ABSTRACT

BONNEY, SIERRA. Regenerative Medicine: Effects of Plant Bioactives on Muscle Protection and Wound Healing. (Under the direction of Dr. Debora Esposito).

Brassinosteroids (BR) are phytohormones frequently used in agriculture to enhance crop yield and quality. While most BR research is conducted in plants, recent administration to animal and cell models has shed light on their wound healing and anabolic potential. The first section in the literature review **(Chapter I)** consists of background information on BR in agriculture and their health-promoting properties observed thus far. The second section expands upon skeletal muscle and how it is impacted by aging, while the third section highlights an excellent model for aging research, the senescence-accelerated mouse prone 8 (SAMP8).

The first experiment in this thesis (**Chapter II**) evaluates the effects of a diet supplemented with cabbage seed extract (CSE), which has high BR content, on fitness, appearance of age-associated behaviors, and myogenic gene expression in an accelerated aging mouse model. Parameters measured include food intake, body weight, body composition, endurance, and assessment of senescence-associated behaviors. Both male and female mice fed the CSE diet exhibited enhanced endurance, and female CSE mice had an increase in lean mass compared to the control group. Additionally, CSE mice exhibited reduced senescent behaviors compared to control groups. Gene expression analysis of gastrocnemius muscle was performed to determine effects of diet on markers associated with muscle regeneration. Gastrocnemius muscle from both male and female CSE mice had increased expression of myogenic markers MyoD1, MyoG, and satellite cell Pax7, and female CSE mice had significant downregulation of proteolytic markers associated with age-related muscle atrophy. These results offer insight into a potential therapeutic option for maintaining muscle mass and improving physical fitness with age.

The second experiment (**Chapter III**) investigates the cell migration properties of human dermal fibroblasts (HDFa) upon BR administration as well as their free radical scavenging abilities in murine macrophages. With age there is a decrease in wound healing efficiency and an increase in oxidative stress, which can exacerbate diseased states and contribute to the $50 billion spent yearly on treating chronic wounds. Previous work by Esposito et al. (2013) showcased BR ability to promote cell migration in murine fibroblasts and accelerate cutaneous wound healing in mice. In plants, BR have proven to be potent scavengers of free radicals and make significant contributions to defense and repair mechanisms. However, it is unknown if this also true in mammalian cells. Lipopolysaccharide (LPS) was used to stimulate murine macrophages (RAW 264.7) which were subsequently treated with BR analogues to determine antioxidant capacity. Three BR compounds were found to significantly reduce reactive oxygen species production ($p < 0.0001$) and five reduced the production of nitric oxide species ($p < 0.05$). Furthermore, BR compounds applied to human dermal fibroblasts (HDFa) at a concentration of 2.0μM, showed that eight out of nine BR compounds significantly increased fibroblast migration ($p < 0.05$) over a 48 hour period, with Homobrassinolide and Homocastasterone having the largest effect. These results support the idea of translational work in mammalian cells by showcasing the ability of BR compounds to reduce accumulation of reactive oxygen species, thus highlighting the potential for BR use in skin therapeutics and regenerative medicine.

Regenerative Medicine: Effects of Plant Bioactives on Muscle Protection and Wound Healing

by
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DEDICATION

I dedicate this body of work to my family and my husband. Thank you all for helping me to retain my sanity, and for your endless love and support throughout this journey.

BIOGRAPHY

Sierra Bonney was born and raised in the small town of New London, North Carolina. Growing up on a farm, a love of animals was instilled in her from an early age. Sierra studied biology at Pfeiffer University, where the guidance and passion of her professors lead to an opportunity to spend a summer as an intern with the Plant Pathways Elucidation Project at the Plants for Human Health Institute where she discovered her love of research. After graduating, she began working as a research associate with Crown Bioscience until their relocation, at which time Sierra began working as a research technician in Dr. Debora Esposito's lab at the Plants for Human Health Institute in Kannapolis, NC. It was there that she was presented with the opportunity to pursue graduate studies as a part of NC State University's Department of Animal Science as a research assistant under the guidance of Dr. Debora Esposito.

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Literature Review

INTRODUCTION

Brassinosteroids

Plants contain compounds called phytohormones, which are essential for growth, development, immune response, and stress tolerance. Phytohormones can work together or independently, and are capable of driving physiological processes at concentrations (Davies, 2010). Currently there are seven known phytohormones: auxins, gibberellins, ethylene, cytokinins, abscisic acid, jasmonic acid, and brassinosteroids (BR) (Clouse and Sasse, 1998). BR were discovered in the 1940s when the United States Department of Agriculture extracted pollen from more than sixty plants in order to identify growth promoting compounds for crop improvement while extract from *Brassica napus* pollen has been shown to enhance growth when sprayed on plant seedlings, and the translocation of this pollen to growing plants stimulates a significant growth response (Kutschera and Wang, 2012; Zullo et al., 2002). The compound responsible for these effects remained unknown.

Using 227kg of *Brassica napus* pollen, the bioactive compound was isolated and identified as a new plant steroid, a polyhydroxylated derivative of 5α-cholestane, and it was termed brassinosteroid (Grove et al., 1979). Extensive research in this field continued through analysis in *Arabidopsis thaliana* which provided further proof of BR importance, as BR deficient plants had significantly stunted growth (Clouse, 2011; Yamamuro et al., 2000). It is now known that BR are found ubiquitously throughout the plant kingdom, with the greatest amounts being found in pollen and immature seeds while also found in other plant tissues, albeit at much lower concentrations (Clouse and Sasse, 1998). See table 1.1 for BR content in various plants tissues. Sixty-two naturally occurring BR have been identified and isolated to date, and numerous

biologically active analogues have been synthesized (Peres et al., 2018; Bishop and Koncz, 2002).

BR are frequently utilized in agriculture for their beneficial properties, including increasing yield and crop quality. Administration of BR is known to be an effective method for improving plant drought-tolerance, which is the number one issue in the agriculture industry resulting in billions of dollars in losses each year (Tanveer et al., 2019). Application of BR to pesticide-treated crops has been demonstrated to degrade pesticide residue through activation and enhancement of the plants' natural antioxidant defense mechanisms (Sharma et al., 2018). Therefore, further investigation into the benefits of BR application, many issues within the agriculture industry may be improved.

Structure

Interestingly, BR shares more structural similarities with mammalian hormones than with other phytohormones (Figure 1). BR are characterized by a poly-hydroxylated 5α-cholestane structure, much like the cholesterol-derived mammalian hormones, and their structures vary based on variations within their A ring, B ring, and side chains (Geuns et al., 1978). Structures featuring A-rings with 2α, 3α-adjacent hydroxyl groups tend to be the most biologically active, as is the case with brassinolide and castasterone (Yokota, 1997). *In-vitro* work has suggested that BR containing β-hydroxyls exhibit reduced bioactivity (Esposito et al., 2011).

BR also share structural resemblance with ecdysteroids, which are arthropod hormones involved in molting, development, and reproduction. Studies of their activity in mammalian systems have shown their ability to exert effects similar to vertebrate hormonal steroids, including immune system enhancement, glucose regulation, anabolic stimulation, hepatoprotection, and radical scavenging abilities (Báthori et al., 2008). Despite structural

similarities, ecdysteroids do not bind to the same cytosolic receptors as vertebrate steroids, but rather to membrane-bound receptors, which was shown using an androgen nuclear receptor binding assay where 20-hydroxyecdysone did not display any significant binding at biological concentrations between 1 and 100 μM (Parr et al., 2015; Gorelick-Feldman et al., 2008). Insect and plant steroids influence signal transduction pathways similarly to anabolic steroids, but they have the potential to be promising alternatives to androgenic steroids due their ability to stimulate protein synthesis and muscle growth in the absence of androgenic side effects (Báthori et al., 2008).

Brassinosteroids in Horticulture

The field of horticulture is vast, with the United States Department of Agriculture reporting a profit exceeding $13 billion in the United States in 2014. However, it is a high-risk and costly industry, as a significant amount of money and resources are spent on growth management, environmental factors, harvest, and technology. BR serve as a valuable resource in this market as they can increase crop yield and enhance nutritional content. Tomatoes treated with 1ppm of BR exhibited increased size, weight, and fruit number as well as greater lycopene and beta carotene content (Ali et al., 2006). BR application in peppers improved size, ripening index, and color in addition to increased antioxidant and phenolic content (Serna et al., 2013). Potatoes treated with BR exhibited greater weight, increased infection resistance, and BR application prevented germination during storage (Zullo et al., 2002). Their ability to improve drought and heat tolerance, disease resistance, and reduce pesticide toxicity has also been validated (Ahammed et al., 2017).

Pathway in Plants

Hormones are signaling molecules essential for growth and regulation in animals and plants. While their physiological roles in plants and animals are similar, the mechanisms are distinct for each (Clouse, 2011). BR signaling has been studied extensively in Arabidopsis, enabling the elucidation of the mechanism, whereby BR bind to cell surface receptor kinase BR Insensitive 1 (BRI1) in plants rather than nuclear receptor family transcription factors in animals.

BR bind to BRI1 which produces a phosphorylation cascade (Kim et al., 2011). Once the receptor is activated, BRI1-Associated Receptor Kinase 1 (BKI1) is phosphorylated and then inhibits BRI1 leading to separation from the plasma membrane and cytoplasmic proteins (Wang, et al. 2011). During this process, BRI1 aid in the phosphorylation of Constitutive Differential Growth 1 (CDG) and BR-signaling kinase-1 (BSK1) and the interaction between these two molecules results in the dephosphorylation of BR-Insensitive 2 (BIN2), a GSK3-like kinase that under normal conditions acts to suppress the BR signal transduction pathway (Kim et al., 2011; Tang et al., 2011). BIN2 deactivation prevents the phosphorylation that would ultimately lead to transcription of BR-associated genes, consequently preventing their expression (Jaillais et al., 2011). This pathway shares many similarities with the WNT signaling pathway in animals in which WNT binds to its receptor and impedes GSK-3B kinase, therefore preventing the accumulation of beta catenin in the cell nucleus and inhibiting gene transcription (Girardi and Le Grand, 2018). The phosphorylation cascade activated by BR binding is also involved in protein homeostasis through activation of Protein Kinase B (AKT) and the subsequent signal transduction pathway (Altomare and Khaled, 2012).

Current Brassinosteroid Research

The Protein Kinase B (Akt) pathway is involved extensively in regulation of protein balance, glucose regulation, and cell survival, and BR was previously shown to activate this pathway through Akt phosphorylation (Esposito et al., 2013). BR-induced stimulation of the Akt pathway has not been fully explored in an aging model, nor have gender-specific effects been analyzed. Therefore, more research is needed to determine if BR administration can attenuate any age-related myogenic or metabolic imbalances that come with age. BR have been deemed safe in animal systems, as BR compound epibrassinolide was found to have a median lethal dose (LD50) exceeding 1000mg/kg in mice and 2000mg/kg in rats for both subcutaneous and oral applications (Kuzmitsky and Mizulo, 1991; Murkunde and Murthy, 2010). Cell culture work has provided insight into their ability to modulate immune response and inhibit several human cancer cell lines, including breast cancer and prostate cancer through interruption of cell proliferation (Malíková et al., 2007). They exhibited anti-proliferative and apoptotic properties that specifically targeted cancerous cells (Steigerová et al., 2010). Additionally, BR were shown to successfully inhibit viral activity including herpes simplex virus type 1 (HSV-1), vesicular stomatitis virus, and arenavirus (Wachsman et al., 2000). These results provide insight into therapeutic implications for BR in the human health field.

Because of the structural similarities between BR, ecdysones, and animal steroids, research interest has piqued in the fields of musculoskeletal systems and human medicine. The *in-vitro* work with arthropod ecdysteroids led to this treatment being used in animal models to investigate anabolic potential. Hirunsai et al. (2016) discovered that 20-hydroxyecdysone administration increased skeletal muscle mass without androgenic side effects in animals experiencing muscle atrophy. Murine myotubes and human primary myotubes treated with

ecdysteroids exhibited an increase of up to 20% in protein synthesis (Gorelick-Feldman et al., 2008). Based on these results, the anabolic potential of BR became of great interest. Esposito et al. (2011) found that BR administration resulted in increased protein synthesis and decreased protein degradation in L6 rat skeletal muscle cells. Bioactivity was dependent upon structure, and BR containing alpha vicinal hydroxyls were found to be essential for anabolic stimulation. Oral administration of BR in animals resulted in an increase in weight, lean mass, gastrocnemius muscle size, and grip strength (Esposito et al., 2011).

Benefits of BR are not limited to protein synthesis, as they have also been found to possess anti-diabetic properties. The BR compound Homocastasterone, was found to lower glucose, triglycerides, and cholesterol levels in both diabetic and healthy animals (Athithan and Srikumar, 2017). In addition, when Homobrassinolide was administered to C57BL/6J obese mice it was shown to improve hyperglycemia (Esposito et al., 2011). These results have been attributed to the ability of BR to modulate glucose metabolism by way of the Phosphoinositide-3-Kinase (PI3K) /Akt pathway, which is involved in glucose metabolism (Zhang et al., 2017). Dysfunction of this pathway can result in type II diabetes and obesity. Mice lacking the gene responsible Akt inhibition displayed increased insulin sensitivity and resistance to obesity, which suggests that targeting molecules responsible for PI3K/Akt pathway activation may strategies for ameliorating diabetes (Chakraborty et al., 2010).

Pharmacogenomic Effect of Brassinosteroids in vivo

The phosphatidylinositiol-3-kinase (PI3K)/Akt pathway is highly coordinated and important for protein balance, metabolic regulation, stress response, and cell survival. BR binding results in phosphorylation and activation of Akt (Esposito et al., 2011). In conjunction with insulin-like growth factor 1 (IGF1), Akt regulates growth of skeletal muscle through a

highly conserved pathway, and this signaling is essential for protein homeostasis, which consists of a balance between protein synthesis and degradation (Frost and Lang, 2007). Conservation of muscle mass is achieved through interactions between glycogen synthase kinase 3b (GSK3b), PI3K, Akt, and mammalian target of rapamycin (mTOR) (Stitt et al., 2004). Protein degradation can be activated through Myostatin binding, Akt suppression, and subsequent activation of the FoxO family of transcription factors associated with atrophy (Tzivion et al., 2011). This pathway does not operate exclusively for skeletal muscle maintenance, as the components are involved in extensive crosstalk with other pathways.

The mechanism that underlies the activation of this pathway is depicted in Figure 1.3. IGF1 binds to its receptor and initiates tyrosine kinase for a phosphorylation cascade resulting in the activation of phosphatidylinositiol-3-kinase (PI3K), production of phosphoinositide-3,4,5-triphosphate which serves as binding location for phosphoinositide-dependent kinase 1 (PDK1) and Akt (Manning and Toker, 2018). Upon phosphorylation, Akt is activated and the continuation of this cascade results in increased expression of genes associated with myogenesis. Binding of Myostatin results in suppression of the protein synthesis and instead increases expression of FoxO transcription factors, which are responsible for the addition of ubiquitin to muscle proteins, ultimately leading to their degradation (Dai et al., 2013).

The extent of the Akt pathway involvement in physiological processes has been thoroughly explored and the essential nature of this pathway is universally accepted. Embryonic Akt-gene knockout mice experience growth retardation, severe skeletal muscle atrophy, and impaired glucose metabolism, but atrophy was rapidly reversed and glucose regulation was improved upon Akt expression (Lai et al., 2004). This work highlights the importance of Akt cross-talk between seemingly distinct pathways.

Homobrassinolide was found to stimulate genes involved in carbohydrate and muscle metabolism, with adrenergic receptor alpha 1d showing the greatest increase in expression (Esposito et al., 2011). This gene plays a role in the mediation of catecholamines, coupling G proteins to interpret and transmit stimuli, and may be involved in maintenance and differentiation of muscle cells (Peng et al., 2018; Strosberg, 1995). Signal transduction through the IGF-1 receptor is crucial for development and regeneration of muscle (Alzhanov et al., 2010). Age-related impairment of this pathway is associated with a decrease in phosphorylation potential (Shay and Hagen, 2009). Furthermore, with age there is an increase in oxidative stress, and the reduced Akt phosphorylation could hinder the ability of this pathway to mitigate this additional stress (Shay and Hagen, 2009). Therefore, more work must be done to identify methods by which to phosphorylate and subsequently activate the Akt pathway to ensure that physiological regulation is maintained through aging. Previous studies have detailed the influence of steroid hormones and their stimulatory effect on the Akt pathway, as it is enhanced by both estrogen and testosterone (White et al., 2013). The decline in circulating levels of these hormones with age negatively impacts signal transduction and contribute to physiological dysfunctions observed in sarcopenia (Kim et al., 2016). BR have been shown to stimulate Akt phosphorylation and protein synthesis without androgenic side effects (Esposito et al., 2011), but these effects have yet to be investigated in models of aging.

Brassinosteroids Promote Wound Healing

Wound healing efficiency can be impacted by an individual's age, sex, lifestyle, stress, and metabolic state (Guo and Dipietro, 2010). Steroid hormones are actively involved in the wound healing process, but the extent of this involvement is dependent upon both age and sex. Males tend to experience slower wound healing, which can be attributed to the increase in

inflammatory response and reduction in extracellular matrix deposition associated with androgens (Kanda and Watanabe, 2005). Estrogens have a number of properties shown to improve skin health and wound healing through modulation of factors involved in matrix deposition, enhancement of collagen synthesis of collagen and preventing its breakdown, as well as contributing to re-innervation, re-epithelialization, and new tissue formation during the wound healing phase (Fimmel and Zouboulis, 2005).

BR have been found to bind to the same receptor as systemin, a plant hormone involved in wound healing, and BR binding to this receptor also results in activation of the wound healing response (Szekeres, 2003). Since BR closely resemble these animal steroids and studies have shown their lack of androgenic side effects, their ability to promote wound healing has been investigated both *in vitro* and *in vivo*. Esposito et al. (2013) used nine structurally diverse BR compounds to treat murine fibroblasts and assess cell migration properties, finding that Homobrassinolide and Homocastasterone contributed to increased cell migration. Based on these results, Homobrassinolide was applied topically to treat cutaneous wounds in C57BL/6J mice and resulted in accelerated wound healing and reduction in wound size (Esposito et al., 2013). BR enhance wound healing through modulation of inflammatory response and enhancement of fibroblast proliferation and migration attributed to Akt phosphorylation (Bujor et al., 2008; Esposito et al., 2013). Wound healing rates decrease dramatically with age, contributing to chronic wounds and eventually morbidity if not effectively treated. Therefore it is necessary to validate whether these results are translatable to human cell lines before BR can be incorporated into therapeutics for regenerative medicine to improve human health.

Free Radical Scavenging Potential of Brassinosteroids

Reactive oxygen species (ROS) and nitric oxide species (NOS) are natural byproducts of metabolism in both plants and mammals. Environmental factors also contribute to ROS generation, and this accumulation can negatively impact physiological processes, including reduced enzymatic activity, altered lipid permeability, and impaired carbohydrate metabolism (Anjum et al., 2012; Gill and Tuteja, 2010; Liemburg-Apers et al., 2015). A dramatic increase in oxidative stress has been found to significantly contribute to the loss of strength associated with sarcopenia as well as other age-related pathologies (Brioche and Lemoine-Morel, 2016). Therefore reduction of ROS has the potential to reduce cell damage and preserve normal cellular function. Plants evolved to have numerous radical-scavenging mechanisms to combat free radicals, and BR have been found to enhance this defense system and contribute to reduced oxidative stress (Verma et al., 2016). Application of BR to rice seedlings exposed to alkaline stressors was shown to significantly reduce ROS while increasing the enzymatic defense network consisting of superoxide dismutase, peroxidase, and catalase (Sharma et al., 2019). Similar to plants, application of BR in a human model with stress-induced dopaminergic neurons resulted in activation of superoxide dismutase and peroxidase with subsequent reduction in ROS and apoptotic events (Carange et al., 2011). Therefore, the radical scavenging capabilities of BR need to be further investigated in cell models, as different BR compounds possess different levels of bioactivity, and identification of BR with the greatest free radical scavenging capacity can be incorporated into therapies for ailments exacerbated by oxidative stress.

SKELETAL MUSCLE

Overview

Healthy skeletal muscle is essential for performing daily activities, maintaining posture, and also serve to protect our vital organs. When an imbalance in skeletal muscle homeostasis occurs resulting in loss of muscle mass and function, simple tasks such as walking can become difficult. Injuries, inactivity, and disease can be a result of these imbalances, but age is also a major contributor to loss of skeletal muscle mass resulting in functional impairment. Sarcopenia is the age-associated loss of muscle mass and strength that can result in additional adverse health effects. This condition affects nearly a third of people over 60 years of age, resulting in healthcare costs in excess of $18 billion yearly in the United States according to the World Health Organization. The disease is a combination of a number of pathophysiological issues stemming from age, inactivity, inflammation, oxidative stress, and insulin resistance resulting in the loss of both muscle mass and function (Walston, 2012). The European Working Group on Sarcopenia in Older People (EWGSOP) referred to sarcopenia as a condition characterized by the gradual decline in both muscle mass and strength occurring with aging that contributes to a poor quality of life and a heightened risk of disability and disease.

There are three stages of sarcopenia: pre-sarcopenia, sarcopenia and severe sarcopenia. The first stage begins with a slow decrease in muscle mass which soon results in the decreased strength in the second stage, and during the final stage both muscle mass and strength are severely affected resulting in poor physical performance (Doherty et al., 2003; Koopman, 2011; Lexell et al., 1988). This impacts other pathways, as skeletal muscle is involved in glucose uptake and glycogen storage, lipid oxidation, and immune response.

The reduction in physical activity due to this condition can contribute to obesity and insulin resistance, as sarcopenia and obesity share several pathophysiological mechanisms and may work synergistically (Choi, 2016). Developed countries have elderly populations that are growing rapidly and living longer, heightening the importance of identifying preventative therapies for age-related muscle loss and improving quality of life. As muscle loss and weakness progresses, there is a sharp increase in the likelihood of falls, metabolic diseases, cardiovascular diseases, compromised immunity, reduction in quality of life, and mortality rate.

Many mechanisms have been proposed for this muscle loss, including high levels of nuclear apoptosis, oxidative stress, and reduced satellite cell population resulting in hindered regenerative capabilities (Marzetti et al., 2012; Johnston et al., 2008; Buford et al., 2010). As previously mentioned, the reduction in muscle mass and strength with age has been attributed to the decrease the number and size of type II muscle fibers, especially in lower extremities (Nilwik et al., 2013). Another issue observed in aging and sarcopenic populations is an increase in the accumulation of reactive oxygen species (ROS). While ROS are a natural byproduct of metabolism, their excessive accumulation negatively impacts the sarcoplasmic reticulum, muscle fibers, and motor neurons, hindering the regenerative potential of muscle. In addition to hindering muscle mass, high levels of ROS have been associated with a reduction in grip strength in older populations of women (Fulle et al., 2004).

Because sarcopenia is a condition characterized by multiple factors, there are currently no effective therapies. Doctors suggest exercise, anti-inflammatory medications, a diet high in protein, hormone therapy, and nutritional supplementation, but there is limited success with these therapies (Greenlund and Nair, 2003). It is difficult to test the efficacy of many therapeutic strategies due to the lengthiness of aging in humans. Many intervention studies have been

performed as to the effects of physical activity on improving age-related dysfunction, but the studies were not long term and thus conclusions regarding long term improvements could not be made (van der Bij et al., 2002; Law et al., 2016). Use of rodent models is often utilized as their skeletal muscle composition is similar to that of humans and studies can be concluded quickly (Skalicky et al., 1996). The debilitating physical affects, high healthcare costs, susceptibility to other diseases observed in sarcopenia, growing aging population, and the lack of effective treatments at this time highlight the need for further research into therapeutic strategies for combatting sarcopenia.

Background on Skeletal Muscle

The system of muscles in the human body is responsible for movement, posture, the generation of body heat, and metabolic regulation. There are more than 700 muscles in the human body, accounting for nearly 50% of body mass. Skeletal muscle attaches to bone and creates a network of tissues, tendons, nerves, and blood vessels which work together to enable movement. Skeletal muscle has a low turnover rate because it is post-mitotic, but it has a high regenerative potential (Collins and Partridge, 2005). Skeletal muscle is highly susceptible to injury due to constant use. Luckily it has an efficient repair system which mirrors the stages of skin wound healing. The first stage is the inflammatory phase which consists of myofiber breakdown and migration of inflammatory cells to the site of interest, soon followed by the repair phase during which the necrotic myofiber undergoes phagocytosis and new muscle fibers are constructed (Järvinen et al., 2005). The final phase is one of remodeling, during which myofibers are reorganized and muscle returns to normal functionality. This repair and regeneration system is highly efficient, and also dependent upon satellite cells, myogenic stem

cells, and the availability of environmental factors within the satellite cell niche (Yin et al., 2013).

Plasticity of muscle is dependent upon its fiber type composition. Type I fibers are slow-twitch fibers, possessing a dense capillary system, high oxidative capacity and mitochondrial content, and are highly resistant to fatigue whereas type II fibers are characterized by reduced capillary density, fewer mitochondria, and generate ATP via glycolysis (Schiaffino and Reggiani, 2011). Muscles associated with endurance and posture, such as the soleus muscle, are comprised primarily of type I fibers, while muscles associated with power and strength like the quadriceps, are primarily composed of type II fibers. The gastrocnemius muscle has a mixed fiber type profile, but it is predominately type II fibers. However, these fibers can convert from one type to another in response to hormonal and growth factor signaling, such as through insulin-like growth factor 1, and exercise training (Glass et al., 2003; Goldspink, 2005). Reduction in size and number of type II fibers and their denervation contribute to the loss of muscle mass and strength observed in the sarcopenic phenotype (Miljkovic et al., 2015), so identifying strategies by for enhancing fiber type conversion in older populations is of great importance. The previously mentioned crosstalk between pathways involved in muscle maintenance and metabolic regulation provides insight into how skeletal muscle importance extends beyond movement. Muscle mass enables proper regulation of both protein metabolism and energy metabolism, so any imbalances in skeletal muscle homeostasis can result in improper function of the metabolic pathways and contribute to disease and obesity. Through identification of ways to maintain efficiency of these pathways with age, it will be possible to improve quality of life for the aging population.

