ACUTE AND CHRONIC EFFECTS OF 12 WEEKS OF COMBINED EXERCISE TRAINING ON IL-6 IN OVERWEIGHT/OBESE POST-MENOPAUSAL WOMEN

By

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Submitted in partial fulfillment of the

requirements for Departmental Honors

in the Department of Kinesiology

Texas Christian University

Fort Worth, Texas

May 4th, 2020

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ABSTRACT

Overweight/obese post-menopausal women exhibit higher serum levels of pro-inflammatory cytokines, including interleukin-6 (IL-6). The purpose of this study was to analyze the acute and chronic effects of 12 weeks of combined exercise training on plasma IL-6 levels in overweight/obese post-menopausal women. Forty-three women were randomly assigned to an exercise (EX, n=22) or an education (ED, n=21) group. EX completed resistance training (2 sets of 8 resistance exercises at 80% of 1RM) followed by aerobic training (25-minute treadmill walk at 70-80% of HRR) three times per week for 12 weeks. ED attended classes and activities two times per week for 12 weeks to control for seasonal variation and social interaction. Blood samples were collected a total of 8 times: 4 times before training (BT) (before the acute exercise bout (PRE), immediately after exercise (PO), 1 hour after exercise (1HR), and 2 hours after exercise (2HR)) and 4 times after training (AT) at the same time points. Lean post-menopausal women were recruited for collection of one resting blood sample to serve as healthy controls (LN, n=11). Baseline IL-6 concentration was significantly higher in the EX ($p<0.001$) and ED ($p<0.001$) groups compared to the LN group. No statistically significant BT/AT x group interaction was observed ($p>0.05$) when the BT and AT PRE time points of the EX and ED groups were compared. In the EX group, the PRE ($p<0.001$), 1HR ($p=0.038$) and 2HR ($p=0.005$) time points were significantly lower than the PO time point. No statistically significant differences were observed when corresponding time points before and after the intervention within a group were compared (i.e., EX BT PRE to EX AT PRE) ($p>0.05$). Exercise training may need to be longer than 12 weeks and paired with weight loss to achieve a significant reduction in inflammatory markers and thus a significant improvement in health in overweight/obese post-menopausal women.

Acknowledgements

First and foremost, I want to thank my family. I truly would not be where I am today without the enthusiastic support, endless patience, and unconditional love of my parents and my younger sisters. Words fail to adequately describe how much my family means to me, and I am so grateful to have such an incredible family in my corner as I pursue my dreams of becoming a physician that makes a difference in the lives of children. Thank you, Mom, Dad, Kaily, and Kelcy. I love you all so much!

Next, I want to thank the extraordinary professor that stepped up to help me complete this project, even though he certainly did not have to. Dr. Esposito, I cannot thank you enough for taking me under your wing, for not letting me give up on earning Departmental Honors, and for allowing me to finish what I started four years ago. Your thoughtful input and infinite support during this project and throughout my four years at TCU meant the world to me, and I hope you know that I could not have done this without you.

Additionally, I want to thank another extraordinary person that helped me complete this project, even though he is no longer a student here at Texas Christian University and is doing amazing things at the Children's Research Institute at UT Southwestern Medical Center. Mike, I cannot thank you enough for all of the incredible expertise and endless encouragement you provided me with over the past four years. Your boundless kindness during a turbulent time meant so much to me, and I hope that you also know that I could not have done this without you.

I also want to thank Dr. Chumley and Dr. Shah for their support of my project. Dr. Chumley, thank you so much for agreeing to be a member of my committee. Dr. Shah, thank you so much for making it possible for me to complete this project and earn Departmental Honors. Additionally, I want to thank the Department of Kinesiology and the John V. Roach Honors

College at TCU for the opportunity to pursue Departmental Honors in the areas of exercise immunology and exercise physiology. I will be forever grateful to TCU for helping me make my dreams come true.

INTRODUCTION

Cardiovascular disease is the leading cause of death in the United States, accounting for 23% of all deaths in 2017 (1). From 2015 to 2016, the prevalence of obesity among American adults was 39.8%, with the prevalence among middle-aged adults (ages 40-59) higher than the prevalence among younger adults (ages 20-39) (2). Obesity is associated with an increased risk of death from several diseases, including coronary artery disease (the most common form of cardiovascular disease), diabetes mellitus (type 2 diabetes), and hypertension (3). In 2017, the American Heart Association reported that approximately 23.4 million individuals in the United States have been diagnosed with diabetes mellitus, 7.6 million individuals are suffering from undiagnosed diabetes mellitus, and 81.6 million have prediabetes, a condition in which blood glucose levels are higher than normal but not high enough to warrant a diagnosis of diabetes mellitus (4). From 2011 to 2014, the prevalence of hypertension among American adults was 34.0%, or approximately 85.7 million individuals (4).

Menopause begins when the ovaries cease production of the hormones estrogen and progesterone, and the absence of these sex hormones is associated with a higher incidence of several diseases, including osteoporosis and cardiovascular disease, among post-menopausal women (5). Post-menopausal women exhibit higher serum levels of pro-inflammatory cytokines, including IL-6, interleukin-1 (IL-1), and tumor necrosis factor alpha (TNF-α) (5). The sex hormone deficiency that characterizes menopause, specifically the estrogen deficiency, is also associated with the up-regulation of cellular receptors and cofactors for IL-6, thus intensifying the effects of increased production of this pro-inflammatory cytokine (6). Menopause is also associated with an accumulation of visceral fat (abdominal fat), which increases the risk of developing obesity and contributes to the systemic inflammation women experience after

menopause (6). Adipose tissue (fat) functions as an endocrine organ, secreting pro-inflammatory cytokines (adipokines), including IL-6 and TNF-α (6).

IL-6 is a cytokine (signaling molecule) that is normally expressed when tissue is inflamed or damaged due to an infection or injury (7). IL-6 induces the acute phase response, a component of the innate immune system, by promoting the production of acute phase proteins, such as C-reactive protein (CRP), serum amyloid A (SAA), and fibrinogen, by hepatocytes (liver cells) (7). In healthy individuals, baseline plasma levels of IL-6, CRP, and fibrinogen can be used to predict an individual's risk of coronary artery disease and death from a myocardial infarction because these molecules are markers of inflammation (8). IL-6 also promotes the production of thrombocytes (platelets), the differentiation of naïve $CD4^+$ T cells (T helper cells), the differentiation of naïve $CD8^+$ T cells (cytotoxic T cells), and the production of antibodies by B cells (7). Thus, IL-6 also plays an important role in activating the adaptive immune system.

