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Low-load resistance exercise during inactivity is associated with greater fibre area and satellite cell expression in older skeletal muscle

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Abstract

Background Age-related sarcopenia is accelerated by physical inactivity. Low-load resistance exercise (LLRE) counters inactivity-induced muscle atrophy in older adults, but changes in muscle fibre morphology are unstudied. We aimed to determine the impact of LLRE during short-term inactivity (step-reduction) on muscle fibre size and capillarity as well as satellite cell (SC) content in older skeletal muscle.

Methods Fourteen older (~71 years) male adults underwent 14 days of step reduction (<1500 steps/day) while performing six sessions of LLRE (~30% maximal strength) with one leg (SR + EX) while the contralateral leg served as an untrained control (SR). Seven healthy ambulatory age-matched male adults (~69 years) served as a comparator group (COM). Muscle biopsies were taken from the *vastus lateralis* after 14 days, and immunohistochemical analysis was performed to determine muscle fibre cross-sectional area (CSA), myonuclear content, SC content ($PAX7^+$ cells), and total (C:F) and fibre type-specific (C:Fi) capillary-to-fibre ratios.

Results Type I and II fibre CSA was greater in SR + EX compared with SR. Whereas there were no differences across fibre types between SR + EX and CON, type II fibre CSA was significantly lower in SR compared with COM. Type II myonuclear domain was greater in SR + EX compared with COM and SR. $Pax7^+$ cells associated with type I and II fibres were lower in SR compared with SR + EX. Type II PAX7+ cells were also lower in SR compared with COM with a similar trend for type I fibres. There were trends for a lower C:Fi in SR compared with SR + EX for both fibre types with no differences for each compared with COM.

Conclusions Minimal LLRE during a period of decreased physical activity is associated with greater muscle fibre CSA, SC content, and capillarization. These results support the use of LLRE as an effective countermeasure to inactivity-induced alterations in muscle morphology with age.

Keywords Sarcopenia; Muscle stem cell; Exercise; Inactivity; Ageing; Muscle fibres

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Introduction

The characteristic loss of muscle mass with ageing, sarcopenia, may precede decrements in muscle function[1] and appears to be accelerated by muscle disuse.[2,3] It is well established that muscle disuse (e.g. bed rest or casting) induces profound muscle loss in older adults[4,5] that is difficult to recover[6,7] and can have significant consequences for older persons.[8] We have recently demonstrated that even a short period of inactivity, created by a reduction in daily steps, results in reductions in leg lean muscle mass in as little as 2 weeks.[9,10] Given that older adults have a delayed and/or

blunted ability to fully recover lost muscle mass and function following disuse-induced muscle atrophy,[6,7] it is imperative to elucidate effective countermeasures for its prevention. We recently demonstrated that minimal (six sessions in 14 days) low-load (high effort) resistance exercise (LLRE) maintained leg lean mass, muscle function, and a robust feeding-induced rise in muscle protein synthesis in older adults.[10] We suggest that these results are related to the ability of LLRE to enhance motor unit recruitment (especially of the higher threshold type II fibres)[11,12] and ultimately highlight the importance of this training paradigm for maintaining muscle mass and function in older adults. Nonetheless, it is unclear whether and to what degree inactivity-induced changes in muscle mass also affect muscle fibre area and whether there may be a fibre-type specific response.

In addition to alterations in protein metabolism that contribute to the loss of skeletal mass with age and disuse, evidence is accumulating that a contraction of the muscle stem cell [i.e. satellite cell (SC)] pool, and or impaired SC function, may contribute to age-related muscle loss.[13] This SC dysregulation also appears to be fibre-type specific, with SC associated with fast twitch type II fibres being more 'susceptible' to age-related impairments.[14,15] This is notable given the generally greater atrophy of these fibres during normal ageing.[16,17] Additionally, SC are also generally located in close proximity to capillaries,[18] which would ostensibly ideally position them to respond to circulating regulatory factors (e.g. IGF-1, IL-6, and hepatocyte growth factor). However, SC in older muscle are located at a greater distance from capillaries than in young individuals, which may impair their biological function and, ultimately, the plasticity of skeletal muscle with age.[19,20] Moreover, the age-related decrease in muscle capillarization may impair nutrient delivery to skeletal muscle,[21] which could be a contributing factor to both an impairment in muscle metabolism and SC function.[19,22,23]

The main aim of the present study was to investigate the impact of minimal LLRE during 2 weeks of inactivity on skeletal muscle fibre morphology in older adults. We also compared the changes in muscle fibre area, SC content, and capillarization with healthy, ambulatory controls to further elucidate the beneficial effects of LLRE during a period of inactivity in ageing. Our main thesis was, consistent with our previous findings at the whole muscle level,[9,10] that LLRE would prove to be effective in preserving muscle fibre area with inactivity.

Methods

Participants

We examined a subset of 14 healthy, moderately active (7011 ± 830 steps/day) older men (71 ± 5 years, 84.3 ± 17.3 kg, 25.3 ± 6.1% body fat) who were recruited to participate in the step-reduction portion of the study, as described previously.[10] A subset of seven older men from separate study[24] served as healthy, ambulatory (5649 ± 2465 steps/day) comparators (COM; 69 ± 3 years, 76.6 ± 7.1 kg, 22.9 ± 4.5% body fat). All baseline characteristics were similar between groups (all $P \geq 0.22$; unpaired *t*-test). Participants provided informed consent with the study conforming to the Declaration of Helsinki and also being approved by the Hamilton Integrated Research Ethics Board.

