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Tannic Acid Crosslinked Collagen Type I for Prevention of Local HER2-Positive Breast Cancer Recurrence

Lauren Gayle Jordan

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TANNIC ACID CROSSLINKED COLLAGEN TYPE I FOR PREVENTION OF LOCAL HER2-POSITIVE BREAST CANCER RECURRENTNESS

A Thesis
Presented to
the Graduate School of
Clemson University

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

by
Lauren Gayle Jordan
December 2016

Accepted by:
Dr. Brian Booth, PhD, Committee Chair
Dr. Sarah Harcum, PhD
Dr. Guigen Zhang, PhD
ABSTRACT

Breast cancer is the most commonly diagnosed cancer among women in the United States, affecting about 12 percent of women nationally (Ma and Jemal, 2013). The American Cancer Society estimates that 246,600 new cases of invasive breast cancer will be diagnosed in women in 2016 (American Cancer Society, 2016b). Breast cancer accounts for almost 30% of new cancer diagnoses and is one of the leading causes of cancer related deaths among women in developed countries. In addition, breast cancer is becoming a worldwide problem with incidence and mortality rates increasing steadily over the past 20 years in developing countries (Ma and Jemal, 2013).

Current treatment options for breast cancer include surgery, chemotherapy, and radiation; however, there are many negative side effects associated with these treatments, and these treatments are not always suitable for every patient. In addition, there is a risk of local breast cancer recurrence, particularly in patients who undergo a lumpectomy. Up to 20% of breast cancer patients experience recurrence, and of these recurrences, around 70% are defined as local (Zimmerman and Mehr, 2014). Therefore, there is a need for the development of a therapeutic agent specifically designed to target the surgical site and minimize the risk of locally recurrent breast cancer.

The overall goal of this research was to develop an injectable soft tissue regeneration matrix for the prevention of local HER2+ breast cancer recurrence. Collagen type I beads were crosslinked with tannic acid (TA) to form the basis for the injectable therapy. The collagen beads were seeded with human adipocytes. The adipocytes attach and grow on the collagen beads, thus remodeling the beads and releasing the tannic acid
to the surrounding environment. The tannic acid acts as an anticancer therapeutic agent by inducing apoptosis in HER2+ breast cancer cells via caspase pathways.

In order to prove the viability of this therapeutic option, several studies were conducted. LIVE/DEAD assays were used to prove successful seeding of the human adipocytes onto the collagen beads. Cell viability studies were conducted to assess the effect of tannic acid on human adipocytes and HER2+ breast cancer cells. In addition, the Folin-Ciocalteu assay was used to analyze the release profile of tannic acid. RNA was isolated from both the human adipocytes and HER2+ breast cancer cells, and Real Time-PCR was conducted to determine the activated pathways involved in cell apoptosis as a result of exposure to tannic acid. Western blotting was used to identify expression of caspases from proteins isolated from the HER2+ breast cancer cell line and normal human breast epithelial cell line.
DEDICATION

I would to dedicate this research to my grandmother, Diane Godwin, and in memory of my cousin, Joshua Loftis.

Breary, you inspire me so much with your strength, courage, and dignity in the face of adversity. I admire the grace and faith with which you have handled the challenges that you have faced in your life. Not only am I amazed by your toughness and will to fight, but I am humbled by your selflessness. You always put others before yourself and show God’s unconditional love to those around you. Thank you for your constant love, support, and interest in my life and academic career. I love you so much and am so grateful to have you in my life.

Josh, you are a shining example of unwavering faith in Christ. I cannot begin to explain the impact that you have had on my life. You have taught me so much about trusting God in every circumstance and motivated me to continue working towards my goals on the days when I’ve been discouraged. I think of you often and strive to be more like you because you were continually growing and transforming to become more like Jesus. Thank you for being my role model. Thank you for the influence that you have had on me and on so many others. Thank you for your beautiful smile and positive attitude. I love you and miss you.

For I know the plans I have for you,” declares the Lord, “plans to prosper you and not to harm you, plans to give you hope and a future.

Jeremiah 29:11
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 wonderful parents, Buddy and Donna Jordan, boyfriend, Carl Bower, and my entire family for their constant love, support, and encouragement. You have helped me stay grounded and motivated me to follow my dreams every day. Next, I would like to thank my advisor, Dr. Brian Booth for his guidance and support. You have created an ideal learning environment for your students in which they are able to grow and develop as researchers. I am so grateful for the opportunity to work under your mentorship and for your patience, guidance, and understanding throughout my thesis completion. I would also like to thank my committee members, Dr. Sarah Harcum and Dr. Guigen Zhang for their time and insight during this process. I would like to thank my fellow Institute for Biological Interfaces of Engineering lab and staff members. In particular, I would like to thank Kendyl Williams, Christopher Moody, and Kerri Kwist for their significant contributions to this work. I would also like to thank Scheen Thurmond for his dedication to ensure that our lab runs smoothly and his constant willingness to help with a smile on his face. I would like to thank the Clemson University Department of Bioengineering for providing me with the necessary foundation and tools for a career in bioengineering. Thank you to my professors and to the departmental staff, in particular Maria Torres, who has provided guidance throughout my entire graduate school career. Lastly, I would like to thank Dabo’s All In Team® Foundation for their funding that made this research possible.
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CHAPTER ONE

INTRODUCTION AND BACKGROUND

1.1 Project Overview

The current standard of care for breast cancer treatment includes surgery in which the cancerous lump and the surrounding tissue are removed, leaving a void as shown in Figure 1.1. The overall goal of this project is to develop an injectable tannic acid crosslinked collagen type I bead matrix to prevent local breast cancer recurrence. The technology shown in Figure 1.2 incorporates the patient’s adipocytes that are seeded onto the tannic acid crosslinked collagen type I beads. As the adipocytes attach and grow on the beads, they cause remodeling of the collagen and release of the tannic acid which then interacts with and kills any residual cancer cells in the tissue surrounding the void.

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

The female breast is composed primarily of adipose tissue and glandular tissue, extending from the clavicle to the center of the sternum or from the second rib to the sixth rib (Zhu and Nelson, 2013). Therefore, the breasts cover the majority of the chest and chest walls. The adult female breast contains 12 to 20 lobes, which are made up of 20 to 40 lobules (Zhu and Nelson, 2013). The breast lobules are formed by groups of secretory units, known as alveoli, which are lined by cuboidal, milk-secreting cells and encircled by myoepithelial cells (Barcellos-Hoff et al., 1989). It is within the alveoli that milk is produced in response to hormonal signals (McKinley and O’Loughlin, 2012). Small ducts which drain milk from the alveoli and lobules join and form 10 to 20 large ducts known as lactiferous ducts (McKinley and O’Loughlin, 2012). Each breast lobe is drained by a single lactiferous duct. Close to the nipple, the lumen of each lactiferous duct expands, forming a lactiferous sinus in which the milk is stored prior to lactation via
the nipple (McKinley and O’Loughlin, 2012). The breast structure is shown in Figures 1.3 and 1.4.

The functional components of the breast are surrounded by adipose tissue. Fibrous connective tissue or fascia, suspensory ligaments known as Cooper’s ligaments, lymph vessels, lymph nodes, and blood vessels are found within this adipose tissue (O’Connell and Rusby, 2015). Superficial and deep fascia, along with the Cooper’s suspensory ligaments, help provide structure, support, and attachment of the breast to the chest (Bland and Copeland, 1998). Lymph vessels carry lymph, a mixture of white blood cells, proteins, fats, and interstitial fluid, to lymph nodes. The lymph nodes are responsible for filtering the lymph fluid to remove any foreign substances or abnormal cells, which is an important component of the immune system (Bland and Copeland, 1998). Lastly, blood vessels provide the breast tissue with necessary oxygen and nutrients, while removing waste.
Figure 1.3: Anterior view of breast structure (McKinley and O’Louglín, 2012)
1.3 Breast Development

Until puberty, the structure of the breast is identical in females and males (Chau et al., 2016). At the onset of puberty in females, thelarche, or secondary breast development, begins (Nakamoto, 2000). In response to female sex hormones and growth hormone, breasts sprout, grow, and develop, changing in size and volume (Javed and Lteif, 2013). About two years after the onset of puberty, females undergo further breast development. The female sex hormone estrogen and growth hormone promote the growth
and development of the fat, glandular, and suspensory tissue structures of the adult female breast (Javed and Lteif, 2013). This development typically continues for about four years until the final breast size, volume, and density is established. It is important to note that breast size and volume does fluctuate in response to hormonal changes during thelarche, menstruation, pregnancy, lactation, and menopause. During pregnancy, estrogen, progesterone, and prolactin cause the growth of ducts and glandular tissue (Javed and Lteif, 2013). In addition, the water, electrolyte, and fat content of the breast as well as its vascularity increase during pregnancy leading to an increase in overall breast volume (Javed and Lteif, 2013). At the onset of menopause, the body stops producing the female sex hormones estrogen and progesterone (Santoro and Randolph Jr., 2011). This loss of hormones results in shrinkage of lobules and glandular tissue (Kalogerakos et al., 2014). In addition, breast density decreases as a result of menopause due to this tissue atrophy.

1.4 Breast Cancer Statistics

Breast cancer is the most commonly diagnosed cancer among women in the United States and affects about 12 percent of women nationally (Ma and Jemal, 2013). The American Cancer Society estimates that 246,660 new cases of invasive breast cancer will be diagnosed in women in 2016 (American Cancer Society, 2016 b). Breast cancer accounts for almost 30% of new cancer diagnoses and is one of the leading causes of cancer deaths among women in developed countries. In addition, breast cancer is
becoming a worldwide problem with incidence and mortality rates increasing steadily over the past 20 years in developing countries (Ma and Jemal, 2013).