IL-6 has been implicated in the onset of osteoporosis because it stimulates the differentiation and activation of osteoclasts, bone cells that absorb bone tissue, and suppresses the activity of osteoblasts, bone cells that create bone tissue (7, 8). Thus, the overproduction of IL-6 leads to excessive bone resorption, resulting in osteoporosis. IL-6 has also been implicated in the pathogenesis of atherosclerosis (9). Atherosclerosis occurs when lipids, including fats and cholesterol, build up in an artery, creating atherosclerotic plaques (lesions) that narrow and harden the artery (9). Atherosclerosis is the most frequent cause of ischemic cardiovascular disease and stroke (9). The smooth muscle cells in the wall of an atherosclerotic artery produce IL-6, which promotes inflammation by inducing the production of CRP by hepatocytes (9). Continuous inflammatory and hemodynamic attacks on a plaque may lead to its rupture or erosion through the wall of the artery, resulting in the recruitment of platelets, which form a

thrombus (clot) that may occlude an artery and cause a myocardial infarction (heart attack) or stroke (9).

Active muscles release IL-6 into the bloodstream, and the production of IL-6 during muscle contraction promotes lipolysis (the breakdown of fats), suppresses the production of TNF-α (a pro-inflammatory cytokine), and increases the production of cortisol (a steroid hormone released by the adrenal glands that promotes the release of glucose into the bloodstream) (10). Thus, IL-6 is also a myokine, or a cytokine released by skeletal muscle tissue, specifically an anti-inflammatory myokine (10). Plasma IL-6 levels increase significantly in response to exercise, and exercise is also associated with the enhanced production of other anti-inflammatory cytokines, such as IL-10, and pro-inflammatory cytokine inhibitors, including IL-1 receptor antagonist (10). A small amount of IL-6 mRNA is present in skeletal muscle biopsies collected before exercise, but skeletal muscle biopsies collected from the same individuals after exercise exhibit a significant increase in the amount of IL-6 mRNA present, thus demonstrating that expression of the IL-6 gene is promoted by the contraction of skeletal muscle (10).

A study that divided its forty participants into an aerobic training group and a resistance training group observed a significant reduction in plasma levels of IL-6 and TNF-α in both groups, with aerobic training producing a more significant decrease in the plasma concentrations of these pro-inflammatory cytokines (11). Participants were obese (body mass index (BMI) between 31 and 35 kg/m^2), of both sexes, between the ages of 34 and 56 years, and type 2 diabetic (11). Each group underwent exercise training for 12 weeks, with the aerobic training group walking on the treadmill for 40 minutes during each session and the resistance training group performing 3 sets of 8 to 12 repetitions for 8 resistance machines (chest press, biceps curl, triceps extension, lower back, abdominals, leg press, leg curl, and leg extension) during each

session (11). Participants in the aerobic training group exercised at 60 to 70% of their maximal heart rate during the first 2 weeks and at 70 to 80% of their maximal heart rate during the last 10 weeks (11). Participants in the resistance training group exercised at 60 to 80% of their one-repetition maximum (1RM) for the entire 12 weeks (11).

Another study that recruited thirty-five participants for 16 weeks of resistance training observed both a significant acute (48 hours post-exercise) and a significant chronic reduction in plasma levels of IL-6 (12). All participants were post-menopausal and elderly females with a BMI equal to or less than 26 kg/m^2 (12). Each participant performed 3 sets of 12 to 14 repetitions in the first 4 weeks, 3 sets of 10 to 12 repetitions in the next 4 weeks, 3 sets of 8 to 10 repetitions in the next 4 weeks, and 3 sets of 6 to 8 repetitions in the last 4 weeks for 10 resistance machines (barbell bench press, 45° leg press, seated row, knee extension, lateral raise, knee flexion, arm extension, hip adduction and abduction, arm curl, and standing calf raise) (12).

A previous study performed in our laboratory that divided its fourteen participants into a low-intensity resistance training group and a high-intensity resistance training group observed a significant elevation of plasma levels of IL-6 immediately after an acute resistance training bout in both groups, with the low-intensity resistance training producing a more significant increase in the plasma concentration of this anti-inflammatory myokine immediately after exercise (13). All participants were healthy and recreationally-active males with a mean age of approximately 22 years and a mean BMI of approximately 22 kg/m^2 (13). Participants in the low-intensity group performed 2 sets of 12 repetitions and a third set to failure at 65% of 1RM for 8 resistance machines (chest press, seated row, leg extension, leg curl, shoulder press, lateral pulldown, leg press, and chest fly) (13). Participants in the high-intensity group performed 2 sets of 12 repetitions and a third set to failure at 85% 1RM for these 8 resistance machines (13).

A subsequent study performed in our laboratory that divided its twenty-three participants into a resistance training group and a social interaction (control) group observed a significant reduction in plasma levels of IL-6 in the control group (14). All participants were obese (BMI between 30 and 40 kg/m^2), post-menopausal, and elderly females. Participants in the resistance training group performed 2 sets of 8 repetitions and a third set to failure for 10 resistance machines (chest press, seated row, leg extension, leg curl, shoulder press, lateral pulldown, leg press, chest fly, leg adduction, and leg abduction) three times a week for 12 weeks (14). The authors hypothesized that a significant reduction in plasma levels of IL-6 was not observed in the exercise group because a longer training intervention (longer than 12 weeks) may be required to observe statistically-significant changes in plasma IL-6 levels (14). Additionally, the authors hypothesized that a significant reduction in plasma levels of IL-6 was observed in the control group because attending lectures covering such topics as maintaining a healthy diet and incorporating physical activity into one's daily activities may have prompted participants in the control group to make lifestyle changes that improved their health and reduced systemic inflammation, thus lowering their plasma IL-6 levels (14).

