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Effectiveness of resistive vibration exercise and whey protein supplementation plus alkaline salt on the skeletal muscle proteome following 21 days of bedrest in healthy males

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Abstract

Muscle atrophy is a deleterious consequence of physical inactivity and is associated with increased morbidity and mortality. The aim of this study was to decipher the mechanisms involved in disuse muscle atrophy in 8 healthy men using 21-day bed rest with a cross-over design (control, with resistive vibration exercise (RVE) or RVE combined with whey protein supplementation and an alkaline salt (NEX)). The main physiological findings show a significant reduction in whole body fat free mass (CON -4.1%, RVE -4.3%, NEX -2.7%, $p < 0.05$), maximal oxygen consumption (CON -20.5%, RVE -6.46%, NEX -7.9%, $p < 0.05$) and maximal voluntary contraction (CON -15%, RVE -12% and NEX -9.5%) ($p < 0.05$) and a reduction in mitochondrial enzyme activity (CON -30.7%, RVE -31.3%, NEX -17%, $p < 0.05$). The benefits of nutrition and exercise countermeasure was evident with an increase in leg lean mass (CON -1.7%, RVE +8.9%, NEX +15%, $p < 0.05$). Changes to the vastus lateralis muscle proteome were characterized using mass spectrometry-based label-free quantitative proteomics, the findings of which suggest alterations to cell metabolism, mitochondrial metabolism, protein synthesis and degradation pathways during bed rest. The observed changes were partially mitigated during RVE but there were no significant pathway changes during the NEX trial. In conclusion, resistive vibration exercise, when combined with whey/alkalising salt supplementation, could be an effective strategy to prevent skeletal muscle protein changes, muscle atrophy and insulin sensitivity during medium duration bed rest.

Key words: Bed rest, Exercise, Nutrition, Insulin resistance, Mitochondrial metabolism, Protein synthesis, Protein degradation

Abbreviations

RVE, resistive vibration exercise; NEX, nutrition and exercise; CON, control; MVC, maximal voluntary contraction, LC-MS, liquid chromatography – mass spectrometry; PGC1- α , Peroxisome proliferator-activated receptor gamma coactivator 1-alpha; ERR, estrogen related receptor; NDUF3, nicotinamide adenine dinucleotide hydrogen -Ubiquinone Oxidoreductase Subunit B3; IMM, inner mitochondrial membrane; MICOS, mitochondrial contact site and cristae organizing system; NADP, Nicotinamide adenine dinucleotide phosphate;

AMP, Adenosine monophosphate; IMP, inosine monophosphate; AMPK, Adenosine monophosphate activated protein kinase; PARP, poly ADP ribose polymerase; TCA, tricarboxylic acid

Introduction

Exposure to microgravity, bed rest and aging are associated with physiological alterations that lead to reduced muscle mass¹. Muscle atrophy can impair functional strength, induce weakness and fatigability that can impact whole-body energy metabolism and, in the long term, quality of life. Despite the functional and clinical impacts of muscle atrophy, the underlying mechanisms are relatively understudied. Bed rest is used as an analogue for physiological changes in microgravity and a model of physical inactivity where ambulatory movement is eliminated while energy balance can be rigorously controlled. A reduction of 6-11% in muscle mass has been reported during bed rest studies²⁻⁴ with a pronounced early decline of 0.6% day⁻¹ in the rate of muscle CSA⁵ and 4-5% week⁻¹ reduction in knee extensor torque⁶. Furthermore, bed rest studies have reported a dramatic reduction (~50%) in muscle protein synthesis compared to pre-bed rest levels^{7,8}.

Physiological impairments with bed rest have been well described⁹. The reduction in bed rest induced fat free mass¹⁰ and basal energy expenditure¹¹ is associated with metabolic derangement and reduced insulin sensitivity¹². Others have reported a reduction in resting fat oxidation¹³ and mitochondrial function¹⁰ after 3-months and 21-days of bed rest, respectively. Proteomic and transcriptomic analysis following bed rest demonstrate decreased expression of oxidative pathways and enzymes^{14,15}. Recent progress in muscle proteomics¹⁶⁻²⁰ has notably provided detailed analysis of the protein signature of lower limb muscles in response to disuse during bed rest or weightlessness in space. However, there is relatively little proteomic data for the countermeasures used to prevent bed rest induced physiological changes in skeletal muscle.

Physiological responses to physical inactivity²¹ have some similarities to that of bed rest¹² and the physical activity level measured during bed rest studies is close to that measured in sedentary ambulatory individuals (physical activity level of 1.4-1.5)¹². Considering physical inactivity is now a major cause of metabolic disease¹² and is associated with an increase in morbidity and mortality²², there is a clear need for more in-depth investigation into the metabolic consequences of bed rest and ways to counteract these changes. While many bed rest studies to date have employed nutritional and exercise strategies to preserve metabolic health, many have only had partial success^{23,24}. The present study investigated the effects of two countermeasure protocols; resistive vibration exercise (RVE) and RVE plus whey protein and alkaline salt supplementation (NEX). Previous research has shown that vibration exercise alone has little effect on muscle atrophy during bed rest²⁵, while a combination of vibration exercise and resistance exercise have been shown to have an additive benefit²⁶. Nutritional

supplementation in weightlessness has shown some promise as demonstrated by Paddon-Jones et al. (2006) who reported that essential amino acids stimulated muscle protein synthesis and prevented loss of lower body mass. However, the protein content of the diet during the control period was lower than the current recommended daily allowance (RDA) ($0.8 \text{ g protein}\cdot\text{kg}\cdot\text{d}^{-1}$) and was increased during bed rest (1.2g/kg/d), therefore, it is not clear if the benefits observed were as a result of the increase in protein intake during bed rest compared to the baseline period. This highlights the need for further studies where the dietary intake of protein is controlled from the beginning. Previous reports have demonstrated that protein ingestion is effective in stimulating muscle protein synthesis after exercise ²⁸. Despite significant progress, further refinement of countermeasure protocols is necessary in order to mitigate the negative consequences of bed rest and spaceflight.

In order to develop appropriate countermeasures, a better understanding of the mechanisms involved in skeletal muscle adaptations to bed rest is necessary. The purpose of this study was therefore to determine the impact of 21-days bed rest on whole-body physiological and lower limb skeletal muscle proteomic changes. The study was designed so that nutritional intake and composition was controlled before and during bed rest while subjects were in energy balance during each experimental protocol. Additionally, we aimed to determine the efficiency of RVE or NEX countermeasure protocols in mitigation of the negative physiological effects of bed rest or to assess if they resulted in a vastly different muscle protein signature compared to bed rest alone.

Experimental Procedures

Subject details and study design. A total of 12 subjects were recruited but four volunteers dropped out of the study; therefore, eight subjects, aged 24-44 years, were included in the current analysis as they completed all three trials according to the randomized study design described below. Group assignment is outlined in Supplemental Table 2. Each candidate signed the Information and Consent Form. This study was conducted at the MEDES clinic, Toulouse, France from June 2012 to December 2013 and was approved by the appropriate Ethics Committee (Comité de Protection des Personnes / CPP Sud – Ouest Outre-Mer I) and the French Health Authorities (Agence Française de Sécurité Sanitaire des Produits de Santé). Volunteers were subjected to seven days of ambulatory controlled conditions for baseline data collection. This was followed by 21 days of head-down-tilt bed rest. During the bed rest, the subjects were randomly assigned to three groups (n=8, each): a control group (CON) that underwent head down tilt (-6° HDT) bed rest only, an exercise group subjected to resistive vibration exercise (RVE) twice a week in addition to HDT and the third group was the same as the RVE group, with the addition of whey protein supplementation plus alkaline salt to their diets (0.6 g /kg body weight/day) (NEX). The bed rest period was followed by seven days recovery in the facility. The study used a crossover design (Fig 1) and the wash-out period between the bed rest periods was for a minimum duration of 120 days to allow subjects to return to pre-bed rest physical status.

Resistive Vibration Exercise Protocol. The exercise training was performed on days 2, 5, 12, 16 and 20. All training was performed on an integrated training device (Novotec Medical, Pforzheim, Germany). This device combines a standard leg press machine, already used for bed rest studies²⁹, with a vibration platform located on the foot plate. While the participants were completing the leg press protocol the foot plate was vibrating (8mm peak-to-peak, 25 Hz). Participants performed bilateral squats (10-reps, 75% 1-RM, 8 seconds per rep), single heel raises (x1.3 body weight, contractions performed as fast as possible until fatigue) and bilateral heel raises (x1.8 body weight, contractions performed as fast as possible until fatigue). A 5% load adjustment was made based on the ability of volunteers to complete the set of exercises.