Satellite Cells and Myogenesis

Genes responsible for myogenesis are active during development and childhood, but many of these factors enter a quiescent state in adulthood and activate only during periods of injury or muscle growth. There exists a regenerative cell network called satellite cells which have been identified as one of the primary contributors for the regenerative properties of muscle. Satellite cells are stem cells located between the sarcolemma and basal lamina of muscle fibers and contribute to the repair and regeneration of muscle tissue, possessing the ability to proliferate and differentiate upon muscle growth or injury. Once activated, satellite cells are destined for one of two endpoints: a path of self-renewal that will replenish the population in the satellite cell niche, or differentiation and muscle regeneration (Yin et al., 2013). Satellite cells remain in a dormant state until needed, expressing the paired box transcription factor 7 (Pax7) and neural cell adhesion molecule (CD56) until they are activated at which point they express both Pax7 and Myogenic Differentiation Factor (MyoD), the latter of which is crucial for myogenic differentiation (Snijders et al., 2015). At this stage, the majority of cells downregulate Pax 7 expression and continue the path of differentiation and myogenic regeneration while the other portion continue high to express Pax7 without expression of MyoD, exit the cell cycle and myogenic pathway and return to a dormant state in the satellite cell niche (Zammit et al., 2004; Nagata et al., 2006). Once cells have committed to becoming muscle precursors, they will express MyoD, Desmin, Myogenic Regulatory Factor 4 (Mrf4), and Myogenin, soon starting the process of fusing and regenerating the muscle (Yin et al., 2013). The myogenic regulatory factors (MRF) MyoD and Myogenic Regulatory Factor 5 (Myf5) are transcription factors responsible for differentiation of muscle precursor cells into myoblasts, while Myogenin and MRF4 stimulate the differentiation of myoblasts into myocytes (Le Grand and Rudnicki, 2007).

Myogenin continues to be expressed throughout myotube formation, and expression of Myf6 and Myosin Heavy Chain aid in the fusion of myotubes to form myofibers (Mathew et al., 2011). Satellite cells are not only important for the regenerative capabilities of injured muscle, but also for their ability to undergo a process of self-renewal and return to a dormant state as this is critical for maintaining an efficient muscle repair system throughout one's lifetime.

Successful myogenesis is dependent upon the interaction of many factors and pathway crosstalk, as myogenic factors MyoD, Myf5, MRF4, and myogenin must interact with transcription regulators, modification proteins, and signaling molecules for myogenic activation (Kitzmann and Fernandez, 2001). Satellite cell differentiation and proliferation involve a number of environmental growth factors found in the satellite cell niche, including platelet-derived growth factor, basic fibroblast growth factor, transferrin and hepatocyte growth factor (Chen & Quinn, 1992). Myogenic factors for muscle cells are also produced by the macrophages responsible for entering areas containing damaged tissue (Ceafalan et al, 2018). Some of these growth factors have been observed to stimulate satellite cell chemotaxis *in vitro* (Bischoff, 1997). Myogenesis requires highly regulated coordination between environmental factors, the immune system, and the PI3K-Akt pathway for efficient muscle repair, but age-associated dysfunction in these areas result in impairment of this mechanism.

Muscle regeneration is dependent upon satellite cells being both fully functional and available to work alongside of the myogenic regulator factors, as shown in Figure 4. The age-related physiological changes hinder the regenerative ability of muscle, which suggests that satellite cells may undergo functional alteration which reduces their sensitivity to environmental stimuli and their proliferative potential (Brack and Muñoz-Cánoves, 2016). Satellite cells undergo constant replenishment during muscle growth, however there is a substantial decline in

this supply with age (Bischoff, 1994). The extrinsic factors that promote the proliferation of these muscle precursor cells also appear to decline with age (Sabourin and Rudnicki, 2000). The mechanisms associated with this decline are still being investigated, though oxidative stress is believed to be a major contributor to satellite cell regenerative capabilities regardless of whether the cells are active our quiescent (Beccafico et al., 2007).

Aging Skeletal Muscle

Age is a known contributor to the reduced regenerative potential of skeletal muscle and its population of satellite cells. In older individuals, both satellite cell functional capacity and total numbers are reduced due to an impairment in activation and self-renewal mechanisms attributed to reduced sensitivity to environmental factors (McHugh and Gil, 2018). Function in aging satellite cells can be partially restored through exposure of these cells to juvenile environmental factors, but this cannot be maintained with onset of increasing loss of muscle mass as is observed in conditions like sarcopenia (Bengal et al., 2017). In the later stages of sarcopenia, environmental cellular stress levels are so high that satellite cell function is severely impaired and introduction of juvenile factors can no longer restore function (Yablonka-Reuveni, 2011).

Many of the regenerative issues with aging muscle are related to micro- and macro-environment, including a range of issues from chronic inflammation, reduced sensitivity to stimulatory factors, and impaired pathway signaling. Satellite cell function improvement was observed in cases where IGF-1 was overexpressed, and this increased expression led to an improvement in muscle regenerating in older mice (Musarò et al., 2004). Additionally, parabiosis experiments between young and old mice successfully restored age-related signaling impairments and restored stem cell regenerative capacity (Conboy et al., 2005). Through

identification of other factors associated with aging satellite cells and methods by which these populations can be improved, biomarkers associated with their decline can serve as therapeutic targets for sarcopenia.

SENESCENCE-ACCELERATED MOUSE PRONE 8 (SAMP8) MODEL

Background

The Senescence-Accelerated Mouse (SAM) consists of 18 unique lines 7 of which is senescent-resistant strains (SAMR) and the remaining 11 are mice prone to accelerated senescence (SAMP), both of which are commonly used for sarcopenia research (Table 1.2). A study performed in SAMP8 mice at 10 weeks, 25 weeks, and 60 weeks of age, found they had a shorter life span, and exhibited visual signs of aging (Guo et al., 2015). Established in 1981, the senescence-accelerated mouse is a research model originating through phenotypic selection from a genetic pool of AKR/J mice (Takeda et al., 1981). It was observed that certain littermates of AKR/J mice displayed senility at an early age and had a reduced life span. Mice from litters characterized by accelerated aging were selected to be used for the SAMP strain, and the mice from litters which exhibited a normal aging process were selected for the SAMR strain (Takeda et al., 1981; Miyamoto, 1997). Inbreeding was performed selectively in order to establish phenotypes centered on lifespan, senescence, and specific age-related disorders (Hosokawa et al., 1984).

The senescence accelerated mouse prone 8 (SAMP8) is known to have age-related learning and memory deficits which allows for examination into their impairments at the molecular level (Takeda et al., 1981). The lifespan of SAMP8 mice ranges from 10 to 17 months whereas the lifespan of SAMR-strain mice ranges from 19 to 21 months, enabling studies to progress much more quickly (Flood and Morley, 1998). The SAMR strain lifespan is comparable

to the commonly used C57BL/6J strain which has an average lifespan of 24 months (Graber et al., 2015). Additionally, the SAMP8 model is unique due to the low occurrence of other age-related ailments when the cognitive impairments develop (Flood and Morley, 1998).

Age-related impairments displayed by SAMP8 mice include learning difficulty, behavioral changes, noticeable differences in circadian rhythm, and high oxidative stress (Butterfield et al., 1997). Older SAMP8 mice display obvious learning impairment through foot shock avoidance testing (Flood and Morley, 1993), which is used to assess oxidative stress (Farr et al., 2016). These results are concordant with the free radical theory of aging that proposes modification by reactive oxygen species (ROS) on molecules contribute to age-related cellular dysfunction (Viña , 2013). Oxidative stress was decreased and memory was improved in SAMP8 mice through treatment with antioxidants, supporting the theory that oxidative stress contributes to many cognitive impairments and targeting ROS can lead to improved function (Farr et al., 2016).

Experimental Use

While Sprague Dawley rats had previously been used for aging research, they lack the comorbidities observed in sarcopenia and are therefore not an optimal model organism for researching the condition. SAMP8 mice exhibit characteristics of senescence, both physical and cognitive, making them a much more appealing model. SAMP8 mice are frequently used in studies looking to identify underlying mechanisms of dementia and other learning and memory deficits associated with the aging process (Yasui et al., 2002; Okatani et al., 2002). Beta amyloid protein expression is a significant contributor to the cognitive decline observed in aging SAMP8 mice, as seen with lowering these protein levels in conjunction with reducing reactive oxidative species slows this decline and improves learning ability (Manich et al., 2011). Furthermore,

Onishi et al. (2019) fed SAMP8 mice a high fat diet and determined that the addition of green tea extracts to their dietary intake improved memory, reduced beta amyloid accumulation, and attenuated other diet-induced dysfunctions seen in SAMP8 mice fed an exclusively high fat diet.

C57BL/6 mice have previously been used for age-related muscle atrophy research, but they did not prove to be an optimal model because loss of muscle mass did not occur until 18 months (Hamrick et al., 2006). SAMP8 mice experience loss of muscle mass around 7 or 8 months of age, validating their use as a model for sarcopenia research (Guo et al., 2015). Derave et al. (2005) investigated the use of SAMP8 and SAMP6 mice for skeletal muscle aging compared to a SAMR1 model, and found that male SAMP8 mice experienced muscular senescence nearly twice as fast as other mouse models, with age-related muscle alterations more pronounced in the hind limbs. The SAMP8 model is frequently utilized in Alzheimer's research because of their advanced cognitive senescence, but their accelerated lifespan and early onset of sarcopenia make SAMP8 well-suited model for age-related myogenic research.

Conclusion and Proposed Research

There are proven benefits of plant BR in the agricultural industry and recent exploration has delved into their potential benefits in mammalian systems, with BR administration in juvenile male animals was shown to increase muscle mass and strength. Sarcopenia is a condition characterized by the loss of muscle mass and strength with age, and it results in extensive healthcare costs and a heightened risk of cardiovascular and metabolic diseases. There is no proven therapy to treat sarcopenia, and treatments which are currently available are not only costly but have limited efficiency. Knowing the health-promoting properties of BR, it is important to test its ability to promote muscle regeneration in an aging model consisting of both male and female animals.

BR isolation is time consuming and expensive, requiring a substantial amount of starting material to isolate a small amount. However, it is known that BR content is highest in seeds and plants in the *Brassicaceae* family of cruciferous vegetables, therefore bok choy cabbage seed extract was selected as the treatment source and added to a low fat diet. The animal model selected was the senescence-accelerated prone 8 (SAMP8) mouse since they have been validated as a model for sarcopenia and experience physical and cognitive senescence at an accelerated rate. The mice will be fed one of three diets: a lean control, high fat, or a lean diet supplemented with 2% cabbage seed extract and will have their food intake, weight, and body composition measured throughout the trial and as their physical endurance tested at 6 separate occasions. Additionally, assessment of age-related senescent behaviors will be performed to determine if this diet reduces the appearance of senescent behaviors. Necropsy will be performed at 32 and 50 weeks and skeletal muscle will be collected for gene expression analysis in order to evaluate biomarkers associated with muscle regeneration and degradation as compared to mice fed a lean control diet.

Table 1.1 Brassinosteroid Content in *Brassicaceae* Plants

Plant	Tissue	Brassinosteroid Content (Dry Weight)	Reference
Brassica campestris	leaves	0.5 ng/kg	Adam and Marquardt (1986)
Brassica campestris	seeds	9.4 ng/kg	Adam and Marquardt (1986)
B. oleracea var. capitata	40mg dry juvenile tissue	264 ng/kg	Pavlović, et al. (2018)
B. rapa ssp. pekinensis	40mg dry juvenile tissue	424.44 ng/kg	Pavlović, et al. (2018)
B. oleracea var. acephala	40mg dry juvenile tissue	634.83 ng/kg	Pavlović, et al. (2018)
Brassica napus	pollen	1000 ng/kg	Adam and Marquardt (1986)

Table 1.2 SAMP Mouse Phenotypes (adapted from Takeda et al., 1999)

Strain	Phenotype
SAMP1	Impaired immune response, hearing impaired, hypertension
SAMP2	Impaired immune response, Senile amyloidosis, cataracts
SAMP3	Joint degeneration
SAMP6	osteoporosis, amyloid accumulation in organs
SAMP7	Lymphoma
SAMP8	Cognitive deficits, emotional disorder, reduced immune response
SAMP9	Cataracts, lymphoma, senile amyloidosis
SAMP10	Brain atrophy, Cognitive deficits
SAMP11	Senile amyloidosis

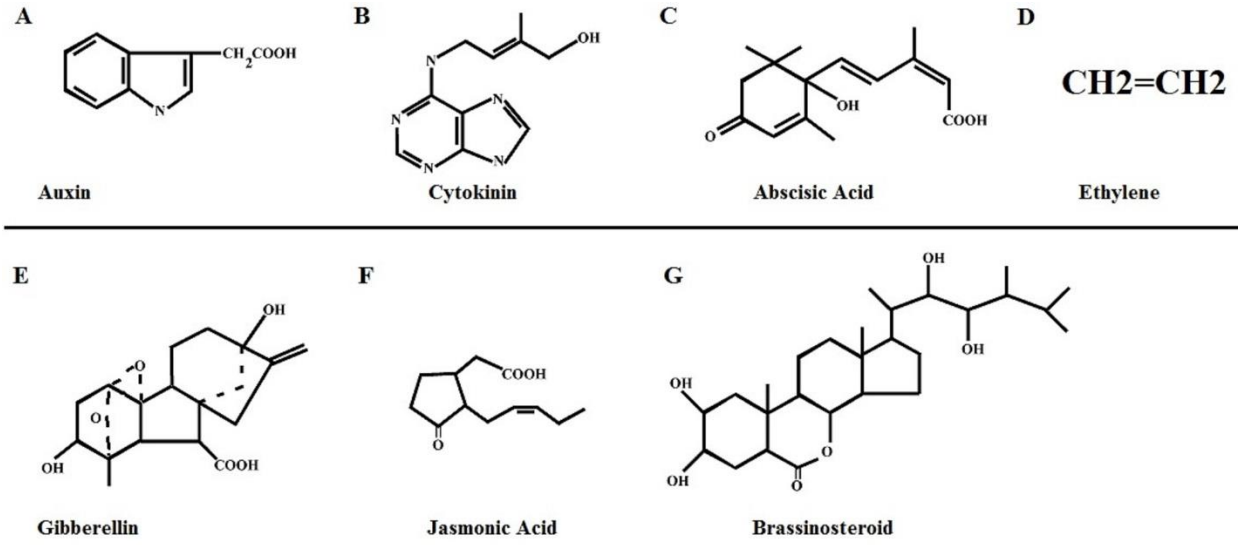

Figure 1. Phytohormone Structure. There are seven phytohormones that are responsible for plant growth, development, and immunity. Shown above are the structures of (A) Auxin, (B) Cytokinins, (C) Abscisic Acid, (D) Ethylene, (E) Gibberellin, (F) Jasmonic Acid, and (G) BR. BR structure differs substantially from that of the other five phytohormones, having a cholestane-based structure more closely resembling the animal sex hormones like testosterone and estrogen.

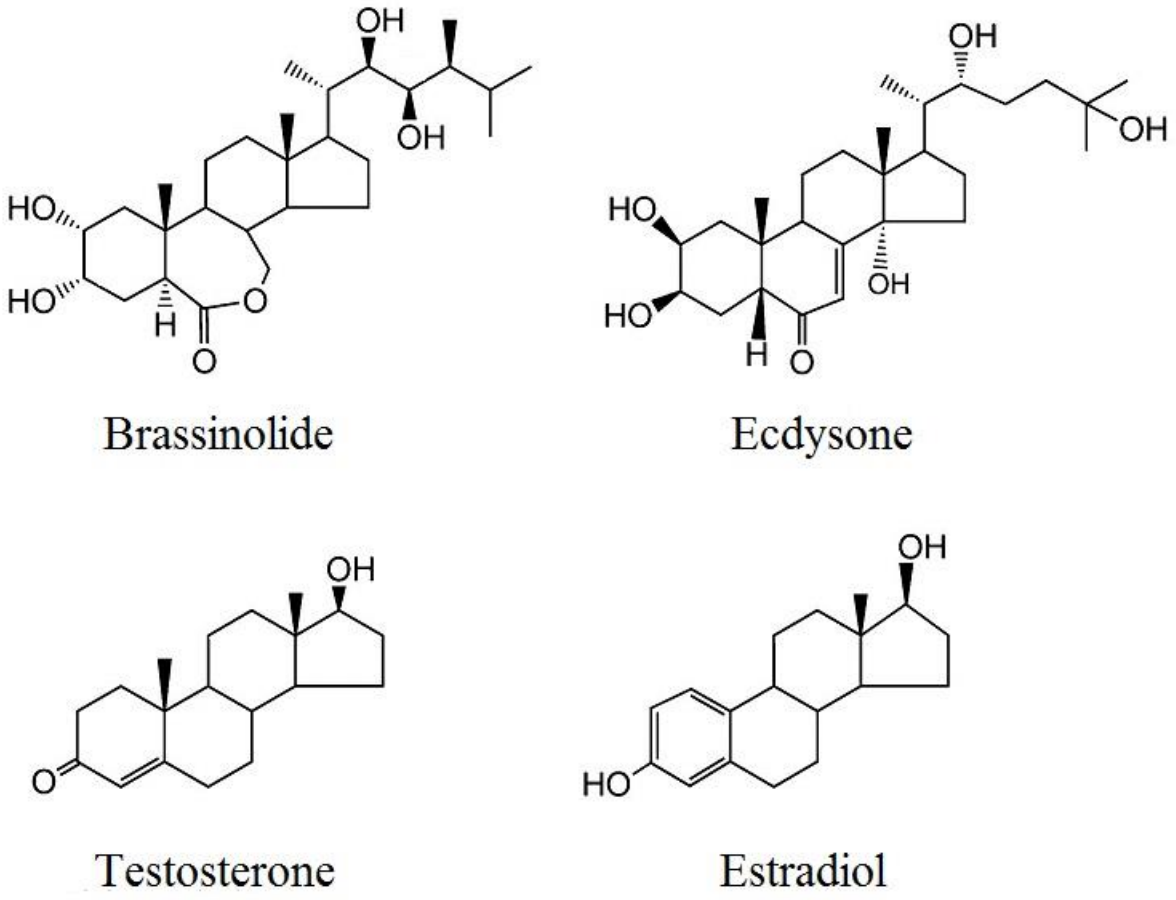

Figure 2. Brassinosteroids and Similarly Structured Hormones. Brassinosteroids (A) share the four-fused ring structure that is signature to animal and insect steroid hormones. (B) Insect hydroxyecdysone is a hormone crucial for insect molting. (C) Testosterone is both an anabolic steroid and a sex hormone in male animals. (D) Estradiol is the primary sex hormone in females during reproductive years.

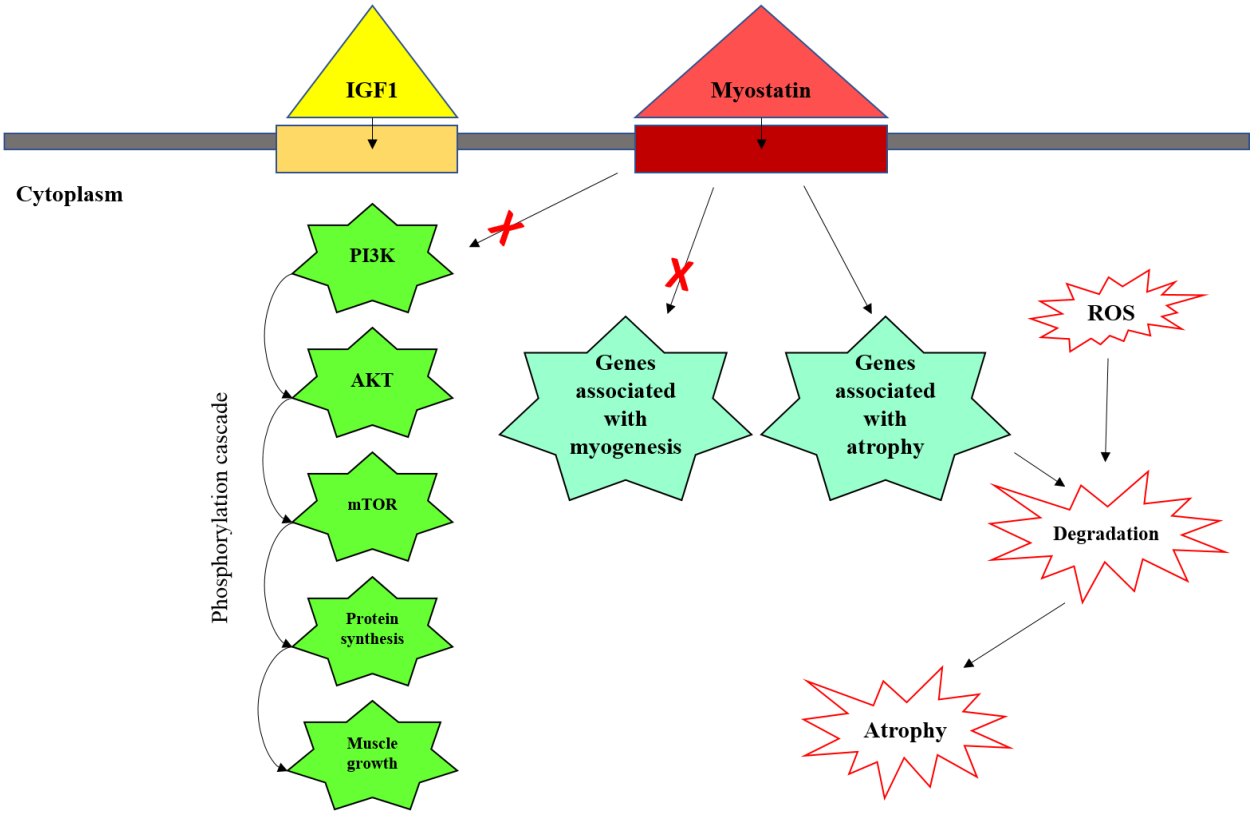

Figure 3. Muscle Growth and Atrophy via the PI3K/Akt Pathway (simplified). The binding of Insulin-like growth factor-1 to its receptor results in muscle growth through phosphatidylinositol 3-kinase (PI3K) and protein kinase B (Akt) signal transduction. Once Akt is activated by PI3K, a phosphorylation cascade occurs and activates mammalian target of rapamycin (mTOR) which is involved protein synthesis and ultimately results in growth of muscle. Myostatin, also known as Growth and differentiation factor 8(Gdf8), negatively regulates myogenesis through suppression of the P13K/Akt pathway. Once myostatin binds to its receptor, it can suppress myogenic gene transcription while activating proteolytic genes such as Atrogin-1 and Trim63. Muscle atrophy can be induced through this pathway, or through protein degradation caused by reactive oxygen species (ROS). Activation of the PI3K/Akt pathway inhibits the suppressive capabilities of Myostatin.

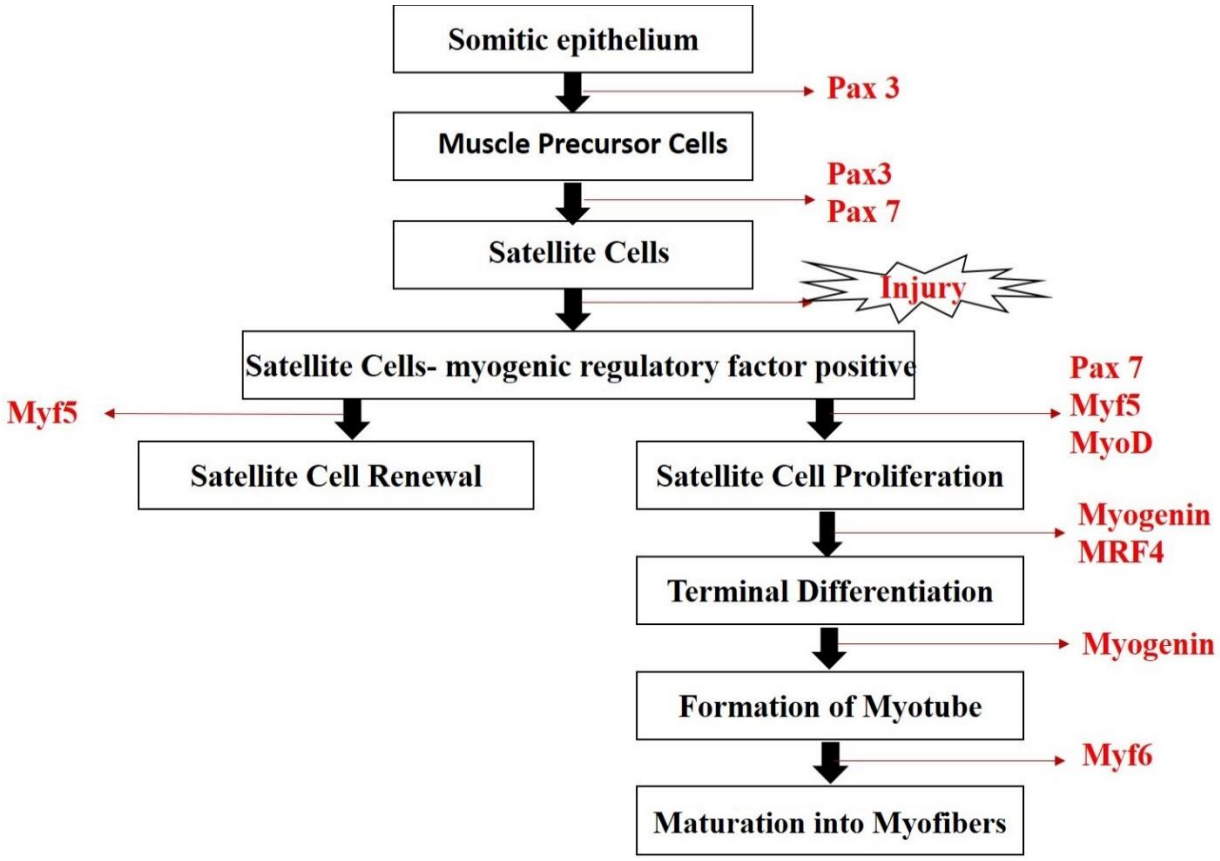

Figure 4. Satellite Cell Activation during Muscle Development and Regeneration. Satellite cells remain in a quiescent state in the niche beneath the sarcolemma until activation upon injury. During development, Pax3 is required for muscle precursor cells to migrate from the somite. Satellite cells express Pax3 and Pax7 during both quiescence and activation, though at activation they begin to express MyoD. Pax7 also plays a role in specification of satellite cells, some of which will be destined for muscle regeneration while others will self-renew and return to the niche for later use. The myogenic regulatory factors (MRF) MyoD and Myf5 are transcription factors that are responsible for differentiation of muscle precursor cells into myoblasts. Myogenin and MRF4 stimulate the differentiation of myoblasts into myocytes. At this stage, cells also express myosin heavy chain which is involved in the maturation of myotubes. Myogenin continues to be expressed throughout myotube formation, and Myf6 expression aids in the maturation of myotubes to form myofibers.