The purpose of this study is to analyze the effects of an acute exercise bout before and after 12 weeks of combined resistance and aerobic exercise training on plasma levels of interleukin-6 (IL-6), a pro-inflammatory cytokine and anti-inflammatory myokine, in overweight/obese post-menopausal women (aged 55 to 75 years). Based upon the body of literature, we hypothesize that baseline plasma levels of IL-6 will be significantly higher in overweight/obese post-menopausal women compared to lean post-menopausal women. Additonally, we hypothesize that the acute exercise bout will result in a significant elevation of plasma IL-6 levels up to 2 hours post-exercise in the participants in the exercise/experimental

(EX) group before the training intervention (BT) but that this response will significantly diminish after the training intervention (AT). We also hypothesize that the 12 weeks of combined exercise training will result in a significant reduction in baseline plasma IL-6 levels in participants in the EX group compared to participants in the education/control (ED) group.

This study is distinct from the studies reviewed because the participants are overweight or obese and post-menopausal, the exercise training protocol is designed to combine resistance and aerobic training, and the effects of an acute exercise bout and chronic training on plasma levels of IL-6 will be assessed. This study will contribute to the body of literature regarding the effects of exercise on inflammatory markers in overweight/obese post-menopausal women, a poorly-studied population at high risk for diseases that IL-6 plays a role in the pathogenesis of, including atherosclerosis and osteoporosis.

METHODS

Forty-three overweight/obese post-menopausal women between the ages of 55 and 75 were recruited from the Dallas-Fort Worth metroplex for this study. BMI, which is calculated by dividing an individual's weight in kilograms by the square of her height in meters, was used to determine if an individual was overweight or obese. Per the Centers for Disease Control Prevention (CDC), when an individual's BMI falls between 25 kg/m^2 and less than 30 kg/m^2, the individual is categorized as overweight (15). When an individual's BMI is 30 kg/m^2 or greater, the individual is categorized as obese (15). Thus, the BMI range for these participants was observed to be 26 kg/m^2 to 47 kg/m^2. Individuals were categorized as post-menopausal if they had not experienced a menstrual cycle in the two years prior to participation in the study after undergoing natural or surgical menopause. Additionally, all participants recruited for this study were sedentary individuals. Individuals were categorized as sedentary if they had not performed regular exercise (defined as more than one mild to moderate bout of exercise per week) in the six months prior to participation in the study. These participants were divided into an exercise group (EX), which underwent a 12-week combined resistance and aerobic exercise training intervention, and an education (control) group (ED), which attended biweekly education sessions regarding a variety of topics, including initiating and maintaining a healthy diet and incorporating physical activity into one's daily activities.

Because the purpose of this study was to measure the levels of several inflammatory markers, including IL-6, individuals were excluded if they suffered from a chronic inflammatory or autoimmune disorder, such as Addison's disease, Graves' disease, multiple sclerosis, systemic lupus erythematosus (SLE), rheumatoid arthritis, psoriatic arthritis, human immunodeficiency virus (HIV) or acquired immunodeficiency syndrome (AIDS), a previous myocardial infarction,

peripheral artery disease (PAD), type 1 or type 2 diabetes mellitus, a previous cerebrovascular accident, a chronic respiratory condition, such as chronic obstructive pulmonary disease or emphysema, a renal or hepatic disease, a blood disorder, or a major affective (mood) disorder.

Furthermore, individuals were excluded if they used tobacco or oral steroids. Individuals were also excluded if they used an anti-coagulant, such as warfarin or Xarelto, in the fourteen days prior to participation in the study or if they underwent surgery in the three months prior to participation in the study. Individuals that regularly ingested over-the-counter non-steroidal anti-inflammatory medications (NSAIDs), such as aspirin, naproxen, ibuprofen, and diclofenac, were asked to refrain from taking these medications for one week prior to blood sample collection with the consent of a primary care physician. Additionally, participants were asked to refrain from ingesting alcohol twenty-four hours prior to blood sample collection. The American College of Sports Medicine (ACSM) Guidelines for Exercise Testing and Prescription were utilized to evaluate potential participants, and any participants with contraindications for exercise testing were excluded from the study. No individual categorized as "high risk" per the ACSM Guidelines was approved for participation in the study.

Additionally, eleven lean post-menopausal women were recruited from the Dallas-Fort Worth metroplex for this study as a control group (LN). In accordance with the inclusion criteria, individuals were categorized as post-menopausal if they had not experienced a menstrual cycle in the two years prior to participation in the study after undergoing natural or surgical menopause. Unlike the forty-three participants initially recruited for the study, these women were moderately-active individuals. Per the CDC, a BMI between 18.5 kg/m^2 and less than 25 kg/m^2 categorizes an individual as of a healthy or normal weight. Thus, the BMI range for these participants was observed to be 18 kg/m^2 to 24 kg/m^2.

Before participant recruitment and data collection for this study commenced, approval from the Institutional Review Board (IRB) at Texas Christian University (TCU) was secured (IRB #1512-104-1601). Participants were recruited from the Dallas-Fort Worth metroplex via word-of-mouth, social media (Facebook) posts, newspaper advertisements, and TCU Announce. Physical flyers were posed at local fitness and community centers, retirement homes, and churches. The study and its requirements were explained in detail to each participant before they were asked to review and sign the informed consent form. After signing the informed consent form, each participant was asked to complete a medical history form and a physical activity questionnaire. These forms were used by the primary investigators to determine if the participant met the inclusion criteria for the study. Before participating in the study, a participant was asked to secure approval from her primary care physician. Additionally, each participant completed a medical screening prior to participation in the study. This screening was performed by the study's physician, Dr. Jay Haynes, and included a review of the participant's medical history and a physical examination to identify any musculoskeletal or flexibility limitations or contraindications to exercise testing, as outlined in the ACSM Guidelines for Exercise Testing and Prescription.

In regards to study design, a three-factor design was utilized to analyze the acute and chronic effects of 12 weeks of combined resistance and aerobic exercise training on plasma levels of IL-6. The first factor was defined as the participant's group. This study had two groups: the exercise (EX) group and the education (ED) group. The second factor was the intervention time point. This study had two intervention time points: before training or education (BT) and after training or education (AT). The third factor was the experimental trial (blood sample collection) time point. This study had four of these time points: before the acute exercise bout or

period of rest (PRE), immediately after the acute exercise bout or period of rest (PO), one hour after the acute exercise bout or period of rest (1HR), and two hours after the acute exercise bout or period of rest (2HR). The sedentary, overweight or obese, and post-menopausal participants assigned to the exercise group or education group were compared to the moderately-active, lean, and post-menopausal participants recruited as controls prior to the 12-week exercise or education intervention. The dependent variable at each time point was the plasma level of IL-6.