Diet and nutrition supplementation. Macronutrient intake included 1.2 g/ kg/ body weight/ day protein (without whey protein supplementation), total fat <30% and 50-60% carbohydrates. Dietary intake was quantified by measuring the weight of each dish before and after (in case of leftovers) each meal, and by calculating the intake

for each nutrient. Total liquid intake was between 35 and 50 ml/kg/day with total water accounting for beverages and food. An isocaloric supplementation of whey protein (0.6 g kg/body weight/ day) was given to the volunteers of the nutritional and exercise intervention group (NEX). For this group, the total protein intake was 1.8 g/kg body weight/ day. Whey protein supplementation was provided using the product Diaprotein®, a powder supplied by Nephrologische Präparate Dr. Volker Steudle (Linden, Germany). The composition was the following: 100 g of Diaprotein® (370kcal) consisted of 90 g protein, 0.2 g fat, 2.5 g lactose, <300 mg sodium, <650 mg potassium, <400 mg calcium, phosphorus < 250 mg, relation phosphorus/protein < 3 mg/g. Since whey protein adds a certain acid load to the diet, supplementation of 90 mmol potassium bicarbonate (KHCO₃)/day, applied in 6 portions (with main meals and snacks) was used to compensate for that. Effervescent tablets of potassium bicarbonate were provided by Krüger GmbH & Co (Bergisch Gladbach, Germany).

Body mass and composition. Body mass was measured daily. Fat mass and fat free mass was measured at 4 time points throughout each study period (baseline data collection, Day-7, bed rest day 1, 10 and 20) by dual-energy x-ray absorptiometry (DEXA) on Hologic, QDR 4500 C scanner using the version software 11.2 (Hologic, Roissy Charles-de-Gaulle, France).

Aerobic Fitness Assessment. Peak oxygen consumption (VO_{2peak}) was assessed before and after each study period using a cycle ergometer in upright position using an incremental protocol. Subjects were connected to the metabolic cart (Oxycon Pro™, Jegger™) for gas exchange determination in the breath by breath mode.

Maximal Isometric Voluntary Contraction. Maximal isometric voluntary contraction (MVC) of four muscle groups; the knee extensor and flexors and plantar flexors and dorsiflexors was recorded before and after each bed rest period using CON-TREX isokinetic dynamometer (Medimex, France). The isometric knee extension and flexion were performed with the subject in a sitting position and in a prone position for ankle extension and flexion, using the dominant limb. Three sets of extension/flexion were recorded for 30 seconds with a two-minute rest period between each set. The measured parameters were the maximal isometric torque (units = Nm) for extension and flexion for the different tested muscle groups.

Hyperinsulinemic-Euglycemic Glucose Clamp. A two stage hyperinsulinemic-euglycaemic glucose clamp was performed at baseline prior to the first bed rest campaign and on day 19 of the CON, RVE and NEX trials. Insulin was infused intravenously using a precision pump at 0.25µU kg⁻¹ min⁻¹ and 1µU kg⁻¹ min⁻¹ along with low dose heparin (10,000U/100ml saline). Each stage was 240-mins and blood glucose levels were maintained at 90 mg

/dl using 10% dextrose variable rate infusion that was adjusted every minute according to continuously measured blood glucose values determined in arterialized blood (heated-hand-technique). The glucose infusion rate to estimate insulin sensitivity was calculated over the last 60-mins of the second stage and expressed relative to the circulating insulin concentration (pmol/l) and fat free mass (kgFFM).

Muscle proteomics. Skeletal muscle biopsy samples were obtained from the vastus lateralis before and after each trial using the Bergström technique. Frozen muscle samples (vastus lateralis) were grinded under liquid nitrogen using a ball mill (MM400, Retsch, Eragny sur Oise, France) and total protein was extracted from the resulting powder using 100-200 μ l of extraction buffer (8M urea, 2M thiourea, 4% CHAPS, 30mM Tris pH 8.5, protease inhibitors, TLCK; Sigma-Aldrich, Lyon, France). After solubilisation, proteins were precipitated using 6 volumes of ice-cold acetone over night at -20°C and centrifuged for 15 minutes at $15000 \times g$, 4°C . Resulting protein pellets were re-solubilized in extraction buffer without protease inhibitors and protein concentration was determined using a Bradford assay (Bio-Rad, Hercules, CA, USA). At this stage, a reference sample comprising equal amounts of all protein extracts was made, to be injected regularly during the whole experiment and thus allow QC-related measurements. For the differential analysis, samples were pooled within experimental groups (CON, RVE, and NEX) with each pool comprised of two patients, which were the same in BDC and HDT conditions.

50 μ g of each pool (i.e. 25 μ g per patient) was evaporated to dryness (SpeedVac, Savant, ThermoScientific, Waltham, MA, USA), re-solubilised in sample buffer (50 mM Tris pH 6.8, 1 mM EDTA, 5% β -ME, 2.5% SDS, 10% glycerol and 0.1% Bromophenol blue), incubated at 95°C for 5 minutes and then electrophoresed for 7 Vh at 35 V in a 4% polyacrylamide stacking gel in order to focus proteins into a single “stacked” band. For each sample, both the “stacked” protein-band and the part of the gel above this band were excised and each of them cut into four equal pieces. After de-staining, reduction and alkylation using an automatic pipetting device (MassPrep, Micromass, Waters, Milford, MA, USA), proteins were in-gel digested with trypsin (Promega, Madison, WI, USA; 300 ng per band) over night at 37°C . Tryptic peptides were extracted subsequently in 40% and 60 % acetonitrile/0.1 % formic acid in water as well as 100% acetonitrile for 1 hour each at 450 rpm on an orbital shaker. Organic solvent in resulting peptide solutions was removed in a speed-vac and samples adjusted to 25 μ l using 0.1 % formic acid before LC-MS/MS analysis. A set of reference peptides (iRT kit; Biognosys AG,

Schlieren, Switzerland) was finally added to each sample prior to LC-MS/MS analyses for additional QC-related measurements.

Samples were analysed on a nanoUPLC-system (nanoAcquity, Waters) coupled to a quadrupole-Orbitrap hybrid mass spectrometer (Q-Exactive plus, Thermo Scientific, San Jose, CA, USA). Two μl of each sample were first concentrated/desalted on a trap column (Symmetry C18, $180\mu\text{m} \times 20\text{mm}$, $5\mu\text{m}$; Waters) using 99% of solvent A (0.1 % formic acid in water) and 1% of solvent B (0.1% formic acid in acetonitrile) at a flow rate of $5\mu\text{l}/\text{min}$ for 3 minutes. Afterwards, peptides were eluted from the separation column (BEH130 C18, $250\text{mm} \times 75\mu\text{m}$, $1.7\mu\text{m}$; Waters) maintained at $60\text{ }^\circ\text{C}$ during 150 minutes using a linear gradient from 3-40% of solvent B.

The Q-Exactive Plus was operated in positive ion mode with source temperature set to 250°C and spray voltage to 1.8 kV. Full scan MS spectra (300-1800 m/z) were acquired at a resolution of 140,000 at m/z 200, with a maximum injection time set to 50 ms and an AGC target value set to 3×10^6 charges. The lock-mass option was enabled (polysiloxane, 445.12002 m/z). Up to 10 most intense peptides (with a minimum of 2 charges) per full scan were isolated using a 2.2 m/z window and fragmented using higher energy collisional dissociation (normalised collision energy set to 27 and dynamic exclusion of already fragmented precursors set to 60 seconds). MS/MS spectra were acquired with a resolution of 17,500 at m/z 200, with a maximum injection time of 100 ms and an AGC target value set to 1×10^5 , and the peptide match selection option was turned on. The system was fully controlled by XCalibur software (v3.0.63; Thermo Fisher Scientific). Peak intensities and retention times of reference peptides were monitored in a daily fashion.

MS raw data processing was performed in MaxQuant (v 1.5.3.8). Peak lists were searched against human protein sequences, which were downloaded from SwissProt (09-07-2015; 40420 sequences, Taxonomy ID: 9606) using the MSDA software suite³⁰. Sequences of common contaminants (247 entries) were added to the database using the Andromeda search engine implemented in MaxQuant. Maxquant parameters were set as follows: MS tolerance set to 20 ppm for the first search and 4.5 ppm for the main search, MS/MS tolerance set to 20 ppm, maximum number of missed cleavages set to 1, carbamidomethylation of cysteine residues set as fixed modification, and oxidation of methionine and acetylation of lysine residues set as variable modifications. For protein identifications, false discovery rates (FDR), estimated on the basis of the number of hits after searching a reverse database, were set to 1% for both peptide spectrum matches (minimum length of seven amino acids) and proteins. Data normalisation and protein quantification was performed using the LFQ (label free quantification)

option implemented in MaxQuant³¹ using a “minimal ratio count” of one. “Match between runs” was enabled using a 0.7-minute time window after retention time alignment. All other MaxQuant parameters were set as default. Only proteins with at least three of four valid values per group, and “absent” (i.e. 0 valid values) in the samples from a given group were kept for further analysis. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE³² partner repository with the dataset identifier PXD006882.

Statistical and functional annotation analysis. The experimental data are expressed as mean \pm standard error (SE). The Shapiro-Wilk test was used to determine if the data was normally distributed and, if not, data was log-transformed for analysis. A mixed between-within subjects’ analysis of variance was conducted to assess the impact of the three bed rest trials (CON, RVE and NEX) on the physiological parameters (body mass, body composition, aerobic capacity and muscle strength). There were no significant differences in the baseline values prior to each trial indicating there was no carry-over effects attributable to the cross-over design. This was verified in a separate analysis using independent samples t-tests for each of the physiological parameters. The differences in insulin sensitivity between baseline measures and the post-bed rest CON, RVE and NEX trials were determined using a 1-way ANOVA. The statistical analysis was performed in SPSS 22.0 considering a two-sided 0.05 significance level. Proteomics data was checked for normal distribution and homoscedasticity by Shapiro-Wilk and Bartlett tests, respectively ($p > 0.01$) and differential protein expression between groups was assigned using Student paired t-tests to compare HDT and BDC conditions ($p < 0.05$). All tests were performed in R (v3.0.2). Hierarchical clustering of proteomics data was performed using Cluster v3.0 software³³. Parameters were set as follows: median centering and normalization of genes for adjusting data and centroid linkage clustering for both genes and arrays. Dendrograms and heatmaps were generated and viewed using the Treeview v1.6.6 program³⁴. Enrichment and functional annotation analysis of proteomics data was performed using the desktop version of DAVID (Ease v2.0) and an updated version of Gene Ontology (GO) databases (April 2017). Enriched GO terms were filtered by only considering those with an Ease score lower than 0.1, a Benjamini p-value lower than 0.05 and a fold enrichment higher than 2. On the basis of their closeness in GO graphs and literature examination, retained enriched GO terms were finally grouped together into broad functional categories which were considered as enriched broad functions.

Results and Discussion

The present study examined the benefits of lower body RVE alone or in combination with whey protein plus alkaline salt supplementation (NEX) as potential countermeasures during periods of prolonged bed rest (21 days) in healthy young males. Physiological characteristics are outlined in Table 1. Bed rest alone (CON) resulted in reduced whole-body fat free mass, aerobic capacity, leg strength and insulin sensitivity. The RVE and NEX trials were not sufficient to prevent the reduction in maximal oxygen consumption or leg strength but did preserve insulin sensitivity. Proteomic analysis revealed perturbations to cell metabolism, mitochondrial metabolism, protein synthesis and degradation in the CON group. The RVE and NEX trials demonstrated a partial protective effect at the protein level and prevented many of the skeletal muscle proteomics changes.

Physical and physiological changes

The bed rest studies conducted by the European Space Agency are carefully controlled to investigate physiological and metabolic outcomes. Dietary intake and physical activity are controlled and standardized for 7 days prior to each period of bed rest. In addition, subjects are maintained in energy balance by adjusting dietary intake based on a combination of basal energy expenditure and body fat monitoring (Table 2 and Fig. 2). Therefore, unlike some bed rest studies, fat mass accumulation is not a confounding factor. The 2-3 kg reduction in body mass, in this study are consistent with the literature³⁵⁻³⁸, and can be attributed to a significant loss in whole-body fat free mass (Table 2 and Fig. 2). Segmental analysis of the DEXA revealed a significant reduction in arm lean mass with no change in leg lean mass in the CON group. Interestingly, there was a trend toward increased leg lean mass in the RVE group and a significant increase in the NEX group (Table 2). As five days of bed rest is known to reduce postural stability³⁹, one possibility is that loss of whole-body fat free tissue could be from postural rather than locomotor muscles. However, DEXA analysis cannot differentiate between these muscle groups and future investigation may benefit from CT scanning or MRI to address this disparity. To date, bed rest studies that have used protein supplementation as a countermeasure have had mixed results. Studies that have shown benefits in terms of nitrogen balance improvement, lean body mass maintenance and mitigation of the loss of strength gave subjects a baseline protein load similar to the RDA (0.8 g protein·kg·d⁻¹) or containing extra calories matching pre-bed rest levels of intake and studies that did not show any benefit gave subjects a baseline protein intake much higher than the RDA⁴⁰. It has been suggested that the positive effects observed may

be reflective of the increase in protein intake during bed rest compared to baseline, as the official RDA was 41% underestimated at the time of early studies. By comparison, studies that have shown no benefits gave subjects a higher baseline protein intake during baseline which may conferred a protective effect. The novelty of the present study was to give subjects the correct recommended daily protein allowance throughout baseline and bed rest. Whole body functional outcomes such as reduced aerobic capacity and leg strength (Fig. 3) were consistent with previous reports⁴¹⁻⁴³ and demonstrate the negative effects of inactivity on these physiological variables. These changes represent early and clinically relevant physiological alterations that occur with imposed inactivity, confinement to bed or space flight that may have negative biological consequences in terms of muscle insulin sensitivity. Our previous publication³⁸ demonstrated a reduction in insulin sensitivity in the CON group only, compared to the RVE group, which included eleven subjects in each group. The current analysis includes eight subjects per group from the same bed rest study, but this cohort were part of all three trials (CON, RVE and NEX). The present analysis also reveals a significant reduction in insulin sensitivity in the CON group while the countermeasures (RVE and NEX) were sufficient to partially offset the decrease in insulin sensitivity (Fig. 4a), supporting the role for moderate intensity resistance exercise in alleviating insulin resistance. It is well established that resistance exercise improves muscle protein synthesis⁴⁴, insulin sensitivity⁴⁵, mitochondrial enzymes⁴⁶ and can aid in maintaining muscle mass during bed rest⁴⁷, further highlighting the benefits of resistance exercise during periods of confinement. A 20% reduction in VO₂ peak following bed rest is a clinically relevant finding that is consistent with other studies^{13, 48} and may be linked to the reduction in mitochondrial function³⁸ and citrate synthase activity (Fig. 4b.). However, many central (cardiovascular changes) and peripheral factors (fiber type, lean mass) could also contribute to this reduction in VO₂ peak. The functional impact of prolonged inactivity is apparent with a 15% reduction in knee extension maximal voluntary contraction (Fig. 3). A loss of lower limb function precedes the loss of leg lean mass which is in keeping with other reports where loss of mitochondrial function preceded the loss in muscle mass in mice subjected to hindlimb suspension⁴⁹. In further support of our findings, Trevino et al. (2019) also demonstrated that muscle mass increased in recovery before contractile function suggesting independent mechanisms regulating muscle mass, mitochondrial function and contractile function⁴⁹. These changes represent important physiological adaptations that occur in response to bed rest. In summary, RVE and NEX can have localized effects on muscle mass that translate to whole body effects on insulin sensitivity but not maximal oxygen consumption or maximal strength. To gain a greater understanding of the

underlying mechanisms regulating these physiological processes we performed an analysis of the muscle proteome.

General Overview of Protein Changes

QC-related measurements indicated good stability of the nano LC-MS/MS system during the whole duration of analyses and good reproducibility of protein abundance determination. Indeed, the median coefficient of variation (CV) was 0.8% regarding retention times of iRT peptides when considering all injections, and it was 17% for their raw intensities. Furthermore, the median CV for all quantified proteins across repeated analysis of the reference sample after LFQ normalisation was 17%.

In total, 1500 proteins were identified, of which 1212 could be quantified across experimental groups (see Supplemental Table 1). In the CON group, 43 proteins were differentially expressed after 21-day bed rest compared to baseline, of which 10 were upregulated and 33 were downregulated (Fig. 5A). In the NEX group 31 proteins were changed of which 17 were upregulated and 14 were downregulated (Fig. 5A). The RVE group had the greatest number of protein changes (54 proteins), with 35 proteins up- and 19 proteins down-regulated (Fig. 5A).

