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RESEARCH PROPOSAL

Effect of combined citrulline supplementation and resistance training intervention on muscle mass, mitochondrial respiratory capacity, and ccf-mtDNA in cancer cachexia patients: Research proposal for a randomized control trial

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ABSTRACT

Cancer cachexia is a complex metabolic syndrome affecting approximately 527,100 cancer patients in the US. It results from adaptation to cancer and is characterized by loss of muscle mass with or without loss of fat mass. The two main aims of this research are to investigate the combined effect of resistance training (RT) and citrulline supplementation (CS) on muscle mass and to determine whether this combined regime alter plasma circulating cell free mitochondrial DNA level and mitochondrial respiratory capacity (MRC) in cancer cachexia patients. Eligible participants (Males and Females: Age ≥50 years diagnosed with cancer cachexia) will be divided into four groups (I-IV). Groups III and IV will be supplemented with citrulline (10 g/day × 12 weeks), and Groups II and IV will perform 12 weeks of RT. The outcome variables include muscle mass (muscle hypertrophy and fat free mass using ultrasound), MRC (muscle biopsy using Oxygraph-2K), and quantification of plasma circulating cell free mitochondrial DNA level (using polymerase chain reaction [PCR]). Coupling of RT with CS will provide an innovative intervention to maximize the potential for lean mass increase by mitigating inflammation and protein degradation while concomitantly enhancing protein synthesis and mitochondrial function.

KEY WORDS: Cancer Cachexia; Citrulline Supplementation and Resistance Training; Muscle Mass; Mitochondrial Respiratory Capacity and ccf-mtDNA

INTRODUCTION

In 2015, there were 3 million cancer survivors in the US with a 5-year survival rate of 99%.[1] Cancer cachexia is a complex metabolic syndrome that results from adaptation to cancer and is characterized by loss of muscle mass with or without loss of fat mass.[2] It affects approximately 527,100 cancer patients (of all kinds) in the US (16.5 subjects per 10,000 people) with a prevalence rate of 85% in patients with solid tumors (e.g., gastric/pancreatic cancer) and 50% in those with prostate, colon, or lung cancers. In advanced cancer, its prevalence is between 60% and 80% with a weight loss of ~86% in the past 1–2 weeks of life and is directly responsible for 30% of cancer deaths.[3] In general, the cost of inpatient stay is higher in cachexic versus non-cachexic cancer patients ($14,751 vs. $13,928).[4]

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The long-term goal of this research is to develop an exercise and nutrition-based lifestyle intervention for cancer cachexia patients. There are two objectives in achieving this goal: First, to elucidate the mechanism(s) through which combined resistance training (RT) and citrulline supplementation

(CS) have an additive effect on muscle mass and second, to determine the efficacy of this concurrent intervention on mitochondrial respiratory capacity (MRC) and plasma circulating cell free mitochondrial DNA (ccf-mtDNA) levels. The central hypothesis is that cancer cachexia induces chronic inflammation, which leads to dysregulated mitophagy and dysfunctional mitochondria. The compromised adenosine triphosphate (ATP) production attenuates muscle protein synthesis (MPS) (by turning off the AMP-activated protein kinase [AMPK]-mammalian target of rapamycin [mTOR] switch) and releases ccf-mtDNA into the plasma [Figure 1]. Concurrent RT and CS will possibly have an amalgamated enhancing effect on both MPS (assessed through muscle mass) and mitochondrial biogenesis by stimulating mTOR and PGC1-α signaling pathways and increasing mRNA levels of genes encoding mitochondrial proteins.[1,5] Despite being highly prevalent and an independent risk factor for reduced survival, cancer cachexia remains an underdiagnosed entity. Thus, the rationale for this study is to improve patient survival by determining a non-therapeutic lifestyle intervention for prevention and treatment of cancer-associated muscle wasting. To attain these objectives, the following two specific aims will be pursued.

Aim (1)

The aim of the study was to investigate the combined effect of RT and CS on muscle mass in cancer cachexia patients. The working hypothesis is that ingestion of citrulline with RT will have a larger increase in the rate of MPS (assessed through muscle mass) compared with the independent effects of either citrulline or RT alone.

Aim (2)

The aim of the study was to determine whether combined RT and CS alter ccf-mtDNA and MRC in cancer cachexia patients. The working hypothesis is that the blended (RT + CS) intervention will reduce plasma levels of ccf-mtDNA and help improve MRC in cancer cachexia patients.