Preliminary testing consisted of anthropometric measurements and a dual energy x-ray absorptiometry (DEXA) scan. Prior to this appointment, participants were asked to wear light clothing. For the anthropometric measurements, a participant's height was measured using a stadiometer mounted on the wall. Before her height was measured, the participant was asked to remove her shoes. Height was measured to the nearest tenth of a centimeter. A participant's weight was measured using an electronic scale. Again, before her weight was measured, the participant was asked to remove her shoes. She was also asked to void her bladder. Weight was measured to the nearest tenth of a kilogram. BMI (weight divided by height squared) was calculated manually using the height and weight collected during this appointment. A participant's waist circumference was measured in duplicate and to the nearest quarter of a centimeter two centimeters superior to the navel using a Gullick measuring tape. A participant's hip circumference was measured in duplicate and to the nearest quarter of a centimeter at the largest part of the gluteal region using a Gullick measuring tape. Waist-to-hip ratio (waist circumference divided by hip circumference) was calculated manually using the waist circumference and hip circumference collected during this appointment.

Body composition, including percent fat, percent android fat (defined as fat in the trunk and upper body), percent gynoid fat (defined as fat in the hips, thighs, and breasts), and bone

mineral density, was assessed using a DEXA scan. Prior to the DEXA scan, each participant was asked to remove all metal objects, including jewelry and clothing containing metal. Each participant was asked to lie on a padded table for approximately ten to twenty minutes as the DEXA scanner passed from the head to the feet of the participant.

Participants who met the inclusion criteria for the study based on the primary investigators' review of the participant's medical history form, physical activity questionnaire, anthropometric measurements, and DEXA scan results were asked to secure approval from a primary care physician and undergo a medical screening prior to commencing participation in the study. All participants (EX, ED, and LN) performed a submaximal treadmill exercise test to assess aerobic fitness. Submaximal was defined as exercise at 85% of the participant's heart rate reserve (HRR), which is equivalent to the difference between an individual's maximum heart rate and resting heart rate. Aerobic fitness is assessed by estimating an individual's maximal oxygen consumption (VO_2 max) based on the results of the treadmill exercise test. VO_2 max was estimated using a linear extrapolation method, and the submaximal VO_2 and heart rate were extrapolated to the individual's maximum heart rate, which was calculated by this equation: 208 – (0.7 x age). Heart rate was monitored and recorded throughout and after the exercise test using a chest strap heart rate monitor, while blood pressure was measured manually and recorded throughout and after the exercise test using a blood pressure cuff and sphygmomanometer.

All overweight or obese participants (EX and ED) completed a one-week acclimation period for the resistance exercises. During three acclimation sessions (acclimation day 1, acclimation day 2, and acclimation day 3), each participant performed eight resistance exercises: chest press, seated row, leg extension, leg curl, lateral pulldown, leg press, leg adduction, and leg abduction. As part of acclimation day 1, proper resistance training techniques were demonstrated

and explained, and each participant's 8-repetition maximum (8RM) was determined for each of the eight resistance exercises. As part of acclimation day 2, each participant completed three sets of each of the eight resistance exercises at 50% of their estimated 1-repetition maximum (1RM), which was calculated from the 8RM value collected during acclimation day 1. The first two sets were comprised of eight repetitions, and the third set was to failure or fifteen repetitions. Failure was defined as momentary muscular failure. As part of acclimation day 3, each participant's 8RM for each of the eight resistance exercises was re-determined to assess the accuracy of the first 8RM value.

Before and after the 12-week exercise or education intervention, members of the EX and ED groups underwent an experimental trial (blood sample collection). These participants were asked to refrain from eating 10 hours prior to blood sample collection. Upon their arrival to the Exercise Physiology Laboratory at TCU, participants were instructed to assume the supine position (to lie down) on a padded table for fifteen minutes. After fifteen minutes, the participant's resting heart rate, supine blood pressure, and standing blood pressure were measured, and the PRE blood sample was collected from the participant.

Members of the EX group were immediately escorted to the recreation center to complete a combined resistance and aerobic exercise training session. For the resistance training portion of the session, which was completed first, participants completed two sets of each of the eight resistance exercises at 80% of their 1RM. The first set was comprised of eight repetitions, and the second set was to failure or fifteen repetitions. For the aerobic training portion of the session, which was completed second, participants walked on a treadmill for twenty-five minutes at 70% to 80% of their HRR with a five-minute warm-up period prior to starting and a five-minute cool-down period prior to finishing. Members of the ED group quietly rested in the Exercise

Physiology Laboratory while members of the EX group completed the acute exercise bout. Immediately after the acute exercise bout (within five minutes), a catheter was inserted into the participant's arm to allow for collection of the PO, 1HR, and 2HR blood samples. Only a PRE blood sample was collected from the members of the LN group.

For the 12-week exercise intervention, members of the EX group reported to the Exercise Physiology Laboratory three times a week (Monday, Wednesday, and Friday) for twelve weeks. Each exercise training session began with a five-minute period of walking as a warm-up. For the resistance training portion of the session, which was always completed first, participants completed two sets of each of the eight resistance exercises at 75% to 100% of their 8RM. The first set was comprised of eight repetitions, and the second set was to failure or fifteen repetitions. The number of repetitions completed by the participant during the second set was examined by the primary investigators biweekly. If it was observed that a participant was performing twelve or more repetitions during the third set during three consecutive sessions, the weight was increased by ten pounds for lower-body resistance exercises (leg extension, leg curl, leg press, leg adduction, and leg abduction) and by five pounds for upper-body resistance exercises (chest press, lateral pulldown, and seated row). Each participant's 8RM for each of the eight resistance exercises was re-measured on the last day of training at the end of the exercise intervention. For the aerobic training portion of the session, which was always completed second, participants walked on a treadmill for twenty-five minutes at 70% to 80% of their HRR with a five-minute warm-up period prior to starting and a five-minute cool-down period prior to finishing.

For the 12-week education intervention, members of the ED group reported to the Exercise Physiology Laboratory two times a week (Tuesday and Thursday) for twelve weeks.