Functional Annotation Analysis

Altered metabolic regulation underpins many of the physiological changes during bed rest. To gain a deeper understanding of the biological pathways affected by bed rest, we analyzed the muscle proteome that exhibited differential expression in all three groups (CON, RVE and NEX). Functional annotation enrichment analysis highlighted 89 gene ontology (GO) terms, of which 42 were significantly enriched in the CON group and 49 in the RVE group. The NEX groups had no GO terms that were significantly enriched, which may be due to the overall low number of altered protein changes in the NEX trial. Altogether, significantly enriched GO annotations were distributed into significantly enriched pathways and functions which incorporated protein synthesis and degradation, mitochondrial function, lipid metabolism, and the immune response (Fig. 5B). The RVE trial showed an increase in immune protein abundance (Ig gamma-1 chain C region, 2.1-fold, Ig gamma-3 chain C region, 2.5-fold, Erythrocyte membrane protein band 4.2, 3.6-fold increase). While regular exercise is known to confer substantial benefits on the immune system, acute exercise bouts during periods of bed rest may induce a significant production of antibodies⁵⁰. Recent publications have documented skeletal muscle proteomic

alterations with bed rest in humans and in space flown mice and have reported protein changes relating to energy metabolism, protein synthesis/degradation, myofibrillar protein content¹⁵ and structural and contractile alterations^{19, 51}. After one month, space flown mice exhibited notable proteomics changes in mitochondrial function, metabolic and fiber switching towards a more glycolytic phenotype together with structural protein changes¹⁷. Proteomics changes observed in the current study, particularly in the CON group, are globally similar to these findings. Overall, in-depth proteomics analysis is revealing a multifactorial remodeling of skeletal muscle metabolic proteome with bed rest and inactivity, which provides a closer insight into the molecular response to imposed physical inactivity.

Protein synthesis

Many have attributed the loss of fat free mass with physical inactivity to a reduction in protein synthesis. However, the cellular mechanisms responsible for the decline are poorly understood. It has been well established that skeletal muscle growth with increased loading is mediated by mTOR resulting in increased protein translation and ribosome biogenesis^{52, 53}. Given the established importance of mTOR signaling, it has been suggested that under conditions of disuse, reduced protein synthesis is a result of mTOR inhibition. However, it is also argued that the reduction in muscle protein synthesis can coexist with normal Akt/mTOR signaling^{54, 55}. Furthermore, 2 weeks of denervation caused a dramatic reduction in ribosomal RNA level coupled with an increase in mTOR signaling⁵⁶, suggesting ribosome biogenesis is controlled by factors other than the mTOR pathway in muscle atrophy. Ribosomal biogenesis and efficiency are important components in protein synthesis. Under conditions of bed rest (21 days), we report a significant reduction in ribosomal subunit proteins (40S ribosomal protein S20 and S25 [RPS20 and RPS25], 60S ribosomal protein L7 and L18 [RPL7 and RPL18], 60S acidic ribosomal protein P0 [RPLP0]) in the CON group (Fig. 5C), which may be a result of reduced de novo ribosome biogenesis⁵⁶. Using denervation, hind limb suspension or immobilization, a loss of ribosomal RNA (rRNA) was observed⁵⁷. rRNA is an important component in mRNA translation and catalyzes the attachment of each new amino acid to the growing chain. Additionally, aged mice versus young mice displayed a failure to upregulate rRNA expression in muscle undergoing hypertrophy suggesting that impaired ribosome biogenesis is an important factor in the blunted hypertrophic response in aging⁵⁸. Together, these findings and ours confirm the importance of ribosomes in maintaining skeletal muscle mass. A similar profile was revealed in the RVE group but with a

reduction in the abundance of a lower number of proteins related to ribosomal biogenesis (60S ribosomal protein L18, ribosomal protein S6 kinase alpha-3, 60S ribosomal protein L23 and 40S ribosomal protein S7 [RPL18, RPS6KA3, RPL23, and RPS7], Fig. 5C). In the NEX group, the abundance of only one ribosomal subunit protein (RPL18) remained undetected in the bed rest condition (Fig. 5C). Hence, proteomic data suggest that resistive vibration exercise may improve protein synthesis in the bed rest condition, obviously in a more marked manner when combined with whey protein and alkaline salt supplementation.

Histones play a central role in transcription regulation and DNA repair and were shown to be reduced in the CON group only (histone H2A type 2-C and histone H2A type 1-J [HIST2H2AC and HIST1H2AJ], Fig. 5C). These basic nuclear proteins are essential for nucleosome structure of the chromosomal fiber and the reported reduction maybe linked to potential DNA damage ⁵⁹. Conversely, upregulation of T-complex protein 1 subunit alpha (TCP1), a molecular chaperone involved in protein folding, in response to RVE (Fig. 5C), may attempt to increase leg lean mass. The fact that TCP1 levels were not increased in the NEX condition may reflect that whey protein supplementation already provides protection with regard to protein synthesis and the increase in TCP1 may not be required here. Additionally, the reduction in leucine-rich PPR motif (LRPPRC) and endonuclease G (ENDOG) in the mitochondria could reduce the translation of mitochondrial proteins due to bed rest in the CON group (Fig. 5C). Elongation translation is another key component in protein synthesis, and it is mediated by elongation factor 1 (EF1), which transfers aminoacyl-tRNA to 80S ribosomes fueled by the hydrolysis of GTP. A reduction in elongation factor 1 delta (EEF1D, Fig. 5C), a subunit of EF1, together with elongation factor Tu and Ts (TUFM and TSFM) in the mitochondria may lead to reduced mRNA translation in the CON group. Such regulation could further contribute to the overall reduction in protein synthesis with bed rest.

Protein degradation

Evidence is limited relating to the role of protein degradation during muscle atrophy in humans partially due to difficulty measuring *in vivo* protein degradation ⁶⁰. One study reported an increase in vastus lateralis muscle proteolysis after 72 hours unilateral lower limb suspension, measured by microdialysis ⁶¹. However, many studies infer an increase in muscle protein degradation by reporting an increase in genes associated with degradation pathways ⁶⁰. We report an upregulation of calpain-1 catalytic subunit (CAPN1, RVE) and calpain-2 catalytic subunit (CAPN2, NEX) (Fig. 5D), which are supported by a previous report ⁶². However, calpain 3, the most abundant skeletal muscle calpain, is unchanged in all three groups. No changes in dominant muscle atrophy

degradation proteins could suggest that the overall loss of whole-body fat free mass may be due to reduced protein synthesis. Another possible reason for minimal changes in the protein degradation proteome may be that while whole body fat free mass was reduced, leg lean mass was preserved in the countermeasure trials. Importantly, we report an upregulation in other proteins involved in the protein degradation pathway in the RVE group (proteasome activator complex subunit 2, cullin associated Nedd8-dissociated protein 1, serum amyloid P component [PSME2, CAND1, APCS], Fig. 5D). NEDD8 is a ubiquitin like protein covalently linked to a cullin, an E3 ubiquitin ligase, through neddylation. The conjugation of NEDD8 to CUL1 is necessary to mediate ubiquitination of specific protein substrates⁶³. Serum amyloid P component, significantly increased here in the RVE group, has also been implicated in skeletal muscle atrophy through its interaction with inflammatory cytokine, IL-6⁶⁴. Few proteins related to protein degradation also have increased expression in the NEX group (Fig. 5D). 26S protease regulatory subunit 8 and 26S proteasome non-ATPase regulatory subunit 13 (PSMC5 and PSMD13) are involved in ATP-dependent degradation of ubiquitinated proteins. Taken together, these data reveal an imbalance between muscle protein synthesis and degradation with bed rest, which ultimately results in loss of whole-body fat free mass while leg lean mass is preserved in the RVE and NEX trials. Altered protein turnover may be linked to changes in autophagic flux.

Autophagy is a cellular degradative pathway that involves the delivery of cytoplasmic cargo to the lysosome. All three forms (macroautophagy, microautophagy and chaperone-mediated autophagy) converge on the lysosome, however they differ in their physiological functions and mode of delivery to the lysosome⁶⁵. Lysosomal hydrolytic enzymes create an acidic internal environment that can readily degrade the ubiquitinated cargo selected for degradation by autophagy receptors such as p62 (also named SQSTM1). The inhibition of autophagic flux and subsequent accumulation of autophagosomes within myofibres is a hallmark of skeletal muscle diseases such as Pompe's disease, characterized by the deacidification of the lysosomal compartment causing muscle atrophy⁶⁶. While the present model of muscle atrophy is not as extreme as certain disease states, a change in autophagy proteins in the CON group (increase in p62 and target of Myb protein 1 [SQSTM1 and TOM1], Fig. 5D) and the NEX group (increase in p62 and reduction in cathepsin G [SQSTM1 and CTSG], Fig. 5D) suggests altered autophagic regulation with physical inactivity. TOM1 plays an important role as a myosin VI cargo adapter and has been suggested to be an important component in autophagosome maturation and fusion with the lysosome⁶⁷. While an increase in autophagy markers may be interpreted as an increase in autophagy, it is also possible that

the flux is inhibited due to the accumulation of these proteins. Interestingly, no change in markers of autophagy were noted in the RVE group despite many reports suggesting that resistance exercise stimulates autophagy. It is well established that autophagy participates in the removal of damaged mitochondria in skeletal muscle subject to stress⁶⁸, thereby linking autophagy machinery with mitochondrial metabolism.