Study design

Participants were required to reduce their daily steps to $<$1500/day for 14 days while performing six sessions of unilateral (i.e. single leg) low-load, high effort (i.e. to volitional fatigue or failure to maintain required range of motion) resistance exercise (i.e. three sessions/week with at least 48 h between sessions) with a randomly selected leg, as previously described.[10] This model resulted in a within-participant comparison to determine how exercise during step reduction (SR + EX) influences muscle fibre characteristics and SC content compared with a leg that did not perform exercise (SR). The low-load (30% of maximal strength) and low-volume (three sets each of leg press and knee extension) exercise was performed to volitional fatigue to minimize the potential for musculoskeletal injuries in this older population yet maximize muscle fibre recruitment, especially of the type II muscle fibres, and muscle protein synthesis.[11,25] On average, participants completed ~30 repetitions per set for knee extension and ~35 repetitions per set for leg press. Muscle biopsies were taken from the vastus lateralis of both legs immediately after the 14-day step-reduction, which occurred 72 h after the final exercise, bout to determine muscle fibre characteristics and changes in muscle protein synthesis, as described previously.[10] In order to characterize potential differences from healthy, ambulatory controls, muscle biopsies were taken at rest in a subset of participants from a previous study.[24] All samples were freed of visible blood and connective tissue prior to being mounted in optimal cutting temperature medium and frozen in isopentane cooled by liquid nitrogen. Samples were stored at −80°C until analysis.

Analysis

Muscle cross sections (7 μm) were prepared from unfixed OCT embedded samples, air dried for 15–45 min, and stored at −80°C. Samples were incubated with the following primary antibodies: anti-Pax7 (1:1), anti-laminin (1:500, Abcam), anti-MHCI (1:5, DSHB), and anti-CD31 (1:25, Abcam) followed by washes with phosphate buffered saline +0.1% tween (PBS-T). Primary antibodies where revealed

through immunoflourescent detection with Alexa Flour 488 donkey anti-rabbit (1:500), Alexa Flour 647 donkey anti-mouse (1:500), Alexa Flour 488 donkey anti-mouse (1:500), and Alexa Fluor 555 donkey anti-rabbit (1,1000) (all Invitrogen). Nuclei were labelled with DAPI prior to final washes (3 × 5 min) in PBS-T and cover slipped with fluorescent mounting media. For immunofluorescence stains that detected Pax7, samples were fixed in 4% PFA for 10 min followed by washes with PBS-T and blocking with a solution containing 2% BSA, 5% FBS, 0.2% Triton X-100, 0.1% sodium azide for 60 min as described previously.[14] Immunofluorescence stains that detected CD31 were performed as described previously[19] with slight modifications. Briefly, following air drying, samples were fixed using ice-cold acetone for 10 min and blocked with 5% donkey serum. Additionally, a 0.01% Sudan Black solution (in 70% ETOH) was applied to samples for 10 min prior to final washes and incubation with DAPI. Samples were visualized with a Zeiss LSM710 laser scanning confocal microscope equipped with a high-resolution camera. For each sample, the following parameters were evaluated as described previously[14]: muscle fibre cross-sectional area was calculated from at least 75 type-I and type-II fibres, fibre-type distribution was calculated as number of MHC type I fibres/total fibre number, number of myonuclei was identified as fibre-associated, sub-laminin DAPI+ cells while the myonuclear domain was calculated as the number of myonuclei per square micrometre. The total SC number was identified as fibre-associated, sub-laminin, Pax7+ cells while the fibre-type specific quantity of SC was identified as sub-laminin, Pax7+ cells associated with fibres +/− for MHC type I.

Capillaries were quantified from images using FIJI software by the same, blinded individual using areas of the muscle cross-sections spanning 2000 × 2000 pixels, which were free of longitudinal fibres and freeze fracture. Analysis consisted of quantification of the capillary-to-fibre ratio in mixed muscle and in a fibre-type specific manner, on both a global (C:F) and local (C:Fi) level. These parameters differ as the global measurement gives an indication of the capillarization of the muscle itself, whereas the local parameter reflects the capillarity of the individual fibres.[26] C:F analysis was conducted by counting the total number of capillaries and fibres, along with specific fibre types and their corresponding capillaries.[27] In order to account for edge effects, fibre counts included all whole fibres within the ROI, while total capillary counts included all interior capillaries plus one-half of all peripheral capillaries.[28]

C:Fi analysis was completed using methods previously described by Hepple *et al*.[29] Briefly, 25 fibres of each fibre type were selected within the ROI using the closest 25 fibres of the same type. Capillary contacts (number of capillaries surrounding a fibre) were analysed for each individual fibre, along with each capillary's sharing factor, the number of fibres a capillary comes into contact with. In order to calculate the C:Fi, capillaries of a specific sharing factor are multiplied by the reciprocal of their sharing factor and subsequently summed. The formula reads as

$$\mathrm{C:Fi} = (n_1(1/\mathrm{SF}_1)\,) + (n_2(1/\mathrm{SF}_2)\,)\ldots,$$

where n is equal to the number of capillaries and SF is the corresponding sharing factor.

Statistics

The primary comparison was determining the difference between SR and SR + EX, which was performed using a two-tailed paired *t*-tests with significance accepted at $P \leq 0.05$. To determine differences from COM as a secondary comparison, unpaired *t*-tests were used and adjusted for multiple comparisons (i.e. