Aerobic And Resistance Training Dependent Skeletal Muscle Plasticity In The Colon-26 Murine Model Of Cancer Cachexia

Andy V. Khamoui ^{a, b, 1}, Bong-Sup Park ^a, Do-Houn Kim ^{a, b}, Ming-Chia Yeh ^{a, b}, Seung-Lyul Oh ^a, Marcus L. Elam ^{a, b}, Edward Jo ^{a, b, f}, Bahram H. Arjmandi ^{a, b}, Gloria Salazar ^a, Samuel C. Grant ^{b, c}, Robert J. Contreras ^d, Won Jun Lee ^e, and Jeong-Su Kim ^{a, b, *}

^a Department of Nutrition, Food and Exercise Sciences, Florida State University, Tallahassee, Florida, USA

^b The Center for Advancing Exercise and Nutrition Research on Aging, Florida State University, Tallahassee, Florida, USA

^c Department of Chemical and Biomedical Engineering and The National High Magnetic Field Laboratory, Florida State University, Tallahassee, Florida, USA

^d Department of Psychology and Program in Neuroscience, Florida State University, Tallahassee, Florida, USA

^e Department of Exercise Science, Ewha Womans University, Seoul, Korea

^f Department of Kinesiology and Health Promotion, California State Polytechnic University, Pomona, Pomona, CA, USA

¹ Current Affiliation: Division of Respiratory & Critical Care Physiology & Medicine, Department of Medicine, Los Angeles Biomedical Research Institute at Harbor-UCLA Medical Center, Torrance, CA, USA

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Corresponding Author *

Jeong-Su Kim, Ph.D., FACSM

Associate Professor

Department of Nutrition, Food and Exercise Sciences

Associate Director, Center for Advancing Exercise and Nutrition Research on Aging

Florida State University

432 Sandels Building

Tallahassee, FL 32306-1493

Office: (850) 644-4795 | Email: jkim6@fsu.edu

ABBREVIATIONS

AT; aerobic training

RT; resistance training

C26; colon-26

DXA; dual energy x-ray absorptiometry

CSA; cross-sectional area

IGF-IEa; insulin-like growth-factor I-Ea isoform

IGF-IEb; insulin-like growth-factor I-Eb isoform

MuRF1; muscle ring finger 1

mTOR; mammalian target of rapamycin

ABSTRACT

Purpose: The appropriate mode of exercise training for cancer cachexia is not well-established. Using the colon-26 (C26) mouse model of cancer cachexia, we defined and compared the skeletal muscle responses to aerobic and resistance training. **Methods:** Twelve-month old Balb/c mice were initially assigned to Control, Aerobic Training (AT; wheel running), or Resistance Training (RT; ladder climbing) (n=16-17/group). After 8 weeks of training, half of each group was injected with C26 tumor cells, followed by 3 additional weeks of training. Body composition and neuromuscular function was evaluated pre- and post-training. Muscles were collected post-training and analyzed for fiber cross-sectional area (CSA), Akt-mTOR signaling, and expression of insulin-like growth factor-I (IGF-I) and myogenic regulatory factors. **Results:** Total body mass decreased ($p<0.05$) in C26 (-8%), AT+C26 (-18%), and RT+C26 (-15%) but not Control. Sensorimotor function declined ($p<0.05$) in Control (-16%), C26 (-13%), and RT+C26 (-23%) but not AT+C26. Similarly, strength/body weight decreased ($p<0.05$) in Control (-7%), C26 (-21%), and RT+C26 (-10%) but not AT+C26. Gastrocnemius mass/body weight tended to be greater in AT+C26 vs. C26 (+6%, $p=0.09$). Enlargement of the spleen was partially corrected in AT+C26 (-27% vs. C26, $p<0.05$). Fiber CSA was lower in all C26 groups vs. Control (-32-46%, $p<0.05$); however, the effect size calculated from C26 and AT+C26 was large (+24%, $d=1.04$). Phosphorylated levels of mTOR in AT+C26 exceeded C26 (+31%, $p<0.05$). RT+C26 showed greater mRNA expression ($p<0.05$) of IGF-IEa (+79%) and myogenin (+126%) with a strong tendency for greater IGF-IEb (+127%, $p=0.069$) vs. Control. **Conclusions:** Aerobic or resistance training was unable to prevent tumor-induced body weight loss. However, aerobic training may have preserved function, reduced the inflammatory response of the spleen, and marginally rescued muscle mass possibly through activation of mTOR. Aerobic training may therefore have therapeutic value for patients with cancer cachexia. In contrast, resistance training induced the expression of genes associated with muscle damage and repair. This gene response may be supportive of excessive stress generated by high resistance loading in a tumor-bearing state.

KEYWORDS: Muscle atrophy, Cachectic, Exercise training, Ladder climbing, Myogenin

1. INTRODUCTION

Cachexia is a life-threatening condition that develops in approximately half of all cancer patients and accounts for roughly 20-30% of cancer-related deaths [1-3]. Key features of cancer cachexia include unintended weight loss, skeletal muscle atrophy, and impaired physical function [1]. As a direct consequence of these changes, the ability to perform activities of daily living is compromised and quality of life is reduced [4]. To address this unmet medical need, research has focused on defining the cellular and molecular signals regulating disease onset and progression, as well as the various pharmacologic and nutritional agents with preventive or inhibitory properties [5]. Because cachexia is a multi-factorial syndrome that arises or progresses from a number of events (e.g. tumor-specific products, inflammation, physical inactivity), it has been suggested that effective therapeutic strategies would require multiple arms [5].

