ABSTRACT: Almost no data exist regarding skeletal muscle responses to real or simulated spaceflight in women. We determined the impact of 60-day bedrest (BR, n = 8), 60-day bedrest with exercise-training (BRE, n = 8), and 60-day bedrest with a leucine-enriched, high-protein diet (BRN, n = 8) on muscle protein composition. Vastus lateralis and soleus muscle biopsies were analyzed for global protein fractions (mixed, sarcoplasmic, myofibrillar) and force-specific proteins (myosin, actin, collagen). Concentrations (micrograms per milligram muscle wet weight) of these proteins were maintained (P > 0.05) in BR, despite large changes in quadriceps (∼21%) and triceps surae (∼29%) volume. Neither countermeasure influenced muscle protein content in either muscle (P > 0.05), despite exacerbation (BRN) or prevention (BRE) of atrophy. Pre-bedrest comparisons showed less myofibrillar protein in the soleus (∼16%, P < 0.05), primarily due to less myosin (∼12%, P < 0.05) and more collagen (234%, P < 0.05) than the vastus lateralis. Muscle protein composition is tightly regulated in lower limb muscles of women, despite the most extreme weightlessness-induced atrophy reported in humans. In contrast, men who underwent prolonged unloading were unable to proportionally regulate atrophy of the soleus. These findings have implications for astronauts and clinical conditions of sarcopenia regarding the maintenance of muscle function and prevention of frailty.

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MUSCLE PROTEINS DURING 60-DAY BEDREST IN WOMEN: IMPACT OF EXERCISE OR NUTRITION

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Women comprise approximately 20% of the active astronaut corps at the U.S. National Aeronautical and Space Administration (NASA). Several women have been crew members on short-duration (∼2 weeks or less) Space Shuttle flights, while a few have taken part in long-duration (several months) stays on the MIR and International Space Stations. However, almost no data from flight studies have been published regarding the skeletal muscle responses to microgravity in women, and few ground-based spaceflight simulation studies have included women. Severe muscle atrophy and strength loss occurs after exposure to real or simulated spaceflight in the absence of appropriate countermeasures. Although much attention has been given recently to muscle mass loss with spaceflight, it is important to know if the composition (i.e., “quality”) of the muscle mass is altered. Whole muscle mass (i.e., muscle volume or cross-sectional area) is monitored before and after, and in the future possibly during, spaceflight by methods that do not provide information regarding muscle quality that may influence the muscle functional capacity of astronauts. This information is especially critical when testing and finalizing countermeasures to preserve muscle mass and function during long-duration spaceflights.

Among the most important functional elements of skeletal muscle are the contractile and connective tissue proteins. Short-term bedrest and spaceflight studies in humans and animal studies using real and simulated spaceflight models of unloading have reported disproportionate alterations of global (myofibrillar) and specific (myosin and actin) force-producing proteins in the muscle. Changes in contractile protein interactions have been shown to al-

Muscle Proteins and Bedrest in Women

Abbreviations: ANOVA, analysis of variance; BR, bedrest only; BRE: bedrest with concurrent aerobic and resistance exercise training; BRN, bedrest with a nutritional intervention consisting of a leucine-enriched, high-protein diet; CP, calf press, resistance exercise; FMOC, 9-fluorenylmethylchloroformate; HYP, hydroxyproline; LBNP, lower body negative pressure; MHC, myosin heavy chain; MEDES, Institute for Space Physiology and Medicine (Toulouse, France); NASA, National Aeronautics and Space Administration; OPA, o-pthalaldehyde; SS, supine squat, resistance exercise; VL, vastus lateralis muscle

Key words: actin; collagen; gender; myosin; unloading

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ter the ability of the muscle to generate force and power\textsuperscript{9,18,40} and place additional stress on the remaining myofibrils.\textsuperscript{27} In addition, an accumulation of the force-transmitting connective tissue (collagen) network occurs in muscle following unloading in animals.\textsuperscript{26} Changes in the intramuscular collagen protein fraction have been shown to significantly impact mechanical properties of skeletal muscle in non-unloading conditions.\textsuperscript{14}