Each education session lasted approximately thirty minutes. Sessions covered a variety of topics, including initiating and maintaining a healthy diet and incorporating physical activity into one's daily activities. The ED group was created to control for diurnal variation (24-hour patterns), seasonal variation (seasonal patterns), and the effects of social interaction. The one-week acclimation period for the resistance exercise was repeated for members of the ED group to re-measure each participant's 8RM for each of the eight resistance exercises after the intervention.

Each blood sample at each experimental trial time point was collected in a chilled ethylenediaminetetraacetic acid (EDTA) tube. EDTA is an anti-coagulant and thus prevents the blood sample from clotting. To separate the plasma from the blood sample, each tube was centrifuged. Centrifugation creates a pellet of cells at the bottom of the tube. Thus, after centrifugation, the supernatant (liquid) is plasma, which was carefully removed using a pipette. The plasma collected from each blood sample was aliquoted (divided) into several aliquot containers and stored in a laboratory freezer at less than or equal to 20 degrees Celsius. Per the manufacturer's instructions for the Quantikine® High Sensitivity (HS) Enzyme-Linked Immunosorbent Assay (ELISA) Human IL-6 Immunoassay, repeated freeze-thaw cycles of the samples were avoided (R&D Systems, Minneapolis, MN). In the summer of 2019, six Quantikine® HS ELISA Human IL-6 Immunoassay kits were used to analyze levels of IL-6 in the plasma samples collected during the study.

The Quantikine® HS ELISA Human IL-6 Immunoassay from R&D Systems employs a quantitative sandwich enzyme immunoassay technique. The microplate provided in the kit has already been coated with the monoclonal antibody specific for human IL-6. When the standards and samples are added to the plate, any human IL-6 present in the well binds to the antibody fixed to the floor of the well. The plate is then washed to remove any compounds not bound to

the antibody, isolating the human IL-6. After the first round of washes, a biotinylated polyclonal antibody specific for human IL-6 is added to the wells, thus sandwiching the antigen (human IL-6) between the monoclonal antibody and the polyclonal antibody. The plate is then washed to remove any unbound biotinylated polyclonal antibodies.

After the second round of washes, an enzyme-linked streptavidin is added to the wells because streptavidin has a high-affinity for biotin, which is attached to the polyclonal antibody. The enzyme linked to the streptavidin in this assay is horseradish peroxidase (HRP). The plate is then washed to remove any unbound enzyme-linked streptavidin. After the third round of washes, a substrate solution is added to the wells. This substrate is acted upon by the enzyme linked to the streptavidin (HRP), producing a color change that is proportional to the amount of human IL-6 present in the well. The color change in each well is stopped using a stop solution, and the optical density (color intensity) of each well is measured using a microplate reader.

Before each assay was performed, the reagents were prepared. The Wash Buffer Concentrate was brought to room temperature and gently mixed to dissolve any crystals that formed while the reagent was stored in a laboratory refrigerator. 40 milliliters of the Wash Buffer Concentrate were combined with 960 milliliters of deionized water to create 1000 milliliters of Wash Buffer. Prior to use in step 10 of the assay, color reagents A and B were brought to room temperature and mixed together in equal volumes to create a substrate solution approximately 15 minutes before use. After the substrate solution was created, it was stored in a cabinet to protect it from light. Prior to use in step 8 of the assay, Streptavidin Polymer-HRP (100X) and Streptavidin Polymer-HRP Diluent were brought to room temperature. 215 microliters of the Streptavidin Polymer-HRP (100X) were mixed with the Streptavidin Polymer-HRP Diluent.

The Human IL-6 HS Standard was brought to room temperature and reconstituted using deionized water prior to performance of the assay. Per the instructions on the label of the vial, a certain volume of deionized water was used to create a stock solution of 100 picograms per milliliter. The standard was gently agitated for fifteen minutes using a laboratory rocker before a serial (stepwise) dilution was performed. Seven tubes were labeled: the first tube was labeled 10 pg/mL, the second was labeled 5 pg/mL, the third was labeled 2.5 pg/mL, the fourth was labeled 1.25 pg/mL, the fifth was labeled 0.625 pg/mL, the sixth was labeled 0.313 pg/mL, and the seventh was labeled 0.156 pg/mL.

900 microliters of the Calibrator Diluent RD5-4 were transferred to the first tube using a pipette. 500 microliters from the first tube were transferred into the second tube using a pipette, 500 microliters from the second tube were transferred into the third tube using a pipette, and so on, until 500 microliters from the sixth tube were transferred into the seventh tube using a pipette. The contents of a tube were mixed thoroughly using the pipette before 500 microliters were transferred to the next tube in the series. Per the manufacturer's instructions, the Calibrator Diluent RD5-4 was used as the zero standard (0 pg/mL) (R&D Systems, Minneapolis, MN).

The Assay Diluent RD1W was brought to room temperature. In step 3 of the assay, 100 microliters of the Assay Diluent RD1W were transferred to each well of the 96-well plate using a pipette. In step 4 of the assay, 100 microliters of standard or sample were transferred to its corresponding well using a pipette. A plate map was created prior to the performance of the assay to guide addition of the standards and samples to the plate. After addition of the standards and samples, the plate was covered with an adhesive strip provided in the kit and incubated for two hours at room temperature on a microplate shaker. In step 5 of the assay, the plate was washed four times using an automated microplate washer. Per the manufacturer's instructions,

complete removal of the Wash Buffer after each wash was performed by inverting the plate and blotting it on clean paper towels (R&D Systems, Minneapolis, MN). The Human IL-6 Conjugate was brought to room temperature. In step 6 of the assay, 200 microliters of the Human IL-6 HS Conjugate were transferred to each well using a pipette. After addition of the Human IL-6 HS Conjugate, the plate was covered with an adhesive strip and incubated for an hour at room temperature on the microplate shaker.