Of the therapies currently being investigated, exercise has received the least attention [6]. Based on the well-defined skeletal muscle adaptations to contractile activity in health and disease [7-11], exercise may be an effective therapy for cancer cachexia [12, 13]. In broad terms, exercise can be classified as low muscular tension sustained for a prolonged period (i.e. aerobic exercise), or high muscular tension generated intermittently (i.e. resistance exercise). Typical training adaptations to the former include increased mitochondrial content, capillary density, and exercise capacity [14-16] whereas the latter has been shown to promote myofibrillar protein synthesis, whole muscle and myofiber hypertrophy, and/or enhanced contractility [17-20]. Each exercise mode, therefore, provides benefits that may alleviate cancer cachexia.

At present, exercise therapies in cachectic cancer patients have not been rigorously evaluated [5, 21]. Consequently, limited data exist to inform the application of exercise in clinical practice. Possible explanations for the lack of information include physical frailty in patients that compromises exercise adherence, limited availability of personnel qualified to supervise training in this population, and/or the need for specialized facilities and equipment [13]. To date, experimental approaches have been confined to pre-clinical models, of which studies provide evidence that aerobic training preserves muscle mass in the presence of tumor load [22-24]. Interestingly, resistance training has been implemented to a lesser degree despite being a potent anabolic stimulus that promotes muscle hypertrophy, enhanced contractile function, and signaling events favoring muscle

growth/preservation [6, 25]. For instance, inhibited insulin-like growth factor-I (IGF-I) activity along with enhanced myostatin signaling has been reported in skeletal muscles of several cancer cachexia models [26-28]. Resistance overload on the other hand, promoted IGF-I pathway activation and suppressed myostatin signaling while preserving muscle mass in other cachexia-related syndromes [29]. Such load-mediated events may collectively alter protein turnover in a manner that also protects against cachectic muscle wasting.

Among the few pre-clinical investigations, resistance exercise was modelled using electrical stimulation or the surgical ablation of synergistic muscles [30, 31]. While these highly invasive methods induce rapid hypertrophy (+30-50% in 1-2 wks) [32, 33], they do not accurately reflect the mechanical stress of resistance exercise in humans on the basis of the timeframe required for training-induced muscle growth (>6 wks). As an alternative, we and others have previously achieved skeletal muscle overload in rodents through weighted ladder climbing [34, 35]. In our hands, this form of chronic overload produced a hypertrophic response similar to resistance training in humans [18, 36]. To our knowledge, this method of resistance loading has not been previously investigated in pre-clinical cancer cachexia models, nor has it been evaluated in comparison to the more often-studied aerobic exercise mode. The purpose of this study, therefore, was to define and compare the skeletal muscle responses to aerobic and resistance training in a well-established murine model of cancer cachexia [37-39]. This pre-clinical approach intended to better understand the role of classical exercise training modes for this debilitating condition. We hypothesized that resistance training would: 1) ameliorate the adverse effects of cachexia on body composition, functional performance, and muscle size to a greater extent than aerobic training; and 2) induce changes in muscle gene expression profiles and signaling events to favor protein synthesis and suppress protein degradation.

2. MATERIALS AND METHODS

2.1 Animals and Design

Female Balb/c mice (12-months old, Harlan Laboratories Inc., USA) were randomly assigned to Control (n=17), Resistance Training (RT, n=16), or Aerobic Training (AT, n=16). Mice in the training groups were familiarized to their respective exercises for one week. After familiarization, all mice were assessed on the *in vivo* measurements including neuromuscular function and body composition (i.e. pre-experiment measurements). RT and AT groups then performed their respective exercises for 8 weeks while Control remained in their cages. After 8 weeks, half of the mice in each initial group were inoculated with colon-26 (C26) tumor cells to induce cachexia, followed by 3 more weeks of training. Identical *in vivo* measurements were repeated on all mice after training, with subsequent sacrifice by anesthetic overdose for tissue collection. Six groups were studied overall including Control, C26, AT, AT+C26, RT, and RT+C26 (n=8/group excluding C26, n=9).

The rationale for this design considered: 1) cell latency/tumor growth phase that typically does not extend beyond three weeks post-injection, and 2) the time to observe a training effect with our method of resistance loading (~10 weeks). Thus, exercise was performed for 8 weeks before tumor cell injection followed by an additional 3 weeks after injection. The initiation of treatment before inoculation followed by continuation during the tumor growth/cachexia phase is consistent with previous exercise and nutrition based pre-clinical studies of cancer cachexia [22, 40, 41]. All mice were housed individually and provided standard chow and water *ad libitum* in a room maintained on a 12:12 hour light:dark cycle. Procedures were approved by the Institutional Animal Care and Use Committee at Florida State University.

2.2 Exercise Training

Resistance exercise was conducted 3 days per week on a custom-built ladder [34]. Resistance loading was applied at the base of the tail using a foam clip with washers or paper clips attached for additional weight (Fig. 1). The initial resistance attached was 50% of body weight followed by 10% increases bi-weekly. The specific loading schedule during weeks 1-2, 3-4, 5-6, 7-8, and 9-11 for the two resistance trained groups were: RT) 14 ± 1 , 17 ± 1 , 20 ± 1 , 23 ± 1 , and 26 ± 1 g; and RT+C26) 14 ± 1 , 17 ± 1 , 19 ± 1 , 22 ± 1 , and 25 ± 1 g. The

training protocol consisted of 1 repetition without weight (i.e. warm-up) and 5 sets of 3 repetitions with weight. One and two minutes of rest were provided between repetitions and sets, respectively. Aerobic exercise was implemented using motorized wheels (Model 80800A, Lafayette Instrument, Lafayette, IN). Each session lasted 60 minutes and was performed 5 days per week. Prior to the main session, the mice performed a 15-min warm-up at speeds increased progressively to the main session intensity. The main session speeds were 5.0, 6.0, 7.0, and 6.5 m/min during weeks 1-2, 3-5, 6-7, and 8-11 of training, respectively. The speed was reduced to a fast walk (6.5 m/min) during weeks 8-11 because of difficulty at the prescribed speed.

