprises the majority of the extracellular matrix (ECM), a highly organized scaffold that surrounds the cells. Collagen also plays an important role in maintaining the structural integrity of the ECM [12]. In vivo, collagen is abundant in the tendons, skin, vasculature, and connective tissues making it easy to isolate [11]. For therapeutic purposes, natural collagen can be formed into three-dimensional scaffolds ex vivo that are intrinsically biocompatible, biodegradable, and non-toxic. The three-dimensional scaffolds mimic the basic properties of the extracellular matrix (ECM) to promote reconstruction of native tissues [10].

To provide an effective scaffold for tissue regeneration, a biomaterial must provide structural support for new tissue growth without triggering an immune response. Additionally, it is favored that the material be degradable into natural components that are also biocompatible. Collagen type I is enzymatically biodegradable by matrix metalloproteinases through hydrolysis [12]. The byproducts of this reaction can be further degraded by gelatinases.
Creating a collagen scaffold for therapeutic use requires a method of crosslinking to maintain structural integrity. In solution, TA can diffuse into gelated collagen to create these crosslinks [13]. Specifically, TA forms hydrogen bonds between the phenols of gallic acid and the amines of the collagen peptide backbone as shown in (Figure 1.3) [13]. This project uses this property to create collagen/TA beads and increases the structural integrity to maintain the bead shape in solution.

![Collagen amino acid sequence and gallic acid group](image)

**Figure 1.3:** Hydrogen bonding between collagen and tannic acid.

**1.4 TA crossed linked with collagen type 1 for tissue regeneration**

TA has also been utilized in biomaterials research as a natural crosslinking agent to improve mechanical properties of natural and synthetic hydrogels and polymers, while also
imparting anti-inflammatory, antibacterial, and anticancer activity to the materials [14]. Collagen type I is the most abundant protein in animals and humans and is routinely used as a biomaterial in reconstructive surgeries. The three-dimensional scaffolds mimic the basic properties of the ECM to promote reconstruction of native tissues. To provide an effective scaffold for tissue regeneration, a biomaterial must provide structural support for new tissue growth without triggering an immune response. Additionally, the material should be degradable into natural components that are also biocompatible. However, isolated collagen for clinical applications has poor thermal stability. Creating a collagen scaffold for therapeutic use requires a method of crosslinking to maintain structural integrity. In solution, TA diffuses into gelated collagen to create crosslinks. Specifically, TA forms hydrogen bonds between the phenols of gallic acid and the amines of the collagen peptide backbone (Figure 1.3) [15]. Using collagen/TA beads as an injectable tissue matrix not only acts as a minimally invasive tool for breast reconstruction following lumpectomy, but slowly releasing TA overtime into the surgical void may potentially decrease rates of tumor recurrence in the area [16].

1.5 Tannic acid (TA) applications

Scaffolds play an important role in tissue constructs as a mechanically stable structure for cell adhesion, growth, extracellular deposition, and healing. Tissue constructs can be assembled from cells of specific tissue types, either primary cells or tissue culture lines. Tissue constructs are also useful platforms for basic research and drug discoveries [14]. Properties of a scaffold that affects tissue regeneration include porosity, pore size, surface chemistry, biodegradability, and mechanical properties [15]. The properties that affect the degree of microbial infection include the
host’s immune response and inflammation. Therefore, TA has attracted attention as a natural crosslinker to improve stability of biopolymers used in tissue regeneration [14]. To provide an effective scaffold for tissue regeneration, a biomaterial must provide structural support for new tissue growth without triggering an immune response. Additionally, the biomaterial must be degradable into natural components that are biocompatible. Scaffolds based on natural biopolymers, like collagen and hyaluronic acid are enzymatically biodegradable. Scaffold depolymerization by ROS generated by human tissues can be lowered through complexation with TA [14]. TA is equipped with incredible therapeutic and regenerative properties which make TA a great candidate for wound healing applications. Therefore, most research studies about TA in the tissue and regenerative medicine have been devoted to wound healing. TA can interact with biopolymers and macromolecules by cross-linking due to its hydroxy and carboxy groups, posing it as a promising pharmaceutical candidate [15]. In recent years, a growing number of reports describe new mechanisms of TA activity and possible application not only in primary chemoprevention, but also in sensitization to conventional drugs used in anticancer therapy [3].

1.6 Tannic acid to prevent for cancer recurrence

1.6 TA Induces Apoptosis

Apoptosis, a cell’s natural mechanism for death, is a promising target for anticancer therapy. Both the intrinsic and extrinsic pathways use caspase mediators during apoptosis through the cleavage of hundreds of proteins [17]. Condensed tannins inhibit fatty acid synthase (FAS) to induce apoptosis [18]. The condensed tannins inhibited the β-ketoacetyl reductase domain of FAS activity, and the inhibition was competitively to NADPH. Zhang et al. hypothesized that the
NADPH loading site in the β-ketoacyl reductase domain of FAS is a reaction site for the tannins [18]. The enzyme FAS is involved in the *de novo* synthesis of long fatty acid chains and is highly expressed in breast cancers [19]. This makes FAS an important target for novel cancer therapeutics as inhibition of this enzyme induces apoptosis. When treated with condensed tannins, estrogen receptor positive (ER⁺) breast cancer cells experienced significant growth inhibition and lowered cell viability. Additionally, Shirode *et al.* aimed to determine the action mechanism of cell growth inhibition by hydrolysable tannins in pomegranate extract using ER⁺ breast cancer cells [20]. The researchers confirmed previous results that tannins may induce cell cycle arrest in G2/M to inhibit cell growth and induce apoptosis. Analysis of microarrays demonstrated that the tannins derived from pomegranate extract downregulated genes associated with chromosome organization, RNA processing, DNA replication, mitosis, and DNA repair. These results demonstrate that TA induces apoptosis through multiple mechanisms in ER⁺ breast cancer cells and demonstrate advanced understanding of the apoptotic mechanisms of tannins, furthering research into the chemotherapeutic use [18,20].