1.5 Breast Cancer Classification

1.5.1 Stages of Breast Cancer

After the initial breast cancer diagnosis, the breast cancer is staged based on tumor size and invasiveness, status of lymph nodes, and metastasis (National Breast Cancer Foundation, Inc. a, 2015).

1.5.1.1 Stage 0

Stage 0 describes non-invasive breast cancers in which the abnormal, cancerous cells have not spread beyond their place of origin. Stage 0 breast cancer includes ductal carcinoma in situ, lobular carcinoma in situ, and Paget disease of the nipple (National Breast Cancer Foundation, Inc. b, 2015).

1.5.1.2 Stage I

Stage I is used to describe invasive breast cancers in which the cancerous cells have begun invading the surrounding breast tissue. Stage IA includes tumors measuring up to 2 cm and no lymph node involvement. Stage IB describes small groups of cancer cells between 0.2 mm and 2 mm in the lymph nodes instead of a breast tumor or a breast tumor measuring up to 2 cm and small groups of cancer cells between 0.2 mm and 2 mm in the lymph nodes (National Breast Cancer Foundation, Inc. b, 2015).
1.5.1.3 Stage II

Stage II also describes invasive breast cancer and is subdivided into Stages IIA and IIB. Stage IIA includes tumors larger than 2 mm in 1 to 3 surrounding lymph nodes instead of a breast tumor, breast tumors measuring more than 2 cm that have spread to axillary lymph nodes, and breast tumors between 2 cm and 5 cm that have not spread to the lymph nodes. Stage IIB includes breast tumors between 2 cm and 5 cm and small groups of cancer cells between 0.2 mm and 2 mm in the surrounding lymph nodes, breast tumors between 2 cm and 5 cm that has spread to 1 to 3 surrounding lymph nodes, and breast tumors larger than 5 cm that have not spread to the lymph nodes (National Breast Cancer Foundation, Inc. c, 2015).

1.5.1.4 Stage III

Stage III describes invasive breast cancer and is subdivided into Stages IIIA, IIIB, and IIIC. Stage IIIA includes tumors found in 4 to 9 surrounding lymph nodes instead of a breast tumor, breast tumors larger than 5 cm and small groups of cancer cells between 0.2 mm and 2 mm in the surrounding lymph nodes, and breast tumors larger than 5 cm that have spread to 1 to 3 surrounding lymph nodes. Stage IIIB describes breast tumors of any size that have spread to the chest wall or skin and up to 9 surrounding lymph nodes. Stage IIIB includes inflammatory breast cancer. Stage IIIC describes breast tumors of any size and/or cancer in 10 or more surrounding lymph nodes (National Breast Cancer Foundation, Inc. d, 2015).
1.5.1.5 Stage IV

Lastly, Stage IV describes metastatic breast cancer that has spread outside of the breast tissue and surrounding lymph nodes into other organs of the body (National Breast Cancer Foundation, Inc. e, 2015).

1.5.2 Hormone Receptor and HER2 Classification

In addition to staging, breast cancer is often classified based on the presence of hormone receptors and the status of human epidermal growth factor receptor-2 (HER2) protein. After pathological examination, the cancer is assigned a classification of hormone receptor-positive or receptor-negative, such as estrogen receptor-positive (ER\(^+\)) and progesterone receptor-positive (PR\(^+\)), HER2-positive (HER2\(^+\)), triple positive, or triple negative. Around 70\% of primary breast cancers are classified as hormone receptor-positive/HER2\(^-\), 10\% hormone receptor-positive/HER2\(^+\), 12\% triple negative, and 5\% hormone receptor-negative/HER2\(^+\) (Howlader et al., 2014). These classifications are important in determining the most appropriate treatment options.

1.6 Clinical Treatment Options

Current clinical treatment options for breast cancer include surgery, radiation therapy, chemotherapy, hormonal therapy, and targeted therapy. Typically patients are treated with a combination of these

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<td>I</td>
<td>100</td>
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<td>II</td>
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<td>III</td>
<td>72</td>
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Table 1.1: Breast Cancer Survival Rates, by Stage (American Cancer Society, 2016 a)
therapies based on overall health, location and type of cancer, and disease spreading (Peart, 2015). According to the American Cancer Society’s 5-yr survival rate data, these treatment options have proven to be successful, particularly in treating earlier stages of breast cancer (see Table 1.1) (American Cancer Society, 2016 a).

In spite of the high success rates for these treatment options, there remain many disadvantages associated with each. Surgery is extremely effective at removing a single tumor; however, it is associated with long recovery times, risks of excessive bleeding and infection, as well as psychological trauma (Arroyo and López, 2011). Radiation locally targets the tumor using high energy x-rays but is associated with swelling fatigue, and nerve damage (American Cancer Society, 2016 e). Chemotherapy is a systemic treatment incapable of specifically targeting the tumor, thus damages and kills healthy cells. It is also associated with many negative side effects including, hair loss, nausea, increased risk of infection due to low white blood cell counts, heart damage, and fatigue (American Cancer Society, 2016 c). Hormonal therapy is not capable of killing the cancerous cells of the tumor; it simply slows the progression of the disease (American Cancer Society, 2016 d). In addition, hormonal therapy is obviously not suitable for hormone receptor-negative breast cancer cell types and can lead to mood swings, blood clots, and increased risk of myocardial infarction (American Cancer Society, 2016 d). Lastly, side effects of targeted therapies include severe diarrhea, heart damage, and congestive heart failure (American Cancer Society, 2016 f). Targeted therapies are often coupled with other treatment options such as chemotherapy and/or hormonal therapy which are also associated with heart damage; thus, the potential heart damage is compounded putting the patient at a
higher risk (Bodai and Tuso, 2015). In addition to these side effects, targeted therapies are not suitable for pregnant women because they can result in severe damage to the fetus (Basta et al., 2015). All current treatment options for breast cancer patients have significant limitations and side effects; therefore, there is a need for a novel therapeutic agent against breast cancer.

1.7 Polyphenols

Polyphenols are a structural class of chemicals characterized by multiple hydroxyl groups attached to an aromatic ring (Vermerris and Nicholson, 2006). Polyphenols are classified as either flavonoids or non-flavonoids based on their chemical structure, biological function, and source (Mocanu et al., 2015). The flavonoid group includes flavonols, flavones, flavanols, anthocyanins, flavanones, and isoflavones. The non-flavonoid group is comprised of phenolic acids, stilbenes, lignans, and tannins (Pandey and Rizvi, 2009). Polyphenols are of great scientific interest due to their abundance with over 8000 identified species and their demonstrated health benefits.

1.7.1 Tannins

One polyphenolic subset of interest is the tannin group. Tannins are water-soluble polyphenolic compounds and are classified as either hydrolysable or condensed (Petridis, 2011). Hydrolyzable tannins have a polyhydric alcohol core and hydroxyl groups that have been partially or wholly esterified by gallic acid or hexahydroxydiphenic acid. Condensed tannins are generally products of polymerized flavan-3-ols and flavan-3,4-diols (Chung et al., 1998). The various tannin classifications are shown in Figure 1.5.
Tannins are found in a wide variety of plants including grains, fruits, nuts, and legumes, and also in drinks such as wine, green tea, and coffee (Carlsen et al., 2010; Oliveira et al., 2014; Mojzer et al., 2016). In addition to availability, studies have also shown an inverse relationship between high tannin consumption and cancer development; therefore, tannins are an ideal candidate as a novel, therapeutic anticancer agent and have been widely studied (Stich and Rosin, 1984).

**Figure 1.5:** Tannin Classifications (Khanbabaee and van Ree, 2001)
1.8 Anticancer Properties and Mechanisms of Tannins against Breast Cancer

1.8.1 Tannin Effects on ER$^+$ Breast Cancer

ER$^+$ breast cancer has been shown to be susceptible to the anti-carcinogenic effects of tannins in numerous studies (Babili et al., 2010; Booth et al., 2013; Fujita et al., 2001; Bawadi et al., 2005; Losso et al., 2004; Zhang et al., 2008; Wakil, 1989; Kuhajda, 2000; Shirode et al., 2013). Tannins such as ellagic acid and gallic acid do not possess estrogenic activity (Zand et al., 2000); therefore, recent studies have focused on other mechanisms of interference.

We (Booth et al., 2013) demonstrated that tannic acid (TA) caused a change in morphology of MCF7 ER$^+$ breast cancer cells from spindle shaped cells to round cells. TA induced significant apoptosis in the ER$^+$ breast cancer cells within 24 hours of exposure, indicating their high sensitivity to the apoptotic effects of TA. In addition to demonstrating the decrease in cell viability and increase in apoptotic activity as a result of TA exposure, we determined the mechanism involved in apoptosis initiation in ER$^+$ breast cancer cells. Apoptosis is induced via activation of the caspases 3/7 and 9. We further postulated that the high sensitivity of MCF7 ER$^+$ breast cancer cells to TA treatment is related to the lack of caspase 3 expression by the MCF7 cells (Booth et al., 2013). Caspase 3 can act as a negative feedback regulator of caspase 9 since it is downstream of caspase 9 (Fujita et al., 2001).

Bawadi et al (Bawadi et al., 2005; Losso et al., 2004) treated ER$^+$ breast cancer cells with water-soluble condensed tannins derived from black beans. Through microscopy, it was shown that tannin treatment disrupts cellular integrity and that cell
death occurred via apoptosis. The tannin cytotoxicity was assessed with a ViaLight HS luciferin and luciferase-based assay using ATP bioluminescence as a cell viability and proliferative ability marker. ATP levels steadily decreased with increasing tannin concentrations for 24 hours indicating decreased cell proliferation and migration activities. The condensed tannins were shown to decrease the activities of metalloproteinase-2 (MMP-2) and MMP-9 to less than 50% of their activity compared to the control cancer cells. The researchers hypothesized that this effect could be associated with the metal chelating properties of tannins, allowing them to bind zinc thus making it unavailable for MMP activity. Condensed tannin treatment reduced the levels of vascular endothelial growth factor (VEGF) in the media. MMPs and VEGF are angiogenic factors necessary for new blood vessel formation; therefore, inhibiting their activity inhibits cancer cell proliferation, migration, progression, and metastasis.

