In Vivo Analysis of an Injectable Tannic Acid Crosslinked Collagen Type I Bead Scaffold for Tissue Regeneration Post-Lumpectomy

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IN VIVO ANALYSIS OF AN INJECTABLE TANNIC ACID CROSSLINKED
COLLAGEN TYPE I BEAD SCAFFOLD FOR TISSUE REGENERATION POST-
LUMPECTOMY

A Thesis
Presented to
the Graduate School of
Clemson University

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

by
Kylie King
May 2021

Accepted by:
Dr. Brian Booth, Committee Chair
Dr. Heather Dunn
Dr. Agneta Simionescu
ABSTRACT

Among women, breast cancer is the most commonly diagnosed cancer in the United States, affecting about 1 in every 8 women. It accounts for approximately 30% of new cancer diagnoses and is one of the leading causes of cancer-related deaths among women in developed countries. Current treatment options for breast cancer are surgery, chemotherapy, and radiation. Unfortunately, there are negative side effects associated with each of these, and they may not work for every patient. In addition, local breast cancer recurrence presents a high risk, especially for patients undergoing lumpectomy procedures.

The goal of this project is to alleviate these problems by developing an injectable bead scaffold to promote tissue regeneration in the void created by lumpectomy, as well as to prevent local recurrence and minimize 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 by inducing apoptosis in breast cancer cells via caspase pathways and has demonstrated antimicrobial properties. The collagen/tannic acid beads were seeded with adipocytes to attach and grow onto the matrix, then release the tannic acid into the surrounding environment as the attached cells remodel the collagen.

To prove the viability of this therapy, an in vivo study was conducted using a pig model. Beads were injected orthotopically into the mammary glands, then excised at three time points for histological analysis. Tissue samples were fixed, embedded in paraffin, and sectioned and stained with various methods to determine cell types present.
Additionally, samples of the kidneys and livers were collected for toxicity analysis. Our results indicate that the collagen beads were successful at promoting tissue regeneration, specifically by increasing expression of VEGF and presence of adipocytes, intermediate filaments, and smooth muscle actin.
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CHAPTER ONE
INTRODUCTION AND BACKGROUND

1.1 Project Overview

Breast cancer is the most common cancer for women, and approximately 1 in 8 (12%) of women will develop invasive breast cancer in their lifetime (Ma and Jemal, 2013). The current standard of care is to perform breast-conserving surgery (or lumpectomy) to remove the tumor and a margin of healthy tissue, leaving a void in the breast as shown in Figure 1.1 (Desantis et al., 2019). The goal of this project is to develop an injectable matrix of tannic acid/collagen type I beads to promote regrowth of healthy tissue and prevent cancer recurrence. The process incorporates adipocytes by seeding them onto the collagen bead. As these cells attach, they cause remodeling of the collagen and release the tannic acid that can kill any residual cancerous cells.

Figure 1.1: Lumpectomy Procedure (Keck School of Medicine of USC, 2016).
1.2 Breast Anatomy

The female breast is composed of adipose and connective tissue as well as a network of glands and ducts. Mammary glands serve as the functional unit of the breast, forming branching tree-like structures comprised of epithelial ducts (Zhu and Nelson, 2013). The breasts cover the majority of the chest wall, extending from the clavicle to the middle of the sternum. Adult female breasts contain between 15 and 20 lobes which can be further divided into 20-40 lobules. These lobules end in terminal ductal lobular units (TLDUs), which are tiny bulb-like glands where milk is produced as a response to hormone signaling. The small ducts that drain milk from the alveoli join together to form larger structures called laciferous ducts (McKinley and O’Loughlin, 2012). Each laciferous duct drains a single lobe of the breast. Prior to lactation, the lumen of the laciferous ducts can expand to form a laciferous sinus capable of storing milk (McKinley and O’Loughlin, 2012). The structure of the breast is illustrated in Figure 1.2.