TA combined with other drugs results in significant induction of apoptosis in CaCo-2 colon adenocarcinoma cells [21]. In YD-38 gingival cancer cells, TA promoted cell cycle arrest in the G1 phase by modulating the phosphorylation of STAT3 and JAK2 proteins and mitochondrial apoptosis [22]. TA treatment upregulated the expression of cleaved caspase-3 which confirmed induction of apoptosis in UMUC3 bladder cancer cells when treated with tannic acid. TA also inhibited proliferation and induced apoptosis in UMUC3 cells by altering the AKT pathway in a dose-dependent manner [23]. Together, these results showed that TA can promote apoptosis; therefore, it may have potential as treatment for cancer.
1.7 Effects of tannic acid on various cancer types

1.7.1 Effects of Tannic Acid on Breast Cancer

Breast cancers are classified by the presence or absence of hormone receptors and the human epidermal growth factor receptor-2 (HER2). Breast tumors are examined pathologically to determine classification: ER\(^+\), progesterone receptor positive (PR\(^+\)), HER2 positive (HER2\(^+\)), triple positive, or triple negative. ER\(^+\) breast cancer is sensitive to the anticancer properties of tannins [24]. TA induces changes in morphology, from spindle-shaped cells to round cells in ER\(^+\) MCF-7 breast cancer cells after 24 hours of exposure, showing that ER\(^+\) breast cancer cells are highly sensitive to the apoptotic effects of TA [24]. Activation of caspases 3/7 and 9 initiates apoptosis in ER\(^+\) breast cancer cells [25]. The higher sensitivity to TA treatment is correlated to the lack of caspase 3 expression by MCF-7 cells.

In addition to the effect on ER\(^+\) cells, hydrolysable tannins from pomegranate extracts reduce growth and tumor volume in HER2\(^+\) breast cancer cells [24]. Treatment with pomegranate extract decreased levels of proteins that regulate angiogenesis, cell proliferation, and inflammation, key processes in cell viability [20]. Booth et al., determined TA released from the remodeling of TA cross-linked collagen beads seeded with preadipocytes cells significantly inhibits cell growth of the HER2\(^+\) breast cancer cells [26]. Condensed tannins in green tea leaves, epigallocatechin-3-gallate (EGCG), can be used in treatments to induce a dose-dependent decrease in ATP production in HER2\(^+\) breast cancer cells, specifically those resistant to the chemotherapy trastuzumab [27]. Treatment with EGCG increased p27 expression, a growth regulator. Therefore,
treatment of EGCG could lead to decreased resistance to chemotherapies of HER2+ breast cancer cells.

Triple-negative breast cancer (TNBC) accounts for about 10-15% of all breast cancers. TNBC differs from other types of invasive breast cancer in that it grows and spreads faster, has fewer treatment options, and tends to have a worse prognosis. The effect of TA on TNBC cells has been investigated [24,25]. Although the effects of TA were less significant on TNBC cells compared to other types of breast cancers, there was a change in the morphology of the triple negative cells. The proliferation rate of TA treated cells was lower than that of untreated cells, TA also induced a significant level of apoptosis in treated TNBC [24,25].

1.7.2 Effects of Tannic Acid on Prostate Cancer

Prostate cancer (PCa) is the most common cancer and the second leading cause of cancer death among men in the United States [28]. TA treatment hinders the growth, clonogenicity, invasive, and migratory potential of prostate cancer cells [29]. Endoplasmic reticulum (EDR) stress is a target of interest with significant clinical importance in chemotherapy. TA has the capability to induce EDR stress by reducing the generation of reactive oxygen species (ROS) in PCa cells [30]. Interference with EDR functions leads to the unfolded proteins, as detected by transmembrane sensors that instigate the misfolding protein response (UPR). Therefore, controlling induced UPR via EDR stress with natural compounds could be a novel therapeutic strategy for the management of prostate cancer. TA activates EDR stress response protein kinase R-like endoplasmic reticulum kinase (PERK), and inositol requiring enzyme 1 (IRE1). TA alters expression of various regulatory proteins (ATF4, Bip, and PDI). TA induced the expression of the
apoptosis-associated markers, Bak, Bim, cleaved caspase 3, and cleaved PARP; while also downregulating various pro-survival proteins including Bcl-2 and Bcl-xL. TA increased the efficacy of chemotherapies doxorubicin and docetaxel, decreasing the proliferation of prostate cancer cells [3]. TA also alters lipid metabolism and disrupts cellular and nuclear membranes in PCa cells. Results indicate that TA could be a potential antitumorigenic weapon in treating PCa.