The experimental findings presented herein are a result of a multinational, multi-investigator, 60-day microgravity simulation study using the 6° head-down-tilt bedrest model. The study followed three groups of women during 60 days of bedrest. One group served as a control and completed bedrest only; one group completed a combined aerobic and resistance exercise (i.e., concurrent training) countermeasure regimen during bedrest; and a final group consumed a nutrition countermeasure consisting of a high-protein, high-leucine diet during bedrest.

We have previously reported the results for the muscle volume and muscle strength responses of these women separately.\textsuperscript{33} These results show the women lost a significant amount of quadriceps (~21%) and triceps surae (~29%) muscle volume, which was completely (quadriceps) or partially (triceps surae, ~8%) attenuated with concurrent exercise countermeasures. To our knowledge, these women demonstrate the most extreme weightlessness-induced atrophy reported among men or women, considering the duration of unloading and the amount of muscle loss. Therefore, the primary purpose of this investigation was to determine the response of global (mixed, sarcoplasmic, and myofibrillar) as well as specific force-generating (myosin and actin) and force-transfer (collagen) protein concentrations. We hypothesized that women would be susceptible to disproportionate alterations of these proteins in the vastus lateralis and soleus muscles. These disproportionate changes in protein composition would in turn contribute to muscle functional deficits. A secondary aim was to compare baseline levels of the measured proteins in the vastus lateralis and soleus of all 24 women to gain insight into the differential responses of these two muscles to unloading.

**METHODS**

**Overall Experimental Design and Subjects.** The current investigation was one component of a multi-investigator study designed to elucidate the effects of simulated spaceflight on women. Our specific objective was to examine the impact of simulated microgravity on muscle protein composition in women and to assess the utility of exercise or nutritional interventions to counteract potential alterations in muscle protein composition. As the overall design has been presented in detail elsewhere,\textsuperscript{35} only a brief explanation is provided herein.

Twenty-four women (Table 1) were recruited from the European Union to participate in 60 days of 6° head-down-tilt bedrest at the Institute for Space Physiology and Medicine (MEDES) bedrest facility, Toulouse, France. Subjects were included if they were judged to be healthy by the MEDES medical staff following a medical and psychological screening exam. For the duration of the study, all subjects remained in bedrest and were continuously monitored. All of the procedures, risks and benefits associated with the experimental testing were explained to the subjects, who gave written informed consent prior to study participation. This study was approved by the human use committees of all the participating institutions in France and the USA (Johnson Space Center and Ball State University).

Subjects were assigned to one of three groups: bedrest only (BR); bedrest with concurrent aerobic and resistance exercise (BRE); or bedrest with a nutritional intervention consisting of a leucine-enriched, high-protein diet (BRN). Biopsies of the vastus lateralis and soleus muscles were taken before and on day 59 of bedrest to assess global and specific functional and structural muscle proteins. Methods and results specific to whole muscle volume and function\textsuperscript{35} and single muscle fiber function\textsuperscript{34,35} have been presented elsewhere.

**Exercise Countermeasure.** The BRE group completed both resistance (RE) and aerobic (AE) exercise during the bedrest period. The exercise protocols are presented briefly herein and in detail elsewhere.\textsuperscript{35}

**Resistance Training.** The BRE group trained the thigh and calf muscle groups using supine squat (SS) and calf-press (CP) exercises in the 6° head-down-tilt position on a gravity-independent ergometer.\textsuperscript{1,32} A

