CHEMOTHERAPEUTIC MYOKINES FROM CONTRACTING SKELETAL MUSCLE AND THEIR EFFECTS ON BREAST CANCER

A Dissertation

by

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ABSTRACT

Exercise has been shown to elicit beneficial effects in both the treatment and prevention of cancer, yet the biology potentiating the effects remains ambiguous. The *hypothesis* for this dissertation was that effluent perfusion medium from contracting skeletal muscle contained factor(s) responsible for the cytotoxic effects of exercise on breast cancer cells. To date, no studies exist designed to eliminate confounding humoral factors, *in vivo*, while still mimicking the effects of exercise via neural activation of the muscle. We addressed this issue by stimulating the muscle via neural activation using an *in situ*, hemicorpus hind limb perfusion preparation. To assess the acute impact of muscle contractions on breast cancer, we first collected medium perfused through quiescent (Non-STM) muscle, subsequently followed by medium collected from contracting (STM) muscle of rats. To determine if the acute impact of contracting muscle is altered by prior muscle conditioning, we compared acute responses of muscle contraction on breast cancer from control (Non-EX) vs. treadmill exercised (EX; 5d/w; 1h/d for 5w) rats. We also assessed the impact of a common carrier of molecules in the bloodstream, by preparing the perfusion medium with or without albumin, using Dextran as the oncotic agent for these experiments.

The *results* of these studies confirmed our hypothesis that effluent media prepared with albumin from contracting muscle (STM) displayed an average percent reduction of MCF7 cell counts by 20% ($p = 0.014$) compared to Non-STM, and prior exercise conditioning did not alter this effect (STM = EX; $p=0.563$). Albumin-prepared

medium yielded similar cell counts as cell controls. Interestingly, the chemotherapeutic effect was not present using medium prepared with Dextran, with or without STM, suggesting albumin as a carrier molecule that is necessary for chemotherapeutic activity. To our knowledge, our results support a decrease in MCF7 cell proliferation from a factor secreted from contracting skeletal muscle, independent of humoral contributors; and are the first to demonstrate that the magnitude of the chemotherapeutic effect of contracting skeletal muscle is not altered by prior exercise training, suggesting that muscle contractions are the key contributors to this anticancer effect and not a result of a training adaptation.

DEDICATION

This dissertation is dedicated to my Heavenly Father, my charming and supportive husband, Dusty, and my incredible family for all the encouragement, guidance and support you've blessed me with throughout this journey!

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NOMENCLATURE

HHLP	Hemicorpus Hind Limb Perfusion
Non-STM	Quiescent Muscle/Not Electrically Stimulated
STM	Electrical Stimulation
EX	Exercised (via Treadmill)
Non-EX	Non-Exercised Control/Normal Cage Ambulation
CC	Cell Culture Control
HR±	Hormone Receptor
HER2	Human Growth Factor-neu Receptor
MCF7	Human Breast Cancer Cell Line (Michigan Cancer Foundation)
MDA-MB-231	Human Breast Cancer Cell Line (MD Anderson)

CONTRIBUTORS AND FUNDING SOURCES

Contributors

This work was supervised by a dissertation committee consisting of Professors James D. Fluckey, Stephen E. Riechman, and John M. Lawler of the Department of Health and Kinesiology and Professor Weston W. Porter of the Department of Veterinary Integrative Biosciences.

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CHAPTER I

INTRODUCTION: BACKGROUND AND SIGNIFICANCE

Breast Cancer

According to the World Health Organization, breast cancer is the most common type of cancer that kills women world-wide (for an entire overview, see the full World Cancer Report (108)). In our nation, out of all new cases of cancer diagnosed in females, 14% is attributed to breast cancer, which accounts for more than 235,000 women (84). While the mortality rates of females diagnosed with this disease have steadily decreased by approximately 1.9% each year from 2003-2012, it is still estimated that over 40,000 women will die of breast cancer by the end of 2016.

Breast cancer develops when a normal cell found in breast tissue transforms into a neoplastic (tumor) cell, due to an interaction between a person's genetic factors and various environmental factors. In short, this transformation is a multistage process of abnormal gene expression that leads to dysregulation in the balance between cell proliferation and cell death. The increased cell growth and decreased cell death leads to the expansion of tumor tissue. A benign, or localized, tumor becomes malignant with further alterations in gene expression that eventually lead to decreased attachment from and invasion into surrounding tissue, blood vessels or the lymphatic system: a process known as metastasis.

Breast Cancer Subtypes

The process of mammary tumorigenesis is further complicated as there are different cell types found in breast tissue (*e.g*. adipose, lobes, lobules, ducts, connective tissue, lymph, etc.), and one tumor may exhibit varying genotypic and phenotypic characteristics depending on the original neoplastic cell (24, 95). Even further, the stromal microenvironment can also strongly influence this process (80, 120). This etiological heterogeneity of breast cancer largely affects the clinical heterogeneity (e.g. prognostic characteristics, diagnostic detectability, and response to treatment), which elicited focused research on the characterization of breast cancer subtypes.

There are five widely accepted molecular subtypes of breast cancer (31, 95, 104, 105) generally characterized by the expression of basal (myoepithelial) or epithelial cell markers (32, 47, 86), hormone receptor (HR±) status (35), and expression of human growth factor-neu receptor (HER2±) (103). These subtypes are described as Luminal A (HR+/HER2-), Luminal B (HR+/HER2+), HER2 over-expression (HR-/HER2+), Basal-like (HR-/HER2-) and Normal-like (HR+/HER2-). While Normal-like mammary tumors share the same HR status as the Luminal A subtype, they express more genes characteristic of basal and adipose cells (95).

The Luminal A subtype represents 72.6% of all cases (67), being the most commonly diagnosed breast cancer. In the present study, we chose the MCF7 cell to study breast cancer due to its characterization as a Luminal A type (54, 89). The second most prevalent subtype was found to be the Basal-like, also known as triple-negative breast cancer, accounting for approximately 13% of all breast cancers (67). The MDA-

MB-231 cell line resembles a Basal-like breast cancer (54, 89) and was chosen to represent the opposite end of the spectrum of breast cancer subtypes in terms of metastatic potential and decreased prognosis.

Cost

The persistent research to understand the cell biology of breast cancer in conjunction with the application to the clinical realm has resulted in many new, yet unfortunately costly, clinical therapies that are potentially inaccessible for many diagnosed with breast cancer. The cost of treating breast cancer continues to rise from an estimated national cost of $16.5 billion in 2010, to a 32% increase by 2020 (78). Despite the recent advances in the availability of quality health care and health insurance to help with the direct medical costs of treatment, the financial feasibility of all the indirect costs of treatment (e.g., childcare, housing, loss of productivity or income) makes many of the current treatment modalities nearly impossible for many women diagnosed with breast cancer to participate. Further, that does not account for over half of breast cancer mortalities that occur in low- and middle-income countries due to lack of diagnostic and treatment options (4). This highlights the need for more cost effective treatment modalities as well as the importance of education and further research on prevention and ways to decrease one's risk of developing breast cancer as a long-term cost-effective way to control this disease.

Risk Factors

There are many known risk factors associated with breast cancer, some of which are modifiable and some which are not. Just a few of the non-modifiable risk factors for

breast cancer include aging, age at menarche or menopause, and family history, including heritable risk factors (BRCA1/2, PTEN, and CHEK2 among others). (For a more extensive list of risk factors and correlating studies, see website sponsored by Susan G. Komen (1)). Still other risk factors that are not necessarily modifiable include the age of parity and ability to/duration of breastfeeding. Fortunately, there are some risk factors that an individual can take action against and move towards the prevention of breast cancer. Some of the modifiable risk factors include dietary factors, smoking, some environmental factors, and most importantly for the remaining discussion in this dissertation, physical activity levels.

The Crossroads of Breast Cancer and Exercise

Physical inactivity has been implicated for increased rates of many non-communicable diseases, and is specifically targeted to be the cause of 10% of breast cancer cases worldwide (71). The inverse relationship between physical activity and breast cancer risk in humans has been recognized since the 1980s (44), nevertheless, more recent estimates of relative risk reductions range from 20-80% in post-menopausal women and 15-30% among pre-menopausal women (42, 43, 81). One of the most commonly cited reviews by Lynch *et al*. revealed a 25% risk reduction of breast cancer across 73 studies done worldwide amongst physically active women as compared to the least active women (76). A more recent review by Wu *et al*. detailed a linear dose-response relationship between the intensity of physical activity and breast cancer risk using a meta-analysis of the literature (126). Light intensity physical activity has contradicting results as to whether or not it reduces breast cancer risk (42, 66, 76, 117).

Breast cancer survivors who engage in regular physical activity provide the strongest evidence for an association between physical activity and cancer outcomes when compared with survivors of any other type of cancer (12). Nearly all breast cancer studies observed in the review by Ballard-Barbash *et al*. (12), reported a reduction in breast cancer-specific mortality as well as all-cause mortality. In both breast cancer patients undergoing adjuvant therapy and breast cancer survivors, physical activity has been shown to increase perceived measures of "quality of life", mood, and/or in overall health (10, 21, 40). Further, physical activity is implicated as a method to counteract the negative side effects of chemotherapy, such as reduced physical fitness, depression, anxiety, pain, nausea, negative changes in body composition, increased fatigue, etc. (3, 28, 29, 36, 62, 119).