1.7.3 Effects of Tannic Acid on Lung Cancer

Lung cancer (LC) is the leading cause of cancer deaths worldwide. There are two main types of lung cancer, non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC). About 85% of lung cancers are NSCLC with the remaining SCLC [26]. Despite recent advancements in standard treatment approaches such as surgery, radiation, chemotherapy, and targeted therapy, lung cancer survival rates remain low [31]. Lung fluid (LF) is a major barrier for distribution of drugs to the lungs. TA is a useful diagnostic tool as a dye that is used to stain plasma membranes, particularly binding most prominently to the margins of tumors. This suggests TA binding and targeting efficiency to cancer cells. The extent of adsorption of LF proteins by TA was revealed by fluorescence quenching in fluorescence spectroscopy [31]. TA binds to LF and forms DNA self-assemblies, which profoundly enhance interaction with LC cells [32]. This study also demonstrated many reasons why TA is a novel carrier for pharmaceutical drugs such as gemcitabine, carboplatin, and irinotecan. A dose-dependent effect was observed with these pharmaceutical drugs treated against A549 and H1299 LC cells. Drug-encapsulated TA-LF complexes combined with gemcitabine, carboplatin, and irinotecan increased cell viability in LC cells. Therefore, determining TA-LF Improves pharmaceutical activity in LC cells.
NSCLCs are highly vascularized tumors, and chemotherapy is often hampered by the development of angiogenesis. Therefore, suppression of angiogenesis is considered a potential treatment approach. In lung cancer cells, TA inhibits stemness by triggering caspase-dependent mitochondria-mediated apoptosis. Stemness is the ability of a cell to perpetuate its lineage, to give rise to differentiated cells, and to interact with its environment to maintain a balance between quiescence, proliferation, and regeneration [33]. TA inhibits the migration and invasion potential of NSCLC cells. TA inhibits NSCLC’s invasion and migratory abilities thereby limiting tumor progression and metastasis [31].

1.7.4 Effects of Tannic Acid on Colorectal Cancer

Colorectal cancer (CRC) is the third most common malignancy worldwide and is one of the leading causes of cancer-related death [34]. The chemotherapy, oxaliplatin (OXA) is essential for the treatment of colorectal patients after the surgical resection of tumor masses. OXA, is a third-generation platinum-based chemotherapeutic agent for the treatment of metastatic colorectal cancer [35]. OXA, causes several serious side effects during the treatment of colorectal cancer. Finding effective strategies to mitigate the cytotoxic effect caused by OXA while also enhancing the anticancer effect on colorectal cancer cells is clinically relevant [36]. CRC is one of the earliest types of cancers that has been reported to respond to the anticancer activity of TA [16]. TA has a different antitumor mechanism compared with OXA, therefore the combination of OXA and TA may have a stronger antitumor effect and reduce the possibility that tumor obtains resistance to OXA. The combination of OXA and TA enhanced apoptosis of CT26 cells in colon cancer in vivo. The combination of the two anti-cancers improved the quality of life and prolonged the survival
time of colorectal peritoneal carcinomatosis CT26 mice model [35]. OXA/TA shows promising anti-angiogenesis and antitumor effects and has potential as an application in the treatment of colorectal cancer [36].

Other studies demonstrated that TA decreased the cellular viability of CaCo-2 colon cancer cell lines in a dose- and time-dependent manner by increasing the apoptotic index of these cell lines. TA induced the Bak and Fas-associated protein with death domain (FADD) protein percentage ratios in these CRC cells. TA induces apoptosis in CRC cells through mitochondrial and death receptor pathways [16]. TA acts as a selective inhibitor of pyruvate kinase isoenzyme M2 (PKM2) in CRC cells, resulting in CRC cellular proliferation [37]. These studies collectively support the anticancer activity of TA in CRC both \textit{in vitro} and \textit{in vivo}.

1.7.5 \textit{Effects of Tannic Acid on Liver Cancer}

Liver cancer is a common cancer worldwide and is also one of the most difficult cancers to treat. Liver cancer results in almost one million deaths per year worldwide, and the danger of this cancer is compounded when the tumor is unresectable [38]. Hepatocellular carcinoma (HCC) is the most common type of liver cancer and has the third highest mortality rate worldwide. Cisplatin (cis-dichlorodiamine platinum, CDDP) is an effective and commonly used chemotherapeutic drug for certain advanced carcinomas. However, CDDP clinical application is limited by side effects and the development of drug resistance [16,37]. TA acts in a synergistic manner with CDDP in preventing liver cancer progression \textit{in vitro} through inducing mitochondrial-mediated apoptosis. Combined TA and CDDP treatment suppress HepG2 liver cancer cell viability and affects cell morphology. TA and CDDP synergistically inhibits cell
growth, indicating that TA and CDDP exerted synergistic effects on HepG2 cells. TA and CDDP synergistically increases apoptosis in HepG2 cells [37]. TA induced caspase activation and increased the presence of cellular ROS and reactive nitrogen species (RNS), while down regulating antioxidant expression. TA exposure also increased cell death and increased DNA fragmentation [34].