In step 7 of the assay, the plate was washed four times using the automated microplate washer. In step 8 of the assay, 200 microliters of the Streptavidin Polymer-HRP (1X) were transferred to each well using a pipette. After addition of the Streptavidin Polymer-HRP (1X), the plate was covered with an adhesive strip and incubated for thirty minutes at room temperature on the microplate shaker. In step 9 of the assay, the plate was washed four times using the automated microplate washer. In step 10 of the assay, 200 microliters of the Substrate Solution were transferred to each well using a pipette. After addition of the Substrate Solution, the plate was incubated for thirty minutes on the counter within an upside-down cardboard box to protect it from light. The Stop Solution was brought to room temperature. In step 11 of the assay, 50 microliters of the Stop Solution were transferred to each well using a pipette. Per the manufacturer's instructions, if the color in the wells did not uniformly change from blue to yellow, the plate was gently tapped to ensure thorough mixing of the Stop Solution with the standards and samples. In step 12 of the assay, the optical density of each well was immediately determined using a microplate reader set to 450 nanometers. Wavelength correction was set to 540 nanometers.

Regarding statistical analyses, a dependent t-test was used to determine if there were differences in anthropometric variables within a group (EX or ED) before the training

intervention (BT) and after the training intervention (AT). Additionally, a three-factor analysis of variance (ANOVA) with a Scheffe post hoc test was used to determine if there were differences in plasma levels of IL-6 between the three groups (LN, ED, and EX) at rest (PRE) before the training intervention (BT) and after the training intervention (AT). The results of this statistical analysis were used to evaluate the hypothesis that baseline plasma levels of IL-6 will be significantly higher in overweight/obese post-menopausal women compared to lean post-menopausal women. Additionally, the results of this statistical analysis were used to evaluate the hypothesis that 12 weeks of combined resistance and aerobic exercise training will result in a significant reduction in baseline plasma IL-6 levels in participants in the EX group compared to participants in the ED group.

Furthermore, a repeated measures ANOVA with a Bonferroni post hoc test was used to determine if there were differences in plasma levels of IL-6 within a group (ED or EX) after an acute exercise bout (EX) or rest period (ED) up to 2 hours after the exercise bout or rest period (PRE, PO, 1HR, and 2HR time points) before the training intervention (BT) and after the training intervention (AT). The results of this statistical analysis were used to evaluate the hypothesis that the acute exercise bout will result in a significant elevation of plasma IL-6 levels up to 2 hours post-exercise in the participants in the EX group before the training intervention (BT) but that this response will significantly diminish after the training intervention (AT).

RESULTS

Dependent t-test results: In regards to changes in the anthropometric and fitness variables, the results in the EX group were as follows: a significant decrease in mean percent android fat ($p=0.045$), a significant decrease in mean percent gynoid fat ($p<0.001$), a significant decrease in mean waist circumference ($p<0.001$), a significant decrease in mean hip circumference ($p=0.024$), a significant increase in estimated relative VO2max ($p<0.001$), and no significant changes in height, weight, age, mean BMI, mean percent fat, or mean waist:hip ratio ($p>0.05$). No significant changes in any of the anthropometric or fitness variables were observed in the ED group. Results of this statistical analysis are detailed in Table 1.

Three-factor ANOVA with Scheffe post hoc test results: As expected, baseline IL-6 concentration was significantly higher in the EX (EX BT PRE: 2.8 ± 1.3 pg/mL; LN BT PRE: 1.0 ± 0.5; $p<0.001$) and ED (ED BT PRE: 3.8 ± 1.7 pg/mL; LN BT PRE: 1.0 ± 0.5; $p<0.001$) groups compared to the LN group, supporting the hypothesis that baseline IL-6 plasma levels will be significantly higher in overweight/obese post-menopausal women compared to lean post-menopausal women. No statistically significant BT/AT x group interaction was observed ($p>0.05$) when IL-6 concentration at the BT and AT PRE time points of the EX and ED groups were compared, rejecting the hypothesis that 12 weeks of combined resistance and aerobic exercise training will result in a significant reduction in baseline plasma IL-6 levels of the EX group compared to those of the ED group. Results of this statistical analysis are illustrated in Figures 1 and 2.

Repeated measures ANOVA with Bonferroni post hoc test results: In the EX group, IL-6 concentration at the PO time point was significantly higher than IL-6 concentration at the PRE (PRE 2.6 ± 1.2; PO 4.3 ± 1.8 pg/mL; $p<0.001$), 1 HR (1HR 3.4 ± 1.2 pg/mL; PO 4.3 ± 1.8;

p=0.038), and 2HR (2 HR 3.9 ± 1.6 pg/mL; PO 4.3 ± 1.8; p=0.005) time points, partially supporting the hypothesis that the acute exercise bout will result in a significant elevation of plasma IL-6 levels up to 2 hours post-exercise in the EX group before the training intervention (BT) but that this response will significantly diminish after the training intervention (AT). No statistically significant differences were observed when IL-6 concentration at corresponding time points before and after the intervention within a group were compared (i.e., EX BT PRE to EX AT PRE) (p>0.05), rejecting the second part of this hypothesis. Results of this statistical analysis are illustrated in Figures 3 and 4.

Anthropometric or Fitness Variable	**Before Training (BT)**	**After Training (AT)**
Height (cm)	163.5 ± 4.4	163.5 ± 4.4
Weight (kg)	88.0 ± 14.6	88.0 ± 13.6
BMI (kg/m^2)	32.9 ± 5.1	33.0 ± 4.8
Age (years)	63.2 ± 5.1	63.8 ± 5.1
Percent fat (%)	46.9 ± 4.6	45.9 ± 4.3
Percent android fat (%)	54.5 ± 7.1	53.2 ± 6.5*
Percent gynoid fat (%)	51.4 ± 4.1	49.3 ± 3.3*
Waist circumference (cm)	107.7 ± 12.8	105.3 ± 12.3*
Hip circumference (cm)	119.4 ± 9.4	118.0 ± 8.2*
Waist:hip ratio	0.9 ± 0.1	0.9 ± 0.1
Estimated Relative VO_2max (mL/kg/min)	21.1 ± 3.0	23.8 ± 3.4