2.3 C26 Cell Culture and Injection

Mice bearing the C26 tumor have been used extensively to model cancer cachexia [37-39]. Cells were cultured *in vitro* in RPMI 1640 supplemented with 10% FBS (vol/vol) and 1% penicillin/streptomycin (vol/vol) in a humidified CO₂ incubator. Cells were subsequently harvested using trypsin (0.05%, Gibco) followed by the addition of complete media and centrifugation to pellet the cells. The supernatant was then removed and the pellet resuspended in PBS. Viable cells were counted by trypan blue staining. Mice in C26 groups were gently restrained and injected in the upper back with a 0.1 ml cell suspension containing 5.0×10^5 cells while tumor-free mice were injected with an equivalent volume of PBS [38, 42].

2.4 Dual Energy X-Ray Absorptiometry (DXA)

Total body mass, fat mass, and lean mass were determined by DXA with software provided by the manufacturer (iDXA Lunar, GE). For the pre-experiment measurement, the mice were first anesthetized i.p. with ketamine/xylazine (70 mg/kg and 3 mg/kg body weight) and positioned so that the entire body and tail were measured. Each full length scan lasted approximately one minute. The post-measurement was taken after sacrifice immediately prior to tissue collection. Mice were euthanized by ketamine/xylazine overdose and the tumor rapidly dissected prior to the last scan in accordance with previous work [43, 44].

2.5 Grip Strength and Sensorimotor Function Tests

A force transducer (DFS-101 Force gauge, AMETEK, CA) interfaced to a data acquisition system was used to measure forelimb grip strength [45]. Each mouse performed three trials separated by 5 minutes of

recovery. The greatest force produced was used for subsequent analysis. The greatest force relative to body weight (i.e. relative strength) was also analyzed. Sensorimotor function was determined using the incline plane test, which required the mice to maintain their body position for 5 seconds on an inclined acrylic glass plane. The angle of the surface was increased from 30° to 60° at 2° intervals [46]. A 5-minute recovery was provided between trials. The inability to remain in position for 5 seconds after three attempts was defined as failure, and the greatest angle completed was used for analysis. The same investigator conducted all trials for both tests.

2.6 Tissue Collection

Mice were euthanized by ketamine/xylazine overdose administered i.p. (300 mg/kg and 30 mg/kg) and tissue was collected 48 hours after the last exercise session. After tumor removal and post-experiment DXA scan, the right hindlimb muscles were excised, weighed, sectioned, snap frozen, and stored at -80°C. The spleen, liver, and heart were also removed while the gastrocnemius from the left hindlimb was mounted cross-sectionally in tragacanth gum, followed by freezing in liquid nitrogen-cooled isopentane for histological analysis.

2.7 Fiber Cross-Sectional Area

Procedures for the determination of fiber CSA were conducted as we have previously described [47]. Briefly, transverse sections (7 µm) were cut from the midbelly of the gastrocnemius on a cryostat at -20°C and then stained with hematoxylin. Images were obtained at 40x and analyzed using NIH-ImageJ software by a single investigator blinded to the identity of the sample.

2.8 RNA Extraction and RT-PCR

Total RNA was extracted from frozen gastrocnemius and plantaris muscle samples using RiboZol reagent (AMRESCO, Solon, OH, USA), and one µg of RNA was reverse transcribed for cDNA synthesis [47]. The primer sequences were: IGF-IEa, (fwd) ATCTGCCTCTGTGACTTCTTGA, (rev) CTGGAGCCATAGCCTGTG; IGF-IEb, (fwd) TCCTTATGAATTGGCTTATC, (rev) GTTGTGCATCTTCCATTCTGTT; myostatin, (fwd) ACAATCTAGGCAAGGAAGTGAG, (rev) GAATGGCAATGTGTATGTAAGG; atrogen-1, (fwd) CGTGACGGCCAACAACC, (rev) CCCGCCAACGTCTCCTCAAT; MuRF1, (fwd) GGCTGCGAATCCCTACTGG, (rev) TGATCTTCTCGTCTTCGTGTTTCCT; myogenin, (fwd)

CAGGAGCCCCACTTCTATGATG, (rev) CACTGGCCTGGACAATGCTC; and MyoD, (fwd) CGCCGCCGCCTGAGCAAAGT, (rev) GGGCGCGGCGTCCTGGTC. For each PCR reaction, 18S was coamplified with each target cDNA (mRNA) resulting in a ratio of target mRNA/18S. Amplified products were separated on 2% agarose gel mixed with ethidium bromide, and images were captured under UV light. Band densitometry was performed using Bio-Rad Quantity One® software.

2.9 Western Blotting

Frozen gastrocnemius samples were homogenized in ice cold lysis buffer (150 mM NaCl, 10 mM HEPES, 1 mM EGTA, 0.1 mM MgCl₂, 1% Triton X-100, pH 7.4) that contained freshly added protease/phosphatase inhibitor cocktail (0.5x Sigma P2714, 100 μ M PMSF, 0.1 μ M okadaic Acid, 1 mM orthovanadate). Protein concentration was determined from the whole muscle extracts using the BCA method, and lysate containing 40 μ g of protein was separated on 4-20% SDS-polyacrylamide pre cast gels (BioRad) at 200 V. Electrotransfer of resolved proteins to PVDF membranes was performed using a semi-dry blotting system and the membranes were subsequently blocked for 1 hour in 3% milk in PBS with 0.1% Tween 20 (PBS-T). Primary antibodies for p-Akt, Akt, p-mTOR, and mTOR (Cell Signaling, Beverly, MA) were diluted 1:500-1:1000 in buffer containing 3% BSA and 0.1% azide in PBS and incubated with the membranes overnight at 4°C. After three washes, membranes were incubated for 1 hour with anti-rabbit IgG horseradish peroxidase conjugated secondary antibody (Cell Signaling, Beverly, MA) diluted 1:10,000 in 3% milk in PBS-T. Membranes were visualized by enhanced chemiluminescence and quantification of blots was performed by densitometry.