Table 1.7: TA effects on various cancer cell line

<table>
<thead>
<tr>
<th>Cancer types</th>
<th>TA treated cell lines</th>
<th>Citations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Breast cancer</td>
<td>MCF7, TNBC</td>
<td>[23-27]</td>
</tr>
<tr>
<td>Prostate cancer</td>
<td>BCL-2, MMPs, PERK</td>
<td>[29-30]</td>
</tr>
<tr>
<td>Lung cancer</td>
<td>A549, H1299</td>
<td>[31-32]</td>
</tr>
<tr>
<td>Colorectal cancer</td>
<td>CT26, CaCo-2</td>
<td>[35-36]</td>
</tr>
<tr>
<td>Liver Cancer</td>
<td>HepG2</td>
<td>[37-38]</td>
</tr>
</tbody>
</table>
2.1 Introduction

Breast cancer is the most common cancer in women in the United States and is the second leading cause of cancer death in women. Breast cancer accounts for about 30% of new female cancers [1]. Overall, the average risk of a woman in the United States developing breast cancer sometime in her life is about 13%. Surgical removal of breast tumors is an important step in treating breast cancer at any stage. An estimated 20% to 30% of women diagnosed, treated, and declared free of disease will have a recurrence [27]. Surgical treatment of breast cancer tumors often focuses on breast-conserving surgeries (BCS), lumpectomy, unless total mastectomy is required due to tumor size or tumor aggression [1].

Reconstructive surgery is chosen by many patients following full or partial mastectomy and is an essential part of patient care. Breast reconstruction is a procedure that can restore the shape, symmetry, and size of one or both breasts following a lumpectomy or mastectomy treatment for breast cancer [40]. Reconstruction offers women the opportunity to reclaim control of their body and self-image following the psychological journey through breast cancer diagnosis and treatment. During a lumpectomy, a lump is removed from the patient's breast, often altering the breast's shape and size. For many women, the loss of breast tissue can negatively impact confidence and self-esteem. They also feel dissatisfied with the perceived lack of symmetry and
balance in their body, particularly if only one breast has been affected [40]. Current breast reconstruction procedures use saline or silicone implants, but there are many problems that come with the implants. They can break, rupture causing infection and pain. Scar tissue may form around the implant, which can make the breast hard or change shape. The longer a patient has breast implants, the chance of needing more surgery to remove or replace the implant later increases. There is a need for a minimally invasive medical implant that not only naturally restores the removed breast tissue but can also play a role in prevention of breast cancer recurrence.

Our group has developed a collagen type I-tannic acid bead matrix that could greatly aid in breast tissue regeneration post-lumpectomy and prevention of tumor recurrence [13]. The injectable beads have the potential to improve upon the concept of breast reconstruction as a tissue regenerative device. Using collagen beads seeded with the patient’s cells would promote natural tissue regeneration, unlike the current silicone or saline implants. The small size beads allow for filling of the small voids and scars to create a more natural look, just like lipofilling procedures.

We tested the viability of the tissue regeneration aspect of this potential therapy, by conducting an in vivo study using a pig model. The collagen/tannic acid beads were seeded with pig adipocytes to attach and grow on the matrix. The cells remodel the collagen as they grow thus releasing the tannic acid into the surrounding environment. Beads were injected orthotopically into the mammary glands of the pig, then excised at two time points for histological analysis. Tissue samples were fixed, embedded in paraffin, sectioned, and stained to determine cell types present. Additionally, samples of the kidneys and livers were collected for analysis. Our results indicate that the collagen beads were successful at promoting tissue generation, specifically by increasing expression of VEGFR, presence of adipocytes, intermediate filaments, and fibroblast.
2.2 Materials and Methods

2.2.1 2D Cell Culture

Primary pig pre-adipocytes and adipocytes were isolated from harvested mammary glands of 6-mouth old female pigs. The mammary glands were minced, incubated in collagenase type I, then the pre-adipocytes were isolated via differential centrifugation. The primary cells were cultured under standard cell culture conditions in DMEM/F12 1:1, 2.5mM L-glutamine, 15mM HEPES buffer (ATCC) supplemented with 10.0% fetal bovine serum (Corning; Corning, NY, USA), 1.0% antibiotic/antimycotic (ThermoFisher; Waltham, MA, USA), and 0.2% fungizone (ThermoFisher). Cultures were maintained at 37°C with 5.0% CO₂.

2.2.2 3D Cell Culture

Primary pig pre-adipocytes and adipocytes cells were grown on collagen type I-tannic acid beads using a rotating bioreactor assembly. Briefly, 10 ml of DMEM/F12 was added to 50 ml mini bioreactors (Corning) with vented caps with 0.7 g of TA/collagen beads and 1x10⁶ SW872 cells. The bioreactor tubes were inserted into a rotating roller, set at 3 r/min, inside an incubator for four days at 37°C with 5.0% CO₂.

2.2.3 Collagen Bead Preparation

The methods for collagen bead preparation are as previously published [23]. All storage/media bottles were autoclaved prior to use. All procedures were conducted under sterile conditions in a biological safety cabinet (Labconco; Kansas City, MO).
A 1.4% (mass/volume) alginate solution was prepared by adding 0.28 g of alginate acid sodium salt from brown algae (Sigma Aldrich; St. Louis, MO) to 20 mL of deionized water filtered using a milliQ system (Millipore Direct 8; Darmstadt, Germany). The solution was covered with Parafilm (Sigma Aldrich; St. Louis, MO) and then mixed using a stir bar (VWR; Radnor, PA) on a stirrer/hot plate (Corning; Corning, NY) on setting 2 (30°C) until the alginate had dissolved. The alginate solution was then sterile filtered under vacuum overnight using a 150 mL bottle top filter (Corning; Corning, NY) and a 250 mL storage/media bottle (VWR; Radnor, PA).