Another study showed tannin induction of apoptosis via inhibition of fatty acid synthase (FAS) (Zhang et al., 2008). FAS is an enzyme involved in de novo synthesis of long chain fatty acids in vivo and is highly expressed in human cancers such as breast cancer (Wakil, 1989; Kuhajda, 2000). Therefore, inhibition of FAS can directly lead to apoptosis, making it an important therapeutic target for cancer treatment. After treatment with condensed tannins derived from the catechu plant, an MTT assay for cell viability was conducted to confirm growth inhibition of ER+ breast cancer cells in a dose-dependent manner. It was also shown that the condensed tannins blocked most of the enzymatic activity of FAS by inhibiting the β-ketoacyl reductase domain of FAS. This inhibition was competitive to NADPH; therefore, it was hypothesized that the tannin
reaction site is the NADPH loading site in the β-ketoacyl reductase domain of FAS. It is also likely that condensed tannins react on the acyl transferase domain of FAS (Zhang et al., 2008).

In order to decipher the mechanisms of action of hydrolysable tannins found in pomegranate extract, Shirode et al (Shirode et al., 2013) conducted several studies using ER⁺ breast cancer cells. The group confirmed previous results that tannins inhibit cell growth by inducing cell cycle arrest in G2/M followed by apoptosis induction. DNA microarray analysis showed that tannins from pomegranate extract downregulated genes involved in mitosis, chromosome organization, RNA processing, DNA replication, and DNA repair. Tannins also upregulated genes associated with regulation of apoptosis and cell proliferation. DNA microarray and quantitative RT-PCR showed downregulation of genes involved in DNA double strand break repair by homologous recombination, including BRCA1, BRCA2, BRCC3, RAD50, RAD51, MRE11, and NBS1. In addition, the levels of predicted microRNAs of homologous recombination genes increased indicating regulation of miRNAs involved in DNA repair by tannins. Tannin treatment increased the frequency of DNA double strand breaks, which is attributed to the downregulation of homologous repair that causes cells to become more sensitive to DNA double strand breaks, growth inhibition, and apoptosis.

1.8.2 Tannin Effects on HER2⁺ Breast Cancer

Tannins have proven to be cytotoxic to HER2⁺ and trastuzumab-resistant HER2⁺ breast cancer cells in a dose-dependent manner (Moongkarndi et al., 2004; Sonoda et al., 2006; Signoretti et al., 2002; Huang et al., 2011; Banerjee et al., 2012; Eddy et al., 2007);
therefore, the mechanism of action of tannins has been widely studied in HER2+ breast cancer cells.

Tannins derived from the pericarps of garcinia mangostana were shown to have antiproliferative and antioxidative effects on HER2+ breast cancer cells (Moongkarndi et al., 2004). MTT reduction assays confirmed the antiproliferative effects after 48 hours of exposure to mangostana-derived tannins, and showed a concentration-dependent decrease in percent cell viability. Morphological analysis using Hoechst33342 and propidium iodide (PI) staining revealed nuclear shrinking, DNA condensation, and DNA fragmentation after 48 hours of tannin treatment. Phase contrast microscopy showed cytoplasmic membrane shrinkage, loss of contact with neighboring cells, membrane blebbing, and apoptotic bodies. DNA ladder patterns on agarose gel electrophoresis exhibited oligonucleosomal DNA fragments from cells after tannin treatment. From the results of the morphological analysis and agarose gel electrophoresis, it was concluded that the tannins from the garcinia mangostana extract induced apoptosis in the HER2+ breast cancer cells. High dose treatments resulted in increased reactive oxygen species (ROS) levels at 48 hours after treatment, followed by a decrease in ROS levels at 72 hours. It was hypothesized that the tannins induce apoptosis, at least in part, via high ROS levels. These ROS levels then decrease over time because only cell debris remains.

In many different human cancers, including breast cancer, overexpression of the F-box protein S-phase kinase-associated protein 2 (Skp2) is observed (Sonoda et al., 2006; Signoretti et al., 2002). Skp2 plays a large role in the progression of breast cancer; therefore, numerous studies have been performed to evaluate different chemopreventative
agents in the downregulation of Skp2. One of these agents is 1,2,3,4,6-Penta-O-galloyl-β-D-glucose, a precursor of gallotannins and ellagitannins, which is also referred to as 5gg. Growth of HER2+ breast cancer cells was inhibited by 5gg in a dose and time-dependent manner (Huang et al., 2011). In addition, through flow cytometry it was shown that 5gg results in cell cycle arrest in the G1 phase, inhibiting cell growth. Skp2 is associated with the S-phase promoting kinase cyclin A-cyclin-dependent kinase 2 (Cdk2) and plays an important role in cell cycle progression regulation. Skp2 levels decrease when cells exit the cell cycle and increase when cell re-enter the cell cycle. Skp 2 targets p27 for degradation, thus acting as an oncogene. 5gg treatment causes Skp2 downregulation, which contributes to the induction of cell cycle arrest in HER2+ breast cancer cells.

Pomegranate extract containing hydrolysable tannins reduced growth and tumor volume of HER2+ breast cancer cells (Banerjee et al., 2012). Caspase-3 was activated, indicating apoptosis was induced via caspase-mediated pathways. Treatment with pomegranate extract decreased mRNA and protein levels of Sp transcription factors Sp1, Sp3, and Sp4. Sp regulated genes cyclin D1, bcl2, VEGF, VEGFR-1, and NF-κB were also decreased. These genes are involved in cell proliferation, angiogenesis, and inflammation.

Epigallocatechin-3-gallate (EGCG), a condensed tannin isolated from green tea leaves, treatment results in a dose-dependent decrease in ATP production in HER2+ resistant cancer cells, resulting in decreased cellular growth and proliferation (Eddy et al., 2007). EGCG treatment caused a decrease in cell number after 96 hours. Through nuclear morphology studies and Hoechst 33258 staining, DNA fragmentation and dispersal in the
cytoplasm and loss of nuclear integrity were observed after high dose EGCG treatments, indicating that EGCG induces apoptosis. EGCG treatment reduced Akt signaling in trastuzumab-resistant HER2\(^+\) breast cancer cells in a dose-dependent manner and increased nuclear expression of FOXO3a and its growth regulatory target gene p27. A lack of p27 expression is associated with trastuzumab resistance, whereas induction of p27 indicates trastuzumab sensitivity; therefore, the expression of p27 after EGCG treatment indicates decreased resistance of the HER2\(^+\) breast cancer cells.

1.8.3 Tannin Effects on Triple Negative Breast Cancer

The effects of tannic acid and other chemopreventative agents have been of interest and the subject of many different studies using triple negative breast cancer cell lines. Triple negative breast cancer cells do not respond to normal hormonal therapies, such as tamoxifen and aromatase inhibitors, and therefore different agents and therapies have been studied.

Tannic acid has been investigated in order to determine its effects on breast cancer cells. We published results where we (Booth et al., 2013) performed a study using collagen type-1 beads cross-linked with tannic acid in order to further understand the effects of the tannic acid on different types of breast cancer cell lines. Though the effects were less significant on the triple negative breast cancer cells compared to other types of breast cancer, 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. Tannic acid was shown to induce a significant level of apoptosis in the triple negative cells that were treated as opposed to those that were not. The effect on caspase activation
was also investigated. Once again, though the effects were not as dramatic as other types of breast cancers, it was shown that caspases 9 and 3/7 were elevated once exposed to tannic acid. All of the effects of tannic acid on the triple negative cell line were shown to be both dose- and time-dependent.

Doxorubicin (DXR) is an anthracycline antibiotic that is effective in treating a variety of solid tumors, including breast tumors. However, due to concerns about toxicities, including cardiotoxicity, it has had limited use. A study by Tikoo et al (Tikoo et al., 2010) showed that tannic acid, when combined with DXR, was able to reduce cardiotoxicity of DXR while promoting the anti-cancer properties of DXR against the triple negative cell line MDA-MB-231. In this study tumors were chemically induced in rats with dimethylbenz[a] anthracene (DMBA) then treated with combinations of DXR and tannic acid. DXR reduced the volume of the tumor as compared untreated tumors. DXR enhanced the expression of the tumor suppressor gene p53, which led to an inhibition of tumor growth. The addition of tannic acid was more effective than DXR alone in elevating the expression of p53, resulting in inhibition of tumor growth. Tannic acid gave protection against DXR-induced myocardial damage by preventing cytoplasmic vacuolization.

Huang et al (Huang et al., 2011) determined the effects of 5gg on the cell cycle in triple negative breast cancer cells that were overexpressing Skp2. The group found that 60% of the treated cells were in the G1 phase, as opposed to only 40% among the control cells. After 48 hours of 5gg treatment, all triple negative breast cancer cells were arrested in the G1 phase. These results indicated that 5gg halted the cell cycle of triple negative
breast cancer cells. 5gg was able to modulate the expression of cell cycle regulatory proteins, which then induced the arrest of the cell cycle. This effect was both time- and dose-dependent.

Several studies have shown the ability of polyphenolics from pomegranate (Punica granatum L.) to inhibit cancer cell proliferation and to induce apoptosis, promote cell cycle arrest, and decrease inflammation. There is evidence that suggests this is in part due to their role in decreasing specificity protein (Sp) factors. Banerjee et al extracted mRNA for gene expression analysis from triple negative breast cancer cells treated with pomegranate extract (Pg) after 24 hours and determined there was a concentration-dependent decrease in cell-viability after being treated with Pg (Banerjee et al., 2012). There were not any significant effects on cytotoxicity in cells that were not treated under the same conditions. The cytotoxicity induced by Pg was accompanied by activation of a primary apoptosis-executing enzyme, caspase-3. Pg inhibited the proliferation of cancer cells while inducing apoptosis.