Mitochondria

There was a reduction in mitochondrial proteins in the CON group linked to fatty acid β -oxidation and ketone metabolism. Specifically, reduced expression of the mitochondrial carnitine/acylcarnitine carrier protein (SLC25A20, Fig. 5E) indicates a reduction in mitochondrial fatty acid uptake. The expression of proteins involved β -oxidation (Acetyl-CoA acetyltransferase, enoyl-CoA delta isomerase 1 and 2, delta(3,5)-delta (2,4)-dienoyl-CoA isomerase, D-beta hydroxybutyrate dehydrogenase, hydroxyacyl-coenzyme A dehydrogenase and 3-ketoacyl-CoA thiolase [ACAT1, ECI1 and ECI2, ECH1, BDH1, HADH, and ACAA2],) were also reduced (Fig. 5E). Isocitrate dehydrogenase (NAD) subunit gamma and succinyl-CoA ligase subunit alpha, both involved in Krebs cycle reactions were also reduced (IDH3G and SUCLG1, Fig. 5E), which may result in the diminished supply of NADH for the electron transport chain which could support our previous finding of reduced mitochondrial respiration in permeabilized muscle fibers¹⁰. A reduction in respiration could also be accounted for by a decrease in mitochondrial content. We report a decrease in citrate synthase activity (Fig. 4b), a common marker of mitochondrial content. Proteomics analysis also revealed a reduction in PGC-1 and ERR-induced regulator in muscle protein 1 protein abundance (PERM1, Fig. 5E). which is required for a subset of genes important for oxidative capacity and mitochondrial biogenesis⁶⁹. Moreover, PGC-1 and ERR-induced regulator in muscle protein 1 is known to regulate the expression of PGC1- α and β , transcription factor co-activators regulating mitochondrial biogenesis. Altered expression of PGC-1 α could be a major determinant of metabolic derangement and has been reported in other bed rest studies¹⁵. The combination of decreased mitochondrial biogenesis, Krebs Cycle reactions and fatty acid oxidation could therefore alter substrate regulation and ATP production for normal cell function after 21 days of bed rest.

While there was an increase in the abundance of NADH dehydrogenase [ubiquinone] 1 beta subcomplex subunit 3 (NDUFB3), an NADH dehydrogenase (complex I) accessory subunit, many other subunits remain unchanged in the NEX group. This group also showed an increase in ETC complex I subunit NADH dehydrogenase iron sulfur protein 4 (NDUFS4), which is known to facilitate oxidation-reduction reactions. MICOS complex subunit

MIC10 (MINOS1) is in the inner mitochondrial membrane (IMM) and is increased with RVE (Fig.5E), providing evidence of exercise mediated cristae junction maintenance. These changes may indicate an attempt to increase mitochondrial function in response to the countermeasures. However, the abundance of so few mitochondrial proteins changed that we cannot make any definite conclusions.

While some proteins increased during the RVE trial, the expression of other mitochondrial proteins decreased. Mycophenolic acid acyl-glucuronide esterase (ABHD10) which plays a role in attenuating inflammation ⁷⁰, as well as elongation factor Ts (TSFM) were reduced in the RVE and CON groups (Fig. 5E) suggesting a reduction in mitochondrial translation, supporting a decrease in mitochondrial content during bed rest ¹⁰. Histidine triad nucleotide-binding protein 2 (HINT2) on the inner mitochondrial membrane, facing the matrix, is also reduced in the RVE and NEX groups (Fig. 5E), suggesting a reduction in mitochondrial cholesterol uptake and thereby steroidogenesis. Enoyl-CoA hydratase domain-containing protein 3 (ECHDC3) was also decreased, perhaps inhibiting fatty acid degradation through beta-oxidation which supports previous findings ⁷¹. The NEX group also shows an increase in mitochondrial proteins involved in fatty acid oxidation. NADP-dependent malic enzyme (ME1) was increased which catalyzes the reversible oxidative decarboxylation of malate to pyruvate, thereby linking the glycolytic and citric acid cycle. Overall, the CON group displays changes that not only suggest a shift from oxidative towards glycolytic metabolism but a general impairment of energy metabolism. These findings are supported by previous bed rest studies ^{15, 72} and mitochondrial dysfunction may be a function of changes occurring elsewhere in the cell.

Cell Metabolism

Alterations to the mitochondrial proteome may be as a result of impaired cell metabolism. Fiber type alterations towards more glycolytic phenotype and impaired lipid trafficking may be as a result of a change in protein expression relating to glucose and FA metabolism. Specifically, AMP deaminase 1 (AMPD1) was increased in the CON group (Fig. 5F). AMP deaminase limits AMP accumulation by degrading AMP to IMP. Inhibition of AMP deaminase by metformin has been shown to increase AMP and activate AMPK ⁷³. It is possible therefore, that an increase in AMPD could inhibit fat oxidation and mitochondrial biogenesis due to its inhibiting effect on AMPK. As a metabolic sensor, AMPK has been described to directly increase the intrinsic activity of PGC1- α through phosphorylation ⁷⁴. Amylo alpha 1-6 glucosidase (AGL) is also upregulated in the CON group (Fig. 5F), and it is known to increase glycogen degradation, further supporting the changes in substrate use from fatty acids

to glucose, a metabolic shift that has been reported with bed rest ¹². Emerging data implicate Acyl-protein thioesterases in the pathogenesis of metabolic disease ⁷⁵. Acyl-protein thioesterase 2 (LYPLA2) is localized in the mitochondrial matrix and hydrolyses long-chain fatty acyl-CoAs into free fatty acids. This enzyme is increased during bed rest in the CON group (Fig. 5F) and is poised to manipulate FA oxidation; however, its specific action remains elusive ⁷⁶. A reduction in nicotinamide phosphoribosyltransferase (NAMPT) expression was also evident in the CON group (Fig. 5F). Through its NAD biosynthetic activity, NAMPT influences the activity of NAD-dependent enzymes involved in redox reactions ⁷⁷ and regulates the nicotinamide phosphoribosyltransferase (NAD) pool. Maintenance of NAD⁺ is paramount to support metabolism and cell survival and a reduction in NAMPT-mediated NAD⁺ biosynthesis could elicit poly ADP ribose polymerase (PARP) which is a major consumer of NAD⁺ in the nucleus and result in cellular functional decline ⁷⁸. A reduction in NAD biosynthetic pathway may be linked to reduced mitochondrial respiration and CS activity.

Interestingly, the protein changes in the RVE group relating to metabolism are primarily linked to glucose metabolism. An increase in solute carrier family 2 facilitated glucose transporter member 1 (SLC2A1, Fig. 5F), an isoform which is responsible for basal glucose uptake and 6-phosphogluconate dehydrogenase (PGD, Fig.5F), which is known to function in the pentose phosphate pathway to generate NADPH, together could suggest that there is an increase in glucose oxidation with RVE, perhaps at the expense of fatty acid oxidation. The RVE group also displayed a reduced expression in protein phosphatase 1 regulatory subunit 3A (PPP1R3A) and mannose-6-phosphate isomerase (MPI, Fig. 5F), both of which are involved in glycogen synthesis and glycolysis. Isocitrate dehydrogenase (IDH1, Fig. 5F) was increased in NEX which could suggest an attempt to increase Krebs cycle intermediates in the NEX group, however, previous reports show reduced TCA cycle enzymes in simulated weightlessness in men ⁷⁹. The NEX group also has reduced expression in fatty acid-binding protein (FABP5), a fatty acid chaperone, which was also down regulated following murine hind limb suspension ²⁰. Taken together, the changes in proteins related to mitochondrial function and cell metabolism indicate the development of overall derangement of energy metabolism in response to reduced energy expenditure.