2.10 Statistical Analysis

All data have been reported as mean $\pm$ SE. To evaluate the effect of the C26 tumor, pre-planned analyses were conducted on Control vs. C26 mice using 2-way ANOVA and t-tests for *in vivo* and *in vitro* measurements, respectively. To assess the impact of aerobic and resistance training in tumor-free mice, analysis was performed on Control, AT, and RT groups by 2-way and 1-way ANOVA for *in vivo* and *in vitro* outcomes, respectively. To evaluate potential countermeasure effects of exercise training in tumor bearing mice, analysis was conducted on Control, C26, AT+C26, and RT+C26 groups using 2-way and 1-way ANOVA for *in vivo* and *in vitro*

measurements, respectively. Total food consumed during the three weeks after tumor cell injection was analyzed by 1-way ANOVA. Follow-up testing was conducted using Tukey's HSD *post hoc* or t-tests to localize main or interaction effects. An effect size statistic (d) was also calculated for selected parameters and interpreted according to Cohen's conventions. P-values ≤ 0.05 were considered significant.

3. RESULTS

3.1 Survival

Two RT+C26 mice (n=2/8; 25%) met the endpoints for euthanasia before completing the study. These mice demonstrated weight loss that reached 20% of pre-injection body weight as well as a body condition score consistent with emaciation on days 13 and 15 post-inoculation. Therefore, they were euthanized on those respective days according to institutional guidelines. All other mice completed the experiment.

3.2 Food Intake

The total amount of food consumed during the three weeks after cell inoculation was not significantly different between Control and C26 mice ($p>0.05$). Food intake during the same period was not significantly affected by exercise training in either tumor-free mice or C26 mice ($p>0.05$).

3.3 Effect of C26 Tumor on Body Composition, Neuromuscular Function, and Muscle Mass

From pre- to post-experiment, total body and fat mass remained constant in Control ($p>0.05$) whereas C26 mice underwent significant declines in both parameters (total body mass: -8%, $p=0.006$; fat mass: -33%, $p=0.002$) (Figs. 2A and 2B). At post-experiment, C26 mice showed significantly lower total body mass (-6%, $p=0.048$) and a tendency for less fat (-20%, $p=0.07$) (Figs. 2A and 2B). No significant interaction or main effect was noted for lean mass ($p>0.05$) (Fig. 2C).

Regarding sensorimotor function, a significant main effect of time was driven by similar pre to post reductions in both groups (Control: -16%, $p=0.006$; C26: -13%, $p=0.001$) (Fig. 2D). Absolute grip strength decreased from pre- to post-experiment in both groups (Control: -7%, $p=0.021$; C26: -23%, $p=0.001$). Furthermore, absolute strength at post-experiment was significantly lower in C26 (-19%, $p=0.005$) (Fig. 2E). Similarly, relative strength significantly decreased over time in both groups (Control: -7%, $p=0.01$; C26: -21%, $p=0.005$), with values also being lower in C26 mice at post-experiment (-18%, $p=0.02$) (Fig. 2F).

In comparison to Control, C26 mice exhibited significantly lower ($p<0.05$) gastrocnemius and plantaris mass (Fig 2G). C26 mice also showed significantly greater liver and spleen mass compared to Control (Fig 2H).

Fiber CSA of the gastrocnemius was noticeably smaller in C26 vs. Control (Fig. 3A), with the mean Fiber CSA being significantly lower in C26 mice (Fig. 3B). The overall distribution of myofibers in C26 mice also clearly tended toward smaller sizes (Fig. 3C).

3.4 Effect of C26 Tumor on E3 Ligase, IGF-I, and Akt-mTOR Expression

C26 mice showed tendencies for greater MuRF1, atrogin-1, and myostatin mRNA in the gastrocnemius vs. Controls (Fig. 3D). There was also a tendency for greater IGF-IEa mRNA in the plantaris of C26 mice (+51%, $p=0.066$) but this was not the case for IGF-IEb (+52%, $p=0.248$) (Fig. 3E). With respect to signaling events (Figs. 3F-H), the ratio of phosphorylated to total Akt in the gastrocnemius was not significantly different between Control and C26 mice ($p>0.05$) (Fig. 3G); however, phosphorylated to total mTOR tended to be lower in C26 (-44%, $p=0.07$) (Fig. 3H).

3.5 Body Composition, Neuromuscular Function, and Muscle Mass in Exercise Trained Tumor-Free Mice

Body composition analysis revealed a significant main effect of time on total body mass that was driven by training-induced losses in AT (-6%, $p=0.003$) and RT (-3%, $p=0.02$) but not Control ($p>0.05$) (Fig. 4A). With respect to fat mass, there was a significant main effect of time in which *post hoc* tests revealed declines in AT only (-10%, $p=0.02$) (Fig. 4B). No significant interaction or main effect was observed for lean mass ($p>0.05$) (Fig. 4C). For sensorimotor function, there were significant declines over time in Control (-16%, $p=0.006$) but not AT and RT ($p>0.05$). Furthermore, post-training values were greater in AT (+18%, $p=0.005$) and RT (+16%, $p=0.012$) vs. Control (Fig. 4D). Post-training strength tended to be greater in RT vs. AT (+10%, $p=0.059$) (data not shown).