Table 1. Changes in Anthropometric and Fitness Variables in the EX Group

*** indicates significantly different than BT value (p<0.05)**

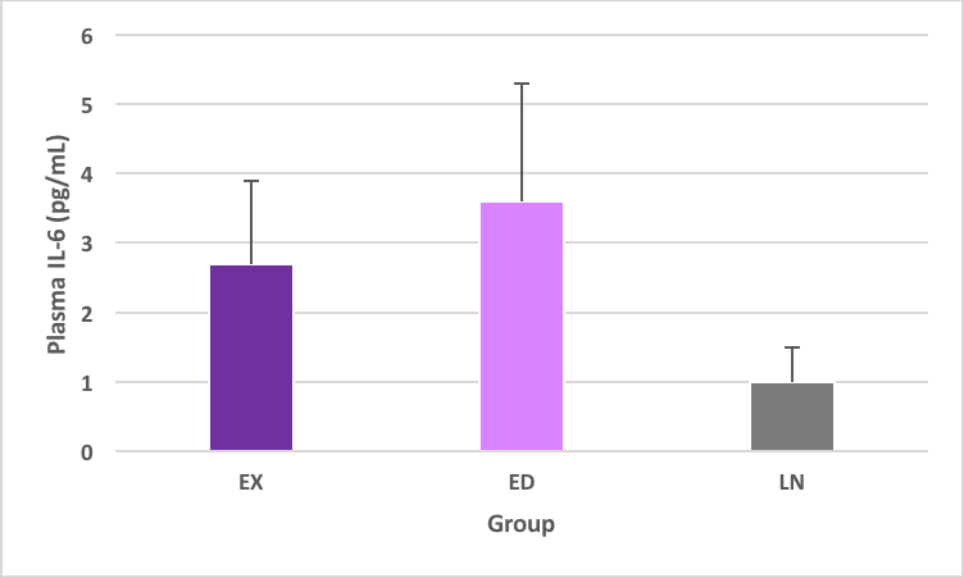

Figure 1. Baseline Plasma IL-6 Levels

***indicates significantly higher than LN ($p<0.05$)**

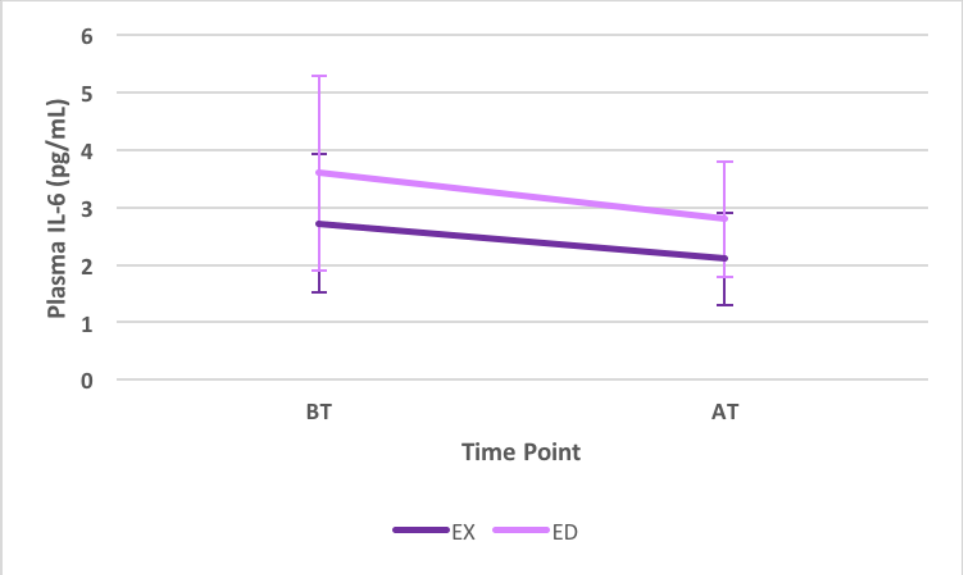

Figure 2. Baseline Plasma IL-6 Levels in EX and ED Groups Before and After Training