### Table 1. Subject characteristics.

<table>
<thead>
<tr>
<th>Group</th>
<th>Age (y)</th>
<th>Height (cm)</th>
<th>Weight (kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BR</td>
<td>8</td>
<td>34 ± 1</td>
<td>163 ± 2</td>
</tr>
<tr>
<td>BRE</td>
<td>8</td>
<td>33 ± 1</td>
<td>165 ± 3</td>
</tr>
<tr>
<td>BRN</td>
<td>8</td>
<td>29 ± 1</td>
<td>170 ± 2</td>
</tr>
</tbody>
</table>

BR, bedrest group; BRE, bedrest with resistance and aerobic exercise group; BRN, bedrest with leucine-enriched, high-protein-diet group.
total of 19 sessions were scheduled for each subject approximately every third day (2–3 days/week), beginning with the second day of bedrest. RE was preceded by 10 minutes of light cycling and submaximal warm-up repetitions. SS exercise involved 4 sets of 7 maximal concentric and eccentric contractions, whereas CP consisted of 4 sets of 14 maximal concentric and eccentric repetitions. Two minutes of rest was provided between sets. This protocol was utilized in order replicate the resistance training program previously used with a 90-day bedrest study in men.1,36

**Aerobic Training.** Aerobic exercise was conducted on a vertically oriented treadmill in a lower-body negative-pressure chamber, which has been previously shown to maintain upright exercise capacity during bedrest.38 Subjects performed AE 2–4 days per week for a total of 29 sessions beginning on bedrest day 1. Each session consisted of 40 minutes of exercise at 40–80% of pre-bedrest V\textsubscript{O\textsubscript{2}} peak followed by 10 minutes of resting LBNP (lower body negative pressure). LBNP produced a foot-ward force of 1.0 BW depending upon subject tolerance.

**Nutritional Countermeasure.** All meals were prepared for each group by the MEDES dietary staff, with controlled amounts of total energy and macronutrients (carbohydrate, fat, and protein), as well as sodium, potassium, calcium, and fluid intake as previously described.39 The specific goal of the nutritional countermeasure was to provide an additional amount of protein and free leucine during the bedrest period for the BRN group. All three groups received similar diets during the 20-day pre-bedrest period, while the BRN group received an additional amount of energy intake equal to the energy expended during the exercise training countermeasure.

**Muscle Biopsy.** Muscle biopsies (≈100–200 mg) were obtained from the vastus lateralis and soleus using the needle biopsy technique before (bedrest minus 8 days) and at the end (day 59) of the simulated microgravity period. Following each biopsy, a portion of the muscle was immediately frozen and stored in liquid nitrogen (−190°C) for analysis of global (mixed, sarcoplasmic, and myofibrillar) and specific functional (myosin and actin) and structural (collagen) muscle protein concentrations. The mixed muscle protein fraction (i.e., all muscle proteins combined and also referred to as the total muscle protein) is composed of both the sarcoplasmic and myofibrillar fractions. The myosin, actin, and collagen proteins reside in the myofibrillar protein fraction.16

**Muscle Protein Quantification.** Mixed, Sarcoplasmic, and Myofibrillar Protein Concentration. For each biopsy sample, a piece of muscle, weighing ≈10 mg (10.11 ± 0.18 mg wet weight), was divided and weighed on a precision microbalance (AD-2Z Autobalance; Perkin-Elmer, Wellesley, Massachusetts) at −35°C. Each sample was homogenized in 40 volumes of cold homogenizing buffer (250 mM sucrose, 100 mM potassium chloride, 20 mM imidazole, and 5 mM ethylene-diamine tetraacetic acid; pH 6.8) in a ground-glass homogenizer (Radnoti Glass Technology, Monrovia, California). Samples were then centrifuged at 20,000 × g for 30 min at 4°C. The supernatant was taken as the sarcoplasmic protein fraction, and the pellet was resuspended in 40 volumes of cold homogenizing buffer and taken as the myofibrillar protein fraction.30 Aliquots of the homogenate (total or mixed protein), sarcoplasmic, and myofibrillar protein fractions were measured for protein concentration using the bicinchoninic acid assay (Pierce Laboratories, Rockford, Illinois) with bovine serum albumin used as the protein standard. The amount of protein in each of the three fractions was normalized to the wet weight of each muscle sample.