Next, a 1.5% (mass/volume) CaCl\(_2\) solution was prepared by adding 3 g of calcium chloride dihydrate (Fisher Scientific; Fair Lawn, NJ) to 200 mL of deionized water filtered using a milliQ system (Millipore Direct 8; Darmstadt, Germany). The solution was mixed using a stir bar (VWR; Radnor, PA) on a stirrer/hot plate (Corning; Corning, NY) at room temperature until dissolved. The CaCl\(_2\) solution was then sterile filtered under vacuum using a 1000 mL bottle top filter (Corning; Corning, NY) and a 500 mL Pyrex storage/media bottle (Fisher Scientific; Fair Lawn, NJ). A 12 mL collagen solution was prepared on ice using a modified version of Vernon and coworker’s method (Vernon et al., 2005). First, 0.444 mL of 10X Dulbecco’s Phosphate Buffered Saline (10X PBS) (Sigma Aldrich; St. Louis, MO) were added to 4 mL of purified bovine collagen solution (PureCol) (Advanced BioMatrix; San Diego, CA). Then, 1.2 mL of fetal bovine serum (FBS) (Corning; Manassas, VA) were added to the solution. Next, 6.356 mL of Dulbecco’s Modified Eagle Medium (DMEM) (ATCC; Manassas, VA) were added to the solution. Lastly, 40μL of 1 N NaOH (Sigma Aldrich; St. Louis, MO) to the solution in order to neutralize the pH. This neutralization was characterized by a color change from clear in color to pink in color, which is attributed to the phenol red present in the DMEM. The collagen solution and the 1.4% alginate
solution were allowed to sit on ice for 30 minutes to ensure better flow. After chilling, the 1.4% alginate solution was added to the collagen in a ratio of 40:60. For my purposes, 8 mL of 1.4% alginate solution were added to the 12 mL of prepared collagen solution.

Next, TA cross-linking solutions were made. A 10.0% (mass/volume) TA solution was made by adding 111 mL of deionized water filtered using a milliQ system (millipore Direct 8; Darmstadt, Germany) to 11.1 g of TA (Sigma Aldrich; St. Louis, MO). The solution was mixed using a stir bar (VWR; Radnor, PA) on a stirrer/hot plate (Corning; Corning, NY) at room temperature until dissolved. The 10.0% TA solution was then sterile filtered under vacuum using a 150 mL bottle top filter (Corning; Corning, NY) and a 1000 mL Pyrex storage/media bottle (Fisher Scientific; Fair Lawn, NJ). Another 150 mL bottle top filter (Corning; Corning, NY) and 1000 mL Pyrex storage/media bottle (Fisher Scientific; Fair Lawn, NJ) were used to sterile filter 189 mL of deionized water filtered using a milliQ system (millipore Direct 8; Darmstadt, Germany). In order to make a 1.0% (mass/volume) TA solution, 11 mL of the sterile filtered 10.0% TA solution were added to 99 mL of the sterile-filtered milliQ water in a new storage/media bottle. Then, 10 mL of the 1.0% TA solution were added to the remaining 90 mL of sterile-filtered milliQ water, yielding a 0.1% (mass/volume) TA solution.

Alginate/collagen beads in the CaCl₂ solution were then strained using an autoclaved strainer. The volume of beads was divided into one half. Each of the 2 storage/media bottles containing, 1.0% TA solution, and 0.1% TA solution received 1/2 of the total number of alginate/collagen beads. The storage/media bottles were then placed on a rocker (VWR; Radnor, PA) overnight at a speed setting of 18 to ensure cross-linking of the collagen within the beads. A 50 mM sodium citrate solution was made by dissolving 3.9 g of sodium citrate (Fisher Scientific;
Fair Lawn, NJ) into 300 mL of deionized water filtered using a milliQ system (millipore Direct 8; Darmstadt, Germany). The solution was mixed using a stir bar (VWR; Radnor, PA) on a stirrer/hot plate (Corning; Corning, NY) at room temperature until dissolved. The solution was then sterile filtered under vacuum using a 1000 mL bottle top filter (Corning; Corning, NY) and a 1000 mL Pyrex storage/media bottle (Fisher Scientific; Fair Lawn, NJ). The sterile-filtered sodium citrate solution was then divided equally into 3 storage/media bottles. The collagen beads from the 3 storage/media bottles containing TA cross-linking solutions were then strained using an autoclaved strainer and transferred to the corresponding storage/media bottle containing 100 mL of 50 mM sodium citrate solution. The storage/media bottles were then placed on a rocker (VWR; Radnor, PA) for 3 hrs.