Conclusion

Due to the logistics of running bed rest studies and the ability to include only a small sample size, a limitation of the present study is that it is a male only study. It is possible that the physiological and proteomic response may be different in females and further investigation will be required to address this. Bed rest triggers numerous

physiological and proteomic alterations spanning a range of biological functions that are partially mitigated with the addition of exercise and protein supplementation. While the countermeasures had different proteomic profiles, NEX appears to confer the greatest protection based on the increased leg lean mass and fewer pathway related changes. RVE and NEX mitigated the reduction in insulin sensitivity but had similar decreases in whole body lean mass. However, when we conducted a segmental analysis of body composition, we found that leg lean mass increased in the NEX group only. As the RVE trial involved lower limb exercise, these results suggest that the addition of whey protein, with an alkalizing agent, can mitigate the local effects of bed rest. These results are supported by the proteomic data where there were no significant pathway changes following the NEX trial. Hence, physical and physiological effects at the organism level as well as the effects on the vastus lateralis muscle proteome following bed rest (CON) were generally mitigated by countermeasures, however, in a more marked manner due to NEX than RVE (Fig. 6). Therefore, future studies may need to consider whole body exercise prescription and not just lower leg RVE. If RVE was applied to other muscle groups, and combined with the whey/alkalizing nutritional supplementation, we may also see a positive outcome on whole body lean mass. However, based on these findings and conflicting evidence from previous bed rest studies, the potential benefits of protein supplementation during bed rest requires further investigation. It is possible that higher intensity exercise and optimal protein doses will need to be established for future bed rest investigations.

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Duality of interest

The authors declare that there is no duality of interest associated with this manuscript.

Contribution statement

HK and DO'G had full access to all the data in the study and take full responsibility for the integrity of the data and the accuracy of the data analysis. FB, GT and AZ completed the proteomics analysis. The study was designed by HK, FR, AZ, IC, M-PB, DO'G, SB and MH. HK, FR, AZ, IC, M-PB, DO'G and SB performed all physiological measurements. The data was acquired by HK, FR, AZ, IC, M-PB, SB and DO'G. The data was interpreted and discussed by all authors. HK, DO'G and FB drafted the manuscript and all authors were involved in revising the article for important intellectual content. Approval of final manuscript was given by all authors.

Data Availability Statement

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Figure Legends

Figure 1. Schematic representation of the study design showing the timeline for subject recruitment, group assignment, recovery and wash-out period. Control (CON), resistive vibration exercise (RVE), nutrition (whey protein and alkalizing salt supplementation) and exercise (NEX).

Figure 2. Changes in body weight and body composition measured by DEXA on Day 1, 10 and 20 of bed rest for 8 subjects during three trials. Energy intake was designed to match energy expenditure in order to maintain whole body fat mass. Body composition measurements include body mass (a), fat-free mass (kg) (b), fat mass (kg) (c) and percent fat mass (%) (d) during the three bed-rest trials with and without countermeasures; CON (grey circle), resistive vibration exercise (RVE) (black square) and nutrition (whey protein supplementation) and exercise (NEX) (orange triangle) trials. Data are presented as mean \pm SE. * $p < 0.05$ vs Day 1, # NEX vs Day 1, ^ RVE vs Day 1 and + CON vs Day 1, $n = 8/\text{group}$

Figure 3. Physiological changes that occur with bed rest. Maximal oxygen consumption was measured by VO_2 peak after a maximal bike exercise test before (pre) and after (post) 21 days of bed-rest with or without the countermeasures (RVE and NEX). VO_2 peak is presented relative to fat-free mass ($\text{ml kgFFM}^{-1} \text{min}^{-1}$) (a) and in absolute values (ml/min) (b). Peak heart rate (HR) (c) and peak power (watts) are also presented. Isokinetic maximal voluntary contraction (MVC) of four muscle groups (knee extensors and flexors, plantar flexors and dorsi flexors) were measured with the subject in a sitting position for the knee exercise (e-f) and prone for the ankle exercise (g-h) before and after each bed-rest trial, CON (black bars), RVE (white bars) and NEX (grey bars). Data are presented as mean \pm SE. * $p < 0.05$ main effect between pre versus post, ++ significant difference between two groups, $n = 8/\text{group}$.

Figure 4. The hyperinsulinemic, euglycemic clamp was done at baseline of the first bed-rest campaign and then after each of the three trials. Each insulin clamp lasted eight hours and blood glucose was measured every 10 minutes. Insulin sensitivity, as measured by glucose infusion rate was determined at baseline (grey bar), post-

CON (white bar), post RVE (brown bar) and post NEX (dark-grey bar). The glucose infusion rates are expressed relative to fat-free mass (kgFFM) and the circulating insulin concentration (pmol/l). Citrate synthase activity, a mitochondrial Krebs Cycle enzyme that provides an indication of mitochondrial enzyme activity was measured in skeletal muscle biopsy samples taken from the *vastus lateralis* before and after each bed-rest trial. Citrate synthase activity (b) was measured using the Sigma kit before and after bed rest. Data are presented as mean \pm SE. * $p < 0.05$ main effect between pre versus post, # pre vs CON post, $n = 8$ /group.

Figure 5. Overview of muscle proteomic response in the three bed rest trials (CON, RVE and NEX).

Changes in the proteome of vastus lateralis muscle due to 21 days of bed rest ($N = 4$ /group) are shown as heatmaps of differentially expressed proteins that were produced by hierarchical clustering (panel A). Signal values between groups were successfully discriminated (green, black and red boxes represent downregulated, intermediate and upregulated proteins, respectively). When considering differential proteins, functional annotation analysis highlighted significantly enriched Gene Ontology terms, which allowed determination of broad functions significantly affected by bed rest (panel B; between brackets are given first the number of enriched GO terms then of differential proteins). Select significant changes in protein abundances are shown as the HDT/BDC ratio calculated from the median abundance of proteins in each group for protein synthesis (panel C), protein degradation (panel D), mitochondrial function (panel E), and cell metabolism (panel F). Detailed protein abundances and fold changes are given in Supplemental Table 1.

Figure 6. Proposed mechanisms linking proteomics, physical and physiological changes observed during the three bed rest trials (CON, RVE and NEX).

Down- and up-regulations induced by bed rest in CON are indicated by green and red arrows, respectively. Similar effects due to RVE and NEX are indicated in black bold, whereas the specific effects observed only with RVE or only with NEX, or the higher effects of one countermeasure over the other are shown in blue.

Table 1: Baseline characteristics of participants

Subject	Age	Height (M)	Weight (Kg)	BMI (kg/M²)	VO2 max (ml/min/Kg)
1	44	1.75	76.3	24.91	33
2	40	1.69	61.1	21.39	38
3	42	1.77	78.5	25.06	37
4	36	1.9	80.7	22.35	48
5	41	1.72	59.0	19.94	46
6	40	1.77	71.2	22.73	34
7	25	1.74	71.8	23.72	39
8	29	1.84	81.8	24.16	40
Mean±SE	35.18	1.76	70.30	22.57	40.55
SD	8.1	0.1	8.2	1.7	4.9
SE	2.43	0.02	2.47	0.516	1.48

Table 2: Body Composition Alterations

	CON		RVE		NEX	
	<i>Pre</i>	<i>Post</i>	<i>Pre</i>	<i>Post</i>	<i>Pre</i>	<i>Post</i>
Weight (kg)	73.4 ± 3	70.5 ± 3.1*	73.1 ± 2.9	70.3 ± 2.7 *	71.8 ± 3.3	70.07 ± 3 *
#WB fat (kg)	15.6 ± 1.7	15.4 ± 1.8	14.7 ± 1.4	14.9 ± 1.4	14.4 ± 1.6	14.6 ± 1.5
#WB lean (kg)	57.8 ± 2.1	55.1 ± 2.3 *	58.5 ± 2.5	55.4 ± 2.2 *	57.4 ± 2.7	55.5 ± 2.5 *
Arm lean (kg)	7.7 ± 0.4	7.2 ± 0.5 *	7.5 ± 0.5	7.3 ± 0.5 *	7.3 ± 0.5	7.2 ± 0.5
Leg lean (kg)	17.4 ± 0.4	17.5 ± 0.5	17.6 ± 0.7	18.3 ± 0.6 (p=0.06)	16.5 ± 0.5	19.1 ± 0.3 *

Data are presented as mean ± SE, *p<0.05 post versus pre, #Whole Body (WB)