**Myosin and Actin Concentration.** Myosin heavy chain (MHC) and actin concentrations were determined by quantitative gel electrophoresis as previously described.18 Aliquots of the myofibrillar protein fraction were diluted with sodium dodecyl-sulfate (SDS) buffer [2% SDS, 125 mM Tris-HCl (pH 6.8), 12.5% glycerol, 5% β-mercaptoethanol, 0.005% bromophenol blue] and heated at 60°C for 4 min. Myofibrillar protein was separated by SDS-polyacryl-
amidite gel electrophoresis (PAGE). MHC was resolved with a 4% stacking gel and a 10% separating gel. Actin was resolved with a 4% stacking gel and a 6–12% gradient separating gel that was allowed to polymerize overnight. For MHC and actin, electrophoresis was performed at a constant current of 20 mA per gel in the stacking gel, and 25 mA per gel in the separating gel with a Tris-glycine electrode buffer at 4°C (Hoeffer SE 600; Amersham Pharmacia Biotech, Piscataway, New Jersey).

The separating gels were silver-stained, digitally photographed (Fluorchem SP; Alpha Innotech, San Leandro, California), and densitometry was completed using NIH Image software (version 1.34s). Each gel was loaded with 5 standards of either MHC or actin (Sigma, St. Louis, Missouri) and subject samples. For each subject sample, appropriate amounts of myofibrillar protein were loaded in each well to approximate the midpoint of the standard curve for MHC and actin. All standards and samples were loaded in duplicate, and an average of the duplicate densities was taken to represent each standard and sample. Each gel contained pre and post samples from the same individual and subjects from each group. Unknown sample amounts of MHC and actin were determined from regression analysis of the standard curves on each gel. Correlation coefficients were 0.98–1.00 for both MHC and actin. MHC and actin amounts were normalized to the muscle wet weight.

**Collagen Concentration.** Skeletal muscle collagen concentration was determined via measurement of the collagen-specific amino acid, hydroxyproline (HYP). The concentration of HYP was quantified by high-performance liquid chromatography and fluorometric detection (1100 Series; Agilent Technologies, Wilmington, Delaware) via the precolumn derivatization method described by Hutson et al., with modifications for human skeletal muscle.

Muscle samples of 10 mg (11.01 ± 0.27 mg wet weight) were weighed at −35°C on a precision microbalance (AD-2Z Autobalance; Perkin-Elmer, Wellesley, Massachusetts) and hydrolyzed in 1 ml of 6 M HCl at 110°C for 30 h. In addition, a sarcosine internal standard prepared in water was added to each vial. The hydrolysates were allowed to cool to room temperature and neutralized with 6 M NaOH. Hydroxyproline standards of 1, 10, 25, 50, 75, 100, and 125 μM were prepared along with the 2-mM sarcosine internal standard, and were used to generate a standard curve.

Derivatization was completed by combining 900-μl aliquots of muscle hydrolyzate or standard with 200 μl of borate buffer (0.7 M boric acid, pH 9.5) and 100 μl of OPA solution (50 mg o-phthalaldehyde dissolved in 1 ml acetonitrile containing 26 μl of β-mercaptoethanol), followed 60 s later by 100 μl of iodoacetamide reagent (140 mg/ml of iodoacetamide in acetonitrile). One minute later, 300 μl of 5 mM FMOC (9-fluorenylmethylchloroformate) in acetone was added. The vials were capped and vortexed between each addition of reagent. One minute after the addition of FMOC reagent, 2 ml of ethylether was added to each reaction vial. The vial was shaken vigorously for 30 s to wash its contents. The organic layer was discarded, and the wash was repeated twice for a total of three washes. Five microliters of the remaining aqueous phase was injected with an autosampler (Agilent Technologies). Separation was achieved through an XTerra RP-18 column of 5 μm, 250 mm × 4.6 mm (Waters, Milford, Massachusetts) using an isocratic mobile phase of 65% acetic acid: 35% acetonitrile (3% glacial acetic acid, sodium acetate buffered to pH 4.3) at a flow rate of 1.0 ml/min. Peaks were monitored at 260/316 nm (excitation/emission) with a gain of 8 and integrated with chromatography software (ChemStation, Agilent Technologies). HYP signals were normalized to the internal standard for each injection and HYP concentration was determined from standard curves of HYP (correlation coefficient of 0.998). All samples and standards were run in triplicate with a mean coefficient of variation of 1.1% and 1.0%, respectively. Collagen content was calculated from the HYP concentration assuming that collagen weighs 7.5 times the measured HYP weight, and the molecular weight of collagen is 300,000.