There were no differences in gastrocnemius mass between Control, AT, and RT ($p>0.05$) (Fig. 4E). However, plantaris mass was significantly greater in RT compared to Control (Fig. 4E). Likewise, plantaris mass in RT significantly exceeded AT (Fig. 4E). Fiber CSA of the gastrocnemius was not different ($p>0.05$) between Control, AT, and RT (Figs. 4F-G). The myofibers from AT did appear to be distributed toward smaller sizes in comparison to Control and RT (Fig. 4H). There were no group differences ($p>0.05$) in liver or spleen mass or any of the mRNAs analyzed (data not shown).

3.6 Body Composition and Neuromuscular Function in Exercise Trained C26 Mice

To evaluate potential countermeasure effects of aerobic and resistance training in C26 mice, comparisons were performed between Control, C26, AT+C26, and RT+C26. Total body mass was maintained over time in Control ($p>0.05$) but not C26 (-8%, $p=0.006$), AT+C26 (-18%, $p=0.001$), or RT+C26 (-15%, $p<0.001$). Post-training values were also lower in AT+C26 (-13%, $p=0.01$) and RT+C26 (-14%, $p=0.01$) when compared to Control (Fig. 5A). For fat mass, there was a significant main time effect in which *post hoc* tests revealed losses in all C26 groups (C26: -33%, $p=0.002$; AT+C26: -40%, $p=0.016$; RT+C26: -40%, $p=0.007$) rather than Control ($p>0.05$) (Fig. 5B). No significant interaction or main effect was detected for lean mass ($p>0.05$) (Fig. 5C).

For sensorimotor function, significant reductions occurred over time in all groups except AT+C26 (Control: -16%, $p=0.006$; C26: -13%, $p=0.001$; AT+C26: -1%, $p=0.769$; RT+C26: -23%, $p=0.003$) (Fig. 5D). Post-training values in AT+C26 were also greater than RT+C26 (+20%, $p=0.01$) while tending to exceed C26 (+12%, $p=0.08$) (Fig. 5D). Absolute strength significantly decreased from pre to post in all groups (Control: -7%, $p=0.021$; C26: -23%, $p=0.001$; AT+C26: -14%, $p=0.003$; RT+C26: -19%, $p=0.002$). Post-training absolute strength was significantly lower only in C26 (-19%, $p=0.004$) vs. Control (Fig. 5E). When strength was normalized to body mass, there were significant pre to post reductions in all groups (Control: -7%, $p=0.01$; C26: -21%, $p=0.005$; RT+C26: -10%, $p=0.004$) except AT+C26 ($p>0.05$) (Fig. 5F).

3.7 Tissue Weights and Fiber Cross-Sectional Area in Exercise Trained C26 Mice

In comparison to Control, gastrocnemius mass was significantly lower ($p<0.01$) in C26 (-13%), AT+C26 (-14%), and RT+C26 (-19%) (Table 1). When normalized to body weight, gastrocnemius mass remained lower in C26 vs. Control (-7%, $p=0.05$); however, there were tendencies for greater mass in AT+C26 compared to both C26 (+6%, $p=0.09$, $d=0.88$) and RT+C26 (+5%, $p=0.11$) (Fig. 5G). At the myofiber level, gastrocnemius CSA was significantly lower ($p<0.001$) in C26 (-46%), AT+C26 (-32%), and RT+C26 (-40%) when compared to Control (Figs. 6A-C). Although no significant differences in fiber CSA were noted between the C26 groups ($p>0.05$), calculation of effect size from C26 and AT+C26 yielded a large effect (+24%, $d=1.04$) (Fig. 6B). Significant liver enlargement ($p<0.01$) was seen in C26 (+34%), AT+C26 (+21%), and RT+C26 (+30%) when

compared to Control (Fig. 5H). There were no differences in liver mass among the C26 groups ($p>0.05$); however, there was a moderate-large effect size calculated from C26 and AT+C26 (-10%, $p=0.186$, $d=0.89$) (Fig. 5H). The spleen was enlarged ($p<0.01$) in C26 (+195%), AT+C26 (+114%), and RT+C26 (+203%) vs. Control (Fig. 5H). However, the spleen in AT+C26 was significantly lower ($p<0.01$) than C26 (-27%, $d=1.82$) and RT+C26 (-29%) (Fig. 5H). There were no differences in tumor mass between the C26 groups ($p>0.05$) (Table 1).

3.8 E3 Ligase, IGF-I, and Akt-mTOR Expression in Exercise Trained C26 Mice

No group differences were detected for MuRF1, atrogin-1, or myostatin mRNA expression in either the gastrocnemius or plantaris muscles ($p>0.05$) (Table 2). IGF-IEa mRNA abundance in the plantaris of RT+C26 significantly exceeded Controls (+79%, $p=0.016$) while its expression in AT+C26 tended to be greater than Controls (+62%, $p=0.053$) (Fig. 7A). There were also tendencies for greater IGF-IEb transcripts in RT+C26 (+116%, $p=0.069$) and AT+C26 (+127%, $p=0.078$) when compared to Control (Fig. 7A). Regarding signaling events (Figs. 6D-F), phosphorylated to total Akt and mTOR were compared between C26 and AT+C26 to determine if Akt or mTOR activation was associated with the partially protective nature of aerobic training on gastrocnemius mass/body weight. When compared to C26, phosphorylated to total Akt was not significantly different in AT+C26 (Fig. 6D); however, phosphorylated to total mTOR levels in AT+C26 significantly exceeded the C26 group (+32%, $p=0.02$) (Fig. 6E).