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CHAPTER 2

Protective Effects of Cabbage Seed Extract in an Accelerated Aging Model

ABSTRACT

Brassinosteroid (BR) is a phytohormone responsible for regulating a number of essential processes in plants including growth, development, and immunity. Their properties are often utilized in agriculture to enhance crop yield, improve crop quality, and reduce pesticide toxicity. All plants contain BR, though the largest amounts are found in seeds and juvenile tissues. Recent research has suggested therapeutic benefits of BR in animals by identifying the ability of plant BR to accelerate wound healing, stimulate protein synthesis, and improve athletic performance in juvenile male animals. Based on this data, an experiment was performed to evaluate the effects of cabbage seed extract incorporated into a lean diet on the physical fitness of an accelerated aging population of male and female mice. Food intake, body weight, endurance, and body composition were monitored throughout the trial period. Mice fed a diet supplemented with cabbage seed extract (CSE) showed improved physical endurance and a significant increase in lean muscle mass when compared to the control group. Age-related behavioral changes were assessed, and CSE mice showed fewer symptoms of cognitive decline associated with senescence as compared to mice fed a strictly low fat diet. Muscle tissue was harvested for gene expression analysis in order to identify biomarkers for satellite cells and genes associated with myogenesis. Both male and female CSE mice had a significant increase in satellite cell and myogenic marker expression in gastrocnemius muscle, and females had significantly reduced expression of proteolytic markers. These results suggest that cabbage seed extract may contribute to improved physical fitness and skeletal muscle regeneration in aging mice, and additionally may serve as a possible therapeutic option for the age-related loss of muscle mass and strength observed in sarcopenia.

INTRODUCTION

The elderly population is increasing rapidly each year, with numbers estimated to reach 2 billion by 2050 according to a 2012 United Nations report. With an aging society comes increased instances of impaired physical performance as well as a hindered ability to perform routine tasks, such as walking at a moderate pace, standing up, and lifting objects weighing more than ten pounds. This impairment often results in hospitalization, frequent falls, co-morbidities, and poor quality of life. Sarcopenia, or age-related loss of muscle and strength, is a signature characteristic of the aging process. This condition is difficult to treat because there are many underlying factors including hormone imbalance, impaired immune system, chronic inflammation, genetics, and lifestyle. These aggravating factors result in a vicious cycle of physical disability and other health problems, which further exacerbate muscle loss.

Sarcopenia is a primary cause of disability in the elderly population within the United States, affecting more than half of people over 50 years of age and resulting in healthcare costs exceeding $18.5 billion dollars each year (Beaudart et al., 2014). Medical recommendations focus on lifestyle changes such as increased activity levels and consuming a healthy diet, but these serve as temporary solutions and do not improve muscle health long-term. Therefore it is of great importance to identify methods for prevention of age-related muscle loss as well as solutions for improving muscle health upon the onset of conditions resulting in muscle loss.

Anabolic steroids are known to stimulate muscle growth, but their use often results in unwanted androgenic side effects like weight gain, acne, and male pattern baldness. Plant brassinosteroids (BR) are similar in structure to animal hormones, such as estradiol and the anabolic hormone testosterone; however, they do not bind to androgen receptors, and therefore do not elicit an androgenic response (Esposito et al., 2011). Brassinosteroid is a phytohormone

involved in regulation of essential processes including growth, cell division, immunity, and stress response. Most BR research has focused on their activity in plants due to their beneficial effects in the areas of crop growth, quality, and yield. Because they exhibit numerous agricultural benefits, BR activity in plants has been thoroughly investigated over the years, enabling new BR compounds to be identified and utilized. Until the last decade, little research had been conducted regarding their effects in cell and animal models.

Recently, many BR compounds have been investigated and found to have a number of potential benefits for human and animal health. Epibrassinolide, a BR compound often used in agriculture, has been shown to have anti-proliferative and apoptosis-inducing effects in various cancer cell lines, including prostate cancer, colon cancer, and small cell lung carcinoma (Obakan et al., 2015; Obakan-Yerlikaya et al., 2017; Sadava et al., 2017). Homocastasterone is another BR compound that has been shown contribute to a number of health benefits, including lower blood glucose, cholesterol, and triglyceride levels in male animals (Athithan et al., 2017). Homobrassinolide, a naturally occurring BR, exhibits low cytotoxicity and its administration in keratinocytes and murine fibroblasts resulted in improved cell migration and proliferation (Esposito et al., 2013). Topical administration of Homobrassinolide was found to improve cutaneous wound healing in male animals and oral administration was found to contribute to improved grip strength and lean muscle mass in juvenile male animals (Esposito et al., 2013; Esposito et al., 2011). However, these studies were performed solely using young male animals, so effects have not yet been identified in female animals nor in aging animals.

Rodents are often the animal model of choice in skeletal muscle studies, but these trials can become exceedingly expensive and time-consuming due to the average lifespan of the animals exceeding 2 years (Skalicky et al., 1996). Therefore, having an animal model that

exhibits the physical characteristics of aging at an accelerated rate would contribute greatly to the pace of research while also saving time and resources. Though the idea of transgenic mice has been proposed, the aging process cannot be replicated by knocking out or altering a gene due to there being a multitude of factors which contribute to the aging phenotype (Kujoth et al., 2005; Mounkes et al., 2003). The senescence-accelerated mouse (SAM) serves as an excellent model for aging studies because they exhibit a number of different age-associated pathologies dependent upon their strain and a shorter than average lifespan (Takeda, 1999). The SAMP-8 strain shows early cognitive decline and has a doubled senescence score at 8 months compared to the strains resistant to premature senescence in addition to a 40% shorter median lifespan (Takeda, 1999). Derave et al. (2005) found that SAMP8 animals displayed a substantial decrease in type-II muscle fibers, a sharper decline in muscle mass at 60 weeks compared to other SAMP strains, and exhibited muscular senescence nearly twice as fast as other animal models.

The purpose of this study is to determine the effects of dietary supplementation of cabbage seed extract (CSE) on animal body weight, body composition, endurance, and muscle health profile in an aging SAMP8 population. It was hypothesized that both male and female mice fed the CSE diet would exhibit improved physical fitness and fewer physical characteristics associated with the aging process as compared to the control groups. Additionally, it was hypothesized that animals fed the experimental CSE diet would have increased expression of markers associated with muscle regeneration and satellite cell activation based on previous work in which BR administration resulted in enhanced Akt activity, thereby increasing protein synthesis. Hence, the overall objective of this study was to evaluate the effects of CSE on the physical fitness and gastrocnemius muscle profile of an accelerated aging population of male and female animals.

MATERIALS AND METHODS

Animals and Diets

All animal experiments were performed according to procedures approved by the NC Research Campus Institutional Animal Care and Use Committee (Protocol 18-0078) in the David H. Murdock Research Institute, an AAALAC accredited animal care facility. The breeding colony of Senescence Accelerated Prone 8 Mice (SAMP8) mice was donated by Dr. Gregory Henderson of Rutgers University. The original breeding pair was purchased from Harlan Laboratories in 2009. The colony is maintained at the David H Murdock Research Institution CLAS animal facility in Kannapolis, NC. The animals were bred to generate multiples litters. Animals (n=48) were given a regular chow diet until 28 days of age, after which they were randomly allocated to one of three treatment groups for experimental purposes. Mice were randomized into groups of n=16 per diet, consisting of 8 females and 8 males. All animals were given *ad libitum* access to Research Diets (New Brunswick, NJ, USA): low 10 kcal% fat diet D12450J (Lean, 3.8 kcal/g); low 10 kcal% fat diet D12450J supplemented with 2% cabbage seed extract (CSE, 3.8 kcal/g); or high 60 kcal% fat diet D12492 (High Fat, 5.2 kcal/g). Mice were housed four animals of the same sex per cage under controlled temperature (24 ± 2 °C) and light under conventional conditions with a 12-hour light/dark cycle with lights turned on at 7:00 a.m. Animal weight and average food intake (animals were group fed) were recorded weekly for the duration of the study, and food spillage was accounted for. Animals were monitored to ensure adequate food and water intake without signs of adverse effects, such as >15% body weight loss or behavioral signs of pain and distress including vocalization, restlessness, lack of mobility, and abnormal resting pose. All animal diets were kept at −20 °C for long-term storage and stability, and freshly thawed food was dispensed to animals every 3–4 days.

Cabbage Seed Extraction for Animal Diet

The source of the cabbage seed was *Brassica rapa* var *pekinensis* seeds (bok choy), donated by Sakata Incorporated (Sacramento, CA). The extraction process, shown in Figure 1, began with weighing out 200 grams of cabbage seeds and grinding them until a homogeneous powder was obtained. The powder was then transferred to a 1 liter glass beaker, defatted with 400mL of hexane, and placed in an incubator-shaker set to room temperature and 150 RPM. The hexane was decanted into a new beaker, evaporated overnight, and the remaining oil was discarded. The beakers containing plant material were occasionally mixed and left in the hood overnight for hexane to evaporate. After evaporation, 200 mL of distilled water was added to the beaker containing plant material and shaken at 150RPM for 1 hour at room temperature. The beakers were then placed in an 80°C water bath for 2 hours. Excess water was poured off, and plant material was placed in an aluminum foil pan and frozen overnight at -20°C. This material was then freeze dried for 3 to 5 days until a dry powder was obtained. The final product was sent to Research Diets (New Brunswick, New Jersey, USA) to incorporate into a low fat animal diet as mentioned previously. All dietary formulations are shown in Table 2.1.

Body Weight and Food Intake

Body weight was measured weekly for each animal. Animals were separated by sex and housed 4 per cage. Food intake measurements were performed weekly for each cage. Food waste was accounted for by separating the cage bedding from unconsumed food.

Body Composition

Body composition was assessed in live, unanaesthetized mice using EchoMRI™ software. This software provided measurements for lean mass and fat mass for each mouse. The EchoMRI was calibrated each day prior to use. Two measurements were taken per animal

throughout the trial: an initial measurement at the beginning of the trial (week 1) and final measurement taken at week 30 to assess changes in lean and fat mass.

Treadmill Performance

Endurance exercise capacity was determined by using a Columbus Instruments rodent treadmill with a shock grid set at <0.34 mA and 1 Hz. Mice were placed into individual compartments and allowed to acclimate for two minutes prior to beginning the endurance test. The speed was set to 16 cm/second and the mice were allowed to warm up for two minutes before the speed was increased by increments of 2 cm/sec every two minutes until a maximum speed of 24 cm/sec was reached. The treadmill intensity was set to 10 and there was no incline during the test. If the animal failed to move during the first two minutes, it was removed. Upon receiving three electrical stimuli from the shock grid, the test was terminated and the animal was removed from the treadmill. The animals were given a maximum of 20 minutes to run. This test was performed at 6 time points throughout the trial based on animal age: 11 weeks, 19 weeks, 30 weeks, 36 weeks, 40 weeks, and 49 weeks.

Senescence Assessment

Degree of animal senescence, or appearance of age-associated behaviors, was assessed based upon the behavioral grading system established by Takeda et al. (1981) specifically for senescence accelerated mice, shown in Table 2. Reactivity and passivity are two categories by which behavioral senescence is determined. Mouse reactivity was determined individually through thirty second behavioral observations of their mobility and energy level. Animals with normal behavior were assigned a score of 0, and mice showing no activity received a score of 4, which was indicative of severe senescent behavior. Passivity was measured individually by lightly pinching the nape of the neck and observing any escape reactions. If no reaction occurred,

the mouse was held by the forelimb in an attempt to elicit a reaction. A mouse with a normal escape response was assigned a score of 0, and a mouse that gave no escape response to either stimuli received a score of 4. The grade of each item has a clear definition, and the overall score represents the degree of senescence.

Necropsy and Tissue Collection

There were two time points at which animal necropsies were performed. The first was at 32 weeks, at which time 4 animals from each group were euthanized and tissues were collected. The second was at 50 weeks, at which time the remaining 4 animals per group were euthanized and tissues were collected. At the time of sacrifice, mice were fasted for a 6 hour period and then euthanized by CO2 inhalation. Whole blood was collected through percutaneous cardiac puncture. Liver, adipose tissue, and skeletal muscle tissue including gastrocnemius, soleus, tibialis, extensor digitorum longus (EDL), and quadriceps were collected, weighed, and immediately snap frozen in liquid nitrogen. The tissues were then stored at -80℃ until further analyses were performed.

Total RNA Extraction, Purification, and cDNA Synthesis

The total RNA was isolated from control and CSE mice gastrocnemius muscle tissue using TRIzol reagent (Life Technologies) following the manufacturer's instructions. RNA was quantified using the Synergy H1/Take 3 spectrophotometer (BioTek, Winooski, VT, USA). The cDNAs were synthesized using 2 μg of RNA for each sample using a commercially available high-capacity cDNA Reverse Transcription kit (Life Technologies), following the manufacturer's protocol on an ABI GeneAMP 9700 (Life Technologies).

Housekeeping Genes and Biomarkers for Quantitative Real-Time Polymerase Chain Reaction

Housekeeping genes were selected based on past success in mouse skeletal muscle. Three housekeeping genes, ribosomal protein L13a (RPL13a), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and hypoxanthine phosphoribosyltransferase (HPRT1), were initially selected and tested, with RPL13a being chosen for normalization of data. Biomarkers associated with satellite cells, myogenesis, and protein degradation were selected for gene expression analysis in the SAMP8 gastrocnemius muscle: Paired box protein-3 (*Pax3*), Paired box protein-7 (*Pax7*), Growth and Differentiation Factor 8 (*Gdf8*; also known as *Myostatin*), Myogenic Factor 5 (*Myf5*), Myogenin (*MyoG*), Myogenic Differentiation 1 (*MyoD1*), Tripartite Motif containing 63 (*Trim63*), and F-box only protein 32 (*Fbxo32*). All primer forward and reverse sequences are list in Table 3**.**

Quantitative Real-Time Polymerase Chain Reaction Analysis

The resulting cDNA was amplified in duplicate by real-time quantitative PCR using PowerUP SYBR green PCR Master Mix (Life Technologies). To avoid interference due to genomic DNA contamination, only intron-overlapping primers were selected using Primer Express version 2.0 software (Applied Biosystems, Foster City, CA, USA). Quantitative PCR (qPCR) amplifications were performed on an StepOnePlus Real-Time PCR System (Life Technologies, Carlsbad, CA, USA) using 1 cycle at 50 °C for 2 minutes and 1 cycle of 95 °C for 10 minutes, followed by 40 cycles of 15 seconds at 95 °C and 1 minute at 60 °C. The dissociation curve was completed with 1 cycle of 1 minute at 95 °C, 30 s at 55 °C, and 30 s at 95 °C. Gene expression was analyzed using the ΔΔCT method and normalized with respect to the expression of the housekeeping gene RPL13a using 7500 Fast System SDS software v1.3.0 (Life Technologies). A value less than 1 indicates transcriptional down-regulation (inhibition of gene

expression) compared with that of the control diet, which shows maximum genetic induction. Values greater than 1 imply overexpression of particular genes. Amplification of specific transcripts were further confirmed by obtaining melting curve profiles.

Statistical Analyses

Analyses were performed using Prism 8.0 (GraphPad Software, San Diego, CA, USA). Body weight and feed intake were analyzed separately for male and female mice. Data were analyzed by two-factor repeated-measures ANOVA, with time and treatment as independent variables. Endurance was analyzed by 2-Way ANOVA with time and treatment as independent variables followed by Dunnett's post-hoc test. Gene expression data were analyzed using two-way ANOVA with treatment and biomarker as independent variables, followed by Dunnet's post-hoc test. Data for body weight, feed intake, and endurance were also analyzed weekly using one-way ANOVA followed by Tukey's multiple comparisons test. All other data were analyzed using one-way ANOVA followed by Dunnett's post-hoc test unless otherwise noted. Male and female data were analyzed separately. The experimental unit is the cage of animals, as all mice were group fed and separated by sex in 4 animals per cage. All data are presented as means ± SEM. Significant differences were accepted when the *P* value was <0.05.

RESULTS

Body Weight and Food Intake

Body weight results are shown in figures 2A and 2B. There were no significant differences in body weight between lean males and CSE males, but high fat males had significantly higher weights than lean and CSE males between weeks 16 and 31 (Table 2.4). A significant difference in body weight in CSE females compared to lean females was only seen at week 38 ($p=0.0195$), but high fat females exhibited a significantly greater weight than lean

females from week 6 to week 50 (Table 2.5). Feed intake is shown in Figure 3. There were no significant differences in feed intake between lean males and CSE males, but increased intake was observed in the high fat males compared to lean males, at a few timepoints (shown in Table 2.6). Similarly, no significant differences in feed intake were observed between lean and CSE females, but high fat females had significantly greater intake periodically between weeks 5 and 15 as shown in Table 2.7.

Body Weight Gain and Feed Efficiency

Body weight gain and feed efficiency data are shown in Figure 4. Figures 4A and 4C show male and female body weight gain, respectively. Figures 4B and 4D show male and female feed efficiency. Feed efficiency was calculated as (body weight gain/food intake) × 100 ratio. (∗) $P < 0.05$ versus respective lean control diet. Shown in Figure 4A, CSE males did not have any significant difference ($p=0.0465$) in body weight gain as compared to lean control animals, but high fat male body weight gain was significantly greater than the control males ($p= 0.0704$). As figure 4C shows, CSE females did not have any significant difference ($p= 0.9611$) in body weight gain as compared to lean control animals, but high fat female body weight gain was significantly greater than the control females ($p= 0.0001$). Feed efficiency from weeks 1 through 32 are shown in figures 4B and 4D. Male feed efficiency is shown in figure 4B. There were no significant differences ($p>0.05$) in feed efficiency in CSE males or high fat males compared to lean control (Figure 4B). There was no difference in feed efficiency between CSE females and control females ($p= 0.9903$), but high fat female feed efficiency was significantly greater ($p= 0.0295$) than the female control group.

Body Composition: Lean Mass

Body composition was measured using EchoMRI in order to determine fat mass and lean mass changes between weeks 1 of the trial to week 30. Changes in lean mass are shown in Figure 5. Male mice on both CSE and high fat diets showed no significant changes in lean body mass by week 30 as compared to lean control males (Figure 5A). Lean males had an average lean mass increase of 1.657%, CSE males had a 5.147% increase, and high fat males had a 6.07% increase between weeks 1 and 30 (Figure 5B). Lean mass changes in female mice is shown in Figure 5C. CSE females showed a significant increase in lean mass ($p= 0.0013$) compared to lean control females at week 30, and high fat females also showed a significant increase in lean mass ($p=0.0016$) compared to lean females. Lean females had an average lean mass increase of 4.058%, CSE females had a 9.527% increase, and high fat females had a 5.335% increase between weeks 1 and 30 (Figure 5D).

Body Composition: Fat Mass

Changes in fat mass between weeks 1 and 30 are displayed in Figure 6. CSE males showed no significant difference in fat mass ($p= 0.0574$) compared to lean males at 30 weeks, but high fat males showed an increase in fat mass ($p= 0.0009$) compared to the lean control group. Lean males had an average change in fat mass of -1.22%, CSE males had a 2.34% increase, and high fat males had a 2.15% increase between weeks 1 and 30 (Figure 6B). Changes in fat mass in female mice are shown in Figure 6C. CSE females did not differ significantly from lean females at week 30 ($p= 0.2492$), but high fat females showed a significant increase ($p=0.0002$). Lean females had an average lean mass increase of 4.673%, CSE females had a 1.167% decrease, and high fat females had an 11.40% increase between weeks 1 and 30 (Figure 5D).

Treadmill Performance

Endurance was assessed 6 times throughout the trial at weeks 11, 19, 30, 36, 40, and 49 using Columbus Instruments rodent treadmill, and results are shown in Figure 7A for male mice and Figure 7B for female mice. Until week 30, there were 8 animals of each sex per treatment group. After week 30, this number was reduced to 4 animals of each sex per treatment group. At week 11, CSE males ran for a longer period ($p= 0.0069$) than control males. At week 19, CSE males ran longer ($p= 0.0044$) while high fat males ran for a significantly shorter period of time ($p= 0.0118$) than control males. The same trend was observed at week 30, with CSE males running for longer ($p= 0.0182$) and high fat males running for a shorter period ($p= 0.0158$) of time compared to lean males. At week 36, there were no notable differences in running time between CSE and lean males ($p= 0.1220$), but high fat males again ran for a shorter time than the control group ($p= 0.0028$). At week 40 no differences were seen in average run time of CSE males ($p= 0.6788$) and high fat males ($p= 0.0891$) compared to lean males. At week 49, no significant differences in average running time were observed in either CSE ($p= 0.8910$) or high fat ($p= 0.3897$) males compared to control. Weekly statistical analyses of male animals is displayed in Table 2.8.

At week 11, no difference was observed in average running time between CSE females and control ($p= 0.9286$), but high fat females ran for a significantly shorter period of time ($p= <0.0001$). At week 19, CSE females ran longer ($p= 0.0450$) while no differences were observed between high fat and control ($p= 0. 0.2673$). At week 30, CSE females ran longer period of time ($p= 0.0363$) while the high fat group ran for a much shorter time ($p= 0.0001$) compared to the lean control group. This trend was again seen at week 36, with CSE females running for longer ($p= 0.0053$) while high fat animals ran shorter ($p= 0.0153$) than the lean group. At week 40 no

differences were seen in average run time of CSE females (p= 0.7647) and high fat males (p= 0.0817) compared to lean males. Again at week 49, no significant differences in average running time were observed in either CSE (p= 0.2454) or high fat (p= 0.1417) females compared to control. Weekly statistical analyses of female endurance data is displayed in Table 2.9.

Senescence Assessment

Figure 8 shows the results of the senescence assessment which measured behavioral changes associated with aging. The two behaviors analyzed were reactivity (activity level) and passivity (natural escape reaction) and these behaviors were scored at 32 weeks of age and again at 47 weeks age for each mouse (4 per sex per group at this time). Figure 8A shows the results of male reactivity assessment. At 32 weeks of age, neither CSE males nor high fat males scored significantly different from control animals (p=0.0613 and p=0.1016, respectively). Reactivity measurements at 47 weeks were similar, with CSE males scoring significantly lower (p= 0.0175) and high fat males not differing by much (p= 0.9439) compared to lean male mice. Female reactivity data is shown in Figure 8B. At 32 weeks of age, no significant differences in reactivity were observed in either CSE females (p= 0.8038) or high fat females (p= 0.0690) as compared to the lean control group. Similar results were seen in reactivity measurements at 47 weeks, with neither CSE females (p= >0.9999) nor high fat females (p= 0.4395) differing significantly from lean female mice. Male animal passivity data is shown in Figure 8C. At 32 weeks of age, no significant differences in passivity were observed (p= 0.5360) in male CSE mice, but high fat mice scored higher on average (p= 0.0115) compared to lean control. At 47 weeks, CSE males scored significantly lower (p= 0.0113) than lean males, but high fat males on average did not differ from the lean control (p= 0.9308). At 32 weeks of age, neither CSE nor high fat females different significantly from lean control (p= 0.9916 and p= 0.6238, respectively), and similar

results were observed at weeks 47 with neither CSE nor high fat female passivity scores differing substantially from lean control females (p= 0.6238 and p= 0.9511, respectively).

Gene Expression Analysis of Gastrocnemius Muscle

Figure 9 shows the gene expression analysis of gastrocnemius muscle from mice fed either the lean control or CSE diet. Gastrocnemius tissues were collected at two time-points: 32 weeks and 50 weeks of age. Analysis of tissue from male CSE mice at 32 weeks (Figure 9A) shows significantly elevated expression levels of *MyoG* ($p<0.0001$), *Pax3* ($p<0.0001$), *MyoD* ($p=0.0002$), and *Fbxo32* ($p=0.0031$), with a 2-fold increase in MyoG expression and a nearly 4-fold increase in Pax3 expression compared to the 32 week old control group. CSE males at 50 weeks had significantly elevated expression of *Gdf8* ($p=0.0023$) and Pax7 ($p=0.0011$), as shown in figure 9B. Analysis of tissue from female CSE mice at 32 weeks (Figure 9C) showed significant upregulation of MyoD ($p <0.0001$), Fbxo32 ($p <0.0001$), Pax3 ($p <0.0001$), and Pax7 ($p=0.0004$). The CSE female gastrocnemius at 32 weeks had a 2-fold increase in MyoD and Pax3 expression. Data from analysis of CSE females at 50 weeks of age showed significantly increased expression of Pax3 ($p <0.0001$) and a significant decrease in proteolytic markers Trim63 ($p=0.0136$) and Fbxo32 ($p=0.0072$). Compared to the female control group, CSE female gastrocnemius showed nearly a 0.5-fold decrease in expression of Trim63, 0.7-fold decrease in Fbxo32, and a 2-fold increase in Pax3 expression.

DISCUSSION

Brassinosteroids (BR) are found ubiquitously throughout the plant kingdom, with highest concentrations found in seeds, pollen, and juvenile tissues. They are essential for normal plant development and are a key component of plant defense systems. Structurally more similar to animal hormones than other phytohormones, BR have been found to elicit an anabolic response

through modulation of the PI3K/Akt Pathway resulting in protein synthesis and an increase in muscle mass. Age-associated loss of muscle mass is one of the greatest contributors to physical disability with age, with sarcopenia being a significant health and economic burden among individuals over 60 years of age. The economic toll of treating the symptoms of this condition as well as the cost of hospitalizations and subsequent care after fall injuries is a major economic burden, surpassing $18.5 billion each year just in the United States (Fuggle et al., 2017). This study aimed to investigate the effects of a lean diet supplemented with 2% cabbage seed extract (CSE) on endurance, body composition, progression of senescent behaviors, and biomarkers associated with satellite cells, myogenesis, and protein degradation in the gastrocnemius muscle.

Previous studied performed by Esposito et al. (2011) highlighted the ability of dietary supplementation of Homobrassinolide over a 24 day period to increase lean mass and gastrocnemius muscle size in juvenile male animals. Similar results were also achieved through ecdysteroid administration leading to an increase in protein synthesis in skeletal muscle cells through activation of the Akt pathway (Gorelick-Feldman et al., 2010). The mechanism behind these alterations was attributed to brassinosteroid-enhanced stimulation of the protein kinase B (Akt) pathway, which is involved in maintaining the balance of protein synthesis and degradation and normal myogenic activity (Esposito et al., 2011). Our results suggest that a 10% low fat diet supplemented with 2% bok choy cabbage seed extract contributed to a healthy body weight, improved body composition, improved endurance, and a healthy muscle profile in an accelerated aging animal model as compared to animals fed a 10% low fat diet.

Our findings demonstrated that the addition of 2% CSE did not result in any significant differences in body weight or food intake compared to the mice fed the low fat control diet, but animals on a 60% high fat diet did exhibit significant increases in body weight throughout the

trial period, specifically between weeks 16 and 31 (Figure 2A, Table 2.4). Similar results were observed with female animals, as overall there were no notable differences between body weight between female control and CSE diets, but those on the high fight diet had higher body weights throughout most of the trial period (Figure 2B, Table 2.5). Feed intake results were similar between lean control and CSE diets for both male and female animals (Figure 3), but high fat feed intake for both sexes showed significant differences (Table 2.6 and Table 2.7). This is similar to body weight and feed intake results observed by Esposito et al. (2013) after oral administration of homobrassinolide to a normal, low fat chow diet provided to juvenile male animals.

Our data confirmed that while no differences were noted in male animals, female SAMP8 mice fed a long-term diet of CSE experienced significant improvements in lean body composition. A previous study investigated the effect of oral administration of 10mg/kg 20-Hydroxyecdysone on body composition, body weight, and blood glucose in obese animals and found that over 13 weeks, animal weights lowered, fat mass was reduced, and blood glucose levels improved through hydroxyecdysone modulation of the PI3K/Akt pathway (Kizelsztein et al., 2009). From the start of the trial to the end (50 weeks), CSE males had a 5.1% increase in lean body composition, high fat males had a 6% lean mass increase, and control animals showed a 1.6% increase. CSE females on the other hand showed a significant increase in lean mass compared to control females, with a 9.5% increase in lean mass during the trial whereas control females had a 4.1% increase and high fat animals had a 5.3% increase during this time. The most striking detail in these results is that while the CSE females had a significant increase in lean mass, they also experienced a decrease in fat mass during this period without significant changes in body weight. Both the control and high fat females had fat mass increases of 4.6% and 11.4%,

respectively. No notable differences in fat mass were observed in any of the male animals. The results show a clear difference in response between the two sexes, confirming the importance of including female animals in studies such as this.

The SAMP8 mouse model is often used for Alzheimer's research due to their rapid cognitive decline with age, but they also exhibit behavioral changes associated with senescence such as diminished activity and reduced escape behaviors (Miyamoto, 1997). Takeda et al. (1981) developed a protocol specifically for SAMP8 mice to assess the severity of these senescent behaviors, and this method was used to rank behavioral differences between treatment groups. Male CSE mice scored significantly lower in the area of animal reactivity compared to control and high fat males at both time-points, and they again showed significantly lower changes in passivity behavior compared to other treatment groups. No significant differences were observed between female animal groups in either reactivity or passivity behaviors at any time-point. Cognitive decline has been attributed to a number of factors including an increase in oxidative stress, and interventions with natural antioxidant compounds such as resveratrol have been shown to improve this state (Lin et al., 2018). Brassinosteroids are known to be an integral part of the plant defense network, protecting plants from viral infection and excessive reactive oxygen species accumulation. Another phytohormone, abscisic acid, has been shown to improve learning, spatial memory, cognitive function, and reduce anxiety-like behaviors in animals when administered as a dietary supplement (Liu et al., 2018; Naderi et al., 2017). As such, more work must be done to investigate the protective effects they exert in animal systems.

Previous studies have shown the ability of animal steroid analogues to elicit an anabolic response and contribute to improvements in animal fitness. One study found that administering 20-hydroxyecdysone to male rats enabled the animals to swim significantly longer during the

forced swim test (Azizov and Seifulla, 1998). As long as the animals have sufficient protein, 20-hydroxyedysone improves animal physical performance without the aid of training (Chermnykh et al., 1988). However, these studies were only performed in young male animals, so for the current study it was important to determine the effects CSE has on endurance in aging male and female animals. In this study, we found that feeding a low-fat 2% CSE diet to accelerated aging male and female animals can contribute to an increase in physical endurance. At weeks 11, 19, and 30, male CSE animals ran for a significantly longer period than the male control animals. Endurance of animals fed the high fat diet was very poor throughout the trial and they often underperformed significantly compared to the lean control group. For the remainder of the trial, there were no significant differences in endurance between male CSE and control animals. CSE males showed the greatest increase in physical performance at week 19.

Female CSE animals exhibited significantly greater endurance than control females at weeks 19, 30, and 36. No significant differences between CSE and control females were observed in week 11, 40, or 49, but high fat female animals performed very poorly in comparison. Female CSE animals had showed the most significant increase at week 36, which differs greatly from male endurance data. Male endurance peaked very quickly, but they also experienced a faster decline in endurance after this period. The drop in female endurance was not as drastic and their peak in endurance came at a much later age. The strong performance displayed by the CSE females may be attributed to their increase in lean mass and reduced fat mass compared to control counterparts. These effects are similar to what is seen with usage of anabolic steroids, though no androgenic side effects have been observed in previous brassinosteroids and ecdysteroid work (Esposito et al., 2011; Gorelick-Feldman et al., 2008) This anabolic response was previously shown to contribute to an increase in gastrocnemius muscle

size, which increases the type II fiber content resulting in increased strength and endurance capabilities (Esposito et al., 2011). Additionally, the structural analogue 20-hydroxyecdysone has been shown to increase ATP, carnosine, calcium, and creatine phosphate content in skeletal muscle cells which all contribute to improved endurance capabilities, therefore it is important to further investigate our data to determine if it follows a similar mechanism of action (Kholodova et al., 1997). Skeletal muscle maintenance is dependent upon the balance of protein synthesis and degradation, and the muscle loss in sarcopenia is due to the imbalance of this system (Fry et al., 2011). The weakness and loss of muscle mass observed in sarcopenia has been attributed to a reduction in number and size of type II muscle fibers, which is the fiber type which primarily comprises the gastrocnemius muscle (Tieland et al., 2018). Based on this data, gene expression analysis was performed using gastrocnemius muscle from mice at 32 weeks and 50 weeks of age to analyze markers associated with myogenesis and satellite cells. The biomarkers selected included those associated with satellite cells: Paired Box 3 (*Pax3*) and Paired Box 7 (*Pax7*); muscle regeneration and regulation: Myogenic Factor 5 (*Myf5*), Myogenic Differentiation 1 (*MyoD1*), Myogenin (*MyoG*), and Growth and Differentiation Factor 8/Myostatin (*Gdf8*); and protein degradation: F Box Protein 32 (*Fbxo32*) and Tripartite Motif containing 63 (*Trim63*). By identifying any changes in expression in these biomarkers, it can be ascertained whether a diet supplemented with cabbage seed extract can contribute to a health muscle profile in an aging model.

Previous work has been done through the feeding of rapeseed meal to pigs in order to improve meat quality traits and the skeletal muscle transcriptome, but results found that markers for proteolysis were actually upregulated while those associated with protein synthesis were downregulated. Potential factors could be the active glucosinolates not removed before being

provided to the pigs (Skugor et al., 2019). Prior research has shown that glucosinolates can have detrimental effects on animal growth and development, so for our CSE diet preparation, the glucosinolates were removed during the extraction process to avoid negative effects (Jeschke et al., 2017).

Male CSE mice showed a significant upregulation of MyoG, MyoD, Pax3, and Fbxo32. MyoG is responsible for myoblast differentiation and fusion to form myofibers during the regeneration process, while MyoD1 is involved in directing muscle precursor cells to begin skeletal muscle development (Tapscott, 2005). MyoG together with MyoD activate myogenic differentiation. MyoD is typically expressed in low levels in adult muscle as compared to the substantial levels observed in neonatal muscle cell nuclei, but higher levels are observed in muscles containing predominately fast-twitch fibers such as gastrocnemius and extensor digitorum longus (EDL) as opposed to slow-twitch muscles like soleus (Hennebry et al., 2009). MyoG is expressed in low levels within adult skeletal muscle, but the highest levels are observed in oxidative muscle fibers, especially muscles containing slow-twitch fibers like the soleus, or a mix of fast and slow-twitch oxidative fibers like the gastrocnemius (Yusuf and Brand-Saberi, 2012).

The female CSE group at 32 weeks had significantly upregulated expression of MyoD, Fbxo32, and both Pax biomarkers indicative of satellite cell activation. At 50 weeks, CSE males had significantly higher expression of Gdf8 and Pax7. CSE females at 50 weeks had increased levels of Pax 3 while showing a significant decrease in the proteolytic biomarkers Fbxo32 and Trim63. Pax3 and Pax7 are both found on the surface of satellite cells, so these results show that there is satellite cell activation occurring, and the increased expression of MyoD and MyoG provide insight into the proliferation and differentiation occurring which contribute to the

skeletal cell regeneration. Most interestingly, the downregulation of proteolytic markers provide a sharp contrast from control animal results at both the earlier and later time-point.

The proteolytic markers Trim63 and Fbxo32 are involved in skeletal muscle homeostasis, but they are overexpressed during age-related atrophy and disease (Gerlinger-Romero et al., 2017). Mice deficient in Trim63 were found to have an increased resistance to muscle atrophy (Glass, 2003), so the reduced expression in the gastrocnemius of CSE females at 50 weeks provides some insight into their improved physical fitness. Previous work has highlighted the ability of endurance exercise to boost satellite cell population and myogenic capacity, so it is possible that the improved endurance abilities in CSE animals contribute to the drastic increase in Pax gene expression (Shefer et al., 2010). Other studies have highlighted the importance of estrogen and estrogen analogues in maintaining satellite cell populations and stimulating their differentiation into muscle fibers, which provides insight into the late endurance peak that was observed in CSE female animals (Collins et al., 2019). The decreased expression of this marker in female animals reveals that there is reduced targeted protein degradation occurring in these muscles and allowing for maintenance or increase of mass, which again highlights the importance of performing these studies in female animals because there are dramatic differences between sexes.

While there are more than 3,000 genes which are regulated distinctly in male and female muscle, sex-specific hormones are also major contributors to the differences in muscle mass, function, and fatigue (Welle et al., 2008). Estrogen and testosterone are both important for skeletal muscle growth, function, fiber type composition, and fatigue (Haizlip et al., 2015). Ovariectomized females had a decrease in muscle fiber cross-sectional area and reduced fiber number, but estradiol supplementation increased these values (Kitajima et al., 2016). Skeletal

muscle is sensitive to circulating estrogen levels, and the reduced sensitivity of these receptors as seen in pre- and post-menopausal females is associated with the reduced regenerative capacity of skeletal muscle (Haizlip et al., 2015). Depletion and reduced sensitivity of these receptors can result in faster muscle fatigue, leading to decreased endurance in females while increasing endurance in male animals (Glenmark et al., 2004).

The PI3K/Akt pathway has been shown not only to induce synthesis but actively suppress pathways associated with catabolism and subsequent activation of factors involved in muscle protein degradation (Wagatsuma et al., 2016). Though this pathway shows a reduced sensitivity to phosphorylation and activation with age, our data suggests that cabbage seed extract may also enhance this pathway similarly to pure brassinosteroid and 20-hydroxyecdysone studies and contribute to a healthy muscle profile in aging individuals. Together this data warrants further research to determine the brassinosteroid profile of the CSE diet and other markers impacted by dietary supplementation.

CONCLUSION

Overall, these data suggest that supplementing SAMP8 mice diet with 2% cabbage seed extract (CSE) can contribute to healthy body weight, improved endurance, and increased lean muscle mass. Furthermore, CSE dietary supplementation may contribute to a healthy muscle profile, an increase in the satellite cell population, and increased myogenic activity in the gastrocnemius, which is primarily affected by sarcopenia and atrophy with age. Cabbage seed extract may also contribute to a reduction in senescence-associated behaviors through antioxidant capabilities, resulting in greater alertness and higher activity levels. These data demonstrate that utilization of cabbage seed extract may serve as a treatment option improving mobility and combatting age-related muscle atrophy. The potential therapeutic applications and

market for cabbage seed extract-containing supplements highlights the need to identify their specific biological activities and elucidate their mechanisms of action in animals. Further studies into their impact on metabolic regulation in an aging animals and effects in other tissues need to be investigated in order to gauge their usefulness in treating diseases of age.

Table 2.1 Diet Composition

	10% Low Fat Diet		**10% Low Fat Diet + 2% Cabbage Seed Extract**		**60% High Fat Diet**	
Product Number	D12450J		D14121802		D12492	
	Gm	**Kcal**	**Gm**	**Kcal**	**Gm**	**Kcal**
Protein	19	20	19	20	26	20
Carbohydrate	67	70	66	70	26	20
Fat	4	10	4	10	35	60
Kcal/gm	3.8		3.8		5.2	
Ingredients	**Gm**	**Kcal**	**Gm**	**Kcal**	**Gm**	**Kcal**
Casein	200	800	200	800	200	800
L-cystine	3	12	3	12	3	12
Corn starch	506.2	2025	506.2	2025	0	0
Maltodextrin 10	125	500	125	500	125	500
Sucrose	68.8	275	68.8	275	68.8	275
Cellulose	50	0	50	0	50	0
Soybean Oil	25	225	25	225	25	225
Lard	20	180	20	180	245	2205
Mineral Mix	10	0	10	0	10	0
Dicalcium Phosphate	13	0	13	0	13	0
Calcium Carbonate	5.5	0	5.5	0	5.5	0
Potassium Citrate	16.5	0	16.5	0	16.5	0
Vitamin Mix	10	40	10	40	10	40
Choline Bitartrate	2	0	2	0	2	0
Cabbage Seed Extract	0	0	21.6	0	0	0
Yellow Dye #5	0.04	0	0.025	0	0	0
Red Dye #40	0	0	0.025	0	0	0
Blue Dye #1	0.01	0	0	0	0.05	0
Total	**1055.05**	**4057**	**1076.65**	**4057**	**773.85**	**4057**

Table 2.2 Protocol for Measuring Senescence, adapted from Takeda et al. (1981)

Behavior	Score 0	Score 1	Score 2	Score 3	Score 4
Reactivity	Typical Behavior	Restlessness, abnormal gait	Reduced agility and changes in behavior	No voluntary movement, only if nudged	Immobile
Passivity	Natural escape reaction from pinching	Reduced escape reaction	No escape reaction, manually turns over	No escape reaction or turnover; only reacts when held by forelimb	No escape reaction.

Table 2.3 Sense and anti-sense primer sequences used in qRT-PCR Analysis (Gene names indicated in footnote)

Gene	Forward Primer Sequence (5'-3')	Reverse Primer Sequence (3'-5')
Ribosomal Protein L13a	CTATGACCAATAGGAAGAGCAACC	GCAGAGTATATGACCAGGTGGAA
Myogenic Factor 5	TGAGGGAACAGGTGGAGAAC	AGCTGGACACGGAGCTTTTA
Myogenin	CTACAGGCCTTGCTCAGCTC	AGATTGTGGGCGTCTGTAGG
Myogenic differentiation 1	AGCACTACAGTGGCGACTCA	GCTCCACTATGCTGGACAGG
Growth Differentiation Factor 8	TGCAAAATTGGCTCAAACAG	GCAGTCAAGCCCAAAGTCTC
Tripartite Motif Containing 63	GAGAACCTGGAGAAGCAGCT	CCGCGGTTGGTCCAGTAG
F-Box Protein 32	AACCGGGAGGCCAGCTAAAGAACA	TGGGCCTACAGAACAGACAGTGC
Paired Box Gene 3	GGGAACTGGAGGCATGTTTA	GTTTTCCGTCCCAGCAATTA
Paired Box Gene 7	CCGTGTTTCTCATGGTTGTG	GAGCACTCGGCTAATCGAAC

Table 2.4 Values from Statistical Analysis of Weekly Male Body Weight Data

Week	1-Way ANOVA	Dunnett's Post-hoc: Lean vs CSE	Dunnett's Post-hoc: Lean vs High Fat
5 (n=8)	P= 0.3257, ns	P= 0.2375, ns	P= 0.7119, ns
6	0.0830, ns	0.2632, ns	0.5846, ns
7	0.0108, *	0.1989, ns	0.1597, ns
8	0.0108, *	0.1708, ns	0.1872, ns
9	0.0076, **	0.1114, ns	0.2121, ns
10	0.0131, *	0.1790, ns	0.2084, ns
11	0.0074, **	0.1817, ns	0.1283, ns
12	0.0074, **	0.1817, ns	0.1283, ns
13	0.0160, *	0.2248, ns	0.1956, ns
14	0.0094, **	0.3142, ns	0.0879, ns
15	0.0101, *	0.3837, ns	0.0751, ns
16	0.0061, **	0.4812, ns	0.0370, *
17	0.0030, **	0.5139, ns	0.0183, *
18	0.0105, *	0.7401, ns	0.0332, *
19	0.0067, **	0.4406, ns	0.0446, *
20	0.0112, *	0.7465, ns	0.0348, *
21	0.0095, **	0.9547, ns	0.0181, *
22	0.0058, **	0.9922, ns	0.0099, **
23	0.0115, *	0.9996, ns	0.0166, *
24	0.0104, *	0.9982, ns	0.0140, *
25	0.0131, *	0.9997, ns	0.0185, *
26	0.0168, *	0.9947, ns	0.0247, *
27	0.0070, **	0.9909, ns	0.0093, **
28	0.0049, **	0.9989, ns	0.0078, **
29	0.0037, **	0.8955, ns	0.0092, **
30	0.0035, **	0.9224, ns	0.0122, *
31	0.0093, **	0.9527, ns	0.0261, *
32 (n=4)	0.1446, ns	0.9892, ns	0.2235, ns
33	0.2798, ns	0.9921, ns	0.3705, ns
34	0.2660, ns	0.9240, ns	0.4334, ns
35	0.3863, ns	0.9149, ns	0.5898, ns
36	0.4509, ns	0.9484, ns	0.6212, ns
37	0.5914, ns	0.9673, ns	0.7321, ns
38	0.6976, ns	0.9761, ns	0.8117, ns
39	0.5849, ns	0.8797, ns	0.8338, ns
40	0.5913, ns	0.9876, ns	0.6921, ns
41	0.4331, ns	0.9497, ns	0.5924, ns
42	0.4460, ns	0.8966, ns	0.5775, ns
43	0.4562, ns	0.8561, ns	0.6273, ns
44	0.3368, ns	0.8473, ns	0.4852, ns
45	0.2614, ns	0.7331, ns	0.4608, ns
46	0.3244, ns	0.8538, ns	0.4642, ns
47	0.2815, ns	0.7491, ns	0.4806, ns
48	0.3972, ns	0.8414, ns	0.5676, ns
49	0.4062, ns	0.8464, ns	0.5747, ns
50	0.4112, ns	0.8020, ns	0.6204, ns

Table 2.5 Values from Statistical Analysis of Weekly Female Body Weight Data

Week	1-Way ANOVA	Dunnett's Post-hoc: Lean vs CSE	Dunnett's Post-hoc: Lean vs High Fat
5 (n=8)	P= 0.8652, ns	P= 0.9913, ns	P= 0.8893
6	0.0082, **	0.9994, ns	0.0118, *
7	0.0016, **	0.7584, ns	0.0015, **
8	0.0021, **	0.9839, ns	0.0031, **
9	0.0005, ***	0.9611, ns	0.0008, ***
10	0.0001, ***	0.8927, ns	0.0002, ***
11	<0.0001, ****	0.9988, ns	<0.0001, ****
12	<0.0001, ****	0.9485, ns	<0.0001, ****
13	<0.0001, ****	0.9179, ns	<0.0001, ****
14	<0.0001, ****	0.9250, ns	<0.0001, ****
15	<0.0001, ****	0.7442, ns	<0.0001, ****
16	<0.0001, ****	0.9928, ns	<0.0001, ****
17	<0.0001, ****	0.8167, ns	<0.0001, ****
18	<0.0001, ****	0.9997, ns	<0.0001, ****
19	<0.0001, ****	0.8585, ns	<0.0001, ****
20	<0.0001, ****	0.7565, ns	<0.0001, ****
21	<0.0001, ****	>0.9999, ns	<0.0001, ****
22	<0.0001, ****	0.9863, ns	<0.0001, ****
23	<0.0001, ****	0.8675, ns	<0.0001, ****
24	<0.0001, ****	0.8489, ns	<0.0001, ****
25	<0.0001, ****	>0.9999, ns	<0.0001, ****
26	<0.0001, ****	0.9999, ns	<0.0001, ****
27	<0.0001, ****	0.8735, ns	<0.0001, ****
28	0.0004, ***	0.8664, ns	0.0005, ***
29	<0.0001, ****	0.9903, ns	<0.0001, ****
30	<0.0001, ****	0.8741, ns	<0.0001, ****
31	<0.0001, ****	0.8887, ns	<0.0001, ****
32 (n=4)	<0.0001, ****	0.8694, ns	<0.0001, ****
33	0.0001, ***	0.5096, ns	0.0001, ***
34	0.0003, ***	0.9969, ns	0.0005, ***
35	0.0001, ***	0.8365, ns	0.0001, ***
36	0.0003, ***	0.8141, ns	0.0004, ***
37	0.0003, ***	0.9341, ns	0.0004, ***
38	<0.0001, ****	0.0195, *	<0.0001, ****
39	0.0003, ***	0.8554, ns	0.0004, ***
40	0.0002, ***	0.7384, ns	0.0002, ***
41	0.0004, ***	0.9932, ns	0.0006, ***
42	0.0008, ***	0.7806, ns	0.0008, ***
43	0.0003, ***	0.7767, ns	0.0003, ***
44	0.0005, ***	0.9847, ns	0.0007, ***
45	0.0001, ***	0.8292, ns	0.0001, ***
46	0.0003, ***	0.9966, ns	0.0005, ***
47	0.0005, ***	0.9899, ns	0.0008, ***
48	0.0004, ***	0.9812, ns	0.0006, ***
49	0.0001, ***	0.9931, ns	0.0002, ***
50	0.0001, ***	0.9955, ns	0.0002, **

Table 2.6 Values from Statistical Analysis of Weekly Male Feed Intake Data

Week	1-way ANOVA	Dunnett's Post-hoc: Lean vs CSE	Dunnett's Post-hoc: Lean vs High Fat
5 (n=2 cages)	0.1061, ns	0.9536, ns	0.1235, ns
6	0.0324, *	0.7596, ns	0.0430, *
7	0.0269, *	0.7774, ns	0.0349, *
8	0.7273, ns	0.8333, ns	0.6576, ns
9	0.0042, **	0.4760, ns	0.0057, **
10	0.6322, ns	0.5755, ns	0.9665, ns
11	0.1031, ns	0.9744, ns	0.1162, ns
12	0.0069, **	0.8628, ns	0.0081, **
13	0.2963, ns	0.8801, ns	0.3819, ns
14	0.3830, ns	0.9953, ns	0.3976, ns
15	0.1772, ns	0.9480, ns	0.2078, ns
16	0.4721, ns	0.9333, ns	0.5566, ns
17	0.8293, ns	0.9444, ns	0.9185, ns
18	0.5367, ns	0.9990, ns	0.5395, ns
19	0.2957, ns	0.8172, ns	0.4146, ns
20	0.2785, ns	0.3322, ns	0.2473, ns
21	0.5646, ns	0.4812, ns	0.7728, ns
22	0.6229, ns	0.6070, ns	0.9995, ns
23	0.6576, ns	0.5831, ns	0.9171, ns
24	0.4877, ns	0.4139, ns	0.6356, ns
25	0.1387, ns	0.2007, ns	0.1153, ns
26	0.1875, ns	0.2308, ns	0.1664, ns
27	0.1945, ns	0.2102, ns	0.1884, ns
28	0.4883, ns	0.5493, ns	0.4391, ns
29	0.9484, ns	0.9817, ns	0.9795, ns
30	0.9185, ns	0.9720, ns	0.9657, ns
31	0.9485, ns	0.9907, ns	0.9685, ns
32 (n=1 cage)	-can't analyze, no SE	- can't analyze, no SE	- can't analyze, no SE