After 3 hours in the sodium citrate solution, the collagen beads were strained using an autoclaved strainer and transferred to corresponding 50 mL centrifuge tubes (Corning; Corning, NY) containing 10 mL of Dulbecco’s Phosphate Buffered Saline (PBS) (Sigma Aldrich; St. Louis, MO). The beads were then stored at 4°C until further use.
Figure 2.3.1: Electrostatic Bead Generator Setup
2.2.4 In Vivo Study

A required step prior to commercialization of any new biological intended for use in humans is to establish biocompatibility in at least two other animal species. We have completed initial biocompatibility studies in a rat model [13]. The objective of this research project is to determine the biocompatibility of our matrix in a pig model that will serve as the second required species prior to commercialization. The pig has been valued in the advancement of human
medicine for years. Surgeons commonly train on pig models before working with human patients, and the pig has been used as a large animal to test pharmaceutical products before starting clinical trials. The pig is currently advancing as a transition model bridging the gap from mouse studies to human trials. Comparing the morphology of developing mammary glands, humans and pigs develop terminal ductal lobular units (TDLU) and epithelial proliferation is concentrated in the TDLU’s.

For this project, beads were injected orthotopically into the mammary glands, a total of 16 injections were done. Each pig received 4 injections, 3 injections of beads seeded with pig adipocytes and 1 bead injection without cells. 24 million total cells were grown, 12 million cells per TA concentration (0.1%, 1%). Each injection was 1.5 million cells per site. Mammary glands were be excised at 4- and 8- weeks for histological analysis. Tissue samples were fixed, embedded in paraffin, sectioned, and stained with various methods to determine cell types present, biocompatibility, inflammation, and tissue growth. Additionally, samples of the kidneys and livers were collected for analysis.
Figure 2.4: Shows experimental design of in vivo study. Each pig received four total injections, three seeded with pig adipocytes and one with no cells seeded.

2.2.5 Tissue Sectioning

After sacrifice, the tissues were harvested and fixed in 10% formalin prior to paraffin embedding. Tissue samples were trimmed to approximately 3mm thick to fit into the cassettes before being loaded into the tissue processor. After processing, the samples were embedded into paraffin blocks for sectioning. The blocks were left to harden overnight. Using a microtome (Leica RM2155), 7 μm sections were taken of the samples and placed onto adhesive microscope slides. In an attempt to locate the injected beads deeper inside the tissue and to also get cleaner sectioned slices. Each tissue sampled was sectioned multiple times, and each embedding block was frozen prior to sectioning. The slides were dried overnight at 56°C prior to staining to ensure excess water was removed and the section was adhered to the slide.
2.2.6 Mammary Tissue Histology Staining

The mammary tissues harvested were also stained with the hematoxylin and eosin (H&E) method as described above. One section from each sample was stained to determine if any beads were in the sample. Masson’s trichrome staining was performed to evaluate connective tissue formation surrounding the beads. The manufacturer’s guidelines were followed for accurate results (Poly Scientific R&D, Bay Shore, NY, USA) and cover slipped using Permount.

Immunohistochemistry was performed on sections that were determined to have beads, as well as one negative control. After deparaffinization and rehydration, endogenous peroxidase activity was blocked by incubating sections with 3% H$_2$O$_2$ for 10 minutes at room temperature. Background blocking was performed using 5% goat serum (Gibco, Penrose, Auckland, NZ) in PBS (ThermoFisher). Afterwards, the primary antibody was added overnight at 4°C. Primary antibodies used were diluted in 5% goat serum and are as follows: anti-adiponectin (1:20), anti-vimentin (1:100), anti- VEGFR-1 (1:100), anti-CD163 (1:100), anti-CD86 (1:50) (all ThermoFisher). Slides were washed in PBS, then incubated with HRP-conjugated secondary antibody (Invitrogen, Carlsbad, CA, USA) for one hour. Slides were washed with PBS again, then ImmPACT DAB Peroxidase Substrate Kit (Vector Labs; Burlingame, CA, USA) was added under a microscope until browning occurred. Slides were rinsed in water to remove excess DAB before nuclear staining with Harris hematoxylin for 5 minutes. Excess hematoxylin was removed by water rinse before standard dehydration and cover slipping for imaging.

2.2.7 Kidney & Liver Staining

32
For toxicity evaluation, the kidney and liver tissue sections from each animal were stained with (H&E). Standard protocol for Harris hematoxylin and eosin was used. The sections were deparaffinized in xylene (NewcomerSupply; Middleton, WI, USA) and a graded series of ethanol (ThermoFisher). The slides were submerged in Harris hematoxylin (ThermoFisher) for 5-10 minutes, followed by bluing reagent (ThermoFisher) and eosin (ThermoFisher) for 45 seconds each. Afterwards, slides were dehydrated in graded ethanol and xylene again, they were cover slipped with Permount mounting medium and imaged for analysis.
3. RESULTS

3.1 Kidney and Liver Analysis

Kidney sections were stained with H&E to assess potential toxicity of TA. Similar to chemotherapy, TA induces apoptosis in cells via caspase pathways. Images of the stained sections from all four pigs where beads were found were sent to Dr. Luigi Strizzi, a board-certified pathologist, and Associate Professor in the Department of Pathology at Midwestern University, for further analysis. He concluded that both the kidney and liver samples showed no signs of cellular or tissue changes that would support significant organ damage (Figure 3.1 and 3.1.2).