Indirect Mechanisms of Exercise on Breast Cancer

There are several postulated mechanisms of how physical activity affects breast carcinogenesis, and subsequently, many reviews have been written on this topic (41, 76, 87, 88, 96, 122). Primary dogma of the benefits of exercise on breast cancer prevention and progression postulates the effects are indirect and are attributed mainly via altering the sex hormones, breast development, insulin resistance, energy balance, body composition, adipokines and inflammatory markers. This presumption is based on observations that alterations to these important biological systems under chronic sedentary conditions, particularly with obesity, have been linked to increases in breast cancer risk (6, 9, 13, 17, 30, 38, 97, 102, 121). More specific prospective mechanisms of exercise protection against cancer include strengthened immune function (5, 46, 74),

enhanced antioxidant and oxidative damage repair capacity (85), DNA repair capacity and epigenetic alterations to DNA (22, 92), and Vitamin D exposure (110, 118).

Direct Mechanisms of Exercise on Breast Cancer

Many of these mechanisms have been further explored with rodent models of chemically induced or injected tumors. Extensive work in this field led Thompson *et al.* (114) to suggest a more direct paradigm of physical activity on the attenuation of tumor initiation and progression. The first two candidate mechanisms proposed stemmed from the effects of exercise on growth factors and hormones, and how the decrease in energy may be attenuating anabolic signaling pathways. However, after further exploring circulating biomarkers, cellular processes and molecular mechanisms within their model of chemically-induced tumors in rats (130), their results did not fully explain the attenuated levels of anabolic activity being directly related to energy status in their model. They then suggested a 3rd potential mechanism that a cytokine may be released through contracting skeletal muscle potentiating these effects. The experiments that follow in this dissertation gives credence to Thompson's hypothesis in that the chemotherapeutic effect of exercise may be modulated through a cytokine, independent of hormones, since our methodology eradicates the influence of endocrine organs (namely the pancreas, liver and reproductive organs) that may be secreting hormones into the blood stream during exercise.

Additional Considerations

Finally, with many of these proposed mechanisms, it is important to bear in mind the etiological heterogeneity of breast cancer and that it is likely that multiple pathways are involved in the carcinogenic process, and may act synergistically under conditions of cancer growth. Thus, the slowing of cancer growth may result from multiple mechanisms impacted by the improved physical conditioning following exercise training. It may be important to note that different types of physical activity or dose may preferentially elicit certain mechanisms over others. Still even further, various subgroups of women may differentially respond to physical activity (25, 75).

Despite the accumulating research done on the effects of physical activity and breast cancer, many of the proposed mechanisms of physical activity are either indirectly mediated through decreased body weight, or are attributed to other whole-body adaptations and benefits of physical activity. Granted, while some of these postulated mechanisms are more fundamental in nature than others, the present series of studies are designed to focus on the direct impact of contracting skeletal muscle on breast cancer using a specific methodology to limit contributions of humoral factors, genetic factors and immune function.

Myokines: The Mechanism of Exercise

To support the notion that contracting muscle directly affects carcinogenesis, skeletal muscle has been identified as a functioning endocrine organ in the secretion of hundreds of various cytokines, peptides and proteoglycans, termed *myokines*, where variable secretion and expression are likely regulated by contractile activity (100, 109, 128). Myokines exert specific autocrine, paracrine or hormone-like effects, and have been proven to effect signaling pathways involved in muscle homeostasis and inflammation (16), endothelial cell function (93) and pancreatic β-cell function (15) to name but a few. Thus, research targeted on the secretion and action of myokines during muscle contractions and their potential effects on the carcinogenic process form a convincing argument for a direct mechanism as to why exercise may be beneficial in cancer prevention and treatment (8, 45, 82, 99).

Two studies, done over the span of 50 years, (52, 82), demonstrated a factor circulating in the blood of fatigued animals has chemotherapeutic effects on tumor-bearing rats. The original paper published in 1962 by Hoffman *et al*. (52) revealed a two-part study to describe the effects of exercise on tumor growth. First, they injected Walker 256 tumors into two groups of Wistar rats and measured tumor growth. The tumors in the control group consistently exceeded the size of the tumors in the group subjected to vigorous exercise, with some tumors showing complete regression. To better explain the source of this chemotherapeutic factor, the second study also injected rats with a tumor, but instead of using an *in vivo* exercise protocol, the experimental group was given an injection of a “fatigue-substance” prepared from a water bath of

electrically stimulated *rectus femoris* muscles. Once again, the tumor weight of the control group exceeded that of the animals given injections.

A more recent study from the same group by Munoz *et al*. (82) prepared the same "fatigue substance" as previously described and further evaluated the substance with a cell proliferation assay and a rat cytokine antibody array. Their results indicated decreased cell proliferation of MCF7 cells upon treatment with "fatigue substance" as compared to the unstimulated muscle bath preparation. Furthermore, they identified six cytokines that were differentially expressed in the substance from stimulated versus unstimulated muscles.

In addition to those studies, a study by Hojman *et al*. (53) collected serum from exercised mice and media from stimulated murine C_2C_{12} muscle cells and saw decreased MCF7 cell proliferation when compared to serum from non-exercised mice and media from non-stimulated C_2C_{12} cells, respectively (53). They also used PCR array analysis to identify oncostatin M (OSM) as a possible candidate of mediating the anti-proliferative effect on breast cancer cells. An analogous study in humans collected serum from 10 adult males before and after 60 minutes of exercise on a bicycle, and observed attenuated growth of a prostate cancer cell line (LNCaP) after exposure to exercised serum (99). This same study demonstrated delayed tumor formation and growth after pre-incubation of LNCaP cells with exercise serum before injection into SCID mice compared to pre-incubation with rest serum. Even though they attribute the mechanism of exercise to be mediating endogenous hormone levels, their model does not disprove the notion that myokines secreted from contracting skeletal muscle may be attenuate malignant growth.

Together, these studies indicate that there may be a direct mechanism of exercise to reduce/slow cancer. The present studies resulting in this dissertation sought to expand on that work by using a methodological approach that directly assessed the cancer therapeutic effect of factor(s) arising directly from intact skeletal muscle, without confounding humoral factors in serum from exercised animals.

Albumin: Implications for Breast Cancer Treatment

Albumin is the most abundant blood protein in mammals with a concentration in humans of 35-50 mg mL^{-1}, which represents about 50-70% of plasma proteins. Not only is albumin the most abundant blood protein, it is also the most abundant protein in the body with approximately 1/2 kg being distributed among the blood circulation, the lymphatic system and the extracellular and intracellular compartments. Albumin is the main protein responsible for creating oncotic pressure in the blood and it is also the most versatile carrier protein for an array of molecules, such as metabolites, ions, amino acids, and fatty acids to name just a few.

As one of the plasma proteins, albumin is a major source of nitrogen and energy, as an amino acid/nutrient source, in the blood in which cells are able to utilize after endocytosis and lysosomal degradation. Tumor cells are characterized by high metabolic activity and thus, demand resources to meet their increased cellular growth and proliferation. Albumin has been proven to be preferentially sequestered by tumor cells at a rate of about 4 times more than the kidneys and about 3 times more than the liver (127). Because of this and its abundance and versatility as a transport protein in the blood circulation, albumin has been targeted as a drug carrier to improve the

pharmacokinetic profile of many therapeutic agents as well as a drug delivery system to the pathogenic site (37, 68, 69). Considering the wide-reaching implications of albumin in chemotherapeutic drug therapy and its necessity for methodologies utilized by this dissertation, we assessed the role of albumin on the chemotherapeutic impact of contracting skeletal muscle. For further information discussing the developments of the four main technologies using albumin in drug delivery within preclinical and clinical phases, see full review by Elsadek and Kratz (37).

Significance, Innovation and Specific Aims

Significance

Since breast cancer is a major cause of morbidity, mortality and health care costs, it has been the focus of many research initiatives. One of the overarching challenges put forth by the United States Department of Defense, among others, is to "revolutionize treatment regimens by replacing them [drugs that have life-threatening toxicities] with ones that are more effective and less toxic (2)." *My research focused on this challenge by applying the use of exercise as a safe, effective, non-toxic intervention to transform the current clinical methodologies for treating breast cancer.*

The background discussion above reflects the research done in the fields of exercise physiology and cancer biology. Each have made vast strides in the understanding of cell signaling and processes, as well as implementing new knowledge to achieve optimal health and treatment methodologies. *The contribution of this dissertation is significant because of the integration of these two fields, cancer biology and exercise physiology, with mutually beneficial outcomes of how exercise affects the*

signaling pathways implicated in cancer initiation and progression. From the perspective of exercise physiology, the consequential acquisition of knowledge pertaining to the mechanism by which exercise affects signaling pathways in neoplastic cells will lead to confidence in the clinical application of exercise within the "cancer population".

The results from this dissertation will also strengthen our understanding of the therapeutic benefits of exercise and potentially identify contraindications for patients with certain types of cancers. The effect of habitual conditioning on the secretion of a "chemotherapeutic exercise-factor" will prove to be a strong impetus for a more active lifestyle. In cancer biology, this dissertation will lead to further discussion on the signaling pathways in cancer cells that can be targeted for treatment. Finally, in the clinical realm, the use of exercise is an inexpensive treatment option that is easily accessible and one that is without the toxic side effects of radiation and chemotherapy.