LITERATURE REVIEW

Cancer disrupts muscle homeostasis (the delicate balance between synthesis and degradation of muscle cell proteins), creates an environment of chronic inflammation, induces anemia related fatigue, and reduces immune function, which subsequently causes anorexia and muscle wasting.[2] Normal muscle protein mass is controlled by the relative balanced rates of MPS and degradation.[2,6,7] In cancer cachexia, this balance is offset with greater muscle protein degradation, thus causing muscle atrophy.[6,7] Cancer cachexia makes it difficult for patients to adhere to and tolerate long anti-cancer therapy regimes, which affects their quality of life and ultimately leads to increased morbidity and mortality.

The role of systemic inflammation in muscle wasting among cancer cachexia patients is well known.[6,8] Chronic inflammation during cancer cachexia is associated with increased circulatory levels of pro-inflammatory cytokines (Interleukin-6, tumor necrosis factor-α, and transforming growth factor-β), which damage mitochondria.[8,9] Dysfunctional mitochondria release aberrant load of reactive oxygen species (ROS) and decrease ATP production.[8-10] Increased ROS targets mtDNA (located near the ROS source,

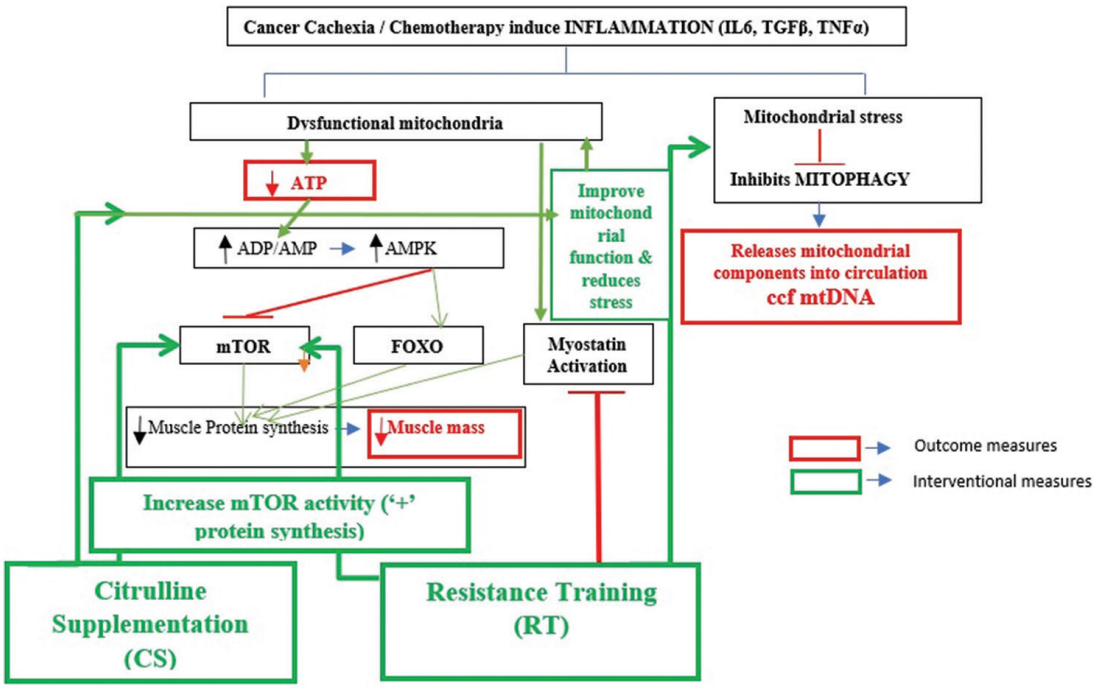

Figure 1: Hypothesis of cancer cachexia and combined effect of citrulline supplementation and resistance training intervention

and electron transport chain) inducing strand breaks, point mutations, and deletions, which ultimately trigger pathologically simulated inflammatory response.[10] Moreover, low ATP production leads to chronic activation of AMPK, which negatively regulates MPS.