Table 2.7 Values from Statistical Analysis of Weekly Female Feed Intake Data

Week	1-Way ANOVA	Dunnett's Post-hoc: Lean vs CSE	Dunnett's Post-hoc: Lean vs High Fat
5 (n=2 cages)	0.0057, **	0.9954, ns	0.0060, **
6	0.0105, *	0.9838, ns	0.0117, *
7	0.0004, ***	0.6249, ns	0.0005, ***
8	0.0080, **	0.7362, ns	0.0100, **
9	0.0002, ***	0.5625, ns	0.0003, ***
10	0.0810, ns	0.9741, ns	0.0915, ns
11	0.0013, **	0.9746, ns	0.0015, **
12	0.2652, ns	>0.9999, ns	0.2693, ns
13	0.0023, **	0.9578, ns	0.0026, **
14	0.5124, ns	0.9991, ns	0.5153, ns
15	0.0103, *	0.9595, ns	0.0105, *
16	0.5137, ns	0.9973, ns	0.5231, ns
17	0.8370, ns	0.9979, ns	0.8423, ns
18	0.2868, ns	0.9932, ns	0.2763, ns
19	0.4286, ns	0.9823, ns	0.4620, ns
20	0.6903, ns	0.9943, ns	0.7069, ns
21	0.4788, ns	0.9930, ns	0.4540, ns
22	0.6333, ns	0.9976, ns	0.6090, ns
23	0.5060, ns	0.9976, ns	0.5146, ns
24	0.6037, ns	0.9911, ns	0.5686, ns
25	0.5640, ns	0.9674, ns	0.5120, ns
26	0.4629, ns	0.7445, ns	0.7113, ns
27	0.6035, ns	0.9997, ns	0.5902, ns
28	0.9137, ns	0.9689, ns	0.8795, ns
29	0.9589, ns	0.9791, ns	0.9896, ns
30	0.7717, ns	0.9810, ns	0.8148, ns
31	0.2460, ns	0.6321, ns	0.1927, ns
32 (n=1 cage)	-can't analyze, no SE	-can't analyze, no SE	-can't analyze, no SE

Table 2.8 Values from Statistical Analysis of Weekly Male Endurance

Week	1-Way ANOVA	Dunnett's Post-hoc: Lean vs CSE	Dunnett's Post-hoc: Lean vs High Fat
11	0.0005, ****	0.0069, **	0.3024, ns
19	<0.0001, ****	0.0044, **	0.0118, *
30	<0.0001, ****	0.0182, *	0.0158, *
36	0.0003, ***	0.1220, ns	0.0028, **
40	0.0363, *	0.6788, ns	0.0891, ns
49	0.2864, ns	0.8910, ns	0.3897, ns

Table 2.9 Values from Statistical Analysis of Weekly Female Endurance

Week	1-Way ANOVA	Dunnett's Post-hoc: Lean vs CSE	Dunnett's Post-hoc: Lean vs High Fat
11	<0.0001, ****	0.9286, ns	<0.0001, ****
19	0.0031, **	0.0450, *	0.2673, ns
30	<0.0001, ****	0.0363. *	0.0001, ***
36	0.0001, ***	0.0053, **	0.0153, *
40	0.0388, *	0.7647, ns	0.0817, ns
49	0.0196, *	0.2454, ns	0.1417, ns

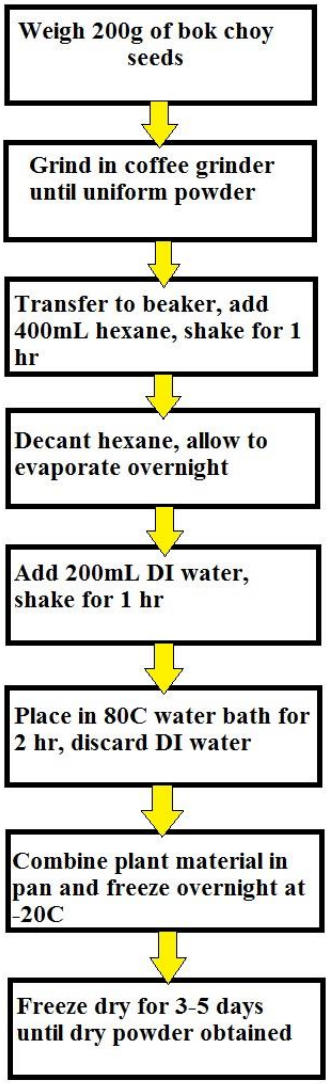

Figure 1. Preparation of Cabbage Seed Extract for Dietary Formulation. Cabbage Extract was prepared according to the protocol used by J Amer Chem Soc (1976) 53: 12-16. Bok choy seeds were used, provided by Sakata Incorporated (California, USA). For each batch, 200 grams of bok choy seeds were weighed out and then ground into a uniform powder using a coffee grinder. The powder was defatted using 400mL of hexane and placed on a shaker for one hour. The hexane was then decanted and the beaker left in the hood overnight to allow hexane to evaporate. 200mL of deionized water was added to the beaker and placed on a shaker for one hour, then placed in an 80°C water bath for 2 hours. The excess water was then discarded. Up to 1.2kg of plant material was then combined into an aluminum pan and frozen overnight at -20°C. The material was then freeze-dried for 3-5 days until a dry powder was obtained. This final product was then incorporated into a specially formulated diet prepared by Research Diets out of New Brunswick, New Jersey.

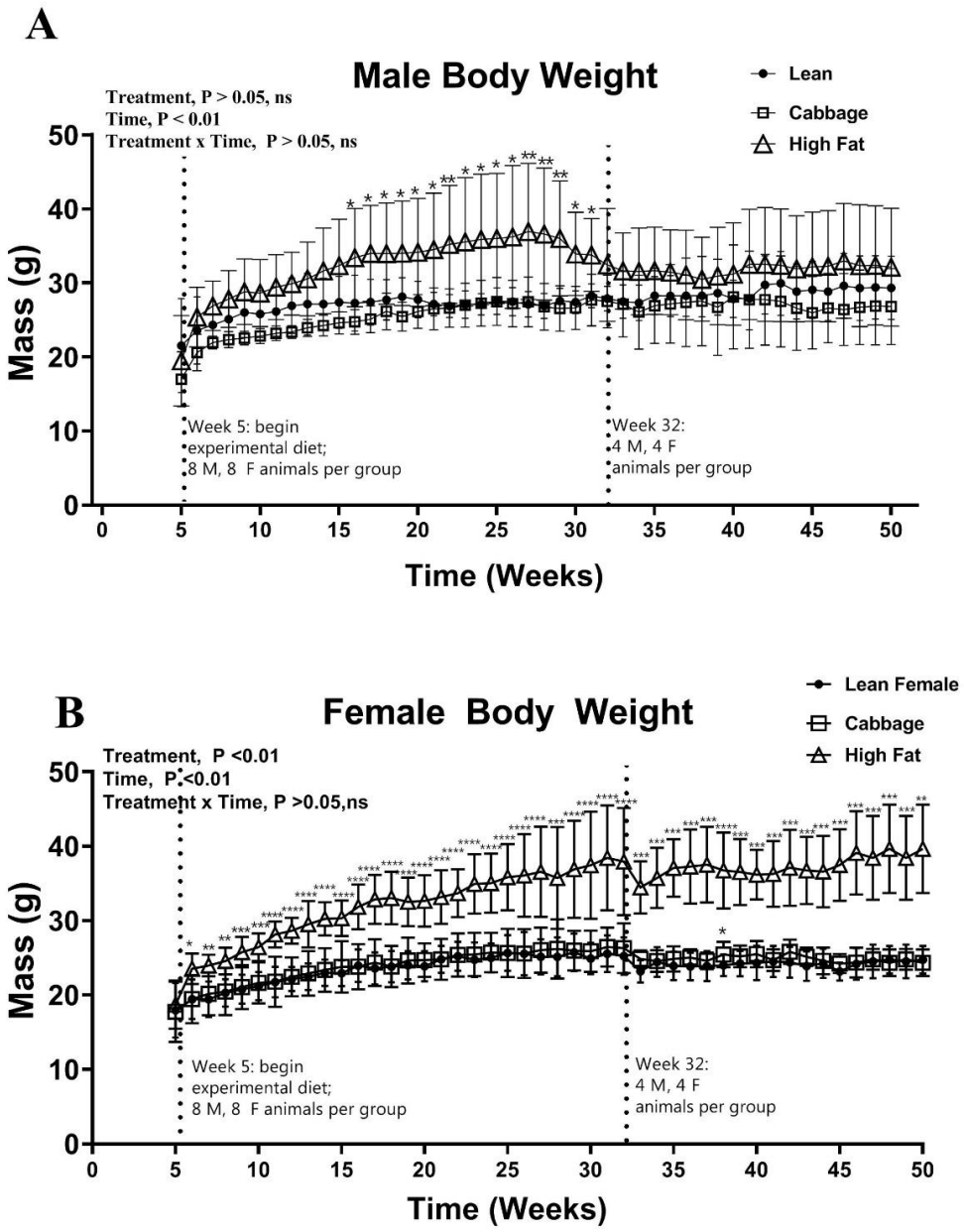

Figure 2. Effect of cabbage seed extract on body weight. Animal body weight was measured weekly over the course of 50 weeks. Body weights of male animals is shown in Figure 2A. Body weights of female animals are displayed in Figure 2B. Weights of males and females on the high fat diet increased, while mice consuming the lean and cabbage-supplemented diets maintained relatively stable weights. Results are expressed as means ± SEM, n=8 animals per treatment and sex up to week 32 (4 animals per cage), and n=4 animals per treatment and sex from weeks 33-50 (4 animals per cage). Body weight was analyzed by 2 factor repeated measures ANOVA with time and treatment as independent variables. Analysis of individual weeks was also performed using 1-way ANOVA followed by Tukey's multiple comparison's test. * P<0.05, ** P<0.01, *** P<0.001, **** P<0.0001.

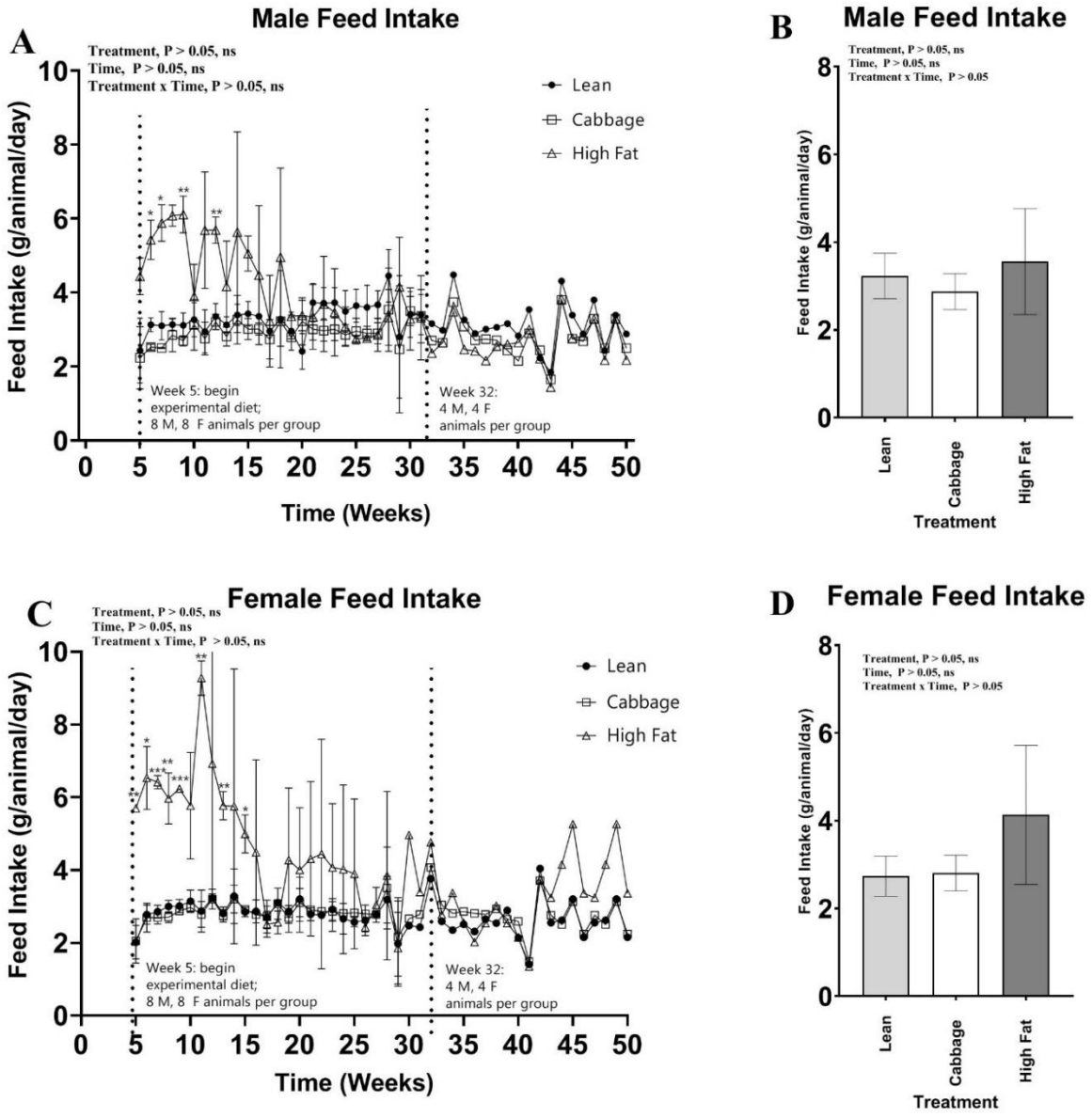

Figure 3. Effect of cabbage seed extract on feed intake. Average food intake was measured weekly throughout the 50 week trial period for male (3A) and female (3C) mice. Figures 3B and 3D show the average daily feed intake for male and female mice until week 32 (n=8 animals per treatment and sex). Results are expressed as means ± SEM, n=8 animals (4 per cage) per treatment and sex until week 32, when n=4 animals (4 per cage) per treatment and sex. No results are shown after week 32 because remaining animals were houses 4 animals per treatment in a single cage and group fed. Food intake was analyzed by 2-factor repeated measures ANOVA, with time and treatment as independent variables. Data were also analyzed weekly using 1-way ANOVA followed by Dunnett's post-hoc test. * indicates $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

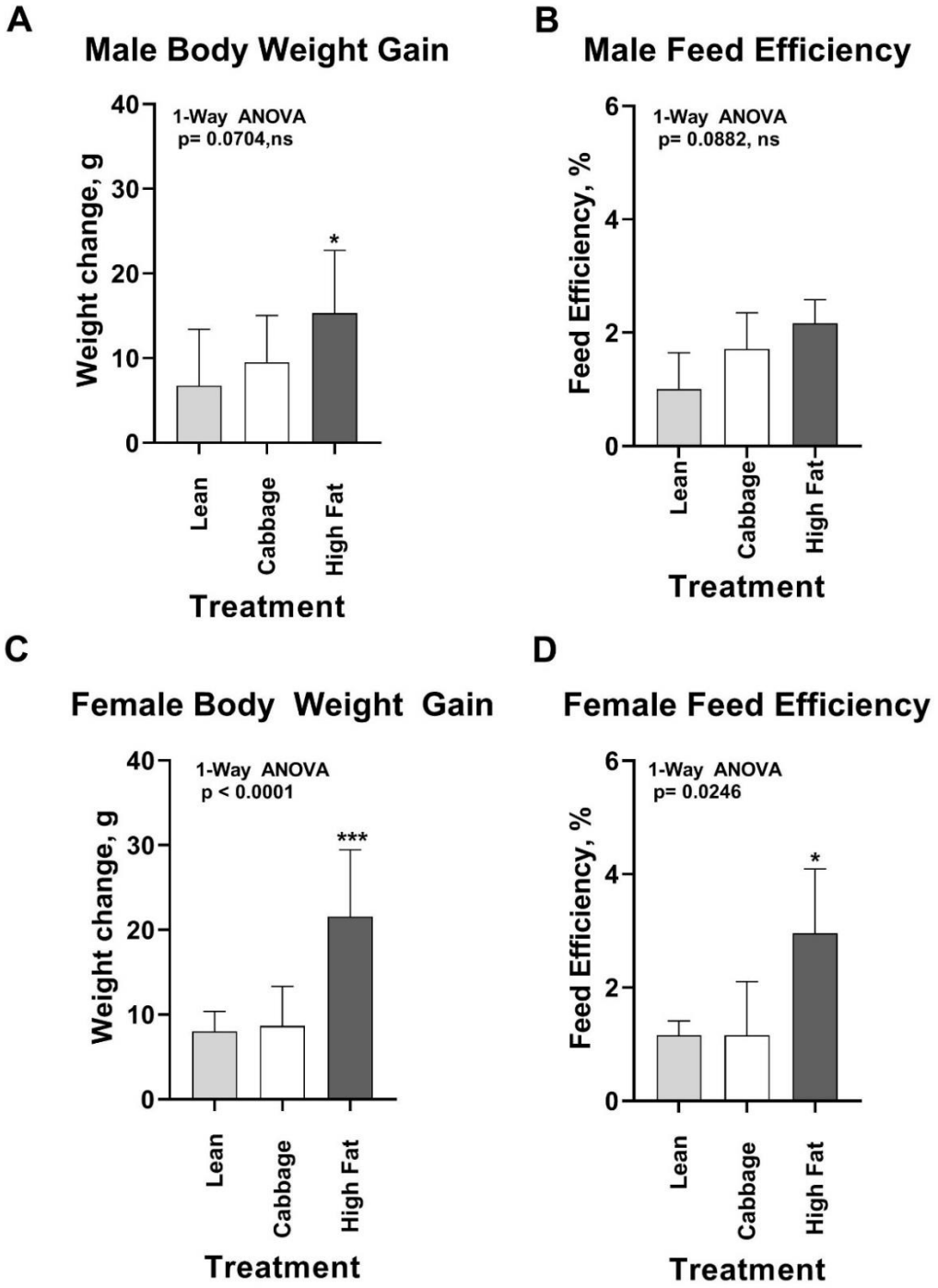

Figure 4. Effect of diet on body weight gain and feed efficiency. Figures 4A and 4C show male and female body weight gain over weeks 1 through 32. Figures 4B and 4D show male and female feed efficiency data from weeks 1 to 32. Feed efficiency was calculated as (body weight gain/food intake) × 100 ratio. (∗) $P < 0.05$ versus respective lean control diet. Body weight gain and feed efficiency were analyzed by one-way ANOVA and Dunnett's post-hoc test. * indicates $P < 0.05$, *** indicates $P < 0.001$.

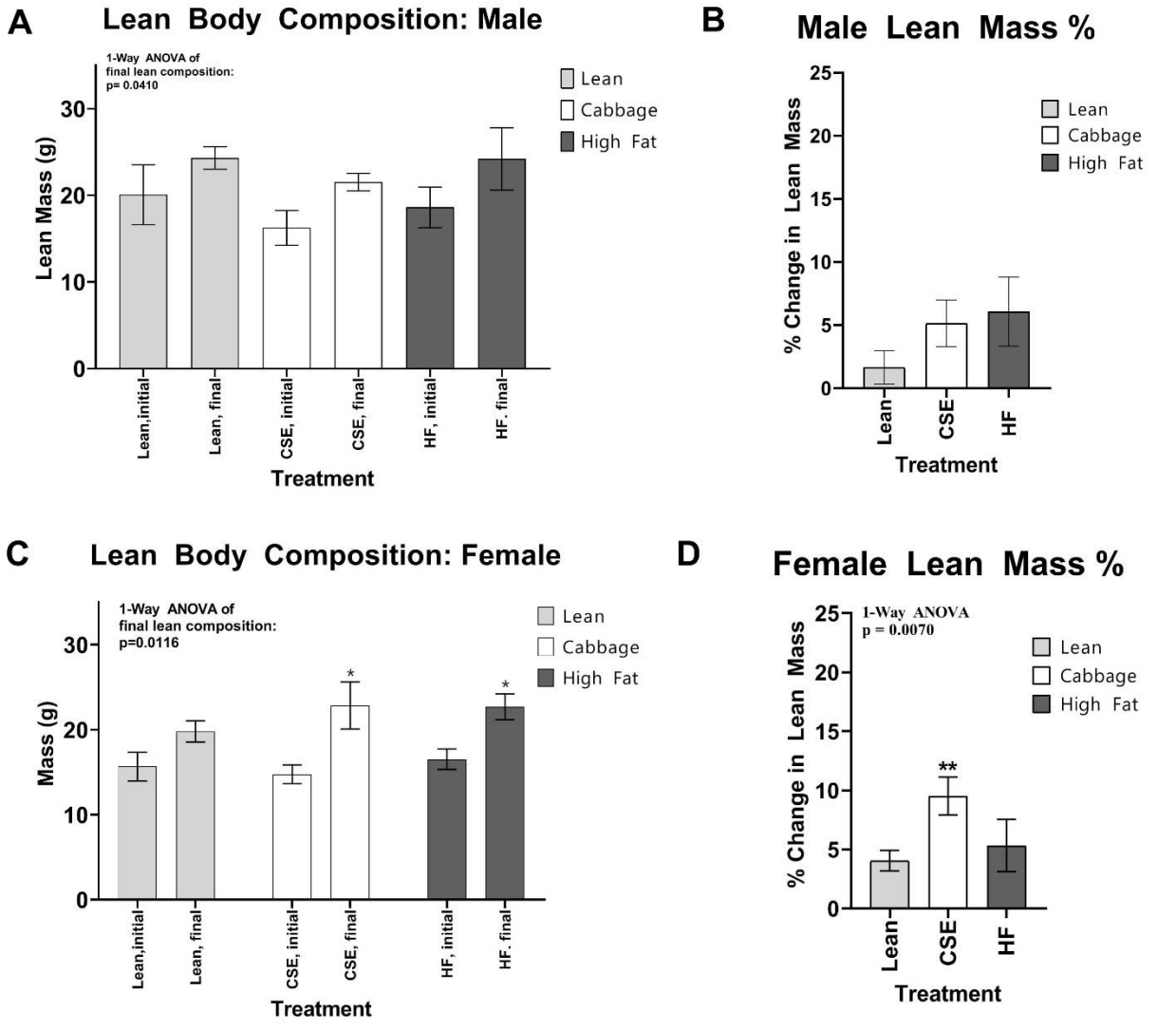

Figure 5. Body Composition: Lean Mass Measurement. EchoMRI software was used to measure lean mass of animals at weeks 1 and 30 of the experimental diet. Figures A and C show the initial measurement at the beginning of the trial (week 1) and the final measurement (week 30). Final measurements were compared to that of lean control diet at week 30. Results are expressed as means ± SEM, n=8 animals per treatment. * P<0.05, ** P<0.01, *** P<0.001, **** P<0.0001. Body composition data were analyzed using one-way ANOVA and Sidak's posttest. Percent change in lean mass (Figures B and D) was analyzed using one-way ANOVA and Dunnett's post-hoc test. % Change was calculated by dividing each animal's lean mass by their body weight and multiplying by 100 for the initial and final values, then subtracting the initial value from the final value.

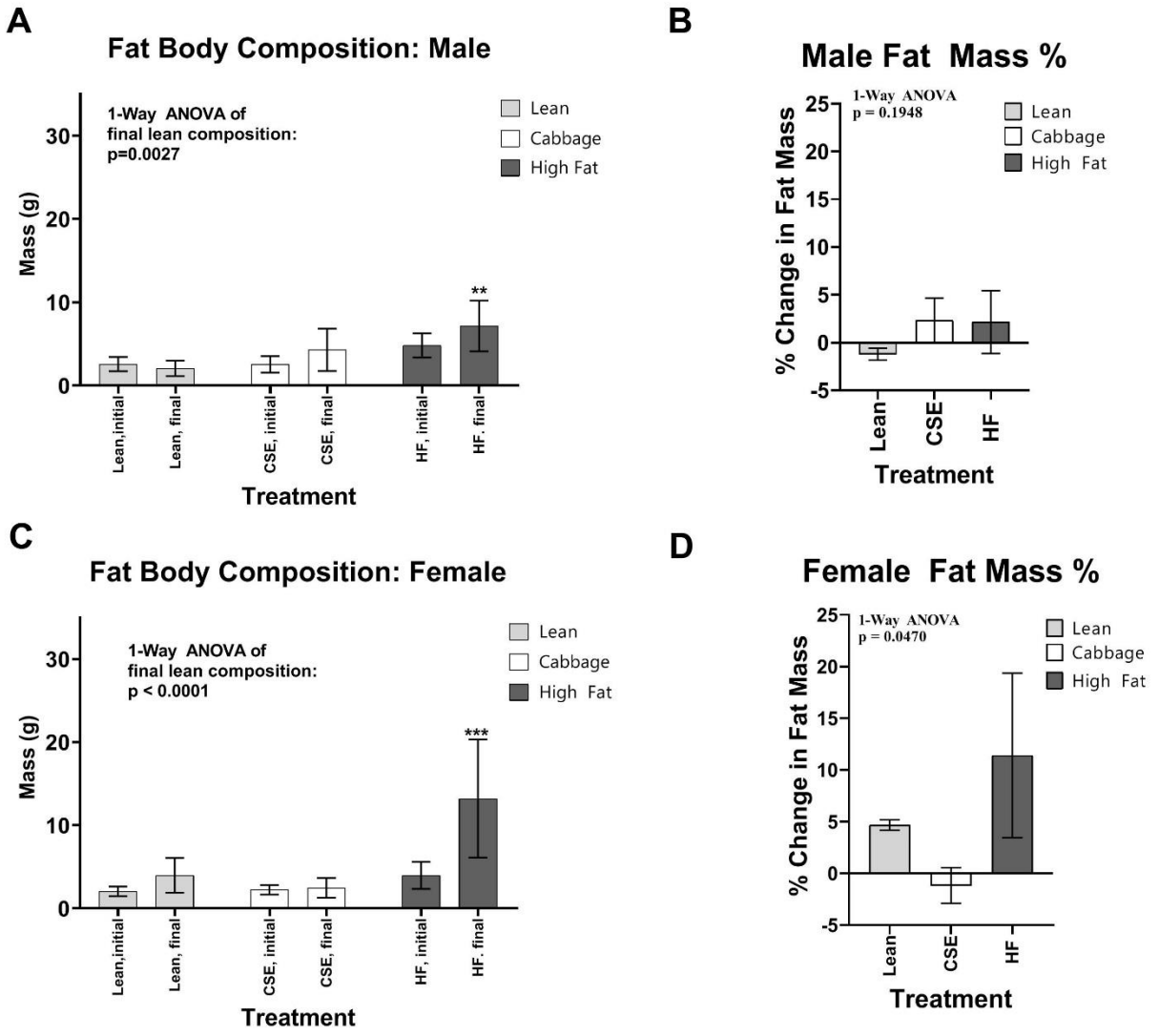

Figure 6. Body Composition: Fat Measurement. EchoMRI software was used to measure fat mass of animals at weeks 1 and 30 of the experimental diet. Figures A and C show the initial measurement at the beginning of the trial (week 1) and the final measurement (week 30). Final measurements were compared to that of lean control diet at week 30. Body composition data were analyzed using one-way ANOVA and Sidak's posttest. Percent change in fat mass (Figures B and D) was analyzed using one-way ANOVA and Dunnett's posttest. Results are expressed as means ± SEM, n=8 animals per treatment. * P<0.05, ** P<0.01, *** P<0.001, **** P<0.0001. % Change was calculated by dividing each animal's fat mass by their body weight and multiplying by 100 for the initial and final values, then subtracting the initial value from the final value.