Innovation

To date, studies have not been performed to control for the potentially confounding humoral factors *in vivo*, while still mimicking the effects of exercise via neural activation of the muscle. In the Hojman study previously mentioned (53), serum was collected in an *in vivo* model where there may be other systemic factors involved, and thus, confounding the mechanism by which exercise exerts its effects. In the opposite extreme, they include an *in vitro* model where myotubes were grown in cell culture. The studies done by Hoffman (1961) and Munoz (2013), utilized an *ex vivo* model where the skeletal muscle has been excised and maintained in a saline bath (52,

82). In both the *in vitro* and *ex vivo* approaches, the skeletal muscles/cells were contracted and maintained in a non-physiological environment. *The innovation of this dissertation was the ability to identify Arterial-Venous differences in non-stimulated and stimulated perfusate from the same animal while maintaining and controlling the physiological environment using an* in situ *model*. To accomplish this, we employed the Hemicorpus Hind limb Perfusion (HHLP) technique to isolate the factor(s) where the event occurs in the desired, optimal location, which in this case is from in-tact, neural activated, contracting skeletal muscle. As described further in the Methodology section, the blood supply to the renal, reproductive and digestive systems are ligated to prevent confounding humoral factors from entering into the perfusate.

The preliminary work that guided the proposal of this dissertation was done to reproduce and verify similar results to work done earlier in collaborative efforts with Kim Westerlind (64). We determined four days of treatment was the optimal length of time to run a cell proliferation experiment on MCF7 cells exposed to three different percentages (5%, 10%, and 20%) of perfusate from HHLP (34). The results in Figure 1 verified our *in situ* model to be effective in releasing chemotherapeutic factor(s) from electrically-stimulated skeletal muscle by the decrease in MCF7 cell growth. In the 10% and 20% treatment group, the MCF7 cells exposed to skeletal muscle perfusate from electrically stimulated skeletal muscle (STM) had significantly lower cell counts than perfusate from quiescent skeletal muscle (Non-STM), $p=0.048$ and $p=0.0025$ respectively.

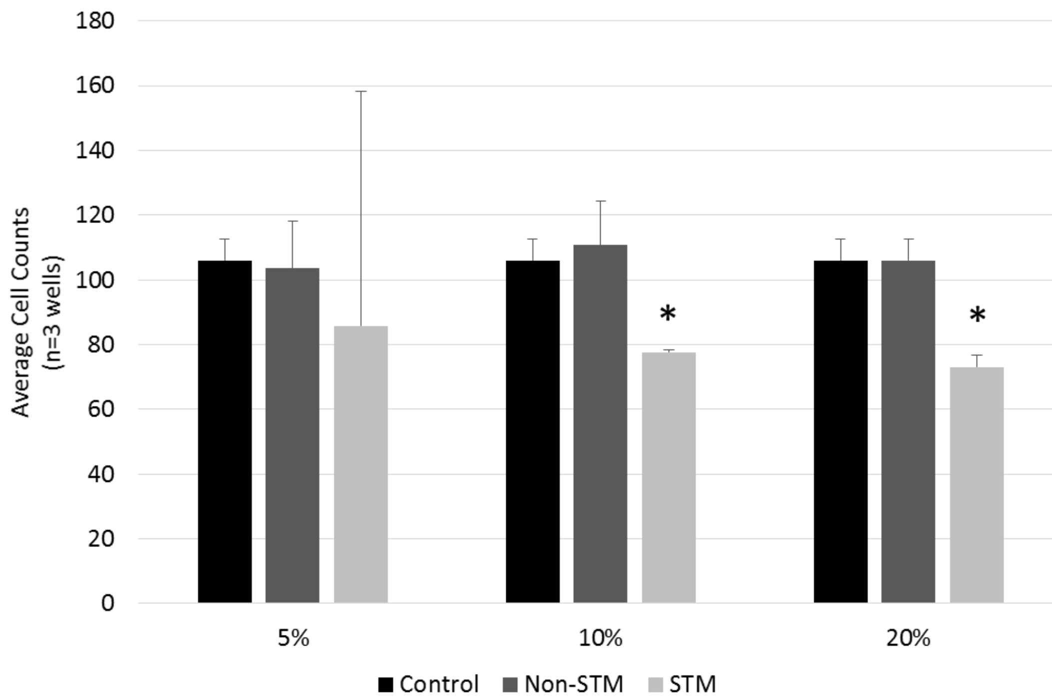

Figure 1. *MCF7 Cell Counts After Treatment with Various Percentages of Skeletal Muscle Perfusate.* MCF7 cells were treated for 5 days with skeletal muscle perfusate from a hemicorpus hind limb perfusion preparation. Perfusate from quiescent skeletal muscle (Non-STM) and from electrically stimulated skeletal muscle (STM) were added to standard cell culture media at various percentages to identify optimal percentage for future experiments. Control consisted of standard cell culture media without the addition of skeletal muscle perfusate. * Denotes statistical significance at $p < 0.05$.

Specific Aims

The *objective* of this dissertation was to characterize the factor(s) released during exercise that have direct, chemotherapeutic effects on breast cancer cells. This objective expanded upon our preliminary work and work done in collaboration with Kim Westerlind (64). The *central hypothesis* for this proposal was that a single myokine is responsible for the cytotoxic effects of exercise on breast cancer cells. Understanding how the secretion of this myokine was associated with activity levels, ranging from normal cage ambulation to moderate, habitual exercise, has wide-reaching ramifications for differences in lifestyles on cancer risk and prognosis.

The following *Specific Aims* guided us in accomplishing the objective and in testing the hypothesis of this dissertation;

1. Identify changes in cellular proliferation of breast cancer cells after treatment with muscle perfusate collected from skeletal muscle during an acute bout of simulated exercise. Additionally, determine whether prior exercise had an effect on the chemotherapeutic response.

The *hypothesis* was that a myokine released from contracting skeletal muscle would have a cytotoxic effect on breast cancer cells. In addition, we hypothesized that prior exercise conditioning would accentuate the cytotoxic effect.

2. Determine the role of albumin on the chemotherapeutic factor within the perfusate.

The *hypothesis* for this aim was that the chemotherapeutic factor required albumin as a carrier protein in the perfusate/blood.

CHAPTER II

METHODOLOGIES

To achieve the specific aims of this dissertation, the following methodologies were used.

Hemicorpus Hind Limb Perfusion (HHLP)

Animals

Female Wistar rats 8-12 weeks old, weighing 275-375g were used. All animals were housed two per cage under a standard 12-hour photoperiod. The animals were provided with normal Rat Chow and water *ad libitum* until the administration of anesthesia. All procedures were approved by the Animal Care and Use Committee of Texas A&M University.

Reagents and Perfusion Medium

A modified Krebs-Henseleit bicarbonate buffer was used as the perfusion medium. Krebs-Henseleit bicarbonate saline (70) was prepared by combining 2.1g $NAHCO_3$ dissolved in 250mL of deionized water that had been gassed for 30 minutes with 95% O_2:5% CO_2, with a working buffer solution containing 118.5 mM NaCl, 4.7 mM KCl, 3.4 mM $CaCl_2.2\ H_2O$, 1.2 mM KH_2PO_4, and 1.2 mM $MgSO_4.\ 7\ H_2O$ per liter. Bovine serum albumin (Fraction V; Amresco, Solon, OH) was dissolved in sterile-filtered Krebs-Henseleit bicarbonate buffer to obtain 3.5g/100mL. Dextrose was added to obtain 90-100mg%, and the solution was refrigerated at 4°C for no longer than 48 hours before the perfusion procedure. We chose an erythrocyte-free preparation to

simplify perfusate preparation and avoid potential confounding effects of erythrocyte metabolism (14, 79, 101). To further study the role of albumin in the perfusate, the same perfusion medium described above was prepared with Dextran (Clinical Grade; MP Biomedicals, LLC, Solon, OH) in place of the bovine serum albumin.

Surgical Preparation and Perfusion of the Hind Limbs

An initial intraperitoneal injection of 0.75mg Ketamine HCl, was administered to anesthetize the rat; additional dosing was administered as needed. Rear foot reflexes were tested to confirm anesthesia before abdominal midline and transverse incisions were performed to expose the abdominal cavity. Evisceration of the digestive and reproductive organs preceded ligation of major arteries branching from the descending aorta to prevent confounding humoral factors from entering the perfusate. This was achieved by first placing a ligature around the base of the uterus and ovaries, along with the neck of the bladder, and excising those organs along with contiguous adipose tissue. Next, two ligatures were tied in the descending colon and incised between the ligatures. Another ligature was placed anterior the stomach and the digestive organs were excised. Two sets of loose ligatures were placed around the descending aorta and the inferior vena cava, one just posterior to the origin of the renal arteries and the other just posterior to the origin of the iliolumbar arteries. The most anterior ligature around the descending aorta was tied and an 18mm gauge FEP polymer cannula (Jelco 4054; Smiths Medical International Ltd., Southington, CT, manufactured in the UK) was immediately inserted and tied into place with both ligatures. A transparent vinyl tube connected the cannula to a Watson-Marlow peristaltic pump (Wilmington, MA, manufactured in the UK), which

was calibrated and set to produce a flowrate of 8-12 ml/min (98), and was increased upon the start of contractions up to 20 ml/min to maintain adequate pressure and flow. The perfusion medium was warmed to 37°C (Warner Instruments Corp., Hamdon, CT, USA) and gassed with 95% O_2:5% CO_2. Perfusion was immediately continued with Krebs-Henseleit bicarbonate buffer after the aortic cannula was in place to make sure cannulation was successful, then was briefly paused to cannulate the inferior vena cava in the same manner of the aorta. A terminal cardiac stick with 0.1mg Ketamine HCl followed the cannulation of the abdominal aorta and vena cava. The perfusate flowed through the hind limbs, out the cannulated vena cava and was collected into pre-labeled vials. A brief 3-5 minute wash out period allowed for the majority of red blood cells and serum to flush out of the hind limbs. The efferent perfusate was then collected for 5-10 minutes from quiescent hind limbs and identified as Non-Stimulated (Non-STM) perfusate.