Kim et al (Kim et al., 2002) studied three pomegranate components: polyphenol-rich fractions from fermented juice, aqueous pericarp extracts, and cold-pressed or supercritical CO$_2$-extracted seed oil of pomegranates. The effects of these three components on human triple negative breast cancer cells were investigated in vitro. The polyphenols inhibited the proliferation of triple negative breast cancer cells in a dose-dependent manner. Although the effects were not as profound as those found in other types of breast cancer, it can be inferred that polyphenols from pomegranates play a role in inhibiting the proliferation of triple negative breast cancer cells. All three components
from which the polyphenols were extracted had an inhibitory effect on the proliferation of the triple negative breast cancer cells.

In recent years, green tea extract has been studied in order to determine the mechanisms of its anti-cancer effects. EGCG, a principal component of green tea, has been shown to aid in both the prevention and the treatment of cancer by inhibiting angiogenesis and tumor invasion, which are required for tumor growth and progression (Khan and Mukhtar, 2010). One study showed suppressed growth and proliferation of triple negative breast cancer cells as a result of EGCG treatment (Braicu et al., 2013). Li et al (Li et al., 2014) reviewed the known effects of green tea extract on different breast cancer cell lines. The findings suggest that the green tea extract containing EGCG is capable of inducing cell cycle arrest and apoptosis in many different types of breast cancer cell lines, including the MDA-MB-231 and MDA-MB-468 triple negative breast cancer cells.
CHAPTER TWO
MATERIALS AND METHODS

2.1 Collagen Bead Preparation

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 coworkers 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, 40 μL 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. Specifically, 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% \( \text{CaCl}_2 \) 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 \( \text{CaCl}_2 \) 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 to 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/h, 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, tannic acid 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 tannic acid (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\textsubscript{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 hrs 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.

2.2 Cell Culture

SW872, BT474, and MCF10A cells obtained from ATCC (Manassas, VA) were cultured in T-75 culture flasks (Corning; Corning, NY) under sterile conditions. For the SW872 cell line, Dulbecco’s Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F12) (ATCC; Manassas, VA) was used. For the BT474 cell line, Dulbecco’s
Modified Eagle Medium (DMEM) (ATCC; Manassas, VA) was used. For the MCF10A cell line, Dulbecco’s Modified Eagle Medium (DMEM) (ATCC; Manassas, VA) supplemented with MEGM SingleQuots (Lonza; Walkersville, MD) was used. In addition, each media type was enhanced with 10.0% fetal bovine serum (FBS) (Corning; Manassas, VA), 1.0% antibiotic-atimycotic (AA) (Gibco; Great Island, NY), and 0.2% fungizone (Gibco; Great Island, NY). The cells were incubated at 37 °C with 5.0% CO₂ in a MCO-18AIC incubator (Sanyo Scientific; Wood Dale, IL).

2.3 Cell Seeding on Collagen Beads

SW872 cells were passaged and resuspended in 10 mL of media. A hemocytometer (Fisher Scientific; Fair Lawn, NJ) and Axiovert 40 CFL microscope (Zeiss; Oberkochen, Germany) were used to determine the concentration of the SW872 cell suspension. The hemocytometer was loaded with 10 μL of the cell suspension and placed on the microscope stage. The 10X objective lens was used with a 10X eyepiece, yielding a total magnification of 100X. The coarse focus and fine focus were adjusted as necessary to bring the hemocytometer counting grid loaded with SW872 cells into focus. Cells within the four large corner squares were counted. This process was repeated for the other counting grid of the hemocytometer, and the two cell counts were averaged. This number was then divided by 4 in order to obtain the average number of cells per large square. The volume of this square is 100 nL; therefore, this number corresponds to the average number of cells per nL. This value was then multiplied by 10,000 in order to convert it to the average number of cells per mL. Finally, the average cell concentration
was multiplied by the total volume of the cell suspension in order to determine the total number of cells in the original sample.

Collagen beads cross-linked with TA in concentrations of 10.0% (mass/volume), 1.0%, and 0.1% and stored in Dulbecco’s Phosphate Buffered Saline (PBS) (Sigma Aldrich; St. Louis, MO) were removed from storage, and centrifuged at 500 rpm for 5 minutes using an Allegra X-12R centrifuge (Beckman Coulter; Indianapolis, IN). The PBS was then aspirated from the 50 mL centrifuge tubes (Corning; Corning, NY). A 100-1000 μL VWR Signature Ergonomic High Performance Single-Chanell Variable Volume pipettor (VWR; Radnor, PA) was used to transfer 500 μL of each concentration of collagen cross-linked beads to 50 mL TubeSpin Bioreactor 50 roller tubes (Techno Plastic Products AG; Trasadingen, Switzerland) in triplicate, for a total of 9 tubes. The end of the pipette tip was cut using scissors to prevent any damages to the collagen beads during pipetting.

From the SW872 cell suspension, 1.5 x 10^6 cells were added to each roller tube based on the determination of the cell concentration from the hemocytometer. Supplemented (as described above) Dulbecco’s Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F12) (ATCC; Manassas, VA) was added to each tube for a total volume of 10 mL per tube.

The roller tubes were then placed in a WSR Bot Drive 7"R 5 x 03 Deck rotating wheel apparatus (Wheaton; Millville, NJ) and spaced appropriately to ensure even rotation. This encouraged even cell attachment to the collagen beads and prevented adhesion of the beads to the tubes. Centrifuge tubes containing water were used for
balance when necessary. The rotating wheel was placed in the MCO-18AIC incubator (Sanyo Scientific; Wood Dale, IL) at 37 °C with 5.0% CO₂. The rotating switch was then turned on to initiate rotation. The roller tubes remained in the WSR Bot Drive 7"R 5 x 03 Deck rotating wheel apparatus (Wheaton; Millville, NJ) for 7 days to allow for attachment and proliferation of the SW872 cells on the beads.

This procedure was repeated using BT474 cells.

2.4 LIVE/DEAD Viability-Cytotoxicity Assay

After 7 days of incubation time in the WSR Bot Drive 7"R 5 x 03 Deck rotating wheel apparatus (Wheaton; Millville, NJ), roller tubes containing collagen beads cross-linked with TA in concentrations of 10.0% (mass/volume), 1.0%, and 0.1% and seeded with SW872 cells were removed from the incubator. The roller tubes were centrifuged at 500 rpm for 5 minutes using an Allegra X-12R centrifuge (Beckman Coulter; Indianapolis, IN), and the media was aspirated from the tubes. Next, 25 mL of Dulbecco’s Phosphate Buffered Saline (PBS) (Sigma Aldrich; St. Louis, MO) were added to each roller tube. The roller tubes were centrifuged again at 500 rpm for 5 minutes using an Allegra X-12R centrifuge (Beckman Coulter; Indianapolis, IN), and the PBS was aspirated from the tubes.

Using a Molecular Probes LIVE/DEAD Viability/Cytotoxicity kit for mammalian cells (Fisher Scientific; Fair Lawn, NJ), a 10 μM Ethidium homodimer-1 (EthD-1) and 10 μM Calcein AM working solution was made by adding 25 μL of 2mM EthD-1 to 5 mL of Dulbecco’s Phosphate Buffered Saline (PBS) (Sigma Aldrich; St. Louis, MO) in a 15 mL
centrifuge tube (Corning; Corning, NY). Then 12.5 μL of 4 mM calcein AM were added to the solution. The working solution was the vortexed for 10 s using a VWR Analog Vortex Mixer (VWR; Radnor, PA).

Next, 300 μL of the working solution were added to each roller tube containing collagen beads cross-linked with TA in concentrations of 10.0% (mass/volume), 1.0%, and 0.1% and seeded with SW872 cells. The SW872 loaded collagen cross-linked beads were allowed to incubate in the dark for 45 min at room temperature. After the incubation period was over, 10 μL of the working solution were added to each roller tube.

A 100-1000 μL VWR Signature Ergonomic High Performance Single-Chanell Variable Volume pipettor (VWR; Radnor, PA) was used to transfer 150 μL of collagen cross-linked beads to microscope slides (Fisher Scientific; Fair Lawn, NJ). The end of the pipette tip was cut using scissors to prevent any damages to the collagen beads during pipetting. An Axiovert 40 CFL microscope (Zeiss; Oberkochen, Germany) was used to view the collagen beads under fluorescence, and an Axiocam MRc5 (Zeiss; Oberkochen, Germany) was used image the collagen beads.

This procedure was repeated for collagen beads cross-linked with TA in concentrations of 10.0% (mass/volume), 1.0%, and 0.1% and seeded with BT474 cells.

2.5 Co-Culture of BT474 cells and SW872 seeded TA crosslinked Collagen Beads

BT474 cells were passaged and resuspended in 10 mL of media. A hemocytometer (Fisher Scientific; Fair Lawn, NJ) and Axiovert 40 CFL microscope
(Zeiss; Oberkochen, Germany) were used to determine the concentration of the BT474 cell suspension according to the previously described method on pages 25-26.

From the BT474 cell suspension, 1.0 x 10^5 cells were added to each well in Costar 24-well Transwell® plates (Corning; Corning, NY) for a total of 36 wells based on the determination of the number of cells per mL from the hemocytometer. Supplemented (as described above) Dulbecco’s Modified Eagle Medium (DMEM) (ATCC; Manassas, VA) was added to each well for a total volume of 500 μL per well. Transwell® permeable supports with a 0.4 μm polycarbonate membrane were placed in all wells except the well of the first row. The first row of wells acted as the control groups. The Transwell® plates were then placed in the MCO-18AIC incubator (Sanyo Scientific; Wood Dale, IL) at 37°C with 5.0% CO2 overnight to allow the cells to attach to the surface of the well plates. The experimental setup and design are shown in Figures 2.3 and 2.4.