Samples were normalized to muscle wet weight, and data are expressed as micrograms of collagen per milligram muscle wet weight.

**Statistics.** Subject characteristics were compared with a one-way analysis of variance (ANOVA). A two-way (group × time) ANOVA with repeated measures on the time factor was used to compare all variables except for the comparison of baseline vastus and soleus protein concentration, in which case a paired t-test was used. When necessary, post hoc comparisons were completed with Tukey’s test. Significance was accepted at P < 0.05. Data are presented as mean ± SE.

**RESULTS**

**Mixed, Sarcoplasmic, and Myofibrillar Protein Concentrations.** No changes (P > 0.05) were observed in the concentrations (micrograms/milligram muscle wet weight) of mixed, sarcoplasmic, or myofibrillar
protein of the vastus lateralis or soleus in any of the three groups (Figs. 1 and 2).

**Myosin, Actin, and Collagen Concentrations.** As in the global protein fractions of the muscle, no significant differences ($P > 0.05$) were seen after 60-day bedrest in the concentrations (micrograms/milligram muscle wet weight) of the contractile proteins myosin or actin in the vastus lateralis or soleus (Figs. 1 and 2). Collagen concentration (micrograms/milligram muscle wet weight) was also unaltered ($P > 0.05$) in the vastus lateralis and soleus of all three groups (Figs. 1 and 2).

**Vastus Lateralis vs. Soleus Protein Concentrations.** Baseline protein concentrations (micrograms/milligram muscle wet weight) of the vastus lateralis and soleus ($n = 24$) are presented in Figure 3. Myofibrillar and myosin protein concentrations were 16% and 12% higher ($P < 0.05$) in the vastus lateralis as compared with the soleus, respectively. Conversely, collagen protein concentration was 234% greater ($P < 0.05$) in the soleus. No differences were observed in mixed ($P = 0.059$), sarcoplasmic, or actin concentrations between the vastus lateralis and soleus.

**DISCUSSION**

The current study is the first to characterize muscle protein composition in women after long-duration simulated spaceflight. We have shown that women are able to maintain the concentrations of the global protein pools, as well as the main contractile proteins, myosin and actin, and the main connective tissue protein, collagen, in the vastus lateralis and soleus muscles after 60 days of bedrest. This maintenance occurred despite the most dramatic atrophy, in terms of amount and rate (up to 29% in 60 days), that has been reported in men or women during studies of real or simulated weightlessness. In addition, lower limb skeletal muscles maintained protein composition despite exercise countermeasures that completely or partially offset the muscle atrophy, or nutrition countermeasures that exacerbated muscle loss.