3.9 Myogenic Regulatory Factor mRNA Expression in Exercise Trained C26 Mice

We questioned whether the elevated IGF-I levels possibly related to regeneration and repair mechanisms [48]. If the increased abundance of IGF-I in the muscles of exercised C26 mice represented an effort to initiate a compensatory response to cope with the dual stressors imposed by tumor and mechanical loads, we posited that transcript levels of the myogenic regulatory factors would also be expressed to a greater degree. When compared to Control, mRNA levels of MyoD and myogenin in C26 mice were not significantly different ($p>0.05$), indicating no effect of the tumor on expression of myogenic regulatory factors (Fig. 7B). In contrast, we observed significantly greater myogenin mRNA abundance in RT+C26 versus Control (+126%, $p=0.036$) and C26 (+150%, $p=0.018$) (Fig. 7B). MyoD expression was not affected by exercise training in C26 mice ($p>0.05$) (Fig. 7B).

4. DISCUSSION

4.1 Adaptive Responses of C26 Mice to Aerobic and Resistance Training

The present study defined and compared the skeletal muscle responses to aerobic and resistance training in the C26 mouse model of cancer cachexia. This information contains clinical relevance because it addresses the adaptations of cachectic muscle to classical exercise modes used in therapeutic settings. We did not observe convincing evidence to substantiate a superior countermeasure effect of resistance loading in comparison to aerobic exercise training. Instead, application of high mechanical stress may have contributed to declines in health because 25% of RT+C26 mice became moribund before the end of the experiment whereas all other C26 groups completed the study. It is noteworthy that tumor weight from RT+C26 exceeded C26 mice by 236 mg (+17%, $p>0.05$) (Table 1). Although not statistically significant, it raises the possibility that increased tumor mass could be linked to the health decline of RT+C26 mice that required early euthanasia. In the remaining RT+C26 mice, there were significant reductions in each performance measure including sensorimotor function, grip strength, and relative grip strength (Figs. 5D-F). The hindlimb muscles also atrophied slightly more versus C26 mice although not significantly (RT+C26: -17-19% vs. C26: -13-14%; Table 1). Because the same resistance training prescription caused plantaris hypertrophy in tumor-free mice (Fig. 4E), cachectic muscle appeared to demonstrate anabolic resistance to a mechanical stimulus. It should be noted that RT+C26 mice did show a lesser degree of absolute and relative strength decline compared to their non-exercised C26 counterparts (Figs. 5E-F), and this could be considered a possible benefit of high resistance loading.

In AT+C26, gastrocnemius mass adjusted for body weight was comparable to Controls while tending to be greater than C26 (+9%, $p=0.09$) and RT+C26 (+5%, $p=0.11$) (Fig. 5G). Furthermore, while fiber CSA in AT+C26 was significantly lower than Controls, it exceeded the C26 mean by approximately $280\ \mu\text{m}^2$ (+24%, $p=0.24$) (Fig. 6B). Despite not attaining statistical significance, calculation of an effect size statistic resulted in a d value of 1.04, which could be interpreted as a large effect of aerobic training according to Cohen's conventions. It could be argued that aerobic training may have been at least mildly protective against cachexia-dependent losses of muscle mass and fiber CSA. To explore associated signaling events, we examined Akt and mTOR activation in C26 and AT+C26. When compared to C26, phosphorylated to total mTOR but not Akt was

significantly greater in AT+C26 (Figs. 6D-F). This greater level may be indicative of persistently active mTOR signaling induced by aerobic training which in turn, may have contributed to the moderate rescue of gastrocnemius mass/body weight and CSA. While high resistance loading has often been reported to stimulate mTOR activation both acutely and after chronic training [49-52], there is also precedence for aerobic training-dependent modulation of mTOR activity [53]. Because C26 mice also displayed a lower ratio of phosphorylated to total mTOR when compared to Control (Fig. 3H), mTOR may be of interest as a target for pharmacological therapy.

Perhaps of greater importance, AT+C26 mice maintained sensorimotor function and relative strength (Figs. 5D, F). The preservation of function was impressive because all other groups showed significant deficits (i.e. Controls, C26, and RT+C26) (Figs. 5D, F). Furthermore, AT+C26 mice suppressed the enlargement of spleen and liver mass that occurred in all C26 groups. Although this suppression was not robust enough to reach Control levels, the spleen (-27%, $p=0.008$, $d=1.82$) and liver (-10%, $p=0.186$, $d=0.89$) were smaller than in C26 mice (Fig 5H). Since hepatosplenomegaly has been reported concurrent with systemic inflammation in cancer cachexia [54], aerobic exercise may serve as a therapy to reduce the inflammatory burden. This possibility is supported by the large effect sizes calculated for both surrogate indices of systemic inflammation. Of additional interest, it may be worth mentioning that AT+C26 consumed the most food during the post-injection window (+9 to +17% vs. all groups, $p>0.05$). Although not statistically significant, this may be of practical benefit for patients with reduced food intake.