Figure 1

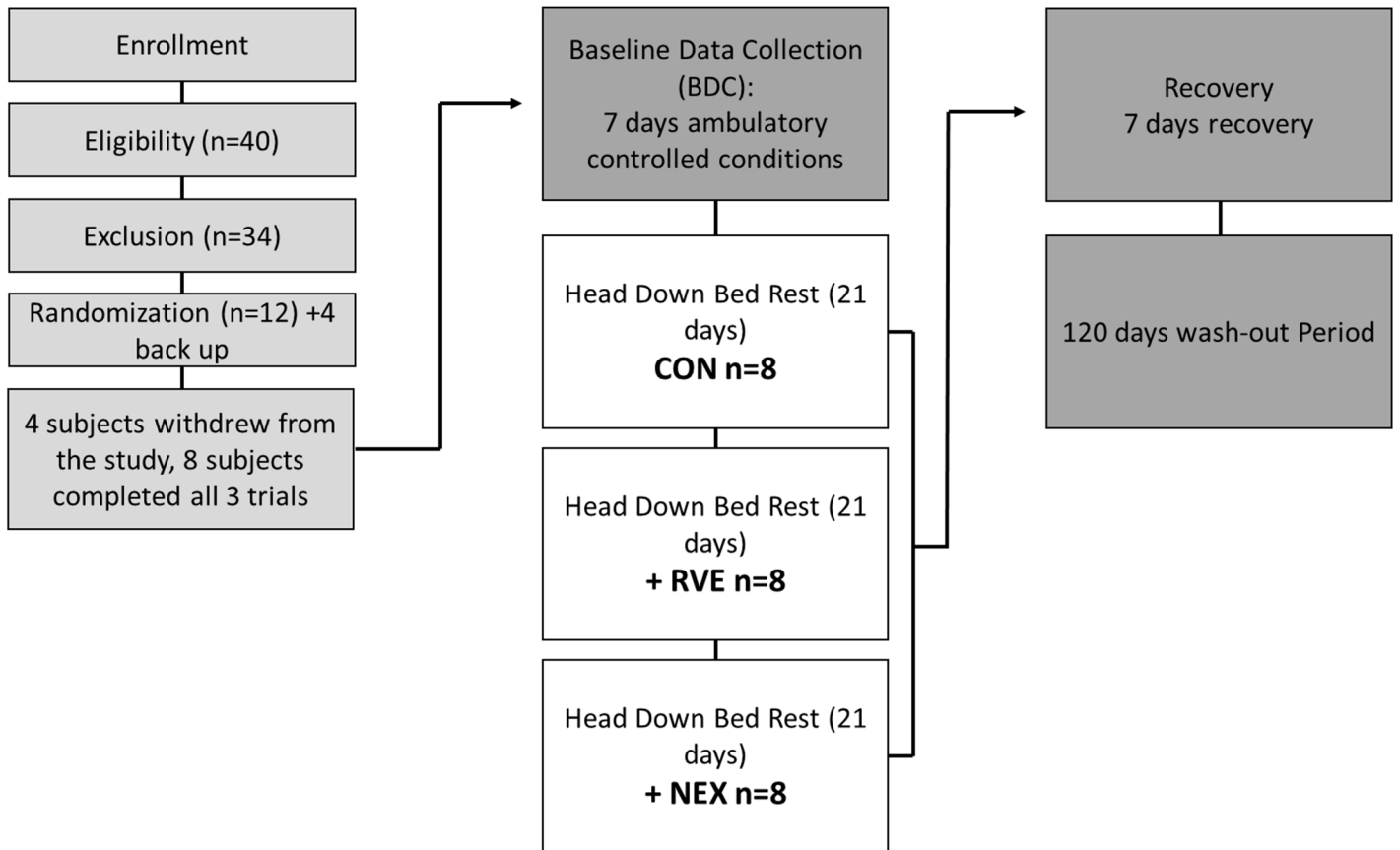


Figure 2

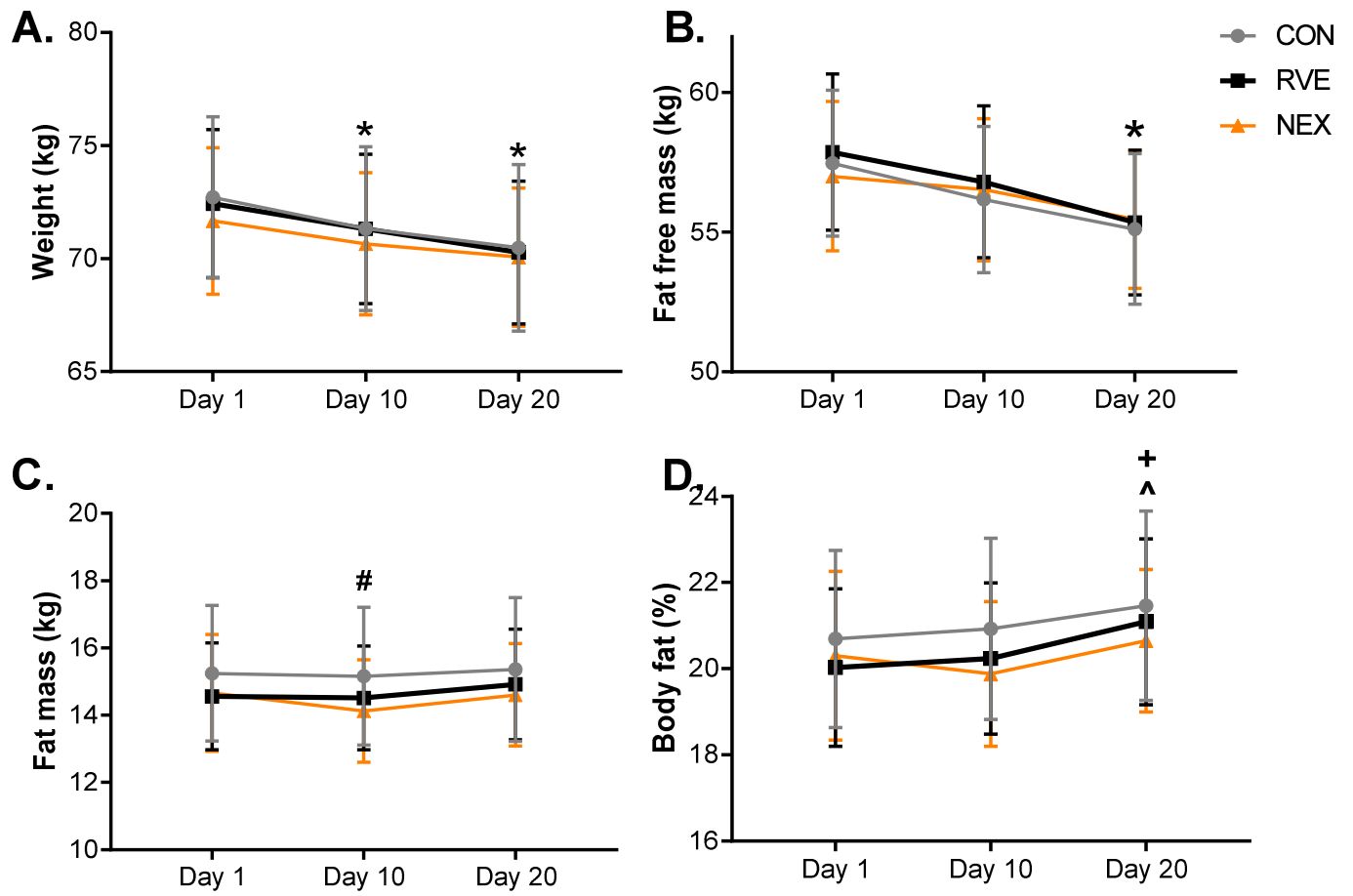


Figure 3

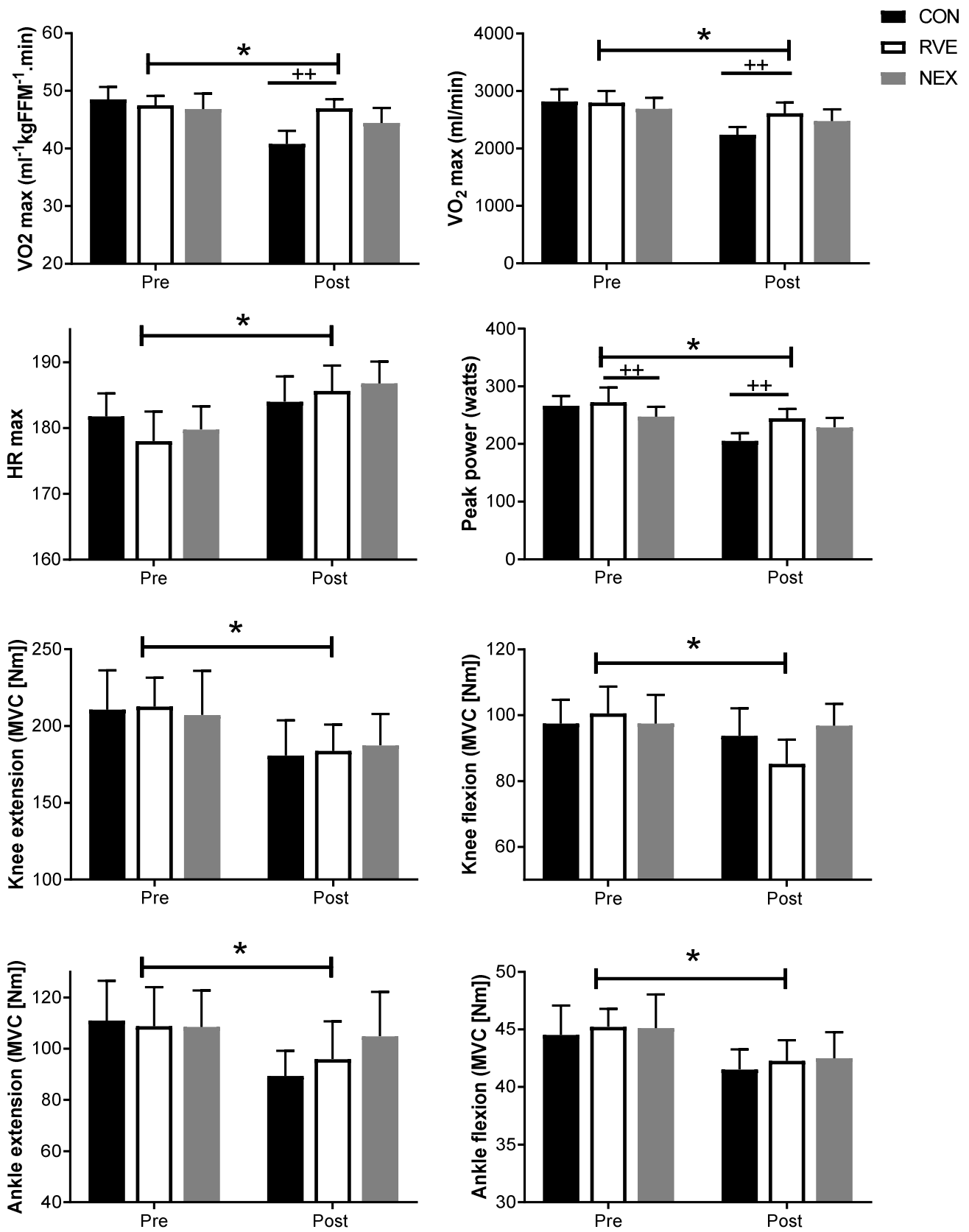


Figure 4

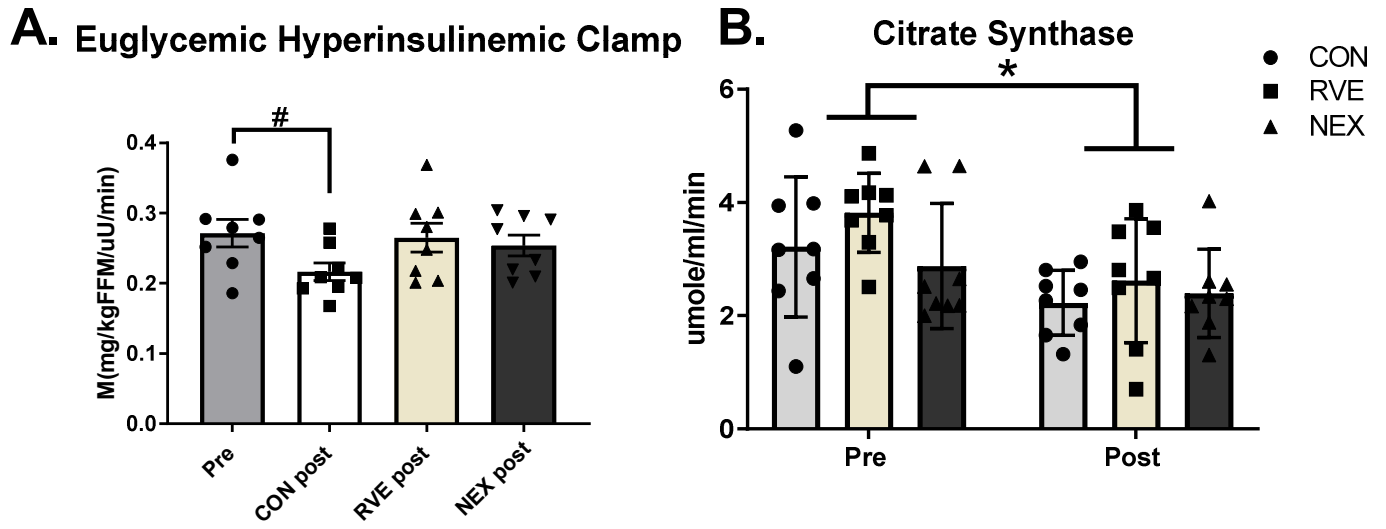
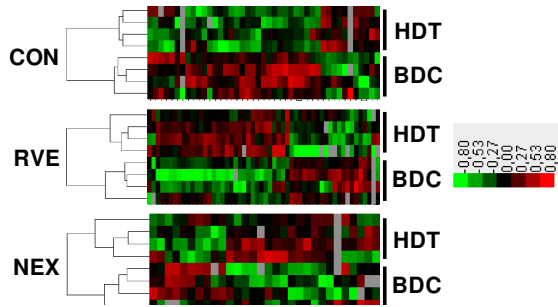
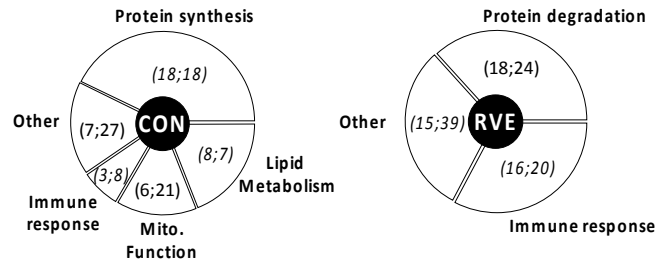


Figure 5.