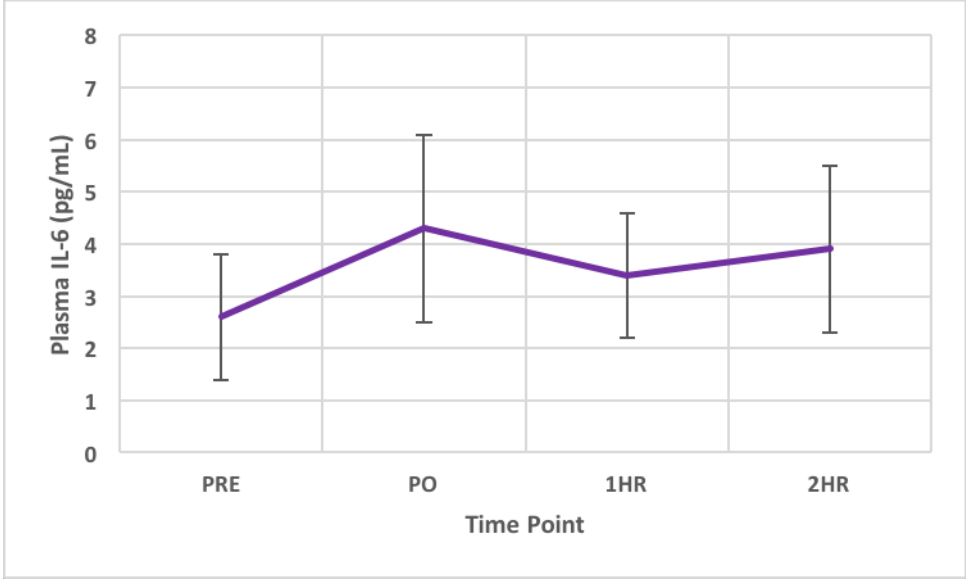

Figure 3. Acute Exercise-Induced IL-6 Response in EX Group

***indicates significantly lower than PO ($p<0.05$)**

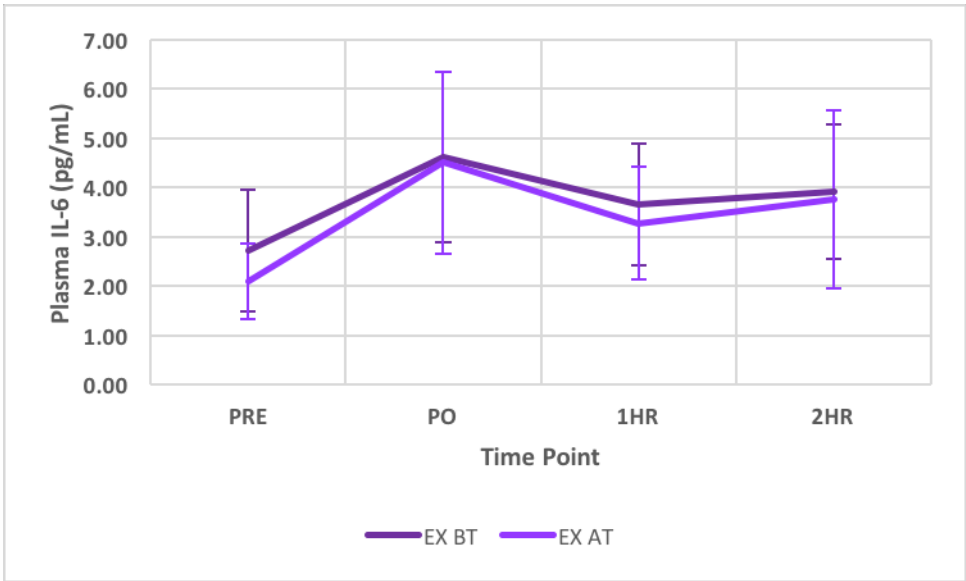

Figure 4. Acute Exercise-Induced IL-6 Response Before and After Training in EX Group

DISCUSSION

Baseline plasma IL-6 levels were significantly lower in the group of lean post-menopausal women (LN) compared to the two groups of overweight/obese post-menopausal women (EX and ED). The EX group did not achieve a significant reduction in mean percent fat, mean BMI, or weight. Additionally, the EX group did not display a significant decrease in plasma IL-6 levels after completion of the training intervention. Participants in the EX group were still classified as overweight or obese after the training intervention, as there was no significant reduction in BMI. The EX group did achieve a significant increase in aerobic fitness (estimated relative VO_2max), but the mean estimated relative VO_2max for this group after the training intervention (23.8 ± 3.4 mL/kg/min) is still classified as below average for females of this age. For females over the age of 50, the average predicted VO_2peak based on a non-exercise regression model for cardiorespiratory fitness developed and validated in a study by researchers in Norway is 32.0 mL/kg/min (17).

We can conclude that 12 weeks of combined resistance and aerobic training produced significant improvements in certain anthropometric measurements, specifically mean percent android fat, mean percent gynoid fat, mean waist circumference, and mean hip circumference, maximal aerobic capacity (estimated relative VO_2max) and thus cardiorespiratory fitness, and strength in overweight/obese post-menopausal women. This type of training intervention did not, however, produce significant improvements in other anthropometric measurements, including weight, mean BMI, mean percent fat, and mean waist:hip ratio, or a significant reduction in plasma levels of IL-6, an inflammatory marker, in this population. Thus, 12 weeks of combined resistance and aerobic exercise training did not appear to reduce systemic inflammation in overweight/obese post-menopausal women, a population at high risk for cardiovascular disease.

A study that compared serum and subcutaneous adipose tissue concentrations of IL-6, TNF-α, and leptin in 14 obese, non-diabetic women before and after 3 weeks of adherence to a very low-calorie diet observed significant reductions in both serum and subcutaneous adipose tissue concentrations of IL-6 and leptin (18). No significant changes in serum and subcutaneous adipose tissue concentrations of TNF-α were observed (18). Additionally, 3 weeks of adherence to this type of diet resulted in significantly weight loss and an approximately three-kilogram decrease in fat mass (18). Baseline levels of serum IL-6, TNF- α, leptin, and CRP were significantly higher in this group of obese non-diabetic women compared to 8 healthy and lean women that were recruited as controls (18).

An 18-month, single-blind, and randomized control trial explored the effects of loss of total and regional fat mass on systemic inflammation in overweight/obese and older adults with knee osteoarthritis (19). 450 adults with a mean BMI of 33.6 ± 3.7 kg/m^2 and a mean age of 65.6 ± 6.2 years were randomized to three treatment groups: a diet-induced weight loss and exercise group (n=150), a diet-induced weight loss only group (n=149), and an exercise only group (n=151) (19). Systemic levels of CRP and IL-6 were measured before the intervention, halfway through the intervention (6 months), and after the intervention (18 months) (19). Additionally, total body and regional (abdominal and thigh) fat mass were measured before and after the intervention. Both groups that adhered to a diet lost significantly more weight than the exercise only group (19). Additionally, both groups that adhered to a diet exhibited significantly lower post-intervention levels of both CRP and IL-6 than the exercise only group (19). The authors of this study concluded that demonstration of healthy and clinically-desirable levels of CRP and IL-6 more than doubles when 5% of total body weight and fat mass is lost via diet-induced weight loss (19).

Since adipose tissue acts an endocrine organ and secretes pro-inflammatory cytokines, including IL-6 and TNF-α, weight loss, which was not achieved during this study, may be necessary to achieve a significant reduction in plasma levels of IL-6 and thus a significant improvement in systemic inflammation (6). Circulating and adipose tissue levels of IL-6, TNF-α, and leptin (adipokines) are positively-correlated with serum CRP levels in non-diabetic obese women, suggesting that the enhanced release of adipokines in obese individuals plays a role in the increased production of acute phase proteins, especially CRP, by the liver (20). CRP is a major risk factor for cardiovascular disease, with a high level of CRP (≥10 mg/L) correlating to a more than 4% risk of experiencing a fatal cardiovascular event, such as a myocardial infarction or stroke, in ten years (21).

Based on the results of this study, we can conclude that adipose tissue plays a significant role in contributing to and maintaining systemic inflammation in overweight/obese post-menopausal women. Based on the current literature regarding the efficacy of exercise in reducing levels of inflammatory markers in post-menopausal women, the training intervention may not have been long enough to observe attenuation of the IL-6 acute exercise response and/or a chronic decrease in plasma IL-6 levels in the EX group. In addition to being longer than 12 weeks, we recommend pairing a combined exercise training intervention with diet-induced weight loss to achieve a significant decrease in inflammatory markers, including CRP, IL-6, and TNF-α, and thus a significant decrease in the systemic inflammation that characterizes overweight/obese post-menopausal women and puts them at higher risk for diseases like atherosclerosis and osteoporosis. Although not all hypotheses were supported, this study contributes to the body of literature regarding the effects of exercise, specifically a 12-week

combined resistance and aerobic training intervention, on levels of inflammatory markers, specifically IL-6, in overweight/obese post-menopausal women between the ages of 55 and 75.

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