An important finding that merits discussion is the total body mass measurements. All three C26 groups showed significant body mass reduction from pre- to post-experiment, suggesting that neither mode of training was effective in preventing weight loss (Fig. 5A). In addition, body mass at the post-experiment timepoint was significantly lower in both aerobic and resistance trained C26 mice compared to Control (Fig. 5A). This finding raises the possibility that regardless of mode, exercise training at the assigned intensities and durations may aggravate body weight loss in the presence of tumor load. In contrast, previous exercise intervention studies using rodent models of cancer cachexia have reported body weight preservation with aerobic or anaerobic training [40, 55, 56]. Nevertheless, some data exists in which aerobic exercise appeared to exacerbate weight loss using

the same C26 mouse model as the present study [21]. In agreement, a follow-up analysis of our exercised non-tumor bearing vs. exercised tumor bearing mice (i.e. AT vs. AT+C26; RT vs. RT+C26) found that C26 mice trained with either mode underwent weight loss significantly greater than their counterparts that performed the same exercise without tumors (-10-15%, $p < 0.05$). These findings suggest that aerobic and resistance training in a tumor bearing state can cause weight loss greater than when the same exercise is performed tumor-free. Possible explanations for the discrepant outcomes here and in the previous literature include the cachexia model studied or the exercise parameters prescribed, which have varied widely.

At the gene level, AT+C26 and RT+C26 mice showed lower mRNA expression of MuRF1, atrogin-1, and myostatin in the gastrocnemius when compared to C26 mice (Table 2); however, this did not reach statistical significance ($p > 0.05$) nor did it coincide with the preservation of muscle mass. In cardiac cachexia, aerobic training has been reported to completely reverse muscle atrophy concurrent with the robust suppression of atrogin-1 and MuRF1 [57] while in a rodent model of cachexia from kidney failure, high resistance loading corrected muscle atrophy and myostatin hyperexpression [29]. These outcomes suggest that exercise can have divergent effects on protein turnover depending on the specific cachectic condition in question.

4.2 Compensatory Mechanisms Associated with Mechanical Overload

RT-PCR analysis of plantaris muscles revealed significantly greater expression of IGF-IEa in RT+C26 and a strong tendency for greater levels in AT+C26 relative to Control (Fig. 7A). The same general pattern of response also occurred for IGF-IEb, where strong tendencies toward greater abundance were found in AT+C26 and RT+C26 relative to Control (Fig. 7A). These IGF-I responses of C26 muscle to exercise could be perceived as a compensatory means to cope with the dual stressors imposed by tumor burden and the mechanical forces applied to muscle by exercise, particularly more so with high resistance overload. This is consistent with the increased muscle IGF-I response following muscle injury due to mechanical strain [58, 59]. The induction of IGF-I consequent to these stressors has been suggested to be a mechanism that initiates the regenerative process through satellite cell activity [59].

In agreement with a possible compensatory regenerative/repair mechanism being executed, we found myogenin expression to be upregulated exclusively in RT+C26 (Fig. 7B). The induction of this myogenic regulatory factor is a unique response that may be attributed to the stress created by high resistance loading coupled with tumor burden. This interpretation is supported by the lack of myogenin induction from the tumor itself (i.e. C26 vs. Control; Fig. 7B), or by exercise alone (i.e. Control vs. AT and RT, data not shown). The specific induction of both IGF-I and myogenin in RT+C26 is supportive of the idea that resistance overload while in a tumor bearing state challenges the host (i.e. widespread muscle damage) such that downstream elements of the regenerative program are being activated to cope, but which ultimately ends up being futile. Indeed, the co-expression of IGF-I and myogenic regulatory factors such as myogenin has been observed in skeletal muscle after damaging resistance exercise [48]. Under this premise, these findings represent gene level data that support the profoundly stressful nature of resistance training in cancer cachexia.

4.3 Perspectives and Conclusions

Therapies for cancer cachexia are likely to consist of multiple arms to target the different defects. We aimed to gain insight into whether or not exercise training merited inclusion as part of multi-modal therapies. Neither mode of exercise training was able to prevent tumor-induced body weight loss. The possibility also exists that training may exacerbate weight loss depending on the specific exercise parameters assigned (e.g. intensity, duration). In addition, several resistance trained C26 mice required euthanasia prior to the end of the experiment as a result of their deteriorating condition. The gene response of these mice also suggests the presence of a damaged skeletal muscle phenotype. In contrast, aerobic exercise training may have provided several benefits such as the mild rescue of muscle size and maintenance of whole body function, which could translate into activities of daily living being performed with greater ease. Additionally, aerobic training may have reduced inflammation, an outcome that may benefit cancer patients as inflammation represents a major regulator of cancer cachexia onset/progression.

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AUTHOR CONTRIBUTIONS

Design of the study: AVK, BHA, SCG, RJC, JSK; Data collection and processing: AVK, BSP, DHK, MCY, SLO, MLE, EJ, GS, WJL; Data interpretation: AVK, BHA, GS, SCJ, RJC, JSK; Manuscript Writing: AVK, JSK

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TABLES

Table 1. Selected tissue weights from exercise trained colon-26 (C26) mice.

Tissue Weight	Control	C26	AT+C26	RT+C26
Gastrocnemius (mg)	117.51 ± 1.99	102.33 ± 3.14*	100.78 ± 3.75*	95.44 ± 1.85*
Plantaris (mg)	12.26 ± 0.38	10.60 ± 0.41*	10.58 ± 0.47*	10.14 ± 0.46*
Tumor (mg)	--	1385.53 ± 154.29	1323.83 ± 302.62	1621.97 ± 204.02

Tissues were collected from the following groups three weeks after cell injection (n=6-9/group): Controls, C26, aerobic trained C26 (AT+C26), and resistance trained C26 (RT+C26). Data are mean ± SE. * Different from Control (p<0.01).

Table 2. Atrogin-1, MuRF1, and myostatin mRNA in skeletal muscles of exercise trained colon-26 (C26) mice.