Electrical Stimulation

For muscle contraction, the sciatic nerve in its gluteal course, distal to the hip joint was carefully exposed and clamped with a low voltage S48 Electrical Stimulator (Astro Med Inc., Warwick, RI, USA). The electrical stimulation protocol from Hespel *et al*. (50) was followed with a few adjustments. The hind limb was electrically stimulated with trains of 100ms at 100Hz, with each train lasting for 10ms. Voltage was adjusted between 4-15V based on contraction strength to ensure constant muscle contractions until fatigue. The trains were delivered at a rate of 60/min for up to 35 minutes. Perfusate was collected during muscle contractions in one minute intervals into pre-

labeled vials. Once the muscle reached fatigue and no longer displayed visible contractions, the electrical current and collection of perfusate was discontinued. Unless otherwise specified, for the majority of experiments, the perfusate from the last 5-7 minutes of muscle contraction were pooled and identified as the Stimulated (STM) group. This methodology is similar to rat hemicorpus perfusion models used in the study of muscle metabolism (50, 59-61, 98). A schematic of the protocol is shown in Figure 2.

All perfusate was kept on ice during collection and centrifuged at 2,500 rpm for 25 minutes in 4°F to spin down any debris and erythrocytes. The supernatant was aspirated and pooled according to predetermined groups and immediately frozen to -80°C. A sample of the Krebs-Henseleit buffer solution used for each experiment was (labeled "K") and preserved at -80°C as a control.

Perfusate samples were thawed and pipetted into pre-labeled 1mL aliquots, and maintained at -80°C until cell culture experiments were prepared.

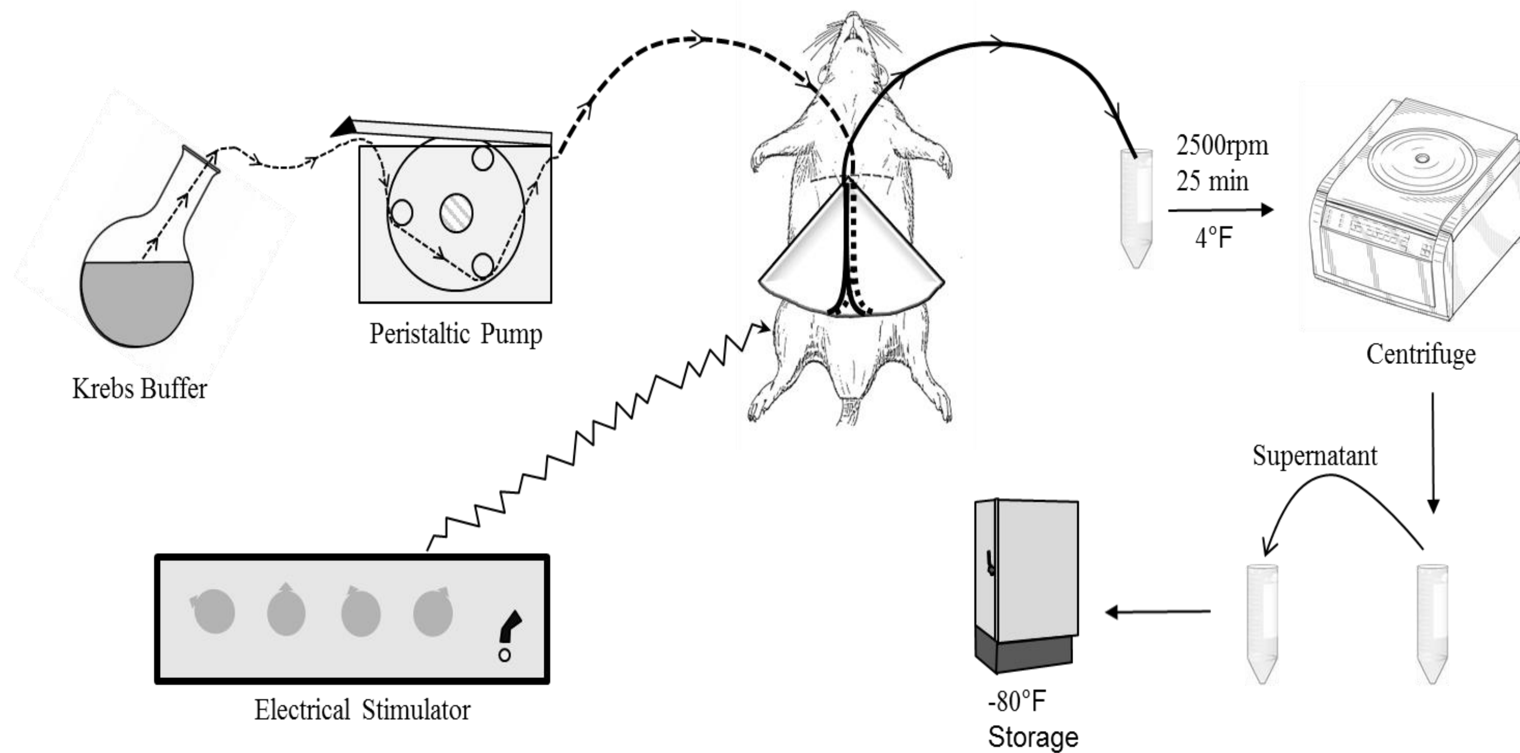

Figure 2. *Schematic of Hemicorpus Hind limb Perfusion Protocol*. Modified Krebs-Henseleit buffer was pumped through the cannulated abdominal aorta. Efferent flow through the cannulated vena cava was collected in conical tubes kept on ice. Perfusate was then spun down to remove RBC or debris. Supernatant was collected and frozen to -80°C for storage. Once perfusate was collected from quiescent hind limbs, electrical stimulation was applied to the same animal's sciatic nerve and perfusate was collected using the identical protocol as previously described.

Exercise Protocol

Moderate-intensity exercise was chosen for this study as it has consistently shown to be beneficial in improving and maintaining health. In fact, the American College of Sports Medicine and the American Heart Association jointly recommend healthy adults to achieve moderate-intensity physical activity for at least 30 minutes per day, 5 days a week or vigorous-intensity physical activity for 20 minutes on 3 days each week (48). In regards to the effects of exercise on breast cancer in rodents, Thompson *et al.* (116) conducted a study targeting exercise intensity on the effects of chemically induced mammary carcinogenesis. They suggest a threshold of 35% maximal exercise intensity to be reached before any beneficial effects of exercise would be realized. In addition, they propose a linear relationship between exercise intensity and the beneficial, protective effects of reduced cancer induction, up to 75% maximal exercise intensity (116). Another study by Westerlind *et al.* (124) reported moderate-intensity exercise to decrease tumor size and growth rate as well as increase tumor latency in adolescent rats. Therefore, the conditioning protocol that follows was chosen to elicit an estimated moderate intensity of 50-75% VO_2max, based on work by others (18, 23, 49, 51, 57, 94).

Our exercise protocol utilized a motorized rodent treadmill, similar to one described by Kimeldorf (65) and further modified by Holloszy (55). Briefly, the treadmill was comprised of a plastic box, partitioned into 20 lanes (2 lanes deep X 10 lanes across) suspended over a wide, continuous belt cycling on metal rollers. The lanes were approximately 10cm by 50cm, allowing each rat a restricted area to run. An air gun was used to enforce compliance to the exercise protocol; after the first few days of

exercise, use of the air gun was seldom needed. Rats were exercised during the first hour of the dark phase within the light-dark cycle each of the 5 days.

After familiarization to the treadmill and belt movement, 8 rats were assigned to the Exercise (EX) group and 4 were assigned as age and weight matched controls (Non-EX). Forty-eight hours after the last familiarization period, rats in the EX group completed a 5-week exercise program consisting of 5 bouts per week. Exercise duration was 60 min/day, while the speed and incline were adjusted as detailed in Table 1. A 5-10 minute warm-up and a 5 minute cool-down period was included in the 60-minute exercise session. The treadmill was initially fixed with no incline, and speed was gradually increased the first three weeks to an average top speed of 20.5 m/min. Once the treadmill was raised to 15% incline at the beginning of Week 4, speed was reduced to maintain a comparable increase in workload. Final top speed with the 15% incline was an average of 15.7 m/min for the last two weeks of exercise. A technical difficulty with one rat on Day 2 in Week 2 prevented us from going the entire 60 minutes.