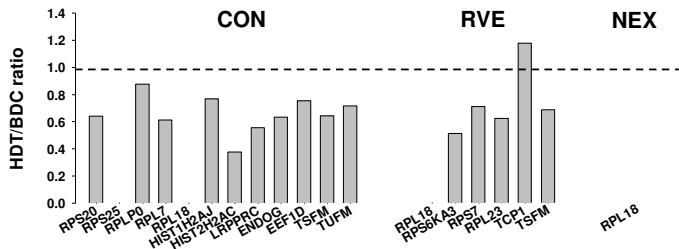
A. Muscle differential proteome



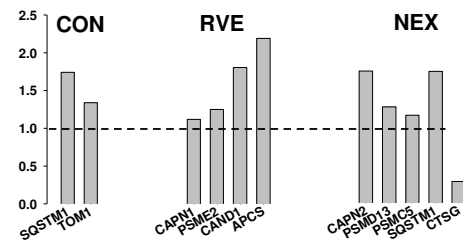
B. Functional annotations



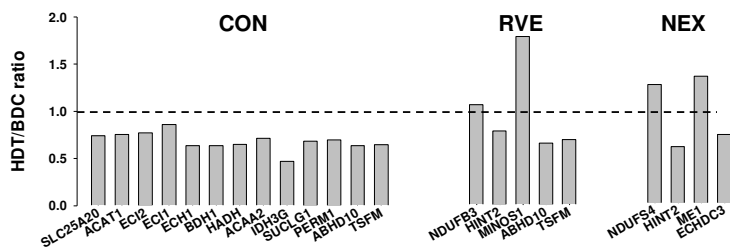
C. Protein synthesis



Protein degradation



E. Mitochondria



F. Cell metabolism

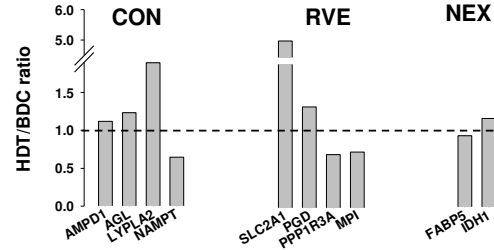


Figure 6