RT is an effective therapeutic intervention known to increase muscle mass and avert muscle atrophy.[11,12] Researchers of late have evaluated the benefits of low-intensity RT with reduced systemic inflammation in context of cancer cachexia.[13-15] For example, Litterini *et al*. (2013), who conducted an randomized controlled trial (RCT) on 66 terminally ill cancer patients, found low intensity individualized RT beneficial and attainable for improving functional mobility.[13] Similarly, Oldervall *et al*. (2011) found a significant increase in functional performance (assessed by hand grip strength and shuttle walk tests) with 8 weeks of circuit training amongst 121 cancer patients.[14]

Most of the RCTs evaluating the efficacy of general nutritional supplements in cancer cachexia have showed inconclusive evidence, which leads researchers to focus currently on individual specific dietary supplementation that benefits the host and is detrimental to cancer growth.[1,6,16,17] Study of amino acids such as glutamine, arginine, and citrulline in a cancer bearing state is an emerging area of research due to their potential role in enhancing nitrogen balance, muscle protein mass, and indices of immunity.[6] The role of citrulline as a positive regulator of MPS was posited by Osowska *et al*. (2006) in energy restriction rat models.[18] Since then, many clinical trials have studied the effect of CS on whole body protein synthesis; however, the precise mechanistic basis involved here remains unclear.[16,18,19] Citrulline potentially exerts its protective effect on muscle mass by improving protein synthesis and shielding myotubes from muscle cell wasting, an effect that is clearly dissimilar to the nitric oxide mediated L-arginine treatment.[19]

Overall, the precise mechanistic basis of weight loss in cancer cachexia is unknown; however, there might be involvement of protein metabolic pathways, inflammatory markers, and dysfunctional mitochondria. Thus, coupling of RT with CS is an innovative and promising intervention to maximize the potential for lean mass increase by mitigating inflammation and protein degradation while enhancing protein synthesis and mitochondrial function.

METHODOLOGICAL APPROACH

Participants

Eligibility criteria

The inclusion criteria are that subjects be males or females, (≥ 50 years) who have been diagnosed with cancer cachexia (cancer of all kinds/stages) as defined by Evans *et al*.;[7] weight loss of at least 5% in the past 12 months; plus any three of the following criteria: decreased muscle strength, fatigue, anorexia, low fat-free mass index, abnormal biochemistry,[7] and permission from the treating physician to participate in exercise.[1] The exclusion criteria will be that subjects have not had chemotherapy or radiotherapy (past 4 weeks), major coronary event or surgery, inability to perform exercise (neurological or musculoskeletal limitation), amino acid supplementation, and current enrollment in structured exercise programs.[1]

Recruitment strategy

Participants will be recruited by referral from the oncologists and staff from the University Medical Center Cancer Center in Lubbock, TX.

Sample size

A sample size of 15 for each group was calculated through Cohen flexible algorithm using Fischer's "F" distribution by comparing differences of means between the four group(s) with "α" = 0.05, "β" = 80%, and effect size (es) = 0.8.[20]

Randomization of groups and blinding

Participants will be randomized in a 1:1 allocation ratio to one of the four study groups, as shown in Table 1. The study personnel and outcome assessors will neither participate in randomization nor have access to group allocation.

Intervention

CS

Participants in the combined (RT+CS) and CS groups will be given CS 10 g/day for 12 weeks (weeks 2–14; refer "study outline") with the participants of combined (RT+CS) receiving it 1 h before their training session.[21] CS will be recorded in a logbook and will be discontinued in case of intolerance or adverse reaction.

RT

Participants in the combined (RT + CS) and RT groups will perform 12 weeks of RT (week 2–14) (refer "study outline" and Table: 2) under certified supervision. Familiarization will be done for 1 week before the actual training (refer "study outline"). Combined (RT + CS) and RT groups will start their training with 1 set (8–15 reps) with further progression in resistance, reps, and sets which will be decided as per their tolerance capacity. Equipment based training will be substituted with free weight training in case of failure to perform the former. The participants in the control and CS group will be asked to maintain their current level of activity.[1,2,13]

Table 1: Randomization of groups

Group	*n*=(no of participants)	Randomization
Group I	15	Control (C)
Group II	15	Resistance training (RT)
Group III	15	Citrulline supplementation (CS)
Group IV	15	Combined (RT+CS)

Outcome measures and their assessment

All outcome measures and their assessment methods are presented in Table 3.