**Figure 3.1**: H&E staining of 0.1% Kidney and liver section. All images taken at 20x magnification harvested at 4- and 8-weeks.
3.2 Mammary Tissue Analysis

3.2.1 Hematoxylin and Eosin Staining Analysis

Beads were observed in all four of the pig models tissue sections. Implant cross sections also vary between the middle and edge sections of the beads due to their spherical shape. Beads observed in the tissue sections are surrounded by normal subdermal tissue consisting of loose connective and adipose tissue. The beads themselves are observed as dark purple due to cells infiltrating and subsequent secretion of ECM and basement membrane (Figure 3.2.1 and 3.2.2).
Figure 3.2.1: H&E staining of 0.1% TA/collagen beads in mammary tissue samples. All images taken at 20x magnification harvested at 4- and 8-weeks. Yellow arrows and circles indicating bead location.
Figure 3.2.2: H&E staining of 1% TA/collagen beads in mammary tissue samples. All images taken at 20x magnification harvested at 4- and 8-weeks. Yellow arrows and circles indicating bead location.

3.2.3 Masson’s Trichrome Staining

Masson’s trichrome staining was used to highlight the collagen composition of the beads and surrounding tissues. This technique stains collagen in blue and fatty and muscle tissue appears red. The beads themselves appear red due to the large number of cells and cytoplasm contained within the cells. There is no dense fibrous capsule of connective tissue surrounding the beads, which would indicate an extreme immune response of encapsulation. The connective tissue surrounding the implants is comparable to healthy subdermal connective tissue. Fibroblasts can be seen as thin, spindle-shaped cells stained blue within the beads. Masson’s trichrome cannot distinguish between newly formed collagen and collagen already present in the beads, but the bright blue fibers infiltrating the beads are loose and unorganized, suggesting newly formed collagen (Figure 3.2.3 and 3.2.4).
Figure 3.2.3: Masson’s Trichrome staining of 0.1% TA/collagen beads in mammary tissue. All images taken at 20x magnification harvested at 4- and 8-weeks. Collagen is stained blue. Yellow arrows and circles indicating collagen fibers within beads.
**Figure 3.2.4:** Masson’s Trichrome staining of 1% TA/collagen beads in mammary tissue. All images taken at 20x magnification harvested at 4- and 8-weeks. Collagen is stained blue. Yellow arrows and circles indicating collagen fibers within beads.

### 3.3. Immunohistochemistry

Immunohistochemistry (IHC) was used to determine the cell types present within and around the beads. Adiponectin is a marker of adipocytes and is a protein hormone produced by adipose tissue. It is involved in the breakdown of fatty acids and regulation of glucose levels. Adiponectin staining was performed on the tissue sections to determine the presence of adipose tissue. Deparaffinization of the tissue samples washes away fatty deposits, leaving residual adiponectin that can be detected via IHC staining. Cells expressing adiponectin can be seen along the surface and within the implants. The existence of adiponectin suggests the presence of newly differentiated adipocytes indicating tissue growth (Figure 3.3.1 and 3.3.2).
**Figure 3.3.1:** Anti-adiponectin IHC staining of 0.1% TA/collagen beads in mammary tissue. All images taken at 20x magnification harvested at 4- and 8-weeks. Yellow arrows and circles are indicating adiponectin positive cells.

**Figure 3.3.2:** Anti-adiponectin IHC staining of 1% TA/collagen beads in mammary tissue. All images taken at 20x magnification harvested at 4- and 8-weeks. Yellow arrows and circles are indicating adiponectin positive cells.

Vimentin staining confirmed the presence of fibroblasts within the samples. Vimentin is a structural intermediate filament protein that is a major cytoskeletal component, and it is a biomarker of fibroblasts. Vimentin mediates the transition of mesenchymal cells into the myofibroblast phenotype, which facilitates wound healing. The presence of Vimentin in cells surrounding and within the injected beads indicates that the wound healing process has begun, as fibroblasts contribute to the formation of the basement membrane (Figure 3.3.3 and 3.3.4).
Figure 3.3.3: Anti-Vimentin IHC staining of 0.1% TA/collagen beads in mammary tissue. All images taken at 20x magnification harvested at 4- and 8-weeks. Yellow arrows and circles are indicating vimentin positive cells.
**Figure 3.3.4:** Anti-Vimentin IHC staining of 1% TA/collagen beads in mammary tissue. All 20x magnification harvested at 4- and 8-weeks. Yellow arrows and circles are indicating vimentin positive cells.

IHC using anti-CD163 was performed to determine the presence of M2 macrophages. M2 macrophages produce either polyamines to induce proliferation or proline to induce collagen production. These macrophages are associated with wound healing and tissue repair. The presence of M2 macrophages within and surrounding the bead indicates that the wound healing process has begun (Figure 3.3.5 and 3.3.6).

**Figure 3.3.5:** Anti-CD163 IHC staining of 0.1% TA/collagen beads in mammary tissue. All images taken at 20x magnification harvested at 4- and 8-weeks. Yellow arrows and circles indicating Anti-CD163 positive cells.
Figure 3.3.6: Anti-CD163 IHC of 1% TA/collagen beads in mammary tissue. All images taken at 20x magnification harvested at 4- and 8-weeks. Yellow arrows and circles are indicating Anti-CD163 positive cells.