Target Gene	Muscle	Control	C26	AT+C26	% Change vs. C26	RT+C26	% Change vs. C26
Atrogin-1	GAS	1.02 ± 0.08	1.91 ± 0.43	1.17 ± 0.25	-39%	1.42 ± 0.42	-26%
	PLT	0.77 ± 0.14	0.99 ± 0.16	1.20 ± 0.14	+21%	1.21 ± 0.17	+22%
MuRF1	GAS	0.93 ± 0.06	1.42 ± 0.22	1.15 ± 0.16	-19%	0.91 ± 0.08	-36%
	PLT	0.78 ± 0.08	0.92 ± 0.13	1.02 ± 0.09	+11%	1.03 ± 0.06	+12%
Myostatin	GAS	3.41 ± 0.36	4.63 ± 0.60	4.17 ± 0.87	-10%	2.93 ± 0.63	-37%
	PLT	2.55 ± 0.23	2.68 ± 0.20	3.04 ± 0.23	+13%	3.13 ± 0.18	+17%

Gastrocnemius (GAS) and plantaris (PLT) muscles were collected from the hindlimbs of the following groups three weeks after cell injection (n=6-8/group): Controls, C26, aerobic trained C26 (AT+C26), and resistance trained C26 (RT+C26). Target mRNAs were determined by RT-PCR and expressed relative to 18S. Data are mean ± SE.

FIGURE CAPTIONS

Figure 1. Resistance overload by weighted ladder climbing. Training was performed 3 days per week for 11 weeks. The initial load was 50% of body weight followed by 10% increases bi-weekly.

Figure 2. Effect of C26 tumor on body composition, function, and tissue weights. Body composition and function were evaluated pre- and post-experiment while tissues were collected at post-experiment in Control and C26 mice (n=8-9/group). Body composition parameters included total body mass (A), fat mass (B), and lean mass (C). Function was indexed by sensorimotor function (D), grip strength (E), and grip strength normalized to body weight (F). Wet weights for the gastrocnemius and plantaris muscles (G) and the liver and spleen (H). Values are mean±SE. Intxn, interaction effect. ME, main effect. NS, not significant. *Significantly less than pre within group (p<0.05). ^Significantly less than Control at post (p<0.05).

Figure 3. Effect of C26 tumor on fiber cross-sectional area, anabolic/catabolic mRNA expression, and Akt-mTOR signaling. The gastrocnemius and plantaris muscles were excised from Control and C26 groups (n=8-9/group) at the end of the experiment. Transverse sections were cut 7 µm thick from the midbelly of mounted gastrocnemius, stained with hematoxylin, and analyzed morphometrically. Representative cross-sections imaged at 40x (A), mean fiber cross-sectional area (B), and fiber size distribution (C) are shown. mRNA expression levels of atrogin-1, MuRF1, and myostatin in the gastrocnemius (D) as well as IGF-IEa and IGF-IEb in the plantaris (E) were normalized to 18S. Western blot experiments probed for phosphorylated and total Akt and mTOR. Representative blots (A), phosphorylated:total Akt (B), and phosphorylated:total mTOR (C) are shown.

Figure 4. Body composition, function, and muscle mass in exercise trained tumor-free mice. Total body mass (A), fat mass (B), lean mass (C), and sensorimotor function (D) assessed at pre- and post-training in Control, Aerobic Trained (AT), and Resistance Trained (RT) (n=8/group). Hindlimb muscles were collected post-training and weighed (E). The gastrocnemius was also sectioned at 7 µm, stained with hematoxylin, and analyzed morphometrically. Representative cross-sections imaged at 40x (F), mean fiber cross-sectional area (G), and fiber size distribution (H). Values are mean±SE. Intxn, interaction effect. ME, main effect. NS, not significant. *Significantly less than pre within group (p<0.05). @Significantly greater than Control at post (p<0.05).

Figure 5. Body composition, function, and tissue weights in exercise trained C26 mice. Body composition and function were evaluated pre- and post-training while tissues were collected at post-experiment in Control, C26, aerobic trained C26 (AT+C26), and resistance trained C26 (RT+C26) (n=6-9/group). Body composition measurements included total body mass (A), fat mass (B), and lean mass (C). Functional indices included sensorimotor function (D), grip strength (E), and grip strength normalized to body weight (F). Tissues were collected at the end of training and measured to determine gastrocnemius mass normalized to body weight (G) and liver and spleen mass (H). Values are mean±SE. Intxn, interaction effect. ME, main effect. NS, not significant. *Significantly less than pre within group (p<0.05). ^Significantly less than Control at post (p<0.05). %Tended to be greater than C26 at post (p=0.08). \$Significantly greater than RT+C26 mice at post (p<0.05).

Figure 6. Fiber cross-sectional area and Akt-mTOR expression in exercise trained C26 mice. The gastrocnemius was excised post-training from Control, C26, aerobic trained C26 (AT+C26), and resistance trained C26 (RT+C26) (n=6-7/group). Transverse sections were cut 7 µm thick from the midbelly of the of mounted gastrocnemius muscle, stained with hematoxylin, and analyzed morphometrically. Representative cross-sections imaged at 40x (A), mean fiber cross-sectional area (B), and fiber size distribution (C) are shown. Western blot experiments probed for phosphorylated and total Akt and mTOR in C26 and AT + C26 to determine if active Akt or mTOR related to the partially protective nature of aerobic exercise. Representative blots (A), phosphorylated:total Akt (B), and phosphorylated:total mTOR (C) are shown. Values are mean±SE.

Figure 7. mRNA expression of insulin-like growth factor-I (IGF-I) and myogenic regulatory factors in exercise trained C26 mice. Plantaris muscles from Control, C26, aerobic trained C26 (AT+C26), and resistance trained C26 (RT+C26) (n=6-8/group) were analyzed post-training by RT-PCR to determine mRNA expression of IGF-IEa and IGF-IEb (A) and MyoD and myogenin (B). Target transcripts were normalized to 18S. Values are mean±SE.

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Figure 7