Rats were inspected for any injuries and none were noted. The rats were also weighed after each exercise session; weights were recorded in a laboratory notebook. For transfer between the cage, rodent treadmill and scale, rats were held at the base of the tail. The treadmill and scale were cleaned and sanitized the end of each training session and were inspected for maintenance/safety concerns. At the conclusion of the 5 week exercise program, EX rats were perfused using the rat hemicorpus perfusion preparation as previously described within 70-80 hours after the last exercise bout. Rats

assigned as age and weight matched controls (Non-EX) were perfused within 72-80 hours of the EX group.

Table 1. *Treadmill Exercise Protocol*

Week	Duration/Day (min)	Average Top Speed/Day	Average Speed/Day	Incline (%)
1	60	12.8	11.0	0
2	57.6*	20.5	16.2	0
3	60	20.3	18.4	0
4	60	15.7	14.4	15
5	60	15.6	14.3	15

Table 1. *Treadmill Exercise Protocol*. Female Wistar rats (n=8) were exercised 60 min/day, 5 days/week for 5 weeks. Average Speeds are noted in meters per minute m/min. * Denotes an issue with one animal that decreased the average exercise duration.

Cell Culture

Cell Lines

MCF-7 and MDA-MB-231 cells were chosen as the cell lines for this dissertation. MCF-7 malignant breast epithelial cells, were originally taken from a pleural effusion of an invasive ductal carcinoma (106) and are estrogen receptor-positive (ER+), and progesterone receptor-positive (PR+) (58, 72). MDA-MB-231 malignant breast epithelial cells were also taken from a pleural effusion (19), however, these cells express more basal-like cell characteristics, are considered metastatic and do not have either the estrogen (ER-) or progesterone receptor (PR-) (58).

Both cell lines were obtained via courtesy from Dr. Weston Porter's laboratory (Texas A&M University, Department of Veterinary Integrative Biosciences, College Station, TX) and grown similar to as described in Noratto *et al*. (91). Briefly, cells were cultured using Dulbecco's Modification of Eagle's Medium (DMEM) 4.5 g/L glucose, L-glutamine, with sodium pyruvate and further supplemented with 5% (v/v) fetal bovine serum (FBS) and 1% (v/v) penicillin/streptomycin. Cells were maintained at 37°C in a humidified atmosphere containing 5% CO_2. Culture media and antibiotics were purchased from Corning (Mediatech Inc., Manassas, VA).

Treatment with Perfusate

To determine the effects of muscle contraction via electrical stimulation, breast cancer cells were treated with perfusates collected from quiescent and contracting skeletal muscle from a hemicorpus hind limb perfusion preparation. Through preliminary studies done by myself, (data shown in Figure 1) we ascertained a 10% (v/v) perfusate in DMEM to be optimal for treatment as it showed a robust response without the potentially confounding effects of over-dilution. Since the experiment was run in triplicate (3 wells for each group), a 10% (v/v) media solution was made for all three wells to maintain consistency of treatment. For example, 1mL of Non-STM perfusate was added to 9mL of DMEM media to obtain 10mL of treatment media. This solution was then used to culture the 3 wells of that group with 2mL each well; any remaining treatment media was discarded. Treatment was done in this manner to maintain consistency within groups; whereby, each well was exposed to the same percentage of perfusate.

Cell Proliferation

Cells were seeded into 6-well plates with 10,000-25,000 cells/well. Each experimental group had 3 wells each. The first treatment, Day 1, was administered 5-7 hours after seeding the plates by adding 200µL/well of perfusate. For subsequent treatments, media was aspirated and fresh treatment media was administered to cells every 24 hours. For the majority of the experiments, cells were treated for 4 days and harvested for cell counts on Day 5.

To evaluate cell proliferation, viable cells were counted using an electronic counter (Z1 Series, Beckman Coulter, Inc.). Cells were prepped for counting by first aspirating the culture media from each well and rinsing with 1mL PBS. Next, 0.5mL trypsin was added to each well until the cells detached. To stop trypsinization and recover total volume, 1.5mL of fresh DMEM was added to each well. Finally, 200µL was taken from each well after vigorously mixing with a pipette to break up potential clumps of cells and then added to pre-labeled vials of 20mL isotonic solution. Cell counts were taken in triplicate from each of these vials.

Impact of Muscle Contraction Perfusate on Breast Cancer Cell Proliferation

Cell Proliferation of Breast Cancer Cell Lines After Exposure to Perfusate from an Acute Bout of Electrical Stimulation

To observe differences in cellular proliferation after treatment of perfusate from stimulated skeletal muscle in two breast cancer cell lines, six rats were perfused using the HHLP protocol as described above. Perfusate was collected during muscle quiescence and labeled NoSTM. Once muscle contraction began, perfusate was collected

each minute in separate vials. Minutes 1-5 were pooled and labeled Stimulation Early (STM-E); the last 5 minutes of contraction were labeled Stimulation Late (STM-L); if muscle contractions exceeded 8 minutes, then the last minutes of contraction were equally divided and pooled in two separate groups, Stimulation Late (STM-L) and Stimulation Late Late (STM-L2). As stated in the above protocol, samples were frozen immediately after electrical stimulation ceased and stored at -80°C for the cell culture component of the experiment. These perfusates were used to treat two rounds each of MCF7 cells and MDA-MD-231 cells as described below.

Fresh 5% DMEM [4.5 g/L glucose, L-glutamine, sodium pyruvate, phenol red] was prepared and MCF7 cells were taken out of liquid nitrogen storage at passage 15. Cells were grown to seed eight 6-well plates at 25,000 cells/well at passage 17. Cells were seeded by 10:00am and the first treatment of perfusate was administered at 4:00pm on Day 1 by adding 200µL of perfusate to each well (3 wells/group). On Days 2-4, treatment consisted of aspirating old media and refreshing the cells with a 10% (v/v) perfusate treatment media as described above. Perfusate from 4 rats were used for the cell culture component of this study. All rats had a NoSTM, STM-E and STM-L group. Only 2 rats had an extra STM-L2 group.

On Day 5, cells were harvested for cell counts and cell proliferation data was recorded and analyzed. (Control cells appeared 90-100% confluent and treatment groups appeared 80-90% confluent).

The same cell culture experiment as described above was duplicated with passage 19 of MCF7 cells, seeded at 20,000 cells/well. In addition, passage 14 of an

MDA-MB-231 cell line seeded at 25,000 cells/ well, were treated concurrently with the MCF7 cells. On Day 5 when cells were to be harvested for cell counts, MCF7 cells were 90-100% confluent and MDA 231 cells were 50-60% confluent.

Perfusate from 5 rats were used to repeat the experiment with passage 13 of the MDA-MB-231 cells. Cells were seeded at 10,000 cells/well and were treated for an additional day. In an attempt to maximize the potential effect, we elected to seed them with 10,000 cells/well as opposed to 25,000 cells/well. Also, because MDA-MB-231 cells tended to grow more slowly, cells were harvested and counted for cell proliferation data on Day 6.

Differences in Cellular Proliferation After Exposure to Perfusate from Skeletal Muscle, with or Without Prior Exercise, During an Acute Bout of Electrical Stimulation

To observe potential differences in cellular proliferation after exposure to perfusate from skeletal muscle, with or without prior exercise conditioning, during an acute bout of electrical stimulation. To accomplish this objective, eight rats were conditioned with the Conditioning Protocol as described above. Four rats served as age and weight-matched controls. All rats were perfused using the HHLP protocol as previously described.

Thirteen 6-well plates were seeded with MCF7 cells with 10,000 cells/well. Perfusates from each of the 12 rats included a Non-STM and a STM group, and the control plate consisted of a cell control with untreated media and a Krebs buffer group. The remainder of the study followed the cell proliferation protocol as previously described.

Albumin as a Required Carrier of the Chemotherapeutic Myokine(s)

To compare cell proliferation between Dextran and BSA perfusate, four rats were perfused using the HHLP protocol as described above, using BSA; a second set of four rats were perfused using the modified HHLP protocol as described above with Dextran in place of the BSA to control for osmotic pressure. Perfusates from each group were pooled to obtain a large volume for proteomics assessment by a collaborator. Approximately twenty milliliters from each group (Non-STM and STM of both Dextran and BSA), were divided into 5mL aliquots and stored at -80ºC.

Four six-well plates were seeded with passage 17 of MCF7 cells. The cell proliferation protocol, as described above, was used for the remainder of this study.

Statistical Analysis

The effect of stimulated skeletal muscle perfusate (STM) compared to non-stimulated skeletal muscle perfusate (NonSTM) on cell proliferation are expressed as average percent reduction using the NonSTM cell count of each individual rat as the control. This was done to reduce the variability between rats and cell culture plates. When comparisons were made regarding fatigue and impact on cellular proliferation of cancer cells, a 2-way ANOVA was performed with repeated measures used across time and treatment. Statistical analyses were completed using SigmaStat version 3.5. Significance was set at $p \leq 0.05$ and all data are presented as means ± SE.