Functional units of the breast include fibrous connective tissue (fascia), lymph nodes and vessels, blood vessels, and Cooper’s suspensory ligaments (O’Connell and Rusby, 2015). These are all found dispersed within the adipose tissue that forms the breast. Superficial and deep fascia, along with Cooper’s ligaments, provide structure and support to connect the breast to the chest wall (Bland and Copeland, 1998). Lymphatic fluid flows through the vessels into lymph nodes, which remove abnormal cells and foreign substances (Bland and Copeland, 1998). Blood vessels are the final functional unit of the breast, providing the tissue with blood and nutrients and removing waste.
1.3 Breast Development

From birth until puberty, there are no morphological differences between male and female breasts (Howard, 2000). Changes occur in both the epithelium and the stroma, where there is an increase in fibrous and fatty tissue. The stroma comprises 80% of the adult female nonlactating breast (Howard, 2000). Under the influence of hormones, specifically estrogen, the breast ducts become elongated and form lobular structures. The end-bud-like structures become a major site for proliferation as they become the TLDUs (Javed and Lteif, 2013). Sex and growth hormones promote further breast development in the glandular, adipose, and suspensory tissues. Development typically will continue for four years after birth until full maturity is reached, although further changes can occur due to hormone fluctuation from pregnancy, menstruation, lactation, and menopause. During pregnancy the glandular tissue and ducts increase in size due to increased levels
of estrogen and progesterone. Additionally, the overall breast volume is increased due to higher amounts of water and electrolytes within the adipose tissue (Javed and Lteif, 2013). Once menopause begins, the body stops producing these sex hormones and the breast volume may decrease as the lobes and glandular tissue shrinks (Santoro and Randolph Jr., 2011). This tissue atrophy may also cause the overall breast density to decrease.

**1.4 Breast Cancer Classification**

**1.4.1 Stages of Breast Cancer**

After the initial diagnosis, the patient will often undergo tests to determine the stage of the breast cancer. The Tumor Nodes Metastasis (TNM) system classifies the cancer based on tumor size and location, size and spread within the lymph nodes, and amount of metastatic spread throughout distant body sites. A grading system is used to describe how likely the tumor is to grow and spread and includes gland formation, nuclear features, and mitotic activity of cells within the tumor. Lastly, the biomarker test determines whether the cancerous cells have receptors for hormones such as estrogen and progesterone, which will help determine the best treatment options.

Stage 0 breast cancer is a non-invasive cancer where abnormal cells have been found in the lining of the milk duct. The cells have not spread outside the ducts or lobes into the surrounding tissue, making it highly treatable. Stages 1-3 describe tumors that have spread to the surrounding breast tissue. The stages are determined by the size of the initial tumor and whether the tumor has invaded surrounding lymph nodes (National Breast Cancer Foundation, Inc b, 2018). Lastly, Stage IV is used to describe metastatic
breast cancer, or breast cancer that has spread outside of the immediate tumor area to other areas of the body. This can include the brain, lungs liver, or extensive spread to lymph nodes (National Breast Cancer Foundation, Inc e, 2018).

1.4.2 Hormone Receptors

Along with staging, breast cancers can be classified by the presence or absence of hormone receptors and the human epidermal growth factor receptor-2 (HER2). Tumor cells can be examined pathologically to determine classification: estrogen receptor positive (ER+) and progesterone receptor positive (PR+), HER2 positive (HER2+), triple positive, or triple negative. Of diagnosed and classified cases, approximately 70% are hormone receptor positive/HER2-, 10% are hormone receptor positive/HER2+, 12% are triple negative, and 5% are hormone receptor negative/HER2+ (Howlander et al., 2014). This classification system is essential for determining the most effective treatment options, as hormone therapies use the receptors to develop treatments targeted specifically for the cancer cells.

1.5 Clinical Treatment Options

Current clinical treatment options include hormone therapy, radiation, surgery, and chemotherapy. Patients will typically undergo one or a combination of these depending on disease progression, location and type of cancer, and the patient’s overall health. These treatments have been successful for treating some stages of breast cancer, with stages 0-2 having survival rates over 93% (Desantis et al., 2019).
Surgeical procedures including lumpectomies or mastectomies can be extremely effective at removing cancerous tumors; however, the complications can be significant. Persistent Post-Mastectomy Pain is a clinically recognized complication and can be described as a burning or aching around the breasts and upper extremities. Initially thought to be rare, it has been reported to have incidence rates of up to 50% (Tait et al., 2018). Additionally, surgery carries an inherent risk of excessive bleeding, infection, and extended recovery times (Arroyo, 2011).