After 7 days of incubation time in the WSR Bot Drive 7"R 5 x 03 Deck rotating wheel apparatus (Wheaton; Millville, NJ), roller tubes containing collagen beads cross-linked with TA in concentrations of 10.0% (mass/volume), 1.0%, and 0.1% and seeded with SW872 cells were removed from the incubator. The roller tubes were centrifuged at 500 rpm for 5 minutes using an Allegra X-12R centrifuge (Beckman Coulter; Indianapolis, IN), and the media was aspirated from the tubes. A 100-1000 μL VWR Signature Ergonomic High Performance Single-Chanell Variable Volume pipettor (VWR; Radnor, PA) was used to transfer 100 μL of collagen cross-linked beads from each roller tube to Transwell® permeable supports in triplicate, for a total of 9 wells per concentration of TA cross-linked collagen beads. The end of the pipette tip was cut using
scissors to prevent any damages to the collagen beads during pipetting. The Transwell® plates were then placed in the MCO-18AIC incubator (Sanyo Scientific; Wood Dale, IL) at 37°C with 5.0% CO₂. Samples were taken on days 3, 5, and 7.

This entire procedure was repeated in order to analyze other sample types.

This method was also modified in order to incorporate the co-culture of MCF10A cells and SW872 seeded TA cross-linked collagen beads in addition to the co-culture of BT474 cells and SW872 seeded TA cross-linked collagen beads. The modified experimental setup in shown in Figure 2.5.
### Figure 2.3: Experimental Setup

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### Day 7

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<tr>
<td>1.0% TA Beads</td>
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<td>0.1% TA Beads</td>
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**Figure 2.4:** Experimental Design

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**Figure 2.5:** Modified Experimental Setup
2.6 Sample Collection

After co-culturing BT474 cells with SW872 seeded TA cross-linked collagen beads, samples were collected on Days 3, 5, and 7. The following methods were conducted for each time point.

2.6.1 Experiment 1

2.6.1.1 Media Collection

The media was removed from the 12 wells in the 24 well-plate that corresponded to the appropriate time point as shown in Figure 2.3 using a 100-1000 μL VWR Signature Ergonomic High Performance Single-Channel Variable Volume pipettor (VWR; Radnor, PA) and placed in corresponding microcentrifuge tubes (Fisher Scientific; Fair Lawn, NJ). The microcentrifuge tubes were then stored in the freezer at 4 °C.

2.6.1.2 Trypan Blue Staining

After the media was removed, 250 μL of 0.05% Trypsin, 0.53 mM EDTA, 1X [-] sodium bicarbonate (Corning; Manassas, VA) were added to each of the corresponding 12 wells. The well-plate was then placed in a MCO-18AIC incubator (Sanyo Scientific; Wood Dale, IL) for 5 min at 37 °C with 5.0% CO₂. Next, 250 μL of Dulbecco’s Modified Eagle Medium (DMEM) (ATCC; Manassas, VA) supplemented with 10.0% fetal bovine serum (FBS) (Corning; Manassas, VA), 1.0% antibiotic-atimycotic (AA) (Gibco; Great Island, NY), and 0.2% fungizone (Gibco; Great Island, NY) were added to each well. A 100-1000 μL VWR Signature Ergonomic High Performance Single-Channel Variable Volume pipettor (VWR; Radnor, PA) was used to resuspend the cells and ensure that they had detached from the surface of the well-plate. Then, 10 μL of each cell suspension
were added to microcentrifuge tubes containing 40 μL of Trypan Blue (Modified) 0.4% solution 1.0N phosphate buffered saline (Trypan Blue) (MP Biomedicals; Solon, Ohio), and the solution was mixed.

2.6.1.3 Protein Isolation

The remainders of the cell suspensions were transferred to microcentrifuge tubes, combining all control cell suspensions, 10.0% TA treated cell suspensions, 1.0% TA treated cell suspensions, and 0.1% TA treated cell suspensions into corresponding tubes. The cell suspensions were centrifuged at 5000 rpm for 5 min using a Sorvall Legend Micro 21R centrifuge (Fisher Scientific; Fair Lawn, NJ). A working solution was made by mixing 800 μL of Mammalian Protein Extraction Reagent (M-PER) (Fisher Scientific; Rockford, IL) with 8 μL of Halt Protease Inhibitor Cocktail, EDTA-free (100X) (Fisher Scientific; Rockford, IL) and 8 μL of Halt Phosphatase Inhibitor Cocktail (100X) (Fisher Scientific; Rockford, IL). Then, 200 μL of the working solution were added to each of the microcentrifuge tubes, and the tubes were allowed to incubate for 10 min at room temperature. The microcentrifuge tubes were then centrifuged for 10 min at 10,000 rpm at 4 °C using a Sorvall Legend Micro 21R centrifuge (Fisher Scientific; Fair Lawn, NJ). After centrifugation, the protein samples were stored in the freezer at -20°C.

2.6.2 Experiment 2

2.6.2.1 Media Collection

The media was collected as previously described on pages 32-33.
2.6.2.2 RNA Precipitation

After the media was removed, 250 μL of 0.05% Trypsin, 0.53 mM EDTA, 1X [-] sodium bicarbonate (Corning; Manassas, VA) were added to each of the corresponding 12 wells. The well-plate was then placed in a MCO-18AIC incubator (Sanyo Scientific; Wood Dale, IL) for 5 min at 37 °C with 5.0% CO₂. Next, 250 μL of Dulbecco’s Modified Eagle Medium (DMEM) (ATCC; Manassas, VA) supplemented with 10.0% fetal bovine serum (FBS) (Corning; Manassas, VA), 1.0% antibiotic-atimycotic (AA) (Gibco; Great Island, NY), and 0.2% fungizone (Gibco; Great Island, NY) were added to each well. A 100-1000 μL VWR Signature Ergonomic High Performance Single-Channel Variable Volume pipettor (VWR; Radnor, PA) was used to resuspend the cells and ensure that they had detached from the surface of the well-plate. The cell suspensions were transferred to microcentrifuge tubes and centrifuged at 1000 rpm for 5 min using a Sorvall Legend Micro 21R centrifuge (Fisher Scientific; Fair Lawn, NJ) in order to lyse the cells. The media was then aspirated from the microcentrifuge tubes, and the cells were resuspended in 200 μL of TRIzol reagent (Fisher Scientific; Fair Lawn, NJ). The tubes were allowed to incubate at room temperature for 10 min. The microcentrifuge tubes were stored in the freezer at 4 °C.

The SW872 seeded TA cross-linked collagen beads were removed from the Transwell® permeable supports using a 100-1000 μL VWR Signature Ergonomic High Performance Single-Channel Variable Volume pipettor (VWR; Radnor, PA) and transferred to corresponding microcentrifuge tubes. The end of the pipette tip was cut using scissors to ensure that all beads were collected. The microcentrifuge tubes were
then centrifuged at 1000 rpm for 5 min using a Sorvall Legend Micro 21R centrifuge (Fisher Scientific; Fair Lawn, NJ) in order to lyse the cells. The media was then aspirated from the microcentrifuge tubes. Next, 200 μL of TRIzol reagent (Fisher Scientific; Fair Lawn, NJ) were added to each microcentrifuge tube, and the tubes were allowed to incubate at room temperature for 10 min. The microcentrifuge tubes were stored in the freezer at 4 °C.

2.6.3 Experiment 3

In addition to BT474 cells, MCF10A cells were co-cultured with SW872 seeded TA cross-linked collagen beads as shown in Figure 2.5.

2.6.3.1 Media Collection

The media was collected as previously described on pages 32-33.

2.6.3.2 Trypan Blue Staining

After the media was removed, cells were stained with Trypan Blue according to the method previously described on page 33.

2.6.3.3 Protein Isolation

Protein was isolated from the cells according to the protocol previously described in section 2.6.1.3.

2.7 Trypan Blue Exclusion Cell Viability Assay

Cell suspensions stained with Trypan Blue were assessed using a hemocytometer (Fisher Scientific; Fair Lawn, NJ) and Axiovert 40 CFL microscope (Zeiss; Oberkochen, Germany) as previously described on pages 25-26. Live cells remained unstained due to
intact cellular membranes while dead cells were stained blue due to lost membrane integrity. Based on this difference in staining, live cells and dead cells within the large center square were counted. This process was repeated for the other counting grid of the hemocytometer, and both the live cell counts and dead cell counts were averaged. These numbers were then multiplied by the dilution factor of 5 since 10 μL of the cell suspensions were added to 40 μL of Trypan Blue. Finally, the average live cell concentration and dead cell concentration were multiplied by the total volume of the cell suspension (0.5 mL) in order to determine the total number of live cells and dead cells in the original sample.

2.8 Folin-Ciocalteu Assay

A Folin-Ciocalteu (F-C) assay was used to quantify the concentration of TA in the media below Transwell® inserts containing SW872 loaded TA cross-linked collagen beads.

First, a 10.0% (mass/volume) TA standard was made by dissolving 1.0 g of tannic acid (Sigma Aldrich; St. Louis, MO) in 10 mL of Dulbecco’s Modified Eagle Medium (DMEM) (ATCC; Manassas, VA). Serial dilutions were then performed in order to obtain TA standards of 5.0%, 2.5%, 1.25%, 0.625%, 0.313%, 0.156%, 0.0781%, 0.0391%, 0.0195%, 0.00977%, 0.00488%, 0.00244%, 0.00122%, 0.000610%, 0.000305%, 0.000153%, and 0.0%.
A 10.0% (volume/volume) F-C reagent (Sigma Aldrich; St. Louis, MO) solution was prepared in milliQ water. In addition, a 700 mM sodium carbonate (Fisher Scientific; Fair Lawn, NJ) solution was prepared in milliQ water.