CHAPTER III

RESULTS

Impact of Muscle Contraction Perfusate on Breast Cancer Cell Proliferation

Cell Proliferation of Breast Cancer Cell Lines After Exposure to Perfusate from an Acute Bout of Electrical Stimulation

To observe differences in MCF7 cellular proliferation after treatment of perfusate from electrically stimulated skeletal muscle, the HHLP was performed on 3 rats and perfusate was collected in 5 minute fractions until fatigue. An additional group, STM-F, resulted from taking the fraction that represented the point of fatigue for each rat regardless of the time point collected. The average percent reduction from Non-STM control was calculated for each rat and the results for the following groups are as shown in Figure 4. Each fraction of electrically stimulated skeletal muscle perfusate (i.e. STM-E, STM-L, STM-L2 and STM-F) had significantly reduced cell counts as compared to the quiescent skeletal muscle perfusate (Non-STM) as the control, $p = 0.003$, $p = 0.003$, $p = 0.023$, and $p = 0.014$, respectively (see Figure 3). A two-way ANOVA to test for differences among the fractions did not show any main effects, $p = 0.217$.

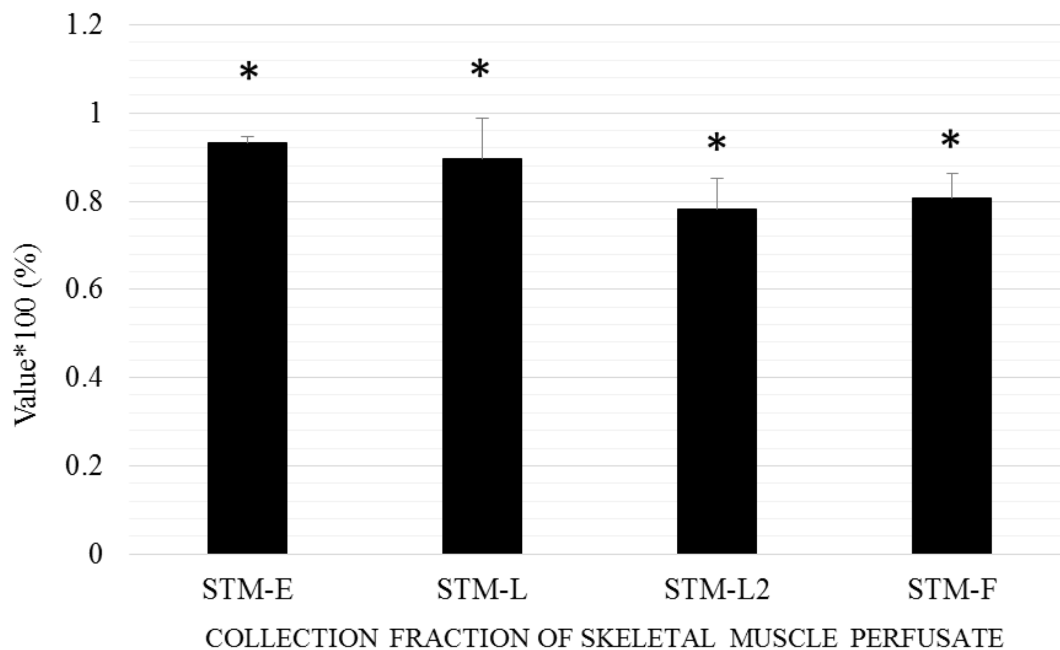

Figure 3. *Average Percent Reduction of MCF7 Cell Proliferation After Treatment with Electrically Stimulated Skeletal Muscle Perfusate from Non-STM Control.* MCF7 cells were treated for 5 days with skeletal muscle perfusate collected from quiescent muscle (Non-STM) and contracting skeletal muscle at different time points for n = 6 rats: the first 5 minutes of contraction (STM-E), minutes 6-10 (STM-L) and if contractions were still present, minutes 11-15 (STM-L2). The STMF fraction represents the fraction at which viable muscle contractions ceased. *Denotes statistical significance when cell counts exposed to the STM muscle perfusates were compared to cells exposed to the Non-STM control, $p < 0.05$. No differences were detected across time ($p>0.05$).

To observe differences in cellular proliferation of MDA-MB-231 breast cancer cells after treatment of perfusate from electrically stimulated skeletal muscle, the HHLP was performed on 4 rats and perfusate was collected in 5 minute fractions until fatigue. The STM-F fraction represents the point of fatigue for each rat regardless of the time point collected. The means for each group of the percent reduction of each rat compared to its Non-STM control are as shown in Figure 4. None of the fractions displayed a significant reduction in cell counts (STM-E, $p = 0.883$; STM-L, $p = 0.347$; and STM-L2 $p = 0.127$); however, the STM-F fraction approached significance at $p = 0.062$.

Differences in Cellular Proliferation After Exposure to Perfusate from Skeletal Muscle, with or without Prior Exercise, During an Acute Bout of Electrical Stimulation

To observe the effects of prior exercise on the effect of perfusate collected from contracting skeletal muscle via electrical stimulation on the cellular proliferation of MCF7 breast cancer cells, the HHLP was performed on rats exposed to 5 weeks of exercise on a treadmill and as well as age and weight matched controls. Results are presented in Figure 5. In both the Non-EX and EX cohorts, treatment with STM perfusate displayed significant average percent reductions in cell proliferation as compared to Non-STM control, ($p = 0.003$ for both cohorts). There was no difference between the average percent reductions in cell proliferation in response to muscle contractions between the control rats when compared to the exercised rats, ($p = 0.895$).

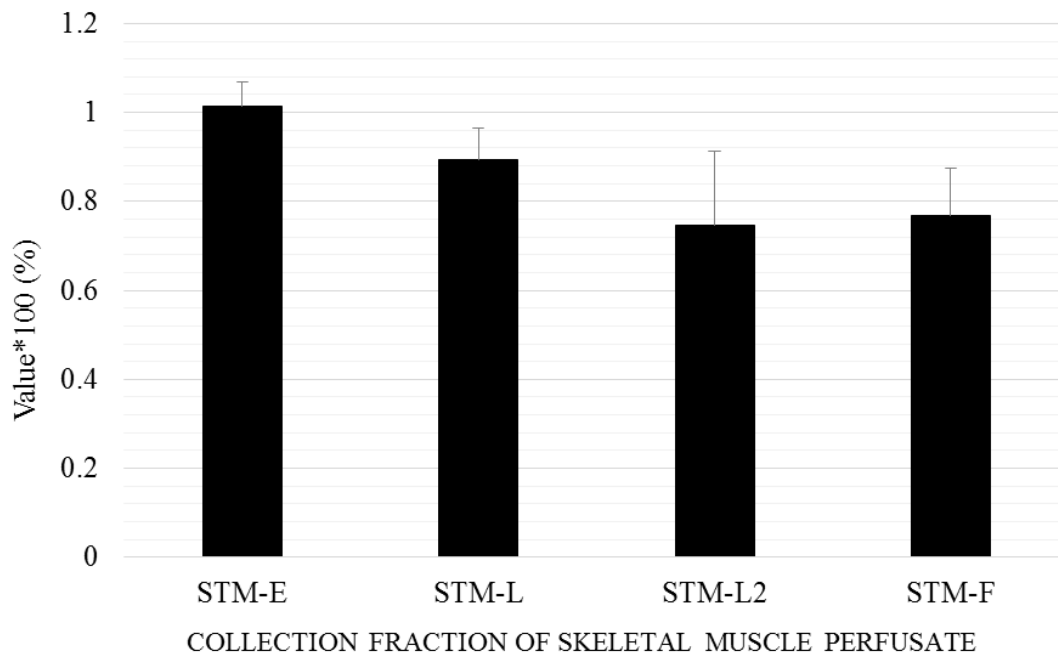

Figure 4. *Average Percent Reduction of MDA-MB-231 Cell Proliferation After Treatment with Electrically Stimulated Skeletal Muscle Perfusate from Non-STM Control.* MDA-MB-231 cells were treated for 5 days with skeletal muscle perfusate collected from quiescent muscle (Non-STM) and contracting skeletal muscle at different time points for $n = 4$ rats: the first 5 minutes of contraction (STM-E), minutes 6-10 (STM-L) and if contractions were still present, minutes 11-15 (STM-L2). The STM-F fraction represents the fraction at which viable muscle contractions ceased. There were no differences detected between cell counts exposed to the STM muscle perfusates as compared to cells exposed to the Non-STM control, $p < 0.05$.