A majority of patients that undergo a lumpectomy or mastectomy procedure elect for reconstructive surgery (Kummerow et al., 2015). Post-lumpectomy reconstruction treatments are purely cosmetic and can involve breast reduction or reconstruction of the breast with silicone implants or autologous fat transplants. While fat grafts are increasing in popularity, this transplantation can lead to tissue calcification and necrosis (Simonacci et al., 2016). Additionally, fat grafts may increase rates of breast cancer recurrence and de novo cancer induction (Largo et al., 2014). Patients that undergo reconstruction procedures have higher incidences of surgical site complications (Beecher et al., 2016). Specifically, postoperative infection occurs in only 12% of patients without reconstruction versus 20% of patients that elect for silicon or autologous adipose implants (Jagsi et al., 2016).

Hormone therapy has been effective in preventing the interaction between estrogens and the estrogen-dependent pathways for stimulation of neoplastic cells, either by blocking the production of estrogens or blocking their action upon tumor cells (Drăgănescu et al., 2017). While useful for preventing recurrence and slowing disease
progression, hormone therapies are not cytotoxic to cancer cells and have been associated with blood clots, irritable mood swings, and increasing risk of heart disease or myocardial infarction.

Radiation can be localized to the tumor and uses high energy X-rays to kill the cancer cells, but can lead to fatigue, swelling, and nerve damage to the affected area (Baskar et al., 2012). Chemotherapy, while effective at killing cancer cells, can also damage or kill healthy breast cells due to its inability to specifically target tumor cells. A wide range of side effects have been well documented: nausea, vomiting, hair loss, decreased white blood cell counts, fatigue, heart damage, and more (Hu et al., 2019).

Every current therapy or treatment option for breast cancer patients has limitations and can lead to severe side effects; therefore, there is a clinical need for a minimally invasive implant that can restore breast tissue while actively preventing cancer recurrence. Recent research has shifted focus from synthetic into natural chemotherapeutic compounds; this project focused on a natural chemotherapeutic compound, tannic acid.

1.6 Tannic Acid

One group of natural chemotherapeutic compound that is of great interest is the tannin group, often called tannic acid (TA). Tannins are water-soluble compounds that can be found in thousands of plant species and are thought to have significant health benefits (Chung et al., 1998). They can be divided into two classifications: hydrolysable or condensed. The main structure of hydrolysable includes a polyhydric alcohol core and hydroxyl groups that are either partially or wholly esterified by hexahydroxydiphenic or
gallic acids. Condensed tannins are a product of polymerized flavan-3-ols and flavan-3,4-diols (Chung et al., 1998). Tannin classifications are shown in Figure 1.3. Tannins are found in grains, fruits, nuts, green tea, and wines (Mojzer et al., 2016). They have also been shown to have anticancer properties, with one study showing an inverse relationship between cancer development and tannin consumption (Stich and Rosin, 1984). The action mechanisms of tannins in breast cancer cells have been widely studied, with many studies showing tannins to be cytotoxic to these cells in a dose-dependent manner (Sonoda et al., 2006; Moongkarndi et al., 2004; Banerjee et al., 2012; Eddy et al., 2007).

![Figure 1.3: Classes of tannins (Khanbabae and van Ree, 2001).](image)

### 1.7 Collagen

Collagen type I is the most abundant protein in animals and humans and is routinely used as a biomaterial in reconstructive surgeries (Chattopadhyay et al., 2014). 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 (Shahrokhi et al., 2014). In vivo, collagen is abundant in the tendons, skin, vasculature, and connective tissues making it easy to isolate (Lodish et al., 2000). 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 (Shahrokhi et al., 2014).

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 (Lauer-Fields et al., 2002). The byproducts of this reaction can be further degraded by gelatinases.