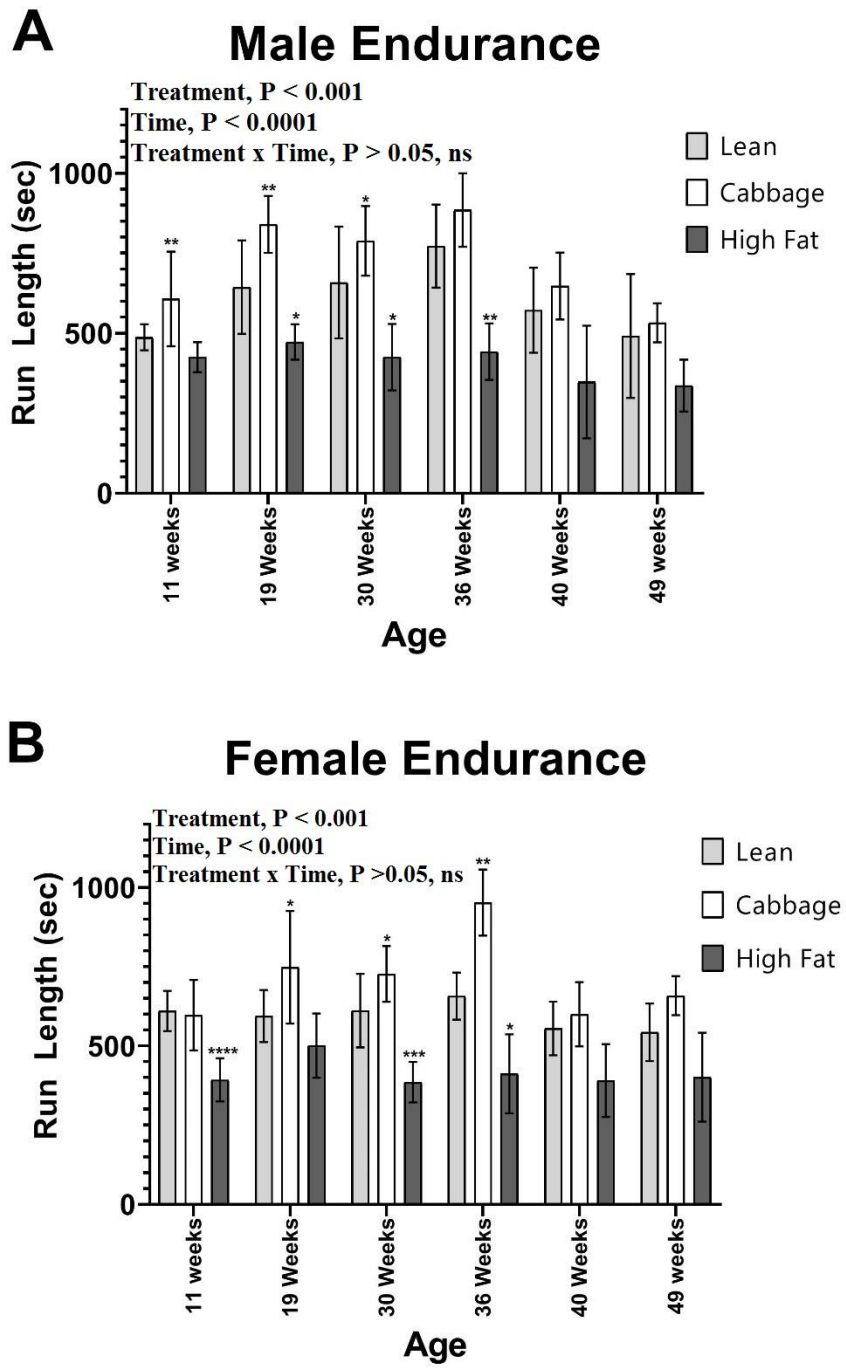

Figure 7. Treadmill Performance Results. Endurance was assessed 6 times throughout the trial using Columbus Instruments rodent treadmill. Male endurance results are shown in figure A and figure B shows female endurance performance. Results are expressed as means ± SEM. *P<0.05 vs lean control diet. Results are expressed as means ± SEM, n=8 male and 8 female animals per treatment up to week 30; n=4 animals per treatment for remaining measurements. * P<0.05, ** P<0.01, *** P<0.001, **** P<0.0001. Data analyzed using 2-way ANOVA with time and treatment as independent variables and Dunnett's posttest.

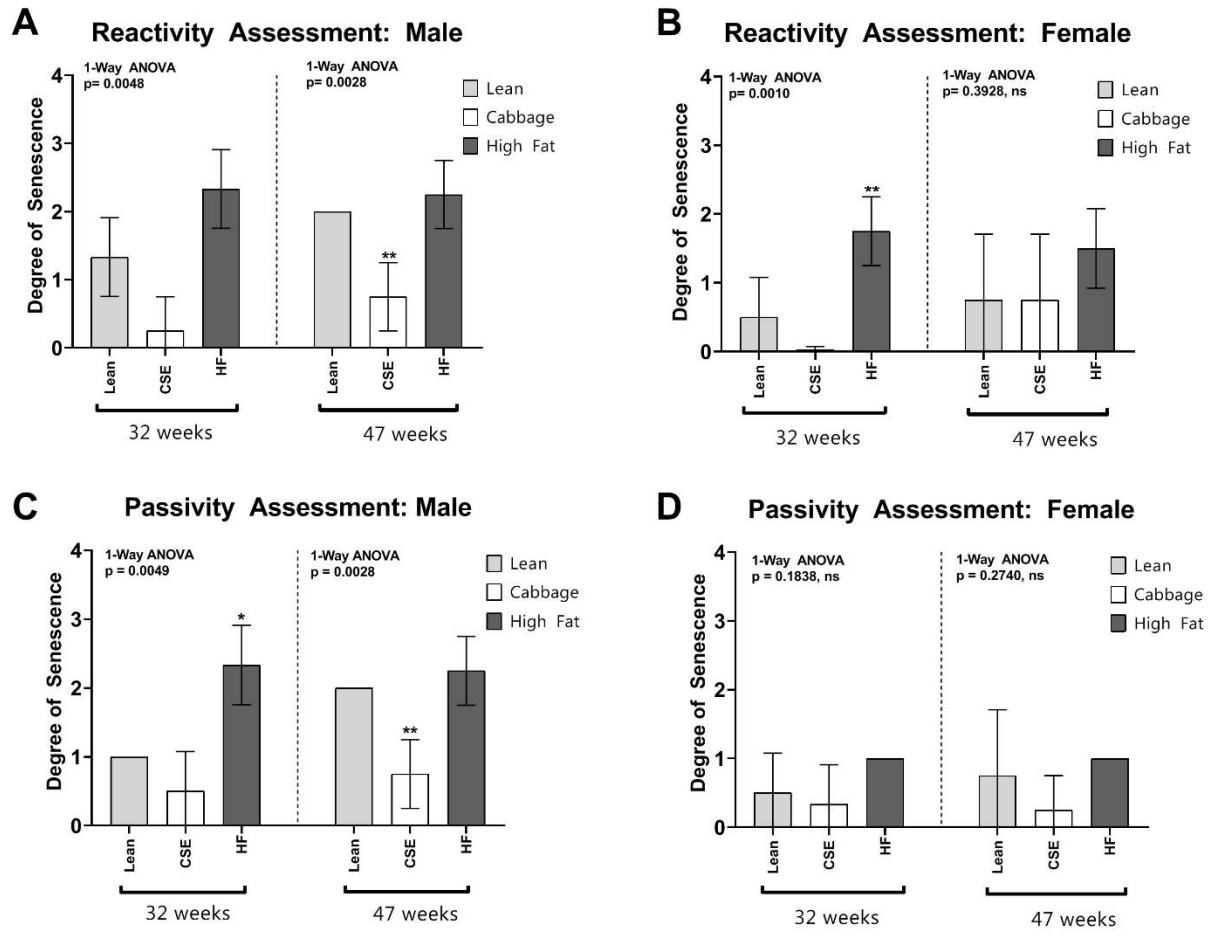

Figure 8. Effects of cabbage seed extract on behavioral senescence. Senescence in aging SAMP8 mice was measured according to the Takeda et al. 1981 protocol based on behavioral changes associated with aging. Animal behavior was assessed at 32 weeks of age and then again at 47 weeks of age. Animal reactivity (Figures 8A and 8B) was determined by observation and each animal was assigned a number from 0 (natural behavior) to 4 (severe) based on response. In figure 8A, all lean males at week 47 received a score of 2 so there was no standard error. Male and female passivity assessment is shown in figures C and D, respectively. Passivity was determined by pinching animals at the nape of the neck and observing the natural escape behavior. Animals were assigned a number from 0 (normal behavior) to 4 (severe) based on response. In figure 8C, lean males at 32 weeks all received a score of 1, while lean males at 47 weeks all received a score of 2, so there was no standard error. In figure 8D, high fat female mice at 32 weeks and 47 weeks all received scores of 1, so there was no standard error. Results are expressed as means ± SEM, n=4 animals per treatment and sex. The same animals were measured at each timepoint. *P <0.05 vs lean control diet at 32 weeks or 47 weeks for the respective comparison. Data were analyzed using 1-way ANOVA and Sidak's post-hoc test. * indicates P <0.05, ** P <0.01.

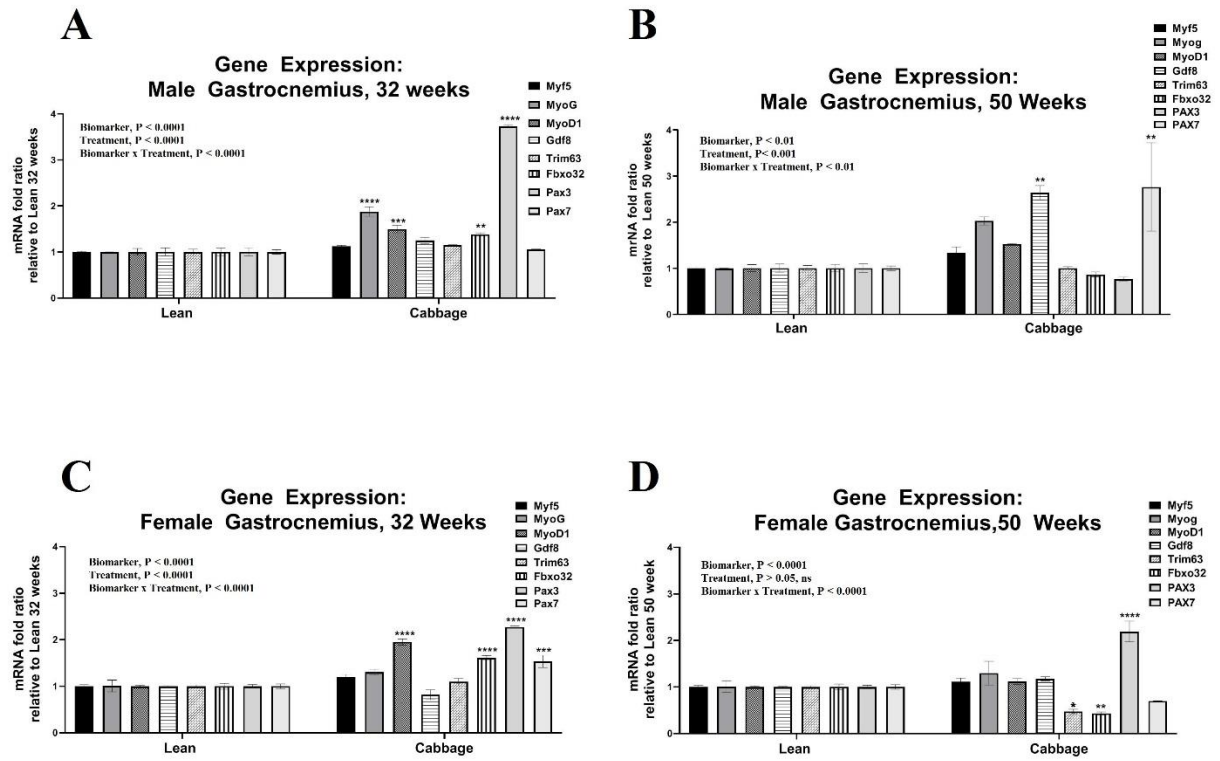

Figure 9. Effects of cabbage seed extract on satellite cell and myogenic gene expression. RNA was isolated from gastrocnemius tissues of male and female SAMP8 fed lean and cabbage seed extract diets. MRNA levels for markers associated with satellite cells, myogenesis, and protein degradation were measured by quantitative real-time PCR. Housekeeping gene RPL13a was used for data normalization. Results are expressed as means ± SEM, n=4 animals per treatment at each time point. *P<0.05, ** P<0.01, *** P<0.001, **** P<0.0001 vs lean control diet at respective time point (either 32 or 50 weeks). Data analyzed using 2-way ANOVA with treatment and biomarker as independent variables, followed by Sidak's post-hoc test.

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CHAPTER 3

Brassinosteroids Accelerate Human Dermal Fibroblast Migration and Reduce Reactive Oxygen Species

ABSTRACT

Identifying the therapeutic potential of plant bioactive compounds has resulted in economical methods by which to treat a number of ailments and enhance the wound healing process. Cell culture assays serve as an exceptional method for screening the wound healing potential and antioxidant capacity exhibited by these natural extracts. Brassinosteroid (BR) is a phytohormone involved in plant development and defense mechanisms, and BR compound Homobrassinolide has exhibited health promoting properties in cellular and animal models. However, with more nearly 70 structurally unique BR compounds exhibiting different bioactivities, it is important to identify those with the most promising benefits to human health (Esposito et al., 2011; Clouse, 2011). In a previous study, our lab selected nine BR compounds and examined their ability to affect cell migration in murine fibroblasts. In this experiment, these nine compounds were investigated for their ability to promote cell proliferation in adult Human Dermal Fibroblast (HDFa) cells to see if similar results could be achieved. Five of these compounds were found to significantly improve fibroblast migration, attributed to the previously observed BR activation of the Akt pathway. Homocastasterone and Homobrassinolide showed the greatest cell migration potential and they were also found to significantly reduce reactive oxygen species in murine macrophages upon stimulation with Lipopolysaccharide, exhibiting potent antioxidant ability similar to BR radical scavenging observed in plants. Cells treated with Epibrassinolide were found to have reduced accumulation of nitric oxide species ($p < 0.01$) The health-promoting properties exhibited by these structurally diverse BR compounds highlight their potential use in regenerative medicine as effective and inexpensive means for improving skin health in aging individuals and reducing occurrences of chronic wounds.

INTRODUCTION

Skin is the largest organ of the body, and it constantly undergoes repair due to damage from environmental exposure. In order to maintain skin integrity and protect the body from infection, efficient wound healing mechanisms are necessary to repair and replace damaged tissue. The efficiency of these mechanisms diminishes with age and chronic wounds, which affect nearly 6 million people worldwide and result in healthcare costs exceeding $20 billion (Järbrink, et al. 2017). It is expected that these numbers will increase not only due to the growing aging population, but also due to the growing prevalence of diabetes, obesity, and cardiovascular disease (Sen et al., 2009).

There are four overlapping stages that occur during wound healing process, with the first being the coagulation stage, followed by the inflammatory stage, then the proliferative stage, and finally the extracellular matrix remodeling stage (Velnar et al., 2009). Abnormal wound healing can result in excessive scar tissue formation or an ulcer. When an injury occurs, tissue factors, platelet growth factors, transforming growth factors, and collagen cause platelets to aggregate. These platelets then degranulate and release inflammatory chemokines and growth factors, followed by migration of neutrophils, lymphocytes, and macrophages to the site of injury in order to remove bacteria and other debris so that wound healing can proceed (Velnar et al., 2009; Thomas et al., 2001). Next is the proliferation stage during which extracellular matrix components are deposited by fibroblasts in order to serve as a scaffold for the final tissue remodeling stage, during which any remaining ECM components are degraded, inflammatory cells leave the site, and apoptosis of excess fibroblasts occurs (Wallace and Zito, 2019).

There are clear impacts of aging on the wound healing process apart from damage caused by ultraviolet rays, namely a decrease in elasticity, diminished re-epithelialization, and reduced cell proliferation.

One of the primary issues with age is the occurrence of chronic wounds due to poor wound healing (Thomas et al., 2001). There are a number of lifestyle factors and comorbidities that also contribute to the slow healing and chronic wounds, such as reduced immune response and antibody production, lower circulation of hormones, cardiovascular disease, and diabetes, with the latter two hindering oxygen and blood circulation (Anderson et al., 2014). Additional factors playing roles in the occurrence of chronic wounds include reduced cell migratory and proliferative abilities, cell senescence, and reactive oxygen species accumulation (Frykberg and Banks, 2015). Reactive oxygen species are beneficial at low concentrations, as they are produced by immune cells to serve as a defense against potential bacterial invasion (Schreml, et al. 2010). However, the chronic inflammatory state experienced with age results in a higher production of ROS which damages the cell and proteins of the extracellular matrix and subsequently activates inflammatory cytokines and proteases, ultimately hindering wound healing (McCarty and Percival, 2013). Previous work in animal models has suggested that potent antioxidants can reduce ROS and NOS to normal levels and reduce wound chronicity and accelerate the healing process (El-Ferjani et al., 2016).

Historically, plants have been used for medicinal purposes for thousands of years and their therapeutic potential in the field of regenerative medicine is evident. Natural extracts are an effective and economical means by which skin health can be maintained and improved, so it is important to identify natural compounds with potent wound healing properties (Thakur et al., 2011). There are myriad treatments on the market, but many have adverse side effects, are costly,

and/or inefficient (Frykberg and Banks, 2015). An efficient and cost-effective treatment that accelerates the healing process and reduces incidence of chronic wounds is needed to counter the rising cost of healthcare and the number of chronic wound patients.

Brassinosteroids play a role in plant defense mechanisms and have also been found to work in a similar manner to systemins, which are heavily involved in the plant wound healing process (Savatin et al., 2014). Brassinosteroids have also exhibited anti-viral, anti-cancer, and antioxidant properties (Malíková et al., 2008). BR bioactivity varies based on structural modifications (Figure 1), so to determine additional health-promoting activities, Esposito et al. (2013) assessed the cell migration potential of nine BR compounds and found that many, particularly Homobrassinolide and Homocastasterone, contributed to the migration of murine fibroblasts. They later determined that topical administration of Homobrassinolide accelerated the healing of cutaneous wounds in male animals. However, until now it was not known whether the results achieved in a murine model would be translatable in a human cell model.

The aim of this study was to expand upon the previous work with the nine BR compounds, but test their migratory abilities in a human cell line and determine whether the results observed are similar to those seen in the murine fibroblast study. Once it has been determined whether the results are translatable in a human cell line, steps can be made toward a clinical trial in which a topical BR can be applied as a cutaneous therapeutic agent for the treatment of wounds. Additionally, as ROS accumulation can hinder the wound healing process, cause cell damage, and exacerbate chronic wounds, therefore it is important to determine the ability of these same nine compounds to reduce ROS and NOS accumulation. As this has not been previously performed, it will first be tested in murine macrophages. Upon completion of the

study, the most promising compounds can be tested in a human cell line before incorporation into a topical treatment for further investigation.

MATERIALS AND METHODS

Reagents

(22S,23S)-Homobrassinolide [Compound 1] was purchased from Waterstone Technology (Carmel, IN) and its structure was confirmed by ESI-LCMS and NMR. Brassinosteroid compounds 2–9 including (22S,23S)-3α-fluoro-homobrassinolide [Compound 2], (22S,23S)-Homocastasterone [Compound 3], (22S,23S)-6-aza-homobrassinolide [Compound 4], (22S,23S)-3α-fluoro-homocastasterone [Compound 5], (22S,23S)-Epibrassinolide [Compound 6], (22R,23R)-Homobrassinolide [Compound 7], (22S,23S)-7v-aza-homobrassinolide [Compound 8], and (22R,23R)-Epibrassinolide [Compound 9] were either synthesized (compounds 4, 5, 6, and 8) or purchased from SciTech (Czech Republic). Modifications to compounds 4, 5, 6, and 8 were made by Dr. Thirumurugan Rathinasabapathy. These modifications were performed to promote compound stability and bioactivity (Esposito et al., 2013; Slavikova et al., 2008). Molecular formulas and structures of each BR compound are listed in Table 3.1.

Cell Lines

Primary human dermal fibroblasts isolated from adult skin (HDFa, Invitrogen C- 013-5C) were cultured in Medium 106 (Invitrogen M-106-500) containing Low Serum Growth Supplement (LSGS, Invitrogen S-003-10) supplemented with Antibiotics Penicillin /Streptomycin Solution 100 IU/100 µg/mL (Fisher MT-30-002-CI). RAW 264.7 Murine Macrophages from American Type Culture Collection (ATCC, Manasses, VA) were maintained using Dulbecco's Modified Eagle Medium (DMEM) containing 10% Fetal Bovine Serum (Gibco A3160602) supplemented with Antibiotics Penicillin /Streptomycin Solution 100 IU/100 µg/mL

(Fisher MT-30-002-CI). The cells were maintained at 37 °C in a humidified atmosphere containing 5% CO_2.

Cell viability and dose range determination

Cell viability was measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay in triplicate and quantified spectrophotometrically at 550 nm using a microplate reader (Molecular Devices, Sunnyvale, CA, USA). The concentrations of brassinosteroid compounds that showed no changes in cell viability compared with that of the vehicle (0.1% ethanol) were selected for further studies. Data not shown.

In vitro inflammatory stimulation

In order to determine the ability of natural and synthetic BR to reduce *in vitro* reactive oxygen species (ROS), an adapted fluorescent dye protocol was used (Choi et al., 2007). RAW 264.7 macrophage cells were seeded at a concentration of approx. 5×10^5 cells/well at a final volume of 1 mL into a 24-well plate and incubated overnight at 37 °C. Fresh fluorescent media, 5 μL of a solution of dichlorodihydrofluorescein diacetate acetylester (H2DCFDA, 5 mg/mL prepared in sterile phosphate-buffered saline, PBS), (Molecular Probes, Eugene, OR, USA), to reach a concentration of 50 μM in each well was added to each well for 30 minutes. After the media was aspirated, 1 μL of sample was added to each well in triplicate for a final concentration of 2 μg/mL and placed in the incubator. After 1 hour, 10 μL of 100 μg/mL lipopolysaccharide (LPS, from Escherichia coli 0127:B8) was added to treatment wells for a final concentration of 1 μg/mL, and then the plates were incubated for 24 hours. The fluorescence of 2′, 7′-dichlorofluorescein (DCF) was measured at 485 nm (excitation) and 515 nm (emission) on a microplate reader (Synergy H1, Biotech, Winooski, VT, USA). Reactive oxygen species

production was normalized to the wells only stimulated with LPS and results were expressed as percent of LPS control.

In vitro nitric oxide radical inhibition assay

The ability of natural and synthetic BR samples to inhibit nitric oxide radical formation was determined in macrophage RAW 264.7 cells. Briefly, cells were seeded at a concentration of 5×10^5 cells/well, final volume: 1 mL, into a 24-well plate, and were exposed to 1 μL of sample extract (final concentrations: 2 μg/mL) and 10 μL of 100 μg/mL lipopolysaccharide (LPS, from *Escherichia coli* 0127:B8) (final concentration: 1 μg/mL). Dexamethasone (DEX) was used as positive control at a concentration of 10 μM. Plates were then incubated for 24 h. The production of nitrite, the stable end-product of NOS generation by macrophages, was assayed colorimetrically as described by Kellogg & Lila (2013). To 100 μL of cell culture medium was added 100 μL of Griess reagent (1% sulfanilamide and 0.1% naphthylethylenediamine in 5% phosphoric acid), and the mixture was incubated in the dark at room temperature for 10 min. The absorbance at 540 nm was recorded (Synergy H1, Biotech, Winooski, VT, USA), and a calibration curve of sodium nitrite (0–100 μM) was used to express results as μM of nitric oxide concentration.