Next, 100 μL of each TA standard and each media sample were transferred to microcentrifuge tubes (Fisher Scientific; Fair Lawn, NJ). Then, 200 μL of the 10.0% (volume/volume) F-C reagent were added to each tube. The tubes were vortexed using a VWR Analog Vortex Mixer (VWR; Radnor, PA). After mixing, 800 μL of the 700 mM sodium carbonate solution were added to each microcentrifuge tube. Tubes were vortexed again using a VWR Analog Vortex Mixer (VWR; Radnor, PA). The tubes were then allowed to incubate at room temperature for 2 hours.

After incubating, 200 μL of each TA standard and each media sample were transferred in triplicate into a 96 well-plate (Corning; Corning, NY). The absorbance was measured at 765 nm on a Synergy Mx microplate reader (BioTek; Winooski, VT). A standard curve was generated using the Gen5 program (BioTek; Winooski, VT), and the total TA concentrations for each sample were outputted.

2.9 RNA Isolation

First, samples resuspended in 200 μL of TRIzol reagent (Fisher Scientific; Fair Lawn, NJ) were removed from the freezer and thawed in a MCO-18AIC incubator (Sanyo Scientific; Wood Dale, IL) at 37 °C with 5.0% CO₂.

Next, the RNA isolation procedure from the TRIzol reagent manual (Fisher Scientific; Fair Lawn, NJ) was followed according to the manufacturer’s guidelines.
Note, the manual provides volumes in terms of volume per 1 mL of TRIzol reagent used. Samples were resuspended in 200 μL of TRIzol reagent; therefore, these volumes were divided by 5 in order to achieve the necessary volumes for my samples.

2.10 DNA Removal

RNA samples were thawed. Then, the TURBO DNA-free procedure from the TURBO DNA-free Kit manual (Fisher Scientific; Fair Lawn, NJ) was followed according to the manufacturer’s guidelines in order to remove any contaminating DNA, remaining DNase, and divalent cations which can catalyze RNA degradation upon heating.

2.11 RNA Concentration Quantification

A NanoDrop 2000 spectrophotometer (Fisher Scientific; Radnor, PA) was used according to the manufacturer’s guideline to determine the concentration of RNA in each sample.

2.12 Reverse Transcription

An Applied Biosystems High-Capacity cDNA Reverse Transcription Kit (Fisher Scientific; Radnor, PA) was used to convert RNA to single-stranded cDNA. The manual was followed according to the manufacturer’s guidelines. The RNA concentrations determined from the Nanodrop were used to calculate the mass of RNA and the volume of RNase-free water to be added to each well for a total RNA sample volume of 10 μL with 3.55 μg of RNA.
2.13 Real-Time Polymerase Chain Reaction

A QuantiTect SYBR Green PCR Kit (Qiagen; Hilden, Germany) and PrimeTime qPCR primers (Integrated DNA Technologies; Coralville, IA) were used. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as the housekeeping gene, and caspases 3, 7, and 9 were targeted. Single-stranded cDNA samples were analyzed in replicates for each gene. The number of samples per gene was determined, and the working master mix for each gene was prepared based on the following calculations:

Samples per gene (including negative control) + 1 = Total Number

Total Number x 10 = μL 2x Qiagen SYBR Green Master Mix

Total Number x 2 = μL PrimeTime assay for each gene

Total Number x 6 = μL Nuclease Free Water

Then, 18 μL of the working master mix was pipetted into each well of a MicroAmp Fast Optical 96-well reaction plate (Fisher Scientific; Fair Lawn, NJ). Next, 2 μL of each cDNA sample and nuclease free water (negative control) were transferred to the appropriate well in duplicate. An optical adhesive cover (Fisher Scientific; Fair Lawn, NJ) was placed on the reaction plate. The plate was run using an Applied Biosystems StepOnePlus Real-Time PCR System (Fisher Scientific; Fair Lawn, NJ) according to the following protocol:

1. Holding: 95 °C for 15 minutes
2. Cycling: 35 cycles
   a. Denature: 94 °C for 15 seconds
   b. Anneal: 60 °C for 20 seconds
c. Extend: 72 °C for 20 seconds

3. Melt: 70-99 °C ramping

2.14 Bicinchoninic Acid Assay

In order to quantify the total amount of protein in a sample, a Pierce BCA Protein Assay Kit (Thermo Scientific; Rockford, IL) was used. The microplate procedure was followed. Diluted albumin standards were prepared according to the protocol, using milli-Q water as the diluent. The BCA working reagent was prepared by mixing BCA reagent A with BCA reagent B 50:1. The diluted albumin standards and the unknown samples were transferred to a microplate in duplicate. Sample size was limited; therefore, only 10 μL of standards and unknown samples were transferred to each well. Next, 200 μL of the working reagent were added to each well, and the plate was mixed on a mini shaker (VWR; Radnor, PA) for 30 seconds. The plate was then incubated at 37 °C with 5.0% CO₂ in a MCO-18AIC incubator (Sanyo Scientific; Wood Dale, IL) for 30 minutes. The plate was removed from the incubator and cooled to room temperature. The absorbance was measured at 562 nm on a Synergy Mx microplate reader (BioTek; Winooski, VT). A standard curve was generated using the Gen5 program (BioTek; Winooski, VT), and the total protein concentrations for each sample were outputted.
2.15 Western Blotting

2.15.1 Protein Separation via SDS-PAGE

Protein lysate samples from Experiment 2 were thawed. The BCA assay data was used to calculate the necessary volume of each sample such that it would contain 20 μg of protein. The appropriate volumes were then measured into microcentrifuge tubes (Fisher Scientific; Fair Lawn, NJ). Laemmli Sample Buffer (Bio-Rad Laboratories, Inc.; Hercules, CA) was added to each microcentrifuge tube for a total volume of 45 μL per tube. The microcentrifuge tubes were incubated in a heatblock (VWR; Radnor, PA) at 95°C for 10 minutes. The tubes were removed using tweezers and were centrifuged briefly using a Spectrafuge Mini (Labnet International, Inc.; Woodbridge, NJ). An 18 well Criterion TGX Precast gel (Bio-Rad Laboratories, Inc.; Hercules, CA) was positioned in a Criterion Cell system (Bio-Rad Laboratories, Inc.; Hercules, CA) containing 1x Tris-Glycine-SDS running buffer (Bio-Rad Laboratories, Inc.; Hercules, CA). Next, 10 μL of the Precision Plus Protein Kaleidoscope (Bio-Rad Laboratories, Inc.; Hercules, CA) were loaded in the first well using a 20-200 μL VWR Signature Ergonomic High Performance Single-Chanell Variable Volume pipettor (VWR; Radnor, PA). The same pipettor was used to load the samples into the subsequent wells. The Criterion Cell system was plugged into the PowerPac Basic (Bio-Rad Laboratories, Inc.; Hercules, CA), and the gel was run for 45 minutes at 200 V.

2.15.2 Protein Blotting

After running, the precast gel was removed, and the case was cracked open. A scalpel was used to cut the gel to size based on the loaded wells. The gel was then placed
in a dish containing 1x Tris-Glycine transfer buffer (Bio-Rad Laboratories, Inc.; Hercules, CA) and allowed to incubate at room temperature for 15 minutes. A Trans-Blot SD Semi-Dry Transfer Cell (Bio-Rad Laboratories, Inc.; Hercules, CA) was used to transfer the blot to the membrane. Extra thick blot paper (Bio-Rad Laboratories, Inc.; Hercules, CA) was soaked in the transfer buffer and placed on the anode plate assembly of the Trans-Blot SD Semi-Dry Transfer Cell. Next, a 0.45 um nitrocellulose membrane (Bio-Rad Laboratories, Inc.; Hercules, CA) was soaked in the transfer buffer and placed on top of the extra thick blot paper. The precast gel was then placed on top of the membrane. Another layer of extra thick blot paper was soaked in transfer buffer and layered on top of the gel. Finally, the cathode plate assembly was latched into place, and the safety lid was secured. The Trans-Blot SD Semi-Dry Transfer Cell was run at 15 V for 30 minutes using a PowerPac HC (Bio-Rad Laboratories, Inc.; Hercules, CA).

2.15.3 Protein Detection

The membrane was then removed and transferred to a petri dish containing 15 mL of 5.0% (mass/volume) milk block solution. The milk block solution consisted of instant nonfat dry milk (Nestlé Baking; Solon, OH) dissolved in Dulbecco’s Phosphate Buffered Saline (Sigma Aldrich; St. Louis, MO). The membrane was incubated in the milk block solution for 1 hour on a mini shaker (VWR; Radnor, PA) at room temperature. The primary antibody was then diluted in the milk block solution 1:1000, and the membrane was incubated on an IKA Vibrax VXR basic (IKA Works; Staufen, Germany) overnight at 4°C. The primary and secondary antibodies (Cell Signaling Technology; Danvers, MA) used are shown in Table 2.1.
The membrane was then washed 2 times with 0.1% Tween20 (Sigma Aldrich; St. Louis, MO) in Dulbecco’s Phosphate Buffered Saline (PBS) (Sigma Aldrich; St. Louis, MO) for 15 minutes on a mini shaker (VWR; Radnor, PA). Next, the secondary antibody was diluted 1:2500 in 15 mL of the milk block solution, and the membrane was incubated on the mini shaker at room temperature for 1.5 hr. The membrane was washed 2 times with 0.1% Tween20 in PBS for 15 minutes on the mini shaker. The chemiluminescence substrate was prepared by mixing 0.75 mL of 20x LumiGlo Reagent (Cell Signaling Technology; Danvers, MA) and 0.75 mL of 20x Peroxide (Cell Signaling Technology; Danvers, MA) with 13.5 mL of milli-Q water. The chemiluminescence substrate was then added to the membrane and incubated on a mini shaker at room temperature for 2 min. The excess substrate was removed using a Kimwipe, and the membrane was imaged using a FluorChem M (Cell Biosciences; Santa Clara, CA). The membrane was washed with 0.1% Tween20 in PBS for 5 minutes on a mini shaker and stored in PBS at 4°C.