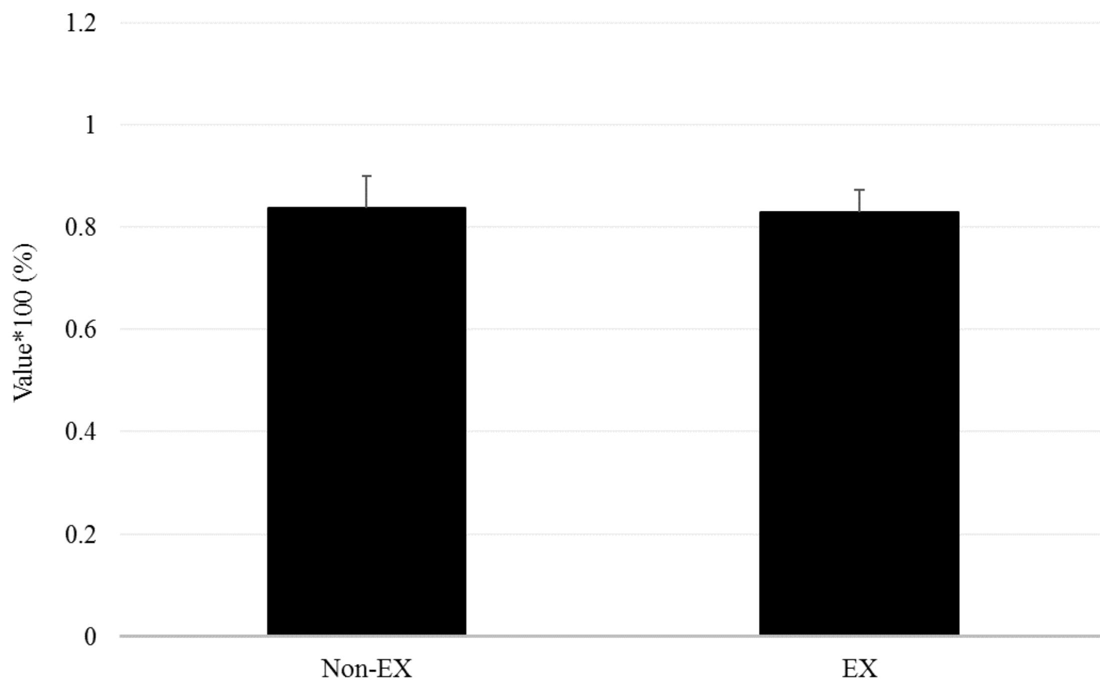

Figure 5. *Average Percent Reduction of MCF7 Cell Proliferation After Treatment with Electrically Stimulated Skeletal Muscle Perfusate from Non-STM Control, with and without Prior Treadmill Exercise.* MCF7 cells were treated for 5 days with skeletal muscle perfusate collected from quiescent muscle (Non-STM) and contracting skeletal muscle via electrical stimulation from rats previously exposed to 5 weeks of exercise on a treadmill (EX; n = 6) and normal cage ambulation control (Non-EX; n = 8). There was no difference between the average percent reductions from Non-STM to STM with prior Exercise (EX) as compared to control (Non-EX), $p = 0.895$. Both had significant average percent reductions as compared to Non-STM control, $p = 0.003$.

Albumin as a Required Carrier of the Chemotherapeutic Myokine(s)

To determine the role of albumin as a carrier protein in the perfusate, cell proliferation of MCF7 cells were compared after treatment of perfusion media prepared with Dextran or BSA. The STM perfusate prepared with BSA demonstrated the expected attenuation in cell proliferation as compared to cell Control (-26%, $p = 0.013$). There was no difference in cell proliferation of MCF7 cells after treatment of Non-STM or STM perfusate prepared with Dextran ($p = 0.402$ and $p = 0.813$, respectively), indicating the importance of albumin as a carrier of the chemotherapeutic factor(s) secreted from contracting skeletal muscle. See data shown in Figure 6.

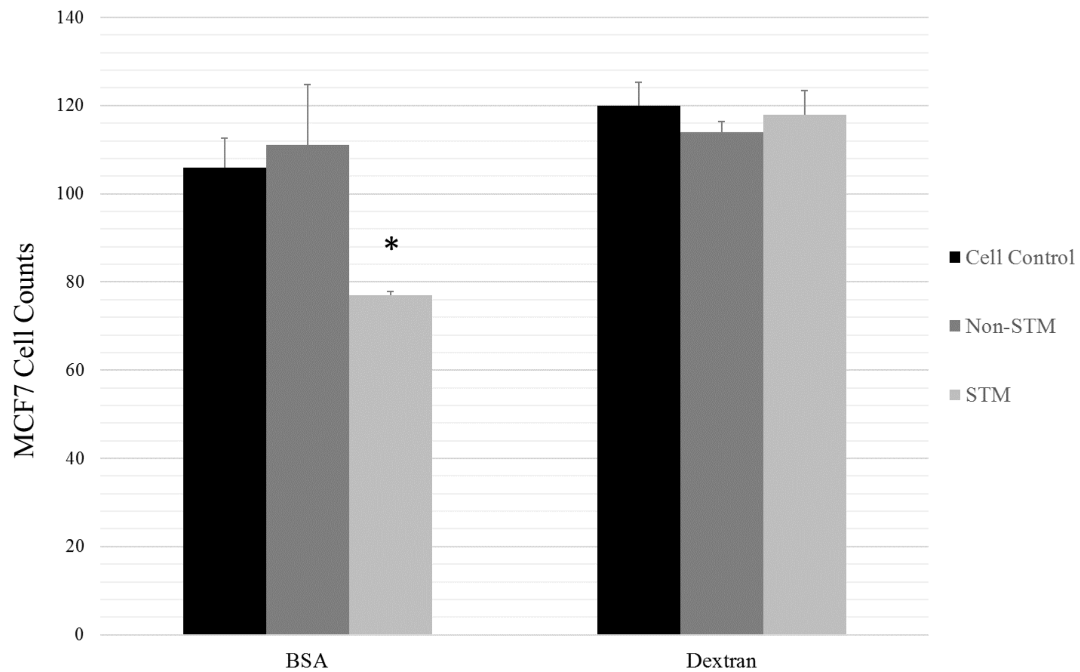

Figure 6. *Albumin as a Required Carrier of the Chemotherapeutic Myokine(s).* MCF7 cells were treated for 5 days with pooled rat skeletal muscle perfusate from n = 3 rats, prepared with BSA or Dextran, collected from quiescent muscle (Non-STM) and contracting skeletal muscle (STM) via electrical stimulation. STM perfusate prepared with BSA attenuated cell proliferation as compared to cell control, ($p = 0.013$). Perfusate from both Non-STM and STM was not different than control when prepared with Dextran, ($p = 0.402$ and $p = 0.813$). Data are expressed as means ± SE. *Denotes statistical significance when cell counts were compared to cell control, $p < 0.05$.

CHAPTER IV

DISCUSSION

The primary aim of this study was to determine if the purported benefits of exercise on breast cancer could be attributed to an intrinsic quality of muscle contraction during the locomotive process, without the confounding influence of compromised metabolism or humoral factors that are attributed to this benefit. To achieve this goal, we utilized a hemicorpus hind limb perfusion model with and without electrical stimulation of the sciatic nerve to collect substances secreted from contracting and quiescent skeletal muscle. In conjunction with this technique, we established a cell proliferation assay with MCF7 and MDA-MB-231 breast cancer cells to determine the chemotherapeutic effects of skeletal muscle perfusate. Others have implicated that muscle contraction, *per se*, is important for this chemotherapeutic action; however, to our knowledge, these data are the first to demonstrate the relationship between longer duration of muscle contractions and increasing cytotoxic effects on breast cancer cells, without the need for fatigue. These data are also the first to offer the notion that prior exercise training does not prove to have additive benefits on the release of (a) chemotherapeutic factor(s) during each acute bout of skeletal muscle contraction, as evidenced by similar cell MCF7 breast cancer cell counts after exposure to STM perfusate from Non-EX and EX.

Impact of Muscle Contraction Perfusate on Breast Cancer Cell Proliferation

Consistent with epidemiological and physiological studies demonstrating an attenuated risk and progression of breast cancer with exercise, our results support the

concept that the very act of locomotion (i.e., contracting skeletal muscle) has a chemotherapeutic influence on breast cancer. Our work is consistent with the notion that a factor, termed myokine, is released from skeletal muscle during exercise that attenuates cancer proliferation (52, 53, 64, 82). To demonstrate that directly, the present study used perfusate from electrically stimulated hind limb skeletal muscles (STM), administered to MCF7 breast cancer cells suppressed cell proliferation by up to 20% compared to Non-STM control ($p = 0.014$). Surprisingly, even though the same experiment with a different breast cancer cell line, MDA-MB-231, displayed close to a 25% reduction in cell proliferation in the STM-F fraction, that outcome was not statistically significant ($p = 0.062$). Early iterations of our experiments performed by Westerlind *et al.* (64) using our perfusates determined reductions of cell proliferation by assessing DNA damage repair and apoptosis. Those studies demonstrated that contracting muscle profoundly elevates apoptosis in two cell lines [+127% in MCF7 and +190% in MDA-MB-231s] when compared to Non-STM cells as determined by morphological criteria. The present study expanded on that work by noting that cell counts are reduced in both cell lines over 5 days of treatment with STM perfusate. Together, our work clearly implicates that substances arising from contracting skeletal muscle being carried through the vascular system remain viable and capable of attenuating cancerous growth.

It is of notable mention that the benefits of exercise may not prove to be equal in every tumor cell type. There have been conflicting studies (27, 33, 63, 83, 107, 115) displaying the varied adverse responses of exercise on tumor growth. Our data clearly

indicate the benefits of exercise in MCF7 cells, which represents the Luminal A subtype. In addition, although it did not quite reach significance, exercise appeared to show a cytotoxic effect on the MDA-MB-231's, a triple negative breast cancer cell type. The present data also support the many studies done in animals proving the beneficial effects of exercise on breast cancer initiation and progression (7, 11, 26, 52, 53, 64, 77, 82, 83, 116, 123-125, 129-131). It is important to keep in mind that many signaling pathways are aberrant in tumorigenesis, and while one particular pathway may be aberrant in some, it may not be in another. This warrants the continued exploration into what signaling pathways are affected in cancer with exercise.