In vitro wound healing assay

Ability of BR samples to promote cell migration was evaluated on a 2-D assay of adherent adult human dermal fibroblasts (HDFa) cell lines using Oris™ 96-well tissue culture treated plates (AMS Biotechnology, Cambridge, MA). For this procedure, 100 μL of HDFa previously treated with fluorescent NucBlue® Live Cell Stain and fluorescent CellTracker™ Red CMTPX Dye were seeded in 96-well plates (max. 3.0×10^5 cells/well) containing stoppers in the center of each plate to create a cell exclusion zone. The plates were incubated for 24 hours at

37C and 5% CO2. After the incubation period, stoppers were removed and treatments were added at concentrations of 0.2 µM and 2µM for each sample to determine dose dependent responses. The progress of cell migration was monitored after 0, 24, and 48 hours after sample addition by measuring the fluorescence at 360 nm (excitation) and 460 nm (emission) on a microplate reader (Synergy H1, Biotech, Winooski, VT). Cells treated with 10 µL of FBS served as a positive control and wells containing no cells or only growth media with 80% ethanol served as negative controls.

Fluorescent image analysis

Bright field and fluorescent images were observed using the EVOS® FL Auto Cell Imaging System (Life Technologies). Images were captured at the center of each well at 0 and 48 hours to have a visual representation of fibroblast migration. The images were analyzed using EVOS software and percentage of wound closure was calculated relative to vehicle control. Area of the space where the insert template was printed was measured at 0 and 48-hour time periods. The percent difference of wound closure was found between the two time points.

Statistical Analysis

Statistics were performed using the software GraphPad Prism version 8.0 (GraphPad Software Inc., La Jolla, CA). All data were analyzed by one-way ANOVA with treatment as factor. All samples were obtained in triplicate and analyzed in three repetitions ($n = 3$) unless otherwise noted. Post hoc analyses of differences between individual experimental groups were made using Dunnett's multiple-comparison tests. Significance was set at $p < 0.05$. Values are reported as means ± SEM.

RESULTS

Reactive Oxygen Species Assay

The potential for BR to inhibit reactive oxygen species (ROS) production was investigated using LPS-stimulated murine RAW 264.7 macrophages. All treatments were compared to an LPS-stimulated untreated control. BR compounds were administered at a concentration of 2µg/mL. Results are shown in Figure 2. Compounds 1, 3, and 4 were found to significantly reduce ROS production by 13%, 11%, and 13.4% respectively ($p < 0.0001$). The remaining compounds (2, 5, 6, 7, 8, and 9) did not make any significant contributions to reducing ROS, with results similar to the untreated LPS control.

Nitric Oxide Assay

The ability of BR to inhibit intracellular nitric oxide production was explored *in vitro* using LPS-stimulated murine RAW 264.7 macrophages. After LPS stimulation, cells were administered BR compounds at a concentration of 2µg/mL. Results are shown in Figure 3. Cells treated with the positive control, dexamethasone, had a 42% reduction ($p <0.0001$) in NOS production. Cells treated with compounds 8 and 9 resulted in a 9% reduction in NOS ($p=0.0054$ and $p=0.0050$, respectively) when compared to the LPS control. Compounds 1, 2, and 5 also contributed to a reduction in NOS species of around 8% when compared to LPS control ($p=0.0161$, $p=0.0174$, and $p=0.0183$, respectively). The remaining compounds (3, 4, 6, and 7) did not make significant contributions to the reduction of NOS.

Cell Migration Assay

Adult human dermal fibroblasts were treated with two doses, 0.2µM and 2µM, of BR compounds and a dose-dependent migration response was observed over a 48 hour period (Figure 4). At the 0.2µM concentration, fibroblasts treated with compounds 1, 2, and 5 showed

significant migration ($p<0.0001$) as compared to cells treated with the vehicle control (80% ethanol). Compound 3 also resulted in significant migration of fibroblast cells ($p=0.0081$). Compounds 4, 6, 7, 8, and 9 did not have any impact on cell migration when compared to fibroblasts treated with vehicle control. At the 2.0μM concentration, fibroblasts treated with compounds 1, 3, 5, 7, and 8 exhibited significantly greater migration than those treated with vehicle control ($p<0.0001$). Treatment with compounds 2 and 4 also produced significantly greater cell migration over 48 hours compared to control ($p=0.0034$ and $p=0.0044$, respectively). The remaining compounds did not result in fibroblast migration that differed significantly from cells treated with the ethanol control.

The dose-dependent migration response within a single compound is shown in Figure 5. After 48 hours, the high dose of compound 3 resulted in significantly greater cell migration than the low dose ($p=0.0209$). After 48 hours, compound 4 had a significant increase in cell migration at 0.2 ($p=0.0026$) and 2.0 ($p<0.0001$). Compound 7 had a significant increase in cell migration at the 2.0 dose after 48 hours ($p=0.0194$). After 48 hours, compound 8 had a significant increase in cell migration at 0.2 ($p=0.0066$) and 2.0 ($p=0.0003$). Compound 9 had a significant increase in cell migration at the 2.0 dose after 48 hours ($p=0.0239$). Compounds 1, 2, 5, 6 did not have any significant differences in migration between the two doses. Figure 6 shows the results observed at the 48 hour time point using the EVOS microscope, and provides a visual of the quantitative data seen in figure 4. Cells treated with the positive control (fetal bovine serum) had high migration over 48 hours, but cells treated with compounds 1, 3, 5, 7, 8 also resulted in a significant migration of cells into the exclusion zone during this period.

DISCUSSION

The skin acts as an environmental barrier, protecting us from chemical, physical, and biological agents that could cause damage if this barrier were to break. However, the constant exposure to these environmental elements results in frequent breaks in the skin (a wound) meaning that our body needs to have proper defenses set in place to prevent further damage. Our bodies have well-coordinated mechanisms that respond to injury and heal wounds in four overlapping phases: (1) the coagulation 2) the migration of immune cells to the site of injury 3) the cell proliferation stage and (4) the remodeling phase during which the collagen is remodeled and the wound closes (Guo and DiPietro, 2010; Reinke and Sorg, 2012). There has been a rapid increase in the elderly population, and it is expected to grow to 2 billion people over the next thirty years. With this sharp rise in aging individuals comes an increase in chronic disease and associated comorbidities which contribute to delayed wound healing and wound chronicity, resulting in frequent hospitalizations and excessive healthcare costs (Avishai et al., 2017; Ashcroft et al., 2002; Gerstein et al., 1993).

Excessive reactive oxygen species (ROS) accumulation also contributes to impaired wound healing. Under normal circumstances, ROS are a natural byproduct of aerobic metabolism and can play important roles in cell signaling. During periods of stress, such as chronic inflammation observed during the aging process, oxidative stress occurs due to the higher levels of ROS that occurs more rapidly than cells can manage. Regulation of ROS through antioxidant mediation is important for normal wound healing, as high levels of ROS and NOS can negatively impact extracellular matrix proteins and impair function of fibroblasts. Low levels of ROS have been shown to stimulate wound healing while unusually high ROS and NOS content can lead to cell damage and diminished wound healing.

While BR antioxidant capabilities have been investigated and confirmed in plants, until this study little work had been done regarding their effects on ROS and NOS in mammalian cells. Our results determined that (22S,23S)-Homobrassinolide, (22S,23S)-Homocastasterone, and (22S,23S)-6-aza-homobrassinolide contributed to significant reduction of ROS. While these BR have previously been shown to reduce oxidative stress in plants, flow-cytometry analysis of cancer cells treated with Homocastasterone showed a 6-fold increase in ROS which resulted in cancer cell apoptosis (Kisselev et al., 2017). In contrast, another study analyzed seven brassinosteroid compounds for antioxidant and neuroprotective abilities and they were found to reduce oxidative stress and protect neurons against MPP+ toxicity, and these activities could be attributed to the B-ring and side chain of each molecule (Ismaili et al., 2011).

Research has shown that BR compounds have attenuated pesticide-based toxicity in plants and significantly reduce oxidative stress due to BR activation of antioxidant defense mechanism (Sharma et al., 2019). Based on these data, it was hypothesized that some of these analogues would possess the ability to reduce reactive oxygen species upon LPS stimulation of the inflammatory response. Compounds 1, 3, and 4 were all validated as significant contributors to the reduction of ROS, while other compounds did not differ much from the LPS-control. Compound 1, Homobrassinolide, has been previously shown to contribute to the ability of plants to combat temperature stress and salt stress through regulating ROS accumulation by increasing the activity levels of catalase, superoxide dismutase, and monodehydroascorbate reductase (Kaur et al., 2018). Compound 3, Homocastasterone, has also exhibited significant radical scavenging capabilities in cancer cells (Kisselev et al., 2017). Similar results were seen when homocastasterone administration modulated antioxidant response and amino acid balance in order to reduce oxidative stress in Chinese mustard plant seedlings (Yadav et al., 2017). The

antioxidant potential of these compounds may contribute to their wound healing capabilities, as oxidative stress can alter the structure and function of the extracellular matrix (Kunkemoller et al., 2017). Age and health-related disruptions like sarcopenia and diabetes can result in altered redox signaling and the subsequent accumulation of these free radicals to a level that does more harm than good for the wound healing process (Sakellariou et al., 2017).

Five BR compounds were found to reduce NOS upon LPS stimulation of inflammatory response. SS-Homobrassinolide (compound 1), SS-3α-fluoro-homobrassinolide (compound 2), SS-3α-fluoro-homocastasterone (compound 5), SS-7v-aza-homobrassinolide (compound 8), and RR-Epibrassinolide (compound 9) reduced inflammation associated with NOS in murine macrophages treated with LPS. However, since BR play a crucial role in stress response and plant defenses, BR can result in NOS accumulation to serves as a signaling molecule for virus and disease resistance (Zou et al., 2018). NOS radicals are produced by macrophages and are involved in many aspects of wound healing, including cell proliferation and the formation of collagen, so it is possible that BR contribute to accelerated wound healing through NOS-signaling mechanisms which also explains why many BR analogues were not as effective at reducing NOS species as they were with ROS reduction (Witte and Barbul, 2002; Anwar et al., 2018). Further investigation into the mechanism behind BR wound healing properties is needed.

Wound healing is affected by both gender and age, and adequate levels of animal sex hormones are needed to maintain efficiency through activation of IGF-1 which accelerates wound healing through cell proliferation and collagen synthesis (Demling, 2005). Both BR and insect ecdysteroids are structural analogues to the animal hormone estrogen, which plays a critical role in the wound healing process. The insect molting hormone ecdysteroid has also shown promising benefits in the areas of protein synthesis and cell migration and proliferation.

Administration of 20-Hydroxyecdysone was shown to promote wound healing in rabbits, stimulating the proliferation of epithelial cells and enhancing the granulation stage (Hou et al., 2007). Phytoecdysteroids have been investigated for their wound healing abilities because of their structural similarity to estrogen, and many were found to promote cutaneous wound healing in both healthy and diabetic animals when administered at concentrations of 10mg/kg (Ramazanov et al., 2016).

The same nine compounds used in Chapter III were previously tested by Esposito et al. (2013) to determine their ability to enhance cell migration in murine fibroblasts, specifically Homobrassinolide, Homocastasterone, and their synthetic analogues. Based on the strong results produced by Homobrassinolide, this compound was used *in vivo* and found to accelerate closure of cutaneous wounds in C57BL/6J mice through Akt modulation and reduction of the pro-inflammatory response (Esposito et al., 2013). These results highlighted the need to investigate whether the same benefits could be observed in a human cell line, and eventually a clinical trial. Human Dermal Fibroblasts (HDFa) are cells involved in the initial injury response and are an integral part of the repair process by rapidly dividing and migrating to the wounded area (Tracy et al., 2016). It was hypothesized that many of the nine BR compounds used to treat murine fibroblasts, particularly Homocastasterone and Homobrassinolide, would promote human fibroblast migration in a similar manner.

A cytotoxicity assay was performed using the BR compounds and it was determined that the most effective, nontoxic concentrations ranged from 0.2mM to 2.0mM, so these two doses were used for the wound healing assay. Over the 48 hour period post-treatment, cells responded to most compounds in a dose-dependent manner (Figure 4, Figure 5). The majority of brassinosteroids tested in this study showed moderate biological activity regardless of structural

changes, though neither Epibrassinolide compounds enhanced cell migration, with (22S,23S)-Epibrassinolide showing little difference from the cells treated with ethanol. Previous studies showed that both RR and SS Epibrassinolide promoted cell proliferation but did not have an effect on migration, whereas cells treated with homobrassinolide-based and Homocastasterone-based compounds resulted in a significant increase of both migration and proliferation (Esposito et al., 2013). This was previously attributed to the ability of Homobrassinolide to induce Akt phosphorylation, so it is likely that Homocastasterone exerts effects similarly (Esposito et al., 2013).

The Akt signaling pathway has been shown to be involved in many different pathways including protein homeostasis, glucose metabolism, cell cycle regulation, as well as the regulation of fibroblast migration, and indeed many of these compounds contributed to a significant increase in fibroblast migration over a 48 hour period as shown in Figure 4 (Li et al., 2016). At the lower 0.2μM dosage, compounds 1, 2, 3, and 5 exhibited substantial fibroblast migration over a 48 hour period. At the higher 2.0μM treatment, compounds 1, 2, 3, 4, 5, 7, and 8 all displayed potent ability to accelerate fibroblast migration. Compounds 6 and 9 did not improve fibroblast migration, mirroring the results observed previously in the treatment of murine fibroblasts. The results seen here reflect those observed in murine fibroblasts, with Homobrassinolide and Homocastasterone possessing the most potent migratory capabilities in HDFa cells. Similarly structured ecdysteroids have been shown to result in Akt activation as well, resulting in anabolic and cell migration responses similar to BR (Gorelick-Feldman et al., 2010). Ecdysteroids isolated from quinoa were found to be potent inhibitors of skin collagenases and to reduce oxidative stress, highlighting their potential use in the skin therapeutic and

regenerative medicine market (Nsimba et al., 2008). These data open the door for further investigation into BR use in the dermatological research.

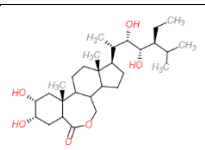
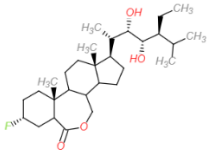
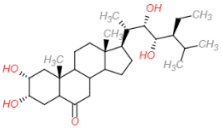
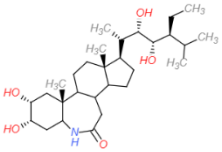
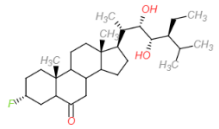
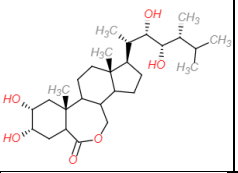
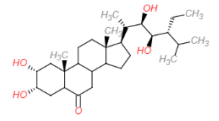
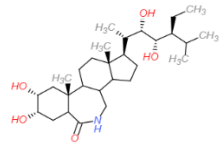
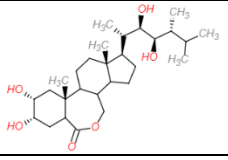
Overall, human dermal fibroblasts treated with BR compounds exhibited accelerated cell migration over 48 hours. Treatment with Homobrassinolide-based and Homocastasterone-based compounds resulted in the greatest migratory activity. Murine macrophages exposed to LPS-induced oxidative stress had significantly lower ROS accumulation when treated with Homobrassinolide and Homocastasterone compounds, while Homobrassinolide compounds and Epibrassinolide contributed to a reduction in NOS. Together these data provides strong evidence for BR ability to promote skin health, particularly through contributions to both cell migration and reducing excessive oxidative stress.

CONCLUSION

To conclude, our results show that many BR analogues exert a positive effect on human dermal fibroblast migration, and some of these compounds contribute to reduced ROS and NOS after LPS-induced oxidative stress in murine macrophages. Eight out of nine BR compounds were found to significantly accelerate human dermal fibroblast migration over a 48-hour period. HDFa cells treated with (22S,23S)-Homobrassinolide and (22S,23S)-Homocastasterone resulted in the most cell migration, whereas (22S,23S)-Epibrassinolide resulted in the lowest cell migration which did not differ from cells treated with the ethanol control. (22S,23S)-Homobrassinolide, (22S,23S)-Homocastasterone, and (22S,23S)-6-aza-homobrassinolide were found to be the most potent scavengers of reactive ROS. Although a natural byproduct of metabolism, ROS can be detrimental with excessive accumulation during aging and diseased states. Oxidative stress plays a role in a number of pathologies, including sarcopenia, cognitive decline, and telomere shortening, and is a major source of cellular damage in aging individuals.

ROS have also been found to exacerbate impaired muscle function observed in sarcopenia sufferers (Fulle et al., 2004). Chapter III highlights the various benefits BR offer to the field of skin health and regenerative medicine, as BR ability to enhance Akt signaling contributes to improved cell migration and ability to act as an antioxidant in some cases. By determining which compounds have the most appealing bioactivity profile, therapeutic options for improving integrity of aging skin and healing chronic wounds can be made available and ultimately improve the quality of life in the rapidly growing aging population.

Table 3.1 Brassinosteroid Compound Identification and Effects on Cell Migration (Adapted from Esposito et al., 2013)

ID	Name	Formula	Structure	Migration at 48 hours 0.2µM, % Control	Migration at 48 hours 2.0µM, % Control
1	(22S,23S)-Homobrassinolide	$C_{29}H_{50}O_6$		55.92±19.86	74.12±11.78
2	(22S,23S)-3α-fluoro-homobrassinolide	$C_{29}H_{49}FO_4$		53.32±13.87	37.92±15.02
3	(22S,23S)-Homocastasterone	$C_{29}H_{50}O_5$		35.22±3.7	85.42±16.95
4	(22S,23S)-6-aza-homobrassinolide	$C_{29}H_{51}NO_5$		-1.35±7.89	37.12±10.56
5	(22S,23S)-3α-fluoro-homocastasterone	$C_{29}H_{49}FO_3$		55.32±15.41	49.62±13.99
6	(22S,23S)-Epibrassinolide	C28H48O6		-1.53±15.14	4.82±21.91
7	(22R,23R)-Homobrassinolide	$C_{29}H_{50}O_6$		10.22±4.23	57.12±13.26
8	(22S,23S)-7v-aza-homobrassinolide	$C_{29}H_{51}NO_5$		-13.23±5.51	52.02±16.59
9	(22R,23R)-Epibrassinolide	$C_{28}H_{48}O_6$		-9.99±14.88	24.52±11.95

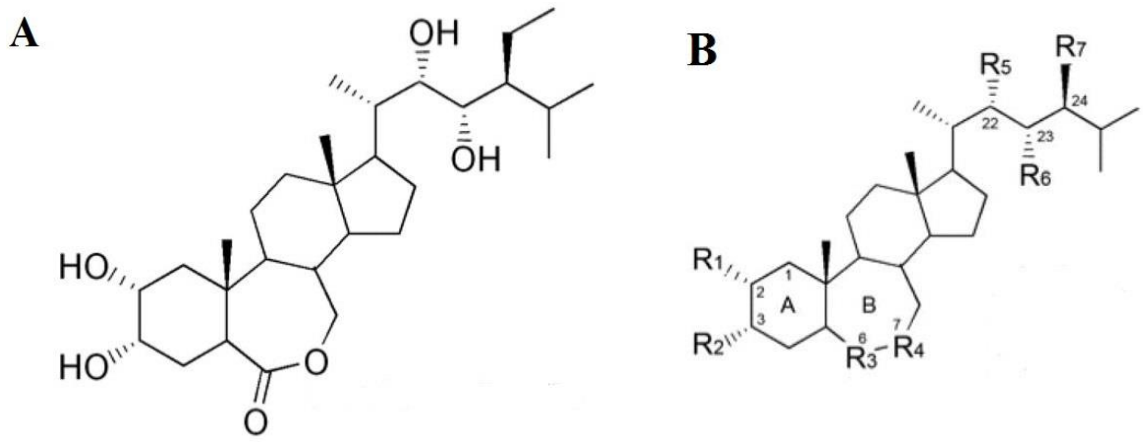


Figure 1. Brassinosteroid Structure and Modification Positions. Figure A shows the structure of natural BR Homobrassinolide. Figure B shows the general BR structure and where structural modifications can occur in other natural and synthetic BR analogues. For the cell culture assays performed in this study, structural modifications were made to either the A or B rings (shown in Table 3.1) to improve molecule stability and bioactivity.

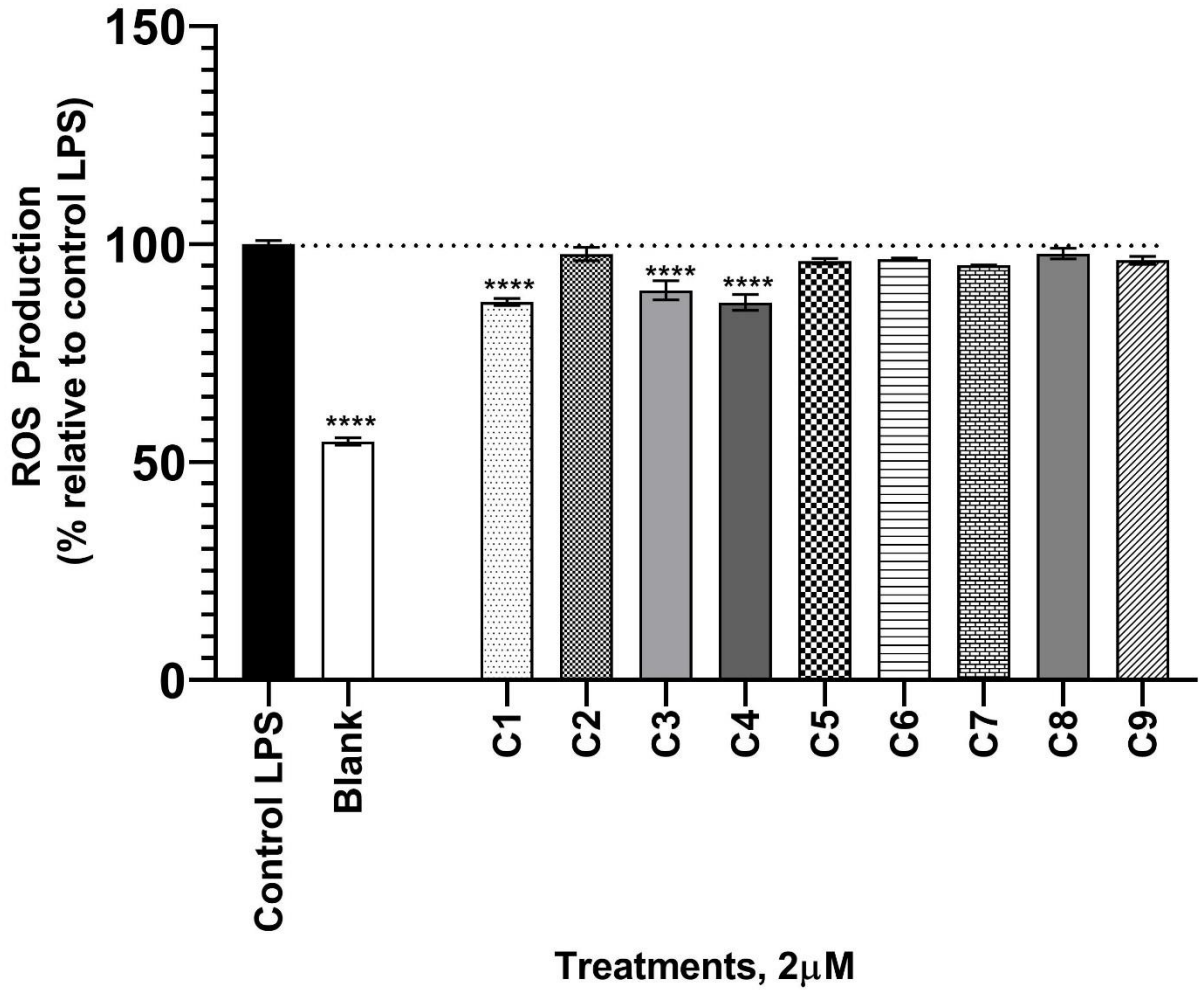


Figure 2. Reactive Oxygen Species Reduction Potential of BR. Effects of structurally different brassinosteroids on the production of reactive oxygen species (ROS) after Lipopolysaccharide stimulation. Samples marked with an asterisk (∗) are significantly different compared to untreated LPS control ($P < 0.05$). ∗$p < 0.05$ vs. the LPS control group. All samples were assayed in triplicate. Data is reported as the mean ± SEM, n = 3. *$p < 0.05$ vs. the LPS-treated group.. One-way ANOVA, Dunnett's post hoc test. ****indicates $p < 0.0001$.