Creating a collagen scaffold for therapeutic use requires a method of crosslinking in order to maintain structural integrity. In solution, TA can diffuse into gelated collagen to create these crosslinks (AB 8). Specifically, TA forms hydrogen bonds between the phenols of gallic acid and the amines of the collagen peptide backbone as shown in Figure 1.4 (Baldwin et al., 2020). This project uses this property to create collagen/TA beads and increases the structural integrity to maintain the bead shape in solution.
1.8 Anticancer Properties of Tannins

1.8.1 Tannin Effects on ER$^+$ Breast Cancer

ER$^+$ breast cancer has been shown to be sensitive to the anticancer properties of tannins in many studies (Booth et al., 2013; Bawadi et al., 2005, Zhang et al., 2008). Our lab has previously demonstrated that TA induced a change in morphology in MCF7 breast cancer cells (ER$^+$) after 24 hours of exposure, showing that they are highly sensitive to the apoptotic effects of TA (Booth et al., 2013). Along with determining the decrease in cell viability and increase in apoptotic activity, we determined the mechanism of inducing apoptosis in ER$^+$ breast cancer cells; the activation of caspases 3/7 and 9.
initiates apoptosis in MCF7 cells (Jordan, 2016). We postulate that the higher sensitivity to TA treatment is correlated to the lack of caspase 3 expression by MCF7 cells (Booth et al., 2013). Caspase 3 is downstream of caspase 9, meaning it can act as a negative feedback regulator for the pathway (Fujita et al., 2001).

Condensed tannins inhibit fatty acid synthase (FAS) to induce apoptosis (Zhang et al., 2008). The enzyme FAS is involved in the de novo synthesis of long fatty acid chains and is highly expressed in breast cancers (Kuhajda, 2000). This makes it an important target for novel cancer therapeutics as inhibition of this enzyme would induce apoptosis. When treated with condensed tannins, ER\(^+\) breast cancer cells showed significant growth inhibition and lowered cell viability. The condensed tannins blocked FAS activity, competitively to NADPH; therefore, they hypothesized that the tannin reaction site is the NADPH loading site of FAS (Zhang et al., 2008).

Additionally, Shirode et al. aimed to determine the action mechanism of hydrolysable tannins in pomegranate extract using ER\(^+\) breast cancer cells (Shirode et al., 2013). The researchers confirmed previous results that tannins may induce cell cycle arrest in G2/M to inhibit cell growth and induce apoptosis. Analysis of DNA microarrays demonstrated that the tannins derived from pomegranate extract downregulated genes associated with chromosome organization, RNA processing, DNA replication, mitosis, and DNA repair. The data from Zhang et al. and Shriode et al. demonstrate that TA induces apoptosis through multiple mechanisms in ER\(^+\) breast cancer cells.

Zhang et al. and Shriode et al. demonstrate advanced understanding of the apoptotic mechanisms of tannins, furthering research into the chemotherapeutic use.
1.8.2  Tannin Effects on HER2+ Breast Cancer

In addition to the effect on ER+ cells, hydrolysable tannins from pomegranate extract had been shown to reduce growth and tumor volume in HER2+ breast cancer cells (Banerjee et al., 2012). Activation of caspase-3 induces apoptosis in the caspase-mediated pathways. Treatment with pomegranate extract decreased levels of proteins that regulate angiogenesis, cell proliferation, and inflammation, key processes in cell viability.

Condensed tannins in green tea leaves called 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 (Eddy et al., 2007). Morphological analysis after treatment revealed DNA fragmentation and decreased nuclear integrity, indicating that EGCG induces apoptosis. Treatment with EGCG also increased p27 expression, a growth regulator. Trastuzumab resistance has been associated with lack of p27 expression while induction of p27 would increase sensitivity to the chemotherapy; therefore, treatment of EGCG could lead to decreased resistance to chemotherapies of HER2+ breast cancer cells.