In the literature, the specifics of intensity, frequency, and duration of exercise to maximize the benefit of exercise on breast cancer remain ambiguous. There was a dose-response association observed with the protective effects of physical activity on breast cancer, in support of moderate activity (>4.5 MET) more than light activities (<4.5 MET) (117). However, in a review of the exercise studies done in breast cancer survivors that targeted the report of findings in view of the principles of exercise training, the limitations in interpreting current literature as the frequency, dose and intensity of exercise, as well as the adherence to exercise protocols, are unclear or not reported (20). Further, a review of the chemically-induced breast cancer model in rodents discussed the differences observed in voluntary physical activity and a more structured form of exercise, and the need for defining laboratory studies in the context of the intensity and duration of exercise (111, 112). The same group also highlights the usefulness and importance of animal models in defining these parameters for highly-

translatable findings in the pre-clinical field of research (113). While the current work was not designed to determine dose-response relationships between exercise and attenuation of breast cancer, our data do provide further insights on the secretion of this factor due to our experimental design. Our study indicates that the benefit of repetitive muscle contractions regarding breast cancer are observed within the first 5 minutes of muscle contractions (STM-E), eliciting a 7% reduction in MCF7 cell proliferation ($p = 0.003$), suggesting that this myokine is already resident in the cell and capable of excretion within 5 minutes of repetitive contractile work. That said, it is also obvious that fractions collected sequentially seem to have a greater effectiveness than the previous fraction, as the impact of the perfusate on cancer cell lines increased with each fraction displaying augmented percent reductions in cell proliferation [10% reduction with STM-L and ~20% with STM-L2 and STM-F], suggesting that either the substance(s) is(are) being manufactured during the contractile process, or that the packaging/release of the substance(s) requires time to be maximized. These data expand on the work of Hoffman and Munoz (52, 82), which ascribes this chemotherapeutic factor to be a "fatigue substance" (named F-substance) because muscles were contracted to fatigue. However, the collection of skeletal muscle secretions with electrical stimulation occurred in a saline bath preparation that collected everything from the start of contractions until fatigue, so postulating that the benefit of exercise on cancer required fatigue was overstated. Our results are the first to clearly implicate that the release of a chemotherapeutic factor is indeed enhanced with the longer duration of muscle contractions; however, fatigue is not necessary to bring about its release. This is

supported by the work of Hojman *et al*. (53) that demonstrated attenuated MCF7 cell growth after exposure to exercise serum in which mice swam for 1 hour, not necessarily until fatigue. In addition, the study by Rundqvist *et al.* (99) only had their human subjects exercise for 1 hour (at moderate intensity of 50-65% VO_2max) before collecting exercise serum in their study displaying diminished prostate cancer growth on cells treated with exercise serum.

The intensity of exercise needed to elicit the chemotherapeutic benefits of exercise cannot be addressed with our data. However, the intensity of muscle contractions in our protocol was based off the strength of contraction needed to maximize glucose uptake in skeletal muscle (39) which results in a dramatic exocytosis of vesicles to plasma membrane surfaces in muscle to elevate glucose transporter content. Although the release of myokines was not the focal point for that study, we suspected that glut4 containing vesicles contain factors that are released into the blood stream during exercise, and our goal was to maximize exocytotic opportunities in the present model. Further work is warranted to explore the emerging mechanism for myokine release with exercise. And once again, our study was not designed to answer specific questions on a clinical prescription of exercise, but is useful in proving the importance of muscle contraction as the mechanism of exercise that is cytotoxic to cancer cells.

It has been long accepted that repetitive bouts of exercise over time elicit a training effect on the body that extend beyond the impact of a single bout. Using this construct of a training effect, it stands to reason that repetitive physical exercise may

improve the benefit of muscle contraction on breast cancer cytotoxicity. Our results clearly indicate that the benefit of exercise on cancer is derived from individual acute bouts of muscle contractions, and not impacted by chronic exercise *per se*. We did not observe a significant difference between the average percent reduction in cell proliferation between rats exposed to 5 weeks of exercise and normal cage ambulation. This study is the first to suggest that consistent/repetitive prior exercise does not improve the secretion of a chemotherapeutic factor from a single bout of exercise. This actually goes against our original hypothesis that previous exercise conditioning would accentuate the cytotoxic effect of STM perfusate on breast cancer cells. Considering the *elasticity* of muscle and its ability to adapt by becoming more metabolically efficient (56), we assumed the same would hold true in the improvement in the release of a chemotherapeutic factor. This strongly suggests that this myokine is normally resident in the cell, and its manufacture is completely independent of physical conditioning. It also stands to reason that any individual expressing this myokine may benefit from physical activity, regardless of prior physical conditioning. Despite the additional effects on skeletal muscle metabolism from prior conditioning, our work highlights the advantages of each acute bout of exercise in the secretion of (a) chemotherapeutic factor(s).

We should note that our capacity to fully understand the impact of a single bout of exercise or physical training on breast cancer is not possible with the current methodologies. Because our studies were not designed to assess dose-response *per se*, we chose to assess the impact of contracting muscle on cancer by exposing the cancer cells to what amounts to a continuous exercise session that spanned 24 hours each day

for 4 days (as they were harvested on day 5). Since it is unlikely that individuals can endure those training behaviors, future studies should address dose-response relationships by allowing cells to be exposed to treatment media similar to what would be seen *in vivo*, by administering treatment media for briefer periods of time and followed by traditional cell media each day. That said, we must reemphasize our findings that perfusate arising from contracting skeletal muscle has a profound impact on breast cancer cell lines, which strongly provide a direct connection between physical activity and reduced cancer cell growth.

The acute exercise model of electrical stimulation via the nerve may be more physiologically correct than stimulation by other means, but this model is still not completely physiological. True neural activation in *in vivo* exercise activates the motor units in a synchronized fashion to match the forces required to meet the demand of the activity. Thus, not all motor units are fired for a given activity. Motor unit recruitment with electrical stimulation of skeletal muscle is an all or nothing response. In order to curb the impact of 'all or nothing' responses resulting from sciatic stimulation, we chose to only stimulate one limb, but perfuse both limbs to reduce the contribution of muscle mass in a reasonable manner. Given that only one limb was activated, it suggests that the myokine(s) involved with this process is (are) potent. Future studies may warrant the use of functional electrical stimulation that mimics locomotion during conditions of hind limb perfusion to determine if the incorporation of both limbs intensifies this response.

Albumin as a Required Carrier of the Chemotherapeutic Myokine(s)

Considering the many physiological functions of albumin in health (90), especially its role in drug-binding and delivery and its use in our methodologies, we assessed the role of albumin on the chemotherapeutic impact of contracting skeletal muscle. Our perfusion medium was designed to mimic the basics of plasma with physiological ratios of electrolytes and similar oncotic properties as well. The modified Krebs-Henseleit buffer solution we used contained 3.5% BSA, which is on the low end of human albumin plasma levels (35-50 mg mL^{-1}) and the high end of rat albumin plasma concentrations (29 mg mL-1; (73)). When we prepared our Krebs-Henseleit buffer for the HHLP without BSA and instead utilized Dextran as our oncotic agent, the chemotherapeutic effect of STM perfusate completely disappeared (Non-STM vs control, $p = 0.402$; STM vs control $p = 0.813$). These results perhaps emphasize albumin's second most important role as a carrier molecule, in that whatever is secreted from contracting skeletal muscle capable of eliciting a chemotherapeutic effect on breast cancer cells requires albumin as a carrier through the vasculature. This notion differs from findings of Hoffman or Munoz (52, 82), where the chemotherapeutic effect of contracting muscle persisted even when BSA was not included in their *in vitro* saline bath preparation of electrically stimulated muscle, suggesting that albumin may be necessary to carry the factor through a vasculature (as in the present study), but not necessary when delivered to the cancer cell directly.

In addition to the systemic role of albumin, it is also a major source of nitrogen and energy for extravascular cells, which is utilized after endocytosis and lysosomal

degradation. The plasma compartment is only estimated to contain about 42% of the total body albumin pool, with the rest abiding in extravascular compartments. This gives rise to the consideration that albumin is preferentially sequestered by tumor cells (127) to serve as a nutrient. Our perfusion medium may actually be providing the cells with an extra source of metabolic substrate to support their high metabolic activity, essentially potentiating their increase in cellular growth and proliferation. If so, this may allow for a glamorous entry of a potentially lethal factor if it indeed is being delivered by albumin to the cell.

CHAPTER V

SUMMARY AND CONCLUSIONS

In summary, we provide evidence that the benefits of exercise on breast cancer, in part, can be attributed to the fundamental properties of contracting skeletal muscle, independent of humoral factors or altered physiological metabolism. Our hemicorpus hind limb perfusion model enabled us to collect substances secreted from skeletal muscle during quiescence and stimulated contraction, directly demonstrating the secretion of (a) chemotherapeutic factor(s) upon contraction. Our results demonstrated a profound 20% reduction of MCF7 breast cancer cells proliferation after 5 days of exposure to STM muscle perfusate. They also expand on previous studies and are the first to clearly implicate that the release of a chemotherapeutic factor is enhanced with the longer duration of muscle contractions, and that fatigue is not necessary to bring about its release. While one could reason that previous exercise conditioning may exaggerate these cytotoxic effects, the results of this study are also the first to offer the importance of muscle contractions as the mechanism of exercise that elicits cytotoxic benefits of exercise on breast cancer, arising from each acute bout rather than chronic exercise *per se*.

Finally, our model highlights the physiological role of albumin as a potential carrier molecule of a variety of substances in mammalian plasma. Our results suggest the presence of albumin to be necessary to transport the chemotherapeutic myokine from contracting skeletal muscle through the vasculature.

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