<table>
<thead>
<tr>
<th>Primary Antibody</th>
<th>Secondary Antibody</th>
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<tbody>
<tr>
<td>β-Actin (13E5) Rabbit mAb (HRP Conjugate)</td>
<td>HRP Conjugated to Primary Ab</td>
</tr>
<tr>
<td>Caspase-3 Rabbit Ab</td>
<td>Anti-rabbit IgG, HRP-linked Ab</td>
</tr>
<tr>
<td>Cleaved caspase-3 (D175) (5A1E) Rabbit mAb</td>
<td>Anti-rabbit IgG, HRP-linked Ab</td>
</tr>
<tr>
<td>Caspase-7 Rabbit Ab</td>
<td>Anti-rabbit IgG, HRP-linked Ab</td>
</tr>
<tr>
<td>Cleaved caspase-7 (Asp198) (D6H1) Rabbit mAb</td>
<td>Anti-rabbit IgG, HRP-linked Ab</td>
</tr>
<tr>
<td>Caspase-9 (Human Specific) Rabbit Ab</td>
<td>Anti-rabbit IgG, HRP-linked Ab</td>
</tr>
<tr>
<td>Cleaved caspase-9 (D330) (Human Specific) Rabbit mAB</td>
<td>Anti-rabbit IgG, HRP-linked Ab</td>
</tr>
</tbody>
</table>

*Table 2.1: Primary and Secondary Antibodies used for Western Blots*
2.15.4 Western Blot Stripping

The PBS was removed from the membrane, and the membrane was washed with 0.1% Tween20 in PBS for 5 minutes. The membrane was stripped by diluting western blot stripping buffer B (Santa Cruz Biotechnology; Dallas, TX) in western blot stripping buffer A (Santa Cruz Biotechnology; Dallas, TX) 1:100. 200 μL of substrate B were added to 20 mL of substrate A and transferred to the membrane. The membrane was incubated with the stripping substrate on a mini shaker at room temperature for 30 minutes. The membrane was washed 3 times with 0.1% Tween20 in PBS for 5 minutes on the mini shaker. The protein detection procedure was repeated for all proteins of interest.
CHAPTER THREE
RESULTS AND DISCUSSION

3.1 LIVE/DEAD Viability-Cytotoxicity Assay

3.1.1 TA crosslinked Collagen Beads Seeded with SW872 cells

After seeding collagen beads with SW872 cells, LIVE/DEAD assays were conducted to assess the cell viability. This assay stains live cells green and dead cells red allowing for visualization and quantification of cell viability. The results of the LIVE/DEAD assay of collagen beads seeded with human adipocytes are shown in Figures 3.1 and 3.2. The LIVE/DEAD assay reveals that the most successful cell seeding of the three groups tested occurs with the 0.1% TA crosslinked collagen beads. It also shows the high toxicity level of the 10.0% TA to the human adipocytes; therefore, confirming the 10.0% TA group as a negative control.

![SW872 Cell Viability Graph]

**Figure 3.1:** SW872 Cell Viability for different TA crosslinking concentrations, *p<0.05 between 10.0% TA and 1.0% TA, **p<0.05 between 1.0% TA and 0.1% TA
Figure 3.2: LIVE/DEAD images of SW872 loaded TA crosslinked Collagen beads, Scale bars = 100 μm

3.1.2 TA crosslinked Collagen Beads Seeded with BT474 cells

The results of the LIVE/DEAD assay of collagen beads seeded with human HER2⁺ breast cancer cells are shown in Figures 3.3-3.6. The results parallel the results of the LIVE/DEAD assay of collagen beads seeded with human adipocytes. The most successful cell seeding occurs with the 0.1% TA crosslinked collagen beads. The 10.0% TA group is very toxic to the HER2⁺ breast cancer cells and again, acts as a positive control. Comparing the results of the LIVE/DEAD assay of collagen beads seeded with
the two different cell lines reveals that TA is significantly more toxic to human HER2+ breast cancer cells than human adipocytes.

**Figure 3.3:** BT474 Cell Viability on Collagen beads Day 3, *p<0.05 between 10.0% TA and 1.0% TA, **p<0.05 between 1.0% TA and 0.1% TA
Figure 3.4: BT474 Cell Viability on Collagen beads Day 6, *p<0.05 between 10.0% TA and 1.0% TA, **p<0.05 between 1.0% TA and 0.1% TA

Figure 3.5: BT474 Cell Viability on Collagen beads Day 9, *p<0.05 between 10.0% TA and 1.0% TA, **p<0.05 between 1.0% TA and 0.1% TA
Figure 3.6: LIVE/DEAD images of BT474 loaded TA crosslinked Collagen beads, Scale bars = 100 μm

3.2 Trypan Blue Exclusion Assay

3.2.1 BT474 cells grown beneath TA crosslinked Collagen Beads

After co-culturing human HER2\(^+\) breast cancer (BT474) cells with human adipocyte (SW872) seeded TA cross-linked collagen beads, a Trypan Blue exclusion assay was conducted. The Trypan Blue exclusion assay allows for visualization and quantification of cell viability due to its permeability of dead cells. The cellular membrane of live cells is intact; therefore, it is impermeable to substances such as the Trypan Blue stain. However, as a cell begins undergoing apoptosis, the integrity of its
cellular membrane is lost, making it permeable to the Trypan Blue stain. In summary, the Trypan Blue penetrates the cellular membrane of dead cells, staining them blue, while live cells remain unstained. This was used to assess the viability of the BT474 cells grown beneath SW872 seeded TA cross-linked collagen beads and to evaluate the effects of the three TA groups on the BT474 cells over time. The results are shown in Figure 3.7. The results indicate that TA released from the remodeling of TA crosslinked collagen beads by SW872 cells inhibits cell growth in BT474 cells at all TA concentrations tested with a p-value < 0.05. It was also shown that TA toxicity increases over time at all TA concentrations tested with a p-value < 0.05.

**Figure 3.7:** BT474 Growth and Proliferation, *p<0.05 between control and TA groups
3.2.2 MCF10A cells grown beneath TA crosslinked Collagen Beads

The Trypan Blue exclusion assay was also used to assess the viability of MCF10A cells grown beneath SW872 seeded TA cross-linked collagen beads and to evaluate the effects of the three TA groups on the MCF10A cells over time. The results are shown in Figures 3.8-3.10. The results indicate that TA released from the remodeling of TA crosslinked collagen beads by SW872 cells do not inhibit cell growth in MCF10A cells at TA concentrations of 1.0% and 0.1% with a p-value > 0.05. The 10.0% TA group does inhibit cell growth in MCF10A cells which is expected due to the high toxicity of 10.0% TA. It was also shown that TA toxicity does not increase over time at all TA concentrations tested with a p-value > 0.05, again indicating the resistance of MCF10A cells to TA toxicity.

**Figure 3.8**: MCF10A Cell Viability after 3 days of TA exposure. *p<0.05 between 10.0% TA group and other groups
Figure 3.9: MCF10A Cell Viability after 5 days of TA exposure, *p<0.05 between 10.0% TA group and other groups

Figure 3.10: MCF10A Cell Viability after 7 days of TA exposure, *p<0.05 between 10.0% TA group and other groups
3.3 Folin-Ciocalteu Assay

A Folin-Ciocalteu assay was conducted in order to quantify the concentration of TA in the media below the Transwell® inserts containing TA crosslinked collagen type I beads of various concentrations. It is important to understand the release profile of TA from the collagen type I crosslinked beads as well as determine the minimum concentration of TA for apoptosis induction. The F-C assay is a colorimetric assay in which the F-C reagent, consisting of phosphomolybdate and phosphotungstate, reacts with phenolic compounds forming a blue complex. The results of the F-C assay are shown in Figure 3.11.

![Tannic Acid Levels in Media](image)

**Figure 3.11:** TA levels in Media below SW872 loaded TA crosslinked Collagen Beads, *p<0.05 between Control and TA groups
Figure 3.11 shows a statistically significant difference (p<0.05) in the concentration of TA is the control group as compared to each of the TA groups. There is not a statistical significance in the TA concentration found in the media among the TA groups tested; however, a decreasing trend is observed as the TA crosslinking concentration decreases.

It is important to note that the F-C reagent reacts with components of the media; therefore, creating a false positive for the presence of phenolic compounds in the media alone. This rationale provides an explanation for a concentration of around 0.0054 %TA (mass/volume) in the control group seen in Figure 3.11. The F-C assay also revealed the relatively small concentrations of TA required to interfere with cell growth and proliferation of BT474 cells. This is important in determining the ideal crosslinking TA concentration, while minimizing toxicity to normal human breast epithelial tissue.

3.4 Real-time Polymerase Chain Reaction

After showing that TA inhibits cell growth in BT474 cells, Real-time PCR was used to evaluate the mechanisms of this inhibition. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a housekeeping gene, and caspase 3, 7, and 9 primers were used as indicators of apoptosis. The results showed no significant expression of caspase 3, 7, or 9 among the 10.0% TA group. This could be attributed to the high toxicity of 10.0% TA, which would yield very little caspase micro-RNA
isolation from the BT474 cells for analyzation through Real-time PCR. Fold change data in caspase 3 expression for the other two TA groups in shown in Figure 3.12.