The global protein fractions of the muscle (mixed, sarcoplasmic, myofibrillar) were studied be-
cause these fractions are commonly examined in metabolic investigations of muscle atrophy, hypertrophy, aging, and unloading. The concentrations of these fractions are ultimately determined by the sum of the synthesis and degradation of the specific proteins contained therein. From the current data, it is clear that women are able to tightly control these processes in both the thigh (vastus lateralis) and calf (soleus) during a period of extreme inactivity-induced atrophy and when an exercise or nutrition stimulus is superimposed during this atrophy process. These findings are in contrast to earlier results of unloading studies of 35 and 90 days that included primarily or exclusively men. Muscle biopsies of the vastus lateralis from these subjects showed a maintenance of the global protein pools (mixed, sarcoplasmic, and myofibrillar) in both the control and exercise countermeasure (no atrophy) subjects. However, the soleus muscle had disproportionate losses of the mixed and myofibrillar protein fractions after 35 (11% atrophy) and 90 (29% atrophy) days of simulated weightlessness. It appears that women are more susceptible than men to unloading-induced muscle loss [greater (vastus lateralis 24% vs. 21%) or similar (soleus 29%) loss in 60 days vs. 90 days]. However, women seem to be better at regulating the protein components of muscles undergoing atrophy. The basis for this gender-specific atrophy and regulation of the muscle composition is unclear and requires further investigation.

Previously, short-term real or simulated spaceflight has been shown to induce contractile protein dysregulation in humans and animals. Data from short-duration (17-day) spaceflight and bedrest in men showed a disproportionate loss of actin protein accompanied by functional alterations in the muscle contractile apparatus. In contrast, we have shown that women maintained the relative amounts of myosin and actin proteins after 60 days of bedrest. These findings are similar to previous data from 35 and 90 days of unloading that also indicate a maintenance of the concentration of the contractile proteins in the thigh and calf muscles. Conservation of the relative amounts of myosin and actin, both in relation to the whole muscle and to each other, appears to be an important aspect of regulating the protein structure of unloaded skeletal muscle. This
response appears to occur even in the presence of countermeasures that completely or partially preserve muscle mass or promote muscle loss. Although whole muscle force and power were significantly altered in the women from the current study and the subjects in the previous unloading studies, alterations in the relative amount of myosin and actin in the muscle did not appear to influence these changes in muscle function.

Although there is a paucity of data regarding the skeletal muscle cellular responses to unloading in women, there are even fewer data that specifically examine the connective tissue component. The connective tissue network plays an important role in muscle function by transferring the force generated by myosin and actin cross-bridge interactions out to the tendon and bone. In the current study, we observed no significant alteration in intramuscular collagen concentration with unloading. These findings conflict with data from animals that show an accumulation of intramuscular connective tissue with unloading, which in turn impacts muscle function. In contrast to the other protein measurements made in the women in the current study, the response of the connective tissue component appeared to be much more variable. This response is also in contrast to measurements of the intramuscular connective tissue component made in men following shorter and longer duration unloading.

A secondary aim of this study was to examine the protein composition of the vastus lateralis and soleus. Very little is known about the differences in protein structure between these two muscles in humans, other than the fiber type differences. Any differences between these two muscles may give insight as to why the soleus atrophies to a greater degree with long-term unloading and is less responsive to exercise countermeasures. From the baseline data of the women from all three groups, the soleus contained less myofibrillar protein, primarily due to less myosin, and greater amounts of connective tissue (Fig. 3). In support of these data, the soleus has been shown to contain a larger amount of slow fibers, which in turn contain less myosin protein than type II muscle fibers in humans. Interestingly, we have shown in a smaller group comprised primarily of men, no differences in the amount of myofibrillar proteins between the vastus lateralis and soleus. These findings suggest a gender-specific difference in the structure of these two muscles.

In conclusion, women are able to maintain the relative proportions of the global protein pools and force-specific proteins during what appears to be the most extreme case of atrophy induced by real or simulated spaceflight. These responses, coupled with previous findings from men, suggest a gender-specific response in skeletal muscle to unloading and add to our understanding of how women and men will respond to long-duration spaceflight. Baseline differences in protein content of the vastus lateralis and soleus muscles may help explain the disproportionate atrophy and response to countermeasures common to the soleus during periods of real and simulated microgravity.

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