IHC was also performed to identify Vascular Endothelial Growth Factor Receptor (VEGF-R). VEGF is produced by fibroblasts to trigger the formation of vasculature, specifically in wound healing. VEGF binds VEGF-R initiating angiogenesis. The presence of VEGF-R is seen within beads surrounding a blood vessel. Increased vasculature within an implant indicates a positive host response and the implanted beads are stimulating angiogenesis and tissue regeneration (Figure 3.3.7 and 3.3.8).
**Figure 3.3.7**: Vascular Endothelial Growth Factor Receptor IHC of 0.1% TA/collagen beads in mammary tissue. All images taken at 20x magnification harvested at 4- and 8-weeks. Yellow arrows and circles are indicating new VEGF-R positive cells.
Figure 3.3.8: IHC Vascular Endothelial Growth Factor Receptor staining of 1% TA/collagen beads in mammary tissue. All images taken at 20x magnification harvested at 4- and 8-weeks. Yellow arrows and circles are indicating new VEGF positive cells.

Finally, to ensure there was not an underlying immune reaction, anti-CD68 IHC was performed. CD68 is a protein highly expressed in monocytic phagocytes including tissue macrophages. These macrophages are essential for the inflammation step of wound healing and their presence is to be expected with all implants, especially at the 4- week time point. However, an overabundance of these macrophages would indicate a larger immune response and potentially non-biocompatible beads. The figures show the results of the anti-CD68 stain, where there are some macrophages localized surrounding the beads. These macrophages have not infiltrated into the beads and are not abundant suggesting a mild immune response (Figure 3.3.9 and 3.3.10).
Figure 3.3.9: Anti-CD68 IHC of 0.1% TA/collagen beads in mammary tissue. All images taken at 20x magnification harvested at 4- and 8-weeks. Yellow arrows and circles are indicating CD68 positive cells.

Figure 3.3.10: Anti-CD68 IHC of 1% TA/collagen beads in mammary tissue. All images taken at 20x magnification harvested at 4- and 8-weeks. Yellow arrows and circles are indicating CD68 positive cells.
4. Discussion

TA crosslinked collagen type I beads provide a matrix for soft tissue regeneration with chemotherapeutic properties against breast cancer. Previous studies have demonstrated their biocompatibility, and this study demonstrated their capability to promote tissue regeneration. Kidney and liver sections stained with H&E showed no signs of cellular or tissue changes that would support significant organ damage (Figures 3.1 and 3.1.2). H&E staining of mammary tissue sections indicated good incorporation into native surrounding tissue with no fibrous encapsulation (Figures 3.2.1 and 3.2.2). As an injectable implant, there is an ease of surgical recovery and lack of surgical void. There was no infection post-injection, and no widespread chronic inflammation or tissue necrosis was observed. Fat tissue growth and collagen fibers were observed within the beads at the 4-week time point, demonstrating incorporation with the native subcutaneous tissue (Figure 3.2.3 and 3.3.4). No beads were located in the 8- or 12-week collected samples in previous studies within the rat model, so we decided to double the dose of beads for each injection in this study and we were able to locate beads in the 8-week samples in our pig model. Adipose tissue growth, shown in Figures 3.3.1 and 3.3.2, is a good indicator of biocompatibility and tissue reconstruction. Beads located showed new adipocytes present despite not being seeded with adipocytes initially.

Additionally, the presence of anti-CD163 positive cells suggest that M2 macrophages are located within the beads (Figures 3.3.3 and 3.3.5). These macrophages are associated with wound healing, tissue repair, and are necessary for the wound healing process as they contribute to proliferation and induce collagen production. Another essential step in tissue regeneration is angiogenesis, which requires the secretion of VEGF. Figure 3.3.7 and 3.3.8 shows cells positive
for VEGF-R around and within the beads at the 4-week time point. Tissue sections from later time points could potentially indicate small blood vessel formation within the implants. Presence of CD68 positive cells indicates a small immune response (Figure 3.3.9 and 3.3.10), although this is to be expected with any non-native implants. It would not affect the performance of the purpose of this device, which is to serve as a scaffold for tissue regeneration and a breast reconstruction material.

5. Future Research

To advance this project towards clinical application, there are key elements that should be assessed. First, correlate histopathology with blood and urine tests from the pig model to evaluate actual compromised organ function, as well as the additional concentrations of TA. Also, we should take brain and heart samples as well for more toxicology studies, comparing the concentrations of blood from the different organs can boost the accuracy of this project. This may require sectioning the remaining tissue sections or potentially repeating the injection model with modifications. Injection amount could be increased for a greater chance of locating beads within the explants. Animals could be given anesthesia for longer post-injection; animal movement immediately after injection could have led to reflux of beads through the injection site. Additionally, since a lumpectomy was not performed this project just tested the tissue generation aspect of this potential therapy. In the future, we should perform a lumpectomy on a model to see if the beads can contribute to new tissue regeneration.

Further IHC analysis could also be performed to fully characterize the tissue response to the implant. Specifically, staining to differentiate between M1 and M2 macrophages throughout
the time period could further demonstrate the process of wound healing and tissue regeneration. The transition to the M2 macrophage phenotype propagates tissue regeneration rather than inflammatory M1 macrophages. Future studies should focus on the anticancer properties of the beads by testing on breast cancer tumors in vivo. Particularly, testing the amount of TA released and its action as a chemotherapeutic agent in vivo. High concentrations of TA have been shown to cause organ failure and death in mice and rats; however, slow release of small amounts of TA localized to the tumor area may prove to be therapeutic without causing toxicity. Determining the half-life of TA in vivo and the release profile would aid in determining the optimal concentration for therapeutic use.
REFERENCES