1.8.3  Tannin Effects on Triple Negative Breast Cancer

Triple negative breast cancer (TNBC) is unique as it does not respond to normal hormone therapies including tamoxifen and aromatase inhibitors, so different approaches to treatments and therapies must be studied. The effects of other chemopreventative agents and TA have been the subject of many studies in recent years. TA has been investigated in order to determine its effects on TNBC cells. Previously, our lab used collagen type-1 beads crosslinked with TA to determine effects of the TA on different
Typical of breast cancer cell lines (Booth et al., 2013; Jordan, 2016). Though the effects were less significant on the TNBC cells compared to other types of breast cancers, there was still a change in the morphology of the triple negative cells. In addition, the proliferation rate of the treated cells was lower than that of untreated cells (Figure 1.5). Tannic acid was shown to induce a significant level of apoptosis in treated TNBC. The effect on caspase activation was also investigated. Once again, though the effects were not as drastic as other breast cancers, caspases 9 and 3/7 were elevated once exposed to TA (Booth et al., 2013; Jordan, 2016). Understanding the apoptotic pathways of TA allows targeted research into development of a clinically therapeutic use.

Figure 1.5: Effect of TA on proliferation of TNBC cells.

One current treatment that has been highly effective in treating breast tumors is Doxorubicin (DXR), an anthracycline antibiotic, however it has had limited use due to high cardiotoxicity. Tikoo et al. demonstrated that when combined with DXR, TA reduced the cardiotoxicity while promoting its anti-cancer properties, specifically against
the TNBC cell line MDA-MB-231. In a breast cancer rat model, DXR was shown to enhance the expression of p53, a tumor suppressor gene, which lead to the inhibition of tumor growth. DXR/TA treatment decreased the overall volume of the tumor. Adding TA to the DXR treatment resulted in increased expression of p53 compared to DXR treatment alone. Also, the addition of TA prevented cytoplasmic vacuolization, which is the main pathway of DXR-induced damage to the myocardium (Tikoo et al., 2010). This indicates that TA is a viable treatment option in combination with current chemotherapies to increase efficacy and decrease cardiotoxicity.

In more recent years, the field of tannin research has focused on EGCG, the extract found in green tea leaves. It has been shown to aid in the treatment and prevention of cancer by inhibiting tumor invasion and angiogenesis (Khan and Mukhtar, 2010). Braicu et al. demonstrated that EGCG treatment of TNBC cells suppresses growth and proliferation (Braicu et al., 2013). All the research demonstrates that TA is a viable anti-cancer option for inclusion in a tissue regeneration matrix to reduce recurrence.
CHAPTER TWO
MATERIALS AND METHODS

2.1 Cell Culture

2.1.1 2D Cell Culture

The human liposarcoma cell lines SW872 and SW872-GFP transfected cell lines were used in this study (ATCC; Manassas, VA, USA). SW872 cells were transfected to constitutively express green fluorescent protein (GFP) as previously described (Jordan, 2016). SW872 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 (Baldwin et al., 2020).

2.1.2 3D Cell Culture

SW872 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 1x10^6 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 Collagen Bead Preparation

This project is ongoing, and the methods for cell culture and collagen bead preparation are as previously published in Booth et al., 2013. 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) CaCl₂ 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 CaCl₂ 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.

Components for the bead generator including the glassware dish, petri dish, electrostatic probe, silicone tubing (5/32” outer diameter and 3/32” inner diameter), nozzle, and stir bar were autoclaved for 40 minutes at 121°C in a large autoclave bag. The Var V1 bead generator (Nisco; Zurich, Switzerland), KDS100 syringe pump (KD Scientific; Holliston, MA), were sterilized using 70% ethanol (EthOH) and Kimwipes (Kimberly-Clark; Roswell, GA).

The petri dish was place on the stage of the bead generator. The glassware dish was then placed within the petri dish, and a stir bar was placed in the glassware dish. The electrostatic probe was then inserted into its holder and tightened using the screw. The 1.5% CaCl₂ solution was poured into the glassware dish until it just overflowed into the petri dish underneath. The probe was then lowered into position so that the tip of the probe was immersed in the CaCl₂ solution and tightened with the screw. The bead generator and the agitator were then turned on so that the stir bar was rotating. The nozzle
was screwed into the Luer lock of the silicone tubing and secured in the holder using the screw. The silicone tubing was then fed through the notch at the top of the bead generator, and the doors to the bead generator were closed. A 20 mL Luer-Lok syringe (BD; Franklin Lakes, NJ) with a 16G 1 1/2 needle (BD; Franklin Lakes, NJ) was used to load the 20 mL of 40:60 alginate: collagen solution, taking care to avoid bubble formation. The syringe was then inverted and any air was forced out of the needle tip. The needle was then removed, and the silicone tubing was attached to the Luer-lock of the syringe. The plunger of the syringe was then used to pump the alginate/collagen solution through the tubing until the solution reached the nozzle. The syringe was then placed in the syringe pump. The pump settings were adjusted to Volume: 20 mL, Rate: 10 mL/hr, Dia: 15, and then the run was started. The voltage of the bead generator was turned on and set to 5.5 V. The bead generator was allowed to run until all of the alginate/collagen solution had flown through the nozzle. The bead generator setup is shown in Figures 2.1 and 2.2.
Figure 2.1: Electrostatic Bead Generator Setup
Figure 2.2: Electrostatic Bead Generator Components
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 CaCl$_2$ solution were then strained using an autoclaved strainer. The volume of beads was divided into thirds. Each of the 3 storage/media bottles containing 10.0% TA solution, 1.0% TA solution, and 0.1% TA solution received 1/3 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 20°C until further use.

2.3 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 (Baldwin et al., 2020). 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 endbuds or TDLU’s.

For this project, 18 female pigs were used with 2 different concentrations of TA at 3 different time points. 9 of these animals received 3 2mL injections of 0.1\% TA-collagen beads and 9 received 3 injections of 1.0\% TA-collagen beads. One of the three injections into each pig was beads without seeding of GFP+ preadipocytes, and the other two were seeded with these cells. 6 animals (3 from each concentration) were sacrificed at each of the timepoints: 4 weeks, 8 weeks, and 12 weeks. At the timepoints listed above, the kidney, liver, and mammary tissue were harvested and embedded for histological examination. Due to problems in fixation, the liver sections were not properly preserved and could not be analyzed.

2.4 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), 5µm sections were taken of the samples and placed onto adhesive microscope slides. In an attempt to locate the injected beads, 3 sections were taken every 100µm from each sample. 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.

2.5 Histological Staining

2.4.1 Kidney Staining

For toxicity evaluation, the kidney explants from each animal were stained with hematoxylin and eosin (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 minutes, followed by bluing reagent (ThermoFisher) and eosin (ThermoFisher) for 45 seconds each. Afterwards, slides were dehydrated in graded ethanol and xylene again, cover slipped with Permount mounting medium and imaged for analysis.

2.4.2 Mammary Tissue Histology Staining

The mammary tissues harvested were also stained with the H&E method as described above. One section from each sample was stained to determine if any beads were located in the sample. Of the 54 tissue samples from mammary glands with injected beads, only 2 samples were determined to have beads. This was likely due to the small amount of beads injected. The rest of the project will focus on these samples, as they were the only ones recovered.
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.

2.4.3 Mammary Tissue Immunohistochemistry

Immunohistochemistry was performed on sections from the two blocks 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% H₂O₂ 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.
CHAPTER THREE

RESULTS

3.1 Kidney 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 both 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 kidney samples showed increased glomerular size with concomitant reduction of the periglomerular urinary space, as shown in Figure 3.1. The affected glomeruli showed increased mesangial cellularity and reduction in glomerular capillary lumen, both indicative of moderate toxicity (Figure 3.1, A and B).

Within the renal tubules, there is not significant inflammatory infiltrates or interstitial edema. These tubules consist of a single layer of normal epithelium. Some non-specific granular material is evident within the tubules as well (Figure 3.1, C and D). This could suggest tubular injury, as mesangial cells use endocytosis to take up and degrade circulating immunoglobulin. This is indicative of an immune response from the TA within the beads, although it is significantly lower than what would be seen in a chemotherapy treatment.
Figure 3.1: H&E staining of the kidney tissue sections. (A,B) 10x magnification, (C,D) 40x magnification. Black arrows in (A) and (B) indicate glomeruli. Red arrows in (C) and (D) indicate nonspecific granular material.

3.2 Mammary Tissue Analysis

3.2.1 Hematoxylin and Eosin Staining

Because of the small size of the beads and the amount of beads in each injection, beads were only observed in two of the explants. Both of these explants were from the four-week time point and 1% TA. Implant cross sections also vary between the middle and edge sections of the beads due to their spherical shape.

Beads observed in the explants 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.
3.2.2 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 (Figure 3.3). 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, indicating newly formed collagen.
Masson’s Trichrome staining of 1% TA/collagen beads in mammary tissue. (A,B) 4x magnification, (C,D) 40x magnification. Collagen is stained blue. Black arrows in (C) and (D) indicating collagen fibers within beads.

3.2.3 Immunohistochemistry Analysis

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 (Figure 3.4). 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 3.4: IHC Adiponectin staining of 1% TA/collagen beads in mammary tissue. (A,B) 4x magnification, (C,D) 40x magnification. Black arrows in (C) and (D) indicating adiponectin positive cells.

Vimentin staining confirmed the presence of fibroblasts within the samples (Figure 3.5). Vimentin is a structural intermediate filament protein that is a major cytoskeletal component, and it is a biomarker of fibroblasts. It mediates the transition of mesenchymal cells into the myofibroblast phenotype, which facilitates wound healing. The presence of this protein 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.
Next, anti-CD163 staining was performed to determine the presence of smooth muscle actin (SMA). Cells positive for SMA are potentially fibroblasts or myofibroblasts, which are cells known to be involved in tissue remodeling. Paired with the vimentin staining, this indicates that native cells are able to infiltrate the beads and begin secreting basement membrane. This is a crucial step in cells’ ability to adhere to the beads, which is a goal in tissue remodeling.
Figure 3.6: IHC anti-CD163 staining of 1% TA/collagen beads in mammary tissue. (A,B) 4x magnification, (C,D) 40x magnification. Black arrows in (C) and (D) indicating SMA positive cells.

IHC was also performed to identify Vascular Endothelial Growth Factor Receptor (VEGF-R), a signaling protein. 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 on the outer edges of the beads and within them (Figure 3.7). Increased vasculature within an implant indicates a positive host response and the implanted beads are stimulating angiogenesis and tissue regeneration.
Finally, to ensure there was not an underlying immune reaction, anti-CD68 staining was performed. CD68 is a protein highly expressed in monocytic phagocytes including tissue macrophages. These cells 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. Figure 3.8 shows 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 3.5: IHC anti-CD68 staining of 1% TA/collagen beads in mammary tissue. (A,B) 4x magnification, (C,D) 40x magnification. Black arrow in (C) indicating CD68 positive cells.
4.1 Conclusions

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 sections stained with H&E showed low inflammatory response and toxicity less than that of standard chemotherapies (Figure 3.1). H&E staining of mammary tissue explants indicated good incorporation into native surrounding tissue with no fibrous encapsulation (Figure 3.2). 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 3.3). Although no beads were located in the 8- or 12-week explants, previous studies within the rat model showed significant collagen remodeling at these time points. This would indicate mature basement membrane formation, an essential element of tissue regeneration. Adipose tissue growth, shown in Figure 3.4, 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 vimentin and SMA positive cells suggest fibroblasts are located within the beads (Figures 3.5, 3.6). Fibroblasts are necessary for the wound
healing process as they contribute to formation of the basement membrane. Secretion of smooth muscle actin is essential for formation of the basement membrane, which allows cells to adhere to the implant. Another essential step in tissue regeneration is angiogenesis, which requires the secretion of VEGF. Figure 3.7 shows cells positive for VEGF-R around and within the beads at the 4-week time point. Explants 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 3.8), 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.

4.2 Future Research

To advance this project towards clinical application, there are key elements that should be assessed. First, beads need to be analyzed at the 8- and 12-week time points, as well as the additional concentrations of TA. 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. Additionally, 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.

Further IHC analysis could also be performed to fully characterize the tissue response to the implant. Specifically, staining for laminin could demonstrate basal lamina growth over the 12 weeks. Also, 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.
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