![Fold Change-caspase 3](chart)

**Figure 3.12:** Fold change in expression of executioner caspase 3, *p<0.05* between 1.0% TA and Control, **p<0.05** between 0.1% TA and 1.0% TA

Figure 3.12 shows a statistically significant fold change in the expression of caspase 3 in the 1.0% TA and 0.1% TA groups as compared to the control with a p-value < 0.05. It also shows a statistically significant difference in the fold change in the expression of caspase 3 between the 1.0% TA group and the 0.1% TA group. It was expected that the fold change would be greater in the 1.0% TA group than the 0.1% TA group; however, the above results could be attributed to the higher toxicity of the 1.0% TA group. The higher toxicity would result in increased levels of caspase proteins earlier during exposure of BT474 cells to TA. The BT474 cell line is known to express X-linked...
inhibitor of apoptosis protein which binds and inhibits active caspases 3, 7, and 9 and ubiquinates them in response to cellular stress (Foster et al.; 2009). The cells exposed to the 1.0% TA group experience higher levels of cellular stress than those exposed to the 0.1% TA group; therefore, inducing the action of X-linked inhibitor of apoptosis protein. As this protein binds and inhibits active caspases, particularly initiator caspase 9, the downstream levels of executioner caspase decrease. If real-time polymerase chain reaction were conducted using RNA isolated from the BT474 cells earlier during their exposure to TA, a higher fold change in the expression of caspase 3 in the 1.0% TA group would be expected as opposed to the 0.1% TA group because the X-linked inhibitor of apoptosis protein would not yet be acting on the caspases. Although the results did not follow the expected trend, the significant fold change in the expression of caspase 3 in both groups indicates induction of apoptosis by TA via the caspase pathways.

3.5 Western Blotting

After showing that TA results in significant fold change in the expression of caspase 3 in the 1.0% and 0.1% TA groups, western blotting was conducted in order to further assess expression of the caspase proteins by the BT474 cells plated below TA crosslinked collagen type I beads. In addition, western blotting was conducted using proteins isolated from MCF10A cells, the normal human breast epithelial cell line, plated below TA crosslinked collagen type I beads in order to compare the effects of TA on HER2+ breast cancer cells and normal human breast epithelial cells. The results are shown in Figures 3.13 and 3.14.
Figure 3.13: Western Blot for caspase 3, caspase 7, and β-actin. Lane 1: Ladder, Lane 2: BT474 cells from the control group, Lane 3: BT474 cells from the 10.0% TA group, Lane 4: BT474 cells from the 1.0% TA group, Lane 5: BT474 cells from the 0.1% TA group, Lane 6: MCF10A cells from the control group, Lane 7: MCF10A cells from the 10.0% TA group, Lane 8: MCF10A cells from the 1.0% TA group, Lane 9: MCF10A cells from the 0.1% TA group

Although western blotting was conducted using primary antibodies for cleaved caspase 3, cleaved caspase 7, caspase 9, and cleaved caspase 9, sufficient expression was not detected for these proteins; therefore, those western blots are not included in Figure 3.13. It is important to note that initiator caspase 9 is upstream of executioner caspases 3 and 7 and is responsible for their activation; therefore, it is likely that there was significant caspase 9 expression earlier in the TA exposure timeframe or that the multiple stripping procedures interfered with the results. The cleaved caspases are smaller than the caspases, and it is possible that those proteins ran off the gel during the protein separation via SDS-PAGE step. Further investigation is necessary to determine the effects of TA exposure on the expression of these proteins by BT474 and MCF10A cells.
The corresponding relative densitometry units (RDU) for caspase 3 and caspase 7 expression are shown in Figure 3.14. The results do not replicate the results determined by real-time PCR. They do not show a significant change in the expression of caspases by BT474 cells exposed to TA in the 1.0% and 0.1% TA groups as compared to the control. However, the results do show a significant difference in the expression of caspase 3 by BT474 cells compared to MCF10A cells when exposed to 10.0% TA. Although it is expected that the fold change in the expression of caspase 3 by MCF10A cells would be greater after exposure to 10.0% TA due to the high toxicity of this concentration, the results confirm the higher toxicity of TA to BT474 cells than MCF10A cells.

In order to further characterize the effects of TA exposure on the expression of caspase proteins by BT474 cells and MCF10A cells, more western blotting analysis is necessary. It is important to analyze the caspase protein expression over time for both cell lines tested in order to determine the ongoing effects of TA exposure and define any resistance to these effects that may exist. It is also important to optimize the western blotting method including the protein loading concentration and washing in addition to minimizing membrane exposure and stripping which can alter expression.

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<tr>
<th></th>
<th>BT474 Control</th>
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<th>BT474 1.0% TA</th>
<th>BT474 0.1% TA</th>
<th>MCF10A Control</th>
<th>MCF10A 10.0% TA</th>
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<tbody>
<tr>
<td>Caspase 3</td>
<td>0.46</td>
<td>0.88</td>
<td>0.33</td>
<td>0.32</td>
<td>0.19</td>
<td>0.16</td>
<td>0.35</td>
<td>0.36</td>
</tr>
<tr>
<td>Caspase 7</td>
<td>0.20</td>
<td>0.42</td>
<td>0.20</td>
<td>0.08</td>
<td>0.20</td>
<td>0.37</td>
<td>0.24</td>
<td>0.27</td>
</tr>
</tbody>
</table>

**Table 3.1: Relative Densitometry Units for caspase expression by BT474 and MCF10A cells exposed to TA**
Figure 3.14: Caspase 3 and 7 expression in BT474 and MCF10A cells exposed to TA
CHAPTER FOUR

CONCLUSIONS

4.1 Conclusions

TA crosslinked collagen type I beads seeded with human adipocyte cells provide a basis for an injectable soft tissue regeneration matrix with chemotherapeutic properties against breast cancer. Adipocytes were successfully loaded onto collagen type I beads with TA crosslinking concentrations of 1.0% and 0.1% as shown using a LIVE/DEAD viability-cytotoxicity assay, with the most successful adipocyte loading occurring in the 0.1% TA group (Figure 3.2). LIVE/DEAD viability-cytotoxicity assays also revealed the higher toxicity of TA to HER2+ breast cancer cells as compared to human adipocytes (Figure 3.6). As adipocytes attach and grow on the TA crosslinked collagen beads, they remodel the collagen, releasing TA which then interacts with the surrounding environment as shown through Folin-Ciocalteu assays (Figure 3.11). The toxicity of TA to HER2+ breast cancer cells was further confirmed using Trypan Blue exclusion assays which revealed a statistically significant decrease in the number of live cells in all three groups tested as compared to the control group across 7 days (Figure 3.7). This reduction in HER2+ cell growth and proliferation is caused by apoptosis induction via the caspase pathways as shown by real-time PCR and western blotting (Figures 3.12 and 3.13). Therefore, TA crosslinked collagen type I beads loaded with adipocytes have demonstrated potential as a novel anti-cancer agent against many breast cancer types including HER2+ breast cancer.
4.2 Future Research

In order to move forward for this research project and advance it towards clinical application, there are several important questions that must be answered through continued investigation.

First, the size of the TA crosslinked collagen type I beads must be optimized for injection. In order to achieve the desired injectable property, the beads must be small enough such that they can be forced through a needle without rupture or compromise of the bead structure. However, this decrease in bead size must be balanced with cell seeding ability and longevity in vivo.

The additional components of the injectable matrix also need to be determined. The current TA crosslinked collagen type I beads must be suspended in a matrix of sorts in order to ensure delivery of the therapeutic TA to the desired target area. This matrix will likely be composed of a hydrogel or sol-gel material that is an injectable liquid at room temperature and a gel or solid at body temperature.

Moving forward, it will also be key to determine the half-life of TA in vivo and the release profile of TA from the collagen crosslinked beads. This is necessary in order to maximize therapeutic effects while minimizing local toxicity over time. It will also aid in determining the optimum TA crosslinking concentration for the collagen crosslinked beads. The ideal TA crosslinking concentration will enable the highest adipocyte cell seeding density without compromising the chemotherapeutic effects of TA against the breast cancer cells.
We have shown that TA has a chemotherapeutic effect against different types of breast cancer by inducing apoptosis via the caspase pathways; however, it is important to better understand the mechanisms of TA action against the breast cancer cell types tested. This will help to determine the ideal candidates for treatment with this therapy. Better understanding the therapeutic action of TA will also provide insight into other potential clinical areas in which this technology could be beneficial such as other soft tissue cancer types.

After optimizing the conformation of the injectable matrix, in vivo studies should be conducted in order to confirm the clinical potential of the matrix as a therapeutic treatment option for breast cancer patients.
APPENDICES
Appendix A

Western Blotting

Figure A-1: Western Blot for β-Actin, Lane 1: Ladder, Lane 2: BT474 cells from the control group, Lane 3: BT474 cells from the 10.0% TA group, Lane 4: BT474 cells from the 1.0% TA group, Lane 5: BT474 cells from the 0.1% TA group, Lane 6: MCF10A cells from the control group, Lane 7: MCF10A cells from the 10.0% TA group, Lane 8: MCF10A cells from the 1.0% TA group, Lane 9: MCF10A cells from the 0.1% TA group
Figure A-2: Western Blot for caspase 3, Lane 1: Ladder, Lane 2: BT474 cells from the control group, Lane 3: BT474 cells from the 10.0% TA group, Lane 4: BT474 cells from the 1.0% TA group, Lane 5: BT474 cells from the 0.1% TA group, Lane 6: MCF10A cells from the control group, Lane 7: MCF10A cells from the 10.0% TA group, Lane 8: MCF10A cells from the 1.0% TA group, Lane 9: MCF10A cells from the 0.1% TA group
Figure A-3: Western Blot for caspase 7, Lane 1: Ladder, Lane 2: BT474 cells from the control group, Lane 3: BT474 cells from the 10.0% TA group, Lane 4: BT474 cells from the 1.0% TA group, Lane 5: BT474 cells from the 0.1% TA group, Lane 6: MCF10A cells from the control group, Lane 7: MCF10A cells from the 10.0% TA group, Lane 8: MCF10A cells from the 1.0% TA group, Lane 9: MCF10A cells from the 0.1% TA group
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