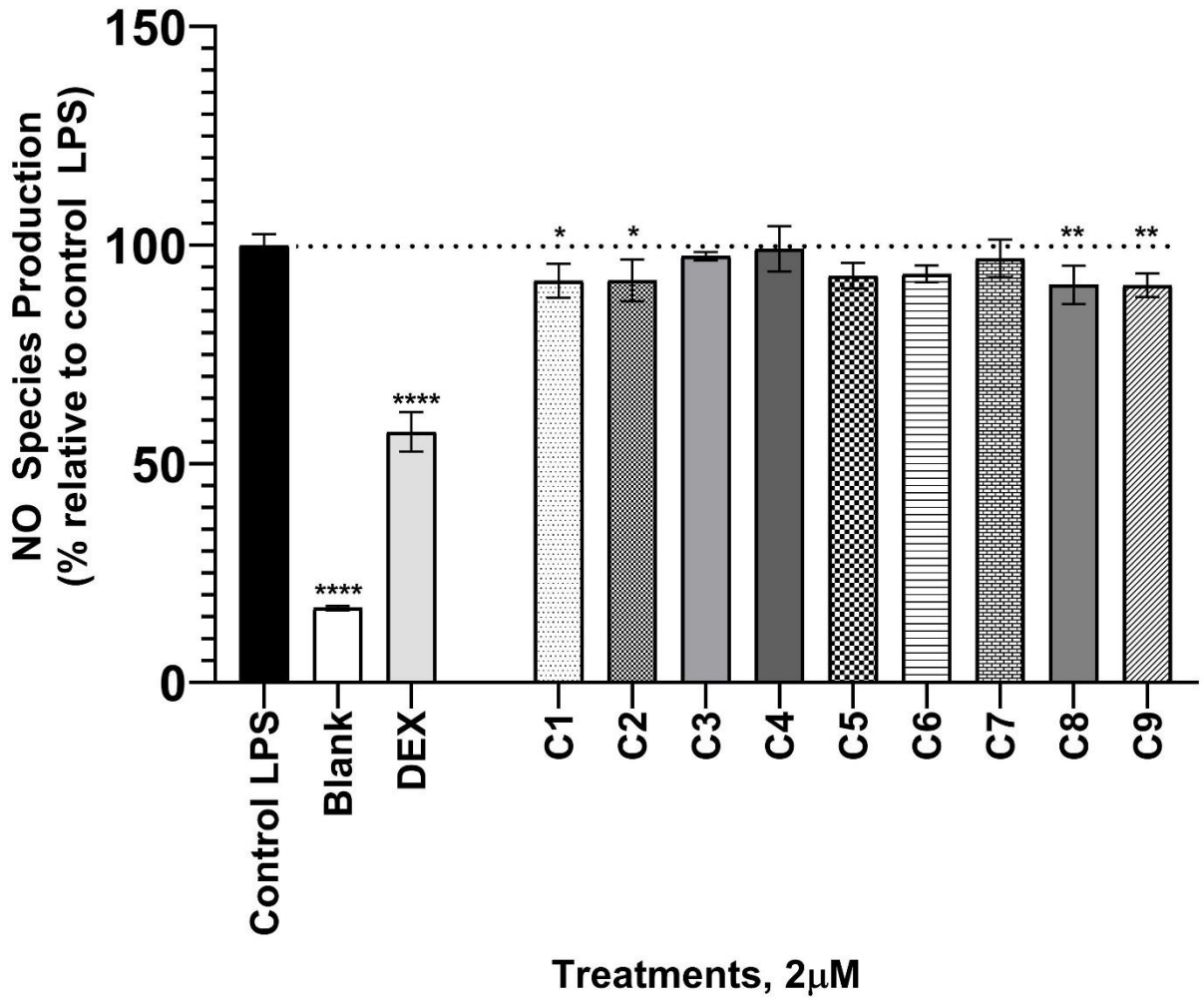


Figure 3. Nitric Oxide Species Reduction Potential of Brassinosteroids. Effects structurally diverse brassinosteroids on nitric oxide (NO) production. Dexamethasone (Dex) at 10 μM was used as a positive control. Samples marked with an asterisk (∗) are significantly different compared to untreated LPS control ($P < 0.05$). All samples were assayed in triplicate. Data is reported as the mean ± SEM, n = 3. One-way ANOVA, Dunnett's post hoc test. * indicates $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and $p < 0.0001$ vs the LPS-treated control group.

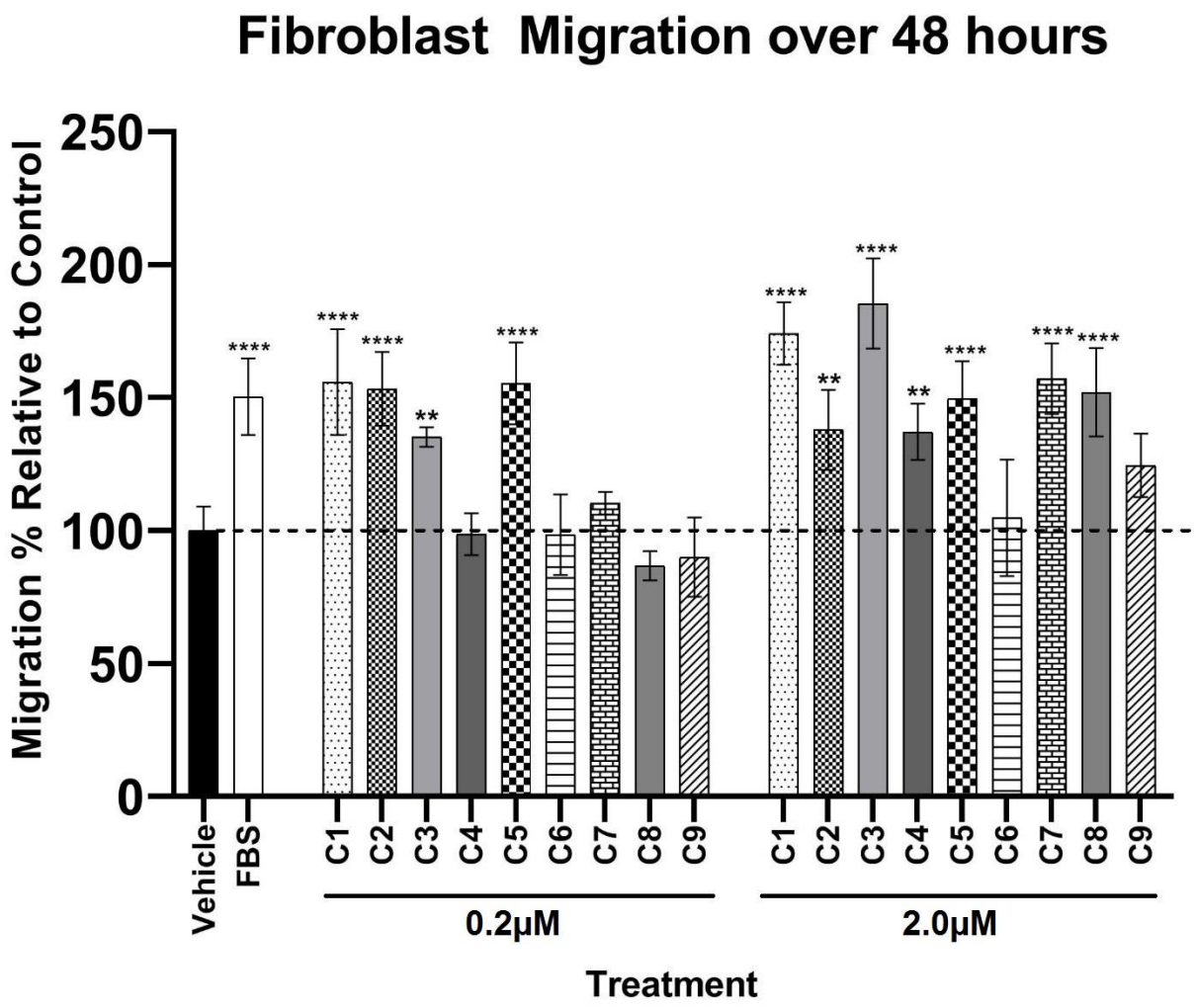


Figure 4. Human dermal fibroblast migration results after 48 hours. Human dermal fibroblast cells (HDFa) in supplemented M106 growth medium were labeled with Cell Tracker Red CMTPX dye and Nuc Blue, then seeded at a density of $3.0x10^5$ in a 96 well Oris Cell Migration Plate. After adherence, stoppers were removed to reveal an exclusion zone devoid of cells. HDFa were treated with BR at concentrations of 0.2µM and 2.0µM in replicates of 4. FBS, fetal bovine serum at 0.5% was used as the positive control. Samples marked with an asterisk (∗) are significantly different compared to vehicle control, 80% ethanol ($P < 0.05$). Data is reported as the mean ± SEM, n=4. One-way ANOVA, Dunnett's post hoc test. * indicates $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and $p < 0.0001$ vs the LPS-treated control group.

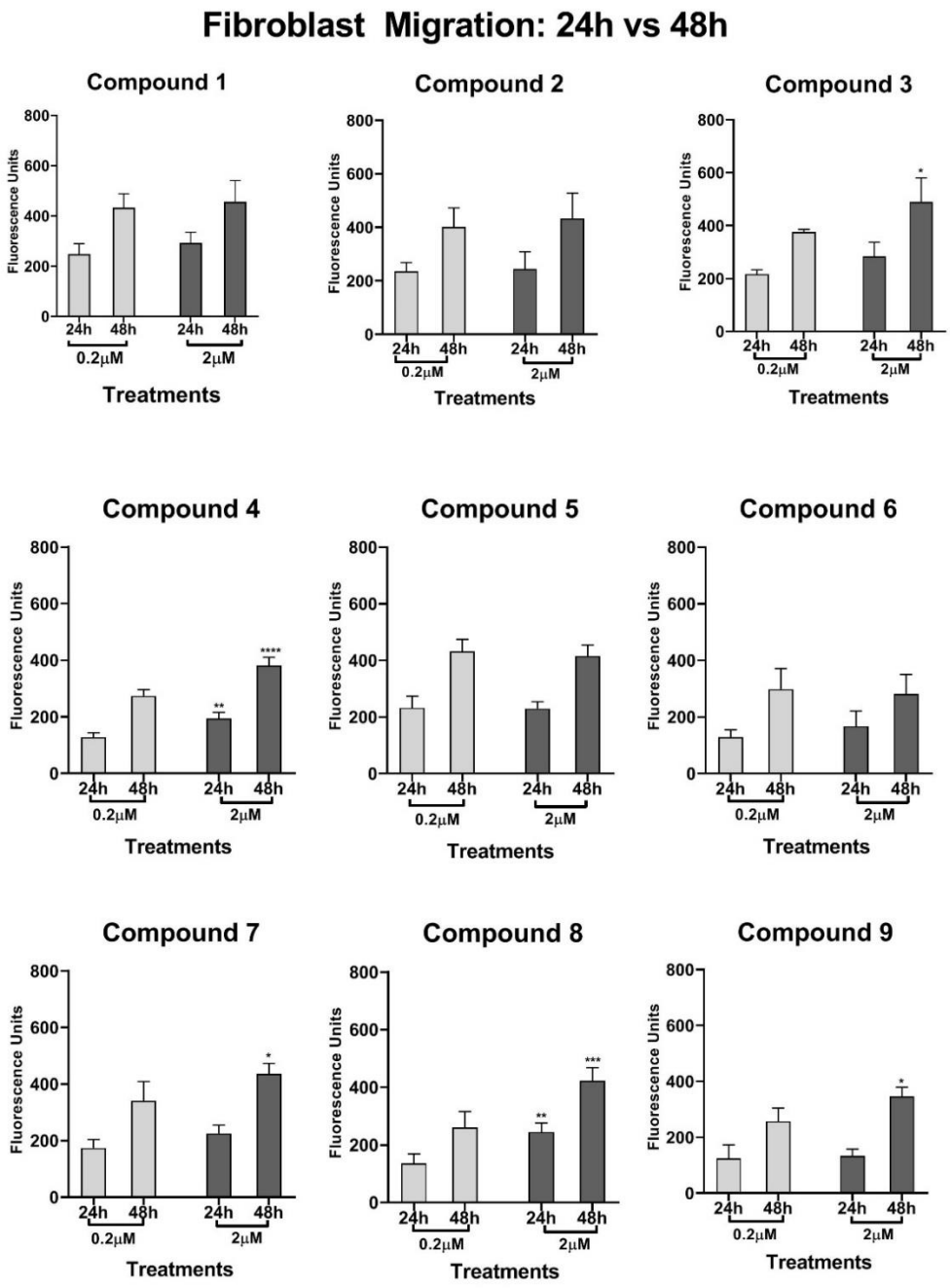


Figure 5. Dose-dependent Effect of Brassinosteroids on Cell Migration. Human dermal fibroblast cells (HDFa) in supplemented M106 growth medium were labeled with Cell Tracker Red CMTPX dye and Nuc Blue and seeded at a density of $3.0x10^5$ in a 96 well Oris Cell Migration Plate. After adherence, stoppers were removed to reveal an exclusion zone devoid of cells. HDFa were treated with BR at concentrations of 0.2µM and 2.0µM. Vehicle was 80% ethanol and 10% fetal bovine serum served as a positive control. This figure shows the migration results at 24 and 48 hours post-treatment. Significant difference between low dose and high dose at different time points indicated ** $p < 0.01$, *** $p < 0.001$, and $p < 0.0001$ vs the LPS-treated control group.

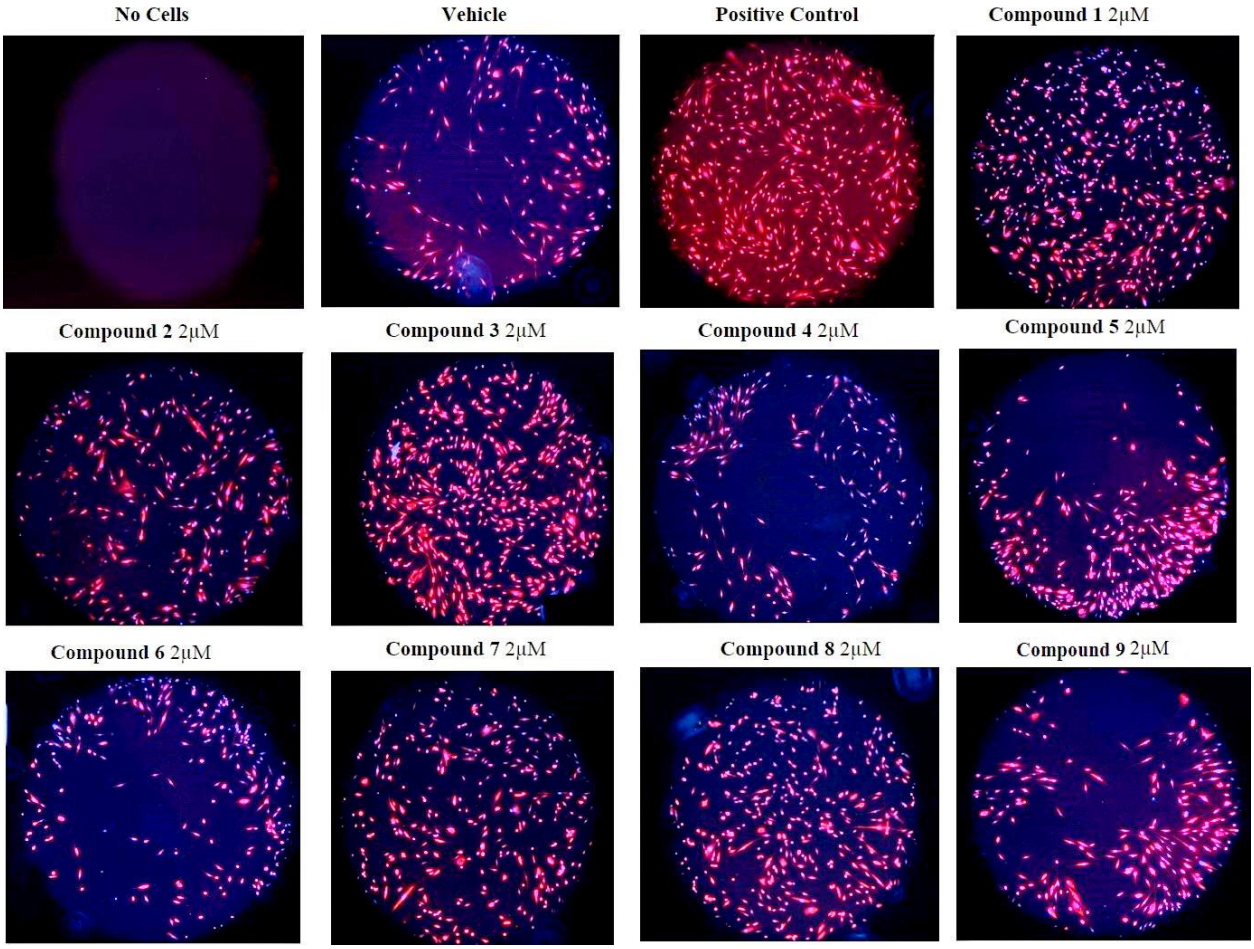


Figure 6. Fluorescent Cell Migration Images. Human dermal fibroblast cells (HDFa) in **2**μM supplemented M106 growth medium were labeled with Cell Tracker Red CMTPX to stain morphology and Nuc Blue to stain nuclei. Cells were seeded at a density of 3.0 x 10^5 in a 96 well Oris Cell Migration Plate. After adherence, stoppers were removed to reveal an exclusion zone devoid of cells. Cells in these images were treated with BR at concentrations of 2.0μM. Fetal bovine serum served as a positive control. An EVOS Fluorescent Imaging Microscope was used to take migration images after 48 hours of fibroblasts migrating into the exclusion zone.

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CHAPTER 4

Conclusions and Future Direction

CONCLUSION

According to the 2018 census, the elderly population in the United States is expected to surpass 75 million individuals by 2034, outnumbering children by nearly half a million. With this sharp rise in aging individuals comes an increase in cases of physical disability due to poor muscle health and impaired wound healing, both resulting in frequent medical treatment and high costs. Sarcopenia, age-related loss of muscle mass and strength, occurs in 10% of individuals over age 60 and more than 33% of those over 80 (Normal and Otten, 2019). There are numerous comorbidities associated with sarcopenia, including diabetes, cardiovascular disease, and chronic inflammation, all of which also contribute to another major problem in the elderly: impaired wound healing (Beaudart et al., 2014; Trierweiler et al., 2018). Sarcopenia results in $18 billion in healthcare costs, while frequent medical care for chronic wounds due to impaired wound healing can drive costs up an additional $30-$50 billion for the US population (Janssen et al., 2004; Sen, 2019).

There are numerous factors associated with the pathophysiology of sarcopenia, including insulin resistance, mitochondrial dysfunction, decreased circulation of anabolic hormones, chronic inflammation, poor nutrition, and genetics, all of which make treating sarcopenia very difficult (Morley 2016). Current treatments include resistance exercise, dietary changes, increased vitamin D intake, and protein supplements. High-dose testosterone has been shown to improve muscle mass and function, but this solution comes with many unwanted androgenic side effects and is not recommended (Morley et al., 2016; Morley et al., 2018). Despite these options, they do not guarantee long-term success in the prevention of sarcopenia or slowing the progress of the condition. Economical methods that are both effective and devoid of unwanted side-effects are needed to treat the growing prevalence of sarcopenia and its comorbidities.

Brassinosteroids are phytohormones responsible for plant growth, immunity, and maintenance. Brassinosteroids promote growth in plants by increasing the rates of protein and nucleic acid synthesis (Bagjuz et al., 2000). Brassinosteroids share structural and physiological similarities with insect ecdysteroids, which have been proven to possess a number of pharmacological benefits in insects and animals, including stimulation of protein synthesis and enhancement of carbohydrate metabolism. Recent work with BR exhibited similar results, with BR administration enhancing protein synthesis in skeletal muscle cells, increasing gastrocnemius muscle size, and improving physical fitness of animals (Esposito et al., 2011). This research focused on the ability of natural compounds, BR-rich cabbage seed extract (CSE) and pure BR, to elicit health promoting properties in new animal and cell models.

The study in Chapter II consisted of evaluating the dietary supplementation of CSE on body weight, body composition, endurance, and behavioral changes in an accelerated aging rodent model. Also investigated was the role of CSE on genes associated with myogenic activity, satellite cells, and protein degradation. The aim of the study was to determine if this cabbage seed extract, known to be rich in brassinosteroids, could contribute to improved physical fitness with age through regulation of myogenesis in both male and female animals. The study confirmed that both male and female animals consuming a low fat diet supplemented with 2% CSE exhibited greater endurance compared to control animals, with males experiencing the endurance boost earlier (19 weeks) whereas female animals had significantly greater endurance later in life (36 weeks). Additionally, CSE females had significantly greater lean mass content compared to control animals. Gene expression analysis of CSE animals' gastrocnemius tissue revealed that at 32 weeks, males had a 2-fold increase in Myogenin and a nearly 4-fold increase in satellite cell marker Pax3, and females had significant upregulation of both Pax3 and MyoD.

At 50 weeks, CSE males had upregulation of Gdf8 and satellite cell marker Pax7, while CSE females had a significant increase in Pax3 expression and a nearly 50% decrease in the proteolytic markers Trim63 and Fbxo32, which provide insight into their improved endurance and increased lean muscle mass. Additionally, male animals at 47 weeks of age showed reduced behavioral changes with age compared to control animals. This work highlights the potential anabolic properties and health benefits offered by dietary supplementation of CSE.

In Chapter III, nine structurally diverse brassinosteroid compounds were used in cell culture studies to first investigate radical scavenging abilities in LPS-stimulated murine macrophage cells, and second to determine if these compounds could contribute to improved cell migration in human dermal fibroblast cells. Based on previous data showcasing the ability of BR compounds Homobrassinolide and Epibrassinolide to regulate ROS levels in plants, it was hypothesized that these compounds and their analogues would impact ROS and NOS accumulation upon stress onset in murine macrophages (Kaur et al., 2018; Tanveer et al., 2018) Our results confirmed that Homobrassinolide, Epibrassinolide, and their analogues contributed to significant reduction of NOS accumulation, while Homobrassinolide and Homocastasterone analogues resulted in the most significant ROS reduction compared to the LPS-stimulated control. Previous work showcased the cell migration potential of these nine BR compounds in murine fibroblasts, with Homobrassinolide, Homocastasterone, and their variants resulting in the most cell migration (Esposito et al., 2013). Our experiment in a human cell line mirrored the previous results, as cells treated with Homobrassinolide and Homocastasterone analogues exhibited the most cell migration over a 48 hour period, with a higher dose of 2µM resulting in greater fibroblast migration. The findings in Chapter III confirm that the previous work

conducted in a murine cell line is in fact translatable in a human line, opening the door to clinical studies in which the therapeutic nature of these BR compounds can be further assessed.

Overall, this work demonstrates the ability of natural compounds, brassinosteroids and BR-rich cabbage seed extract, to contribute to areas of health that are heavily impacted by the aging process. The compound of interest, brassinosteroid, was shown to have potent bioactivity levels within a human cell line in regard to migration ability as well as free radical scavenging capabilities in murine macrophages. Dietary supplementation of brassinosteroid or rich brassinosteroid sources could prove to be a therapeutic agent used in conjunction with diet and exercise to lessen the physical and economic burdens of the aging process.

FUTURE DIRECTION

The research previously described in Chapters II and III was intended to provide alternative approaches to improving the health profile of aging muscle and enhancing wound healing activity, both of which are major issues for the aging population. Sarcopenia and injuries caused by age-related muscle loss result in costs exceeding $18 billion yearly, and this number is expected to grow as the elderly population increases (Beaudart et al., 2014). Cabbage seeds are known to be a rich source of brassinosteroid, which is a phytohormone known to result in an anabolic response in animals through modulation of the Akt pathway (Clouse et al., 2011; Esposito et al., 2011). Cabbage seed extract serves as a potential nutritional intervention that may alleviate the loss of muscle mass and function that is observed in sarcopenia. The phytohormone found within these cabbage seeds, BR, can also contribute to the relief of other symptoms associated with sarcopenia, such as impaired wound healing and excessive ROS and NOS accumulation. The work in these chapters offers insight into therapeutic treatments to ease

symptoms associated with the aging phenotype, specifically those surrounding muscle loss and wound healing.

Presently, suggested treatments for muscle loss observed in sarcopenia include exercise therapy and a higher protein intake. However, the long-term success rates of these are low. The addition of CSE to one's diet is an economically viable option, and our results showed that CSE as 2% of the total intake by weight was sufficient to result in improvements to endurance and myogenic profile to both males and females, and to contribute to an increase in lean mass in female animals. It is important to test other doses of this extract and find a suitable amount that would be feasible and effective upon human consumption. Additionally, it is important to investigate other *Brassicaceae* sources, identifying which have the most beneficial BR profiles and to use other parts of the plant known to be rich in BR.

Our study compared the gastrocnemius muscle profiles of mice fed a 10% low fat diet and a 10% low fat diet supplemented with 2% CSE. Previous studies have shown the ability of BR to regulate blood glucose, lower body weight, reduce cholesterol, and lower triglycerides in animals with diet-induced obesity (Esposito et al., 2012). As diabetes is becoming increasing prevalent and can exacerbate sarcopenia, it is of utmost importance to investigate any benefits offered by a high fat diet supplemented with CSE (Trierweiler et al., 2018). In vitro work has shown the ability of Homobrassinolide to significantly reduce glucose, but the antidiabetic effect of plant sources rich in BR should be further investigated as the number of individuals diagnose with diabetes increases (Esposito et al., 2012).

Another important factor in sarcopenia is bone health. Multiple factors contribute to the onset of both osteoporosis and sarcopenia, and each condition is believed to impact the other (Go et al., 2013). BR is structurally similar to estrogen which is critical for bone growth and

maintenance in young individuals as well as for bone turnover in adults, and estrogen deficiency results in the formation of osteoclasts and bone being resorbed (Väänänen and Härkönen, 1996). Ecdysone is another structural analogue of both estrogen and BR, and previous studies confirmed that beta-ecdysone administration in animals prevented glucocorticoid-induced changes in bone mass and bone cell viability (Dai et al., 2015a). Another study showed that beta-ecdysone administration in gonadal-sufficient and insufficient animals of both sexes increased peak bone mass (Dai et al., 2015b). These studies warrant the investigation of any beneficial effects BR or plant sources rich in BR may have on bone health, adding to the list of their protective properties in the treatment of sarcopenia.

Chapter II focused primarily on the myogenic profile of the gastrocnemius muscle, as this muscle is primarily affected in sarcopenia. Many other tissues were collected for analysis, including other skeletal muscles, adipose tissue, liver, and brain, so it is important to complete analysis of these tissues to determine any effects that the CSE diet exerted upon them. BR were previously shown to increase the type-II fibers of the gastrocnemius muscle, so it is necessary to elucidate the mechanism behind this change in fiber composition and see how muscles of other fiber types are impacted, if at all (Esposito et al., 2011).

Improving human through the supplementation or application of natural, plant-based products could improve quality of life in the rapidly aging population. As the population grows and unhealthy lifestyles result in cardio-metabolic disease, instances of sarcopenia and chronic wounds will surpass the already excessive healthcare costs that we see today. Exercise and diet are important factors for maintaining health, but these alone are insufficient to combat the multifactorial condition of sarcopenia. Treatments that target the core problems associated with these chronic conditions without imparting negative effects seen with anabolic steroids are a

necessity. Brassinosteroids and CSE may prove to be useful in the long-term treatment of these conditions.

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