Assessment of muscle mass

Muscle mass will be assessed using muscle hypertrophy (functional cross sectional area [fCSA]; using ultrasound) and fat-free mass (FFM); using dual energy X-ray absorptiometry [DXA]). Muscle hypertrophy will be measured by analyzing changes in vastus lateralis fCSA using ultrasound (B-mode, 7.5 MHz linear-array probe) by capturing sequential images (from middle-to-lateral direction position) with participants relaxed in a supine position.[12] FFM will be estimated by performing DXA scans on participants using a Lunar Prodigy scanner (General Electric) with enCORE software[22] and Wang *et al*. 4C model equations.[23]

Assessment of MRC

Percutaneous skeletal muscle biopsies from vastus lateralis muscle will be collected with subjects in supine position. A suction adapted five-gauge Bergstöm needle will be used to extract the sample from the subjects under ketamine sedation and aseptic precautions. About 10–20 mg of muscle tissue sample will be immersed in an ice-cold preservation buffer. These muscle tissue samples will then be separated into myofiber bundles with their sarcolemma membranes permeabilized in sucrose buffer containing 5 mM saponin for 10–20 min (4–8°C). 3 mg of muscle tissue will be transferred to Oxygraph-2K respirometer chamber (Oroboros Instruments, Innsbruck, Austria). Mitochondrial substrates and inhibitors will be added to the respirometer chambers of the Oxygraph-2K to determine mitochondrial respiratory states or MRC.[24]

Quantification of ccf mt DNA

10 ml of peripheral blood samples will be collected from antecubital vein and centrifuged immediately at 1600 g for 10 min (temperature) to separate into plasma and serum. Plasma will then be centrifuged again at 16,000 g for10 min (temperature) and stored at −80°C. Plasma ccfmtDNA will then be measured in the extracted isolated DNA through amplification of MTATP 8 gene at locus8446 utilizing real-time PCR (ABI PRISM 7000 Sequence Detection System) with pre-decided forward primer, reverse primer, and probe.[25]

Strengths and Limitations

Strengths of this study include adaptation of specific low-intensity RT for cancer patients and intervention looking at both the individualized and combined effect of RT + CS on muscle mass, MRC, and ccf mtDNA in cancer cachexia patients. However, a probable limitation will be indirect assessment of MPS and ATP synthesis by estimating muscle mass and MRC, respectively. The use of Oxygraph-2K (Oroboros instruments) to measure MRC, which has low-throughput capability and requires greater time per sample assay may emerge as another potential limitation.[26]

Ethical Approval

The study will be submitted to the Institutional Review Board at Texas Tech University, Lubbock, TX for approval before engaging in data collection.

Data Management and Statistical Analysis

Data will be collected on paper charts and stored securely inside patient folders before being entered electronically into

Table 2: Proposed weekly (3 days/week) RT protocol for combined (RT+CS) and RT groups

Day 1			Day 2			Day 3		
Lower body and trunk			**Lower body and upper body**			**Upper body and trunk**		
Exercise	**Intensity**	**Reps/Sets**	**Exercise**	**Intensity**	**Reps**	**Exercise**	**Intensity**	**Reps**
Warm up			Warm up			Warm up		
Leg press	Variable*	(8–15)×1 or 2 sets	Leg press	Variable*	(8–15)×1 or 2 sets	Shoulder press	Variable*	(8–15)×or 2 sets
Leg extension	Variable*	(8–15)×1 or 2	Leg extension	Variable*	(8–15)×1 or 2 sets	Chest press	Variable*	(8–15)×1 or 2 sets
Plank		20 s	Shoulder press	Variable*	(8–15)×1 or 2 sets	Plank		20 s

*Resistance will be set to a level where they will feel to take rest for 1–2 min after the set

Table 3: Outcome measures and their assessment methods

Measure	Assessed entity	Methods (sample)	Pre-intervention assessment (3 days before intervention RT training)	Post-intervention assessment (3 days after intervention RT training)
Muscle mass	Muscle hypertrophy (fCSA)	Ultrasound	✓	✓
	Fat-free mass	DXA	✓	✓
Mitochondrial respiratory capacity	Mitochondrial respiratory states	Oxygraph-2K (Muscle biopsy)	✓	✓
ccf mtDNA	ccf mtDNA level	PCR (Plasma)	✓	✓

the analysis software SPSS (version 26.0, Illinois, Chicago). Normality will be tested using Shapiro–Wilk test. Intention-to-treat models (to avoid the effects of crossover and dropout) will be used to test the outcome variables for both the first and second hypotheses among all four groups using repeated measures analysis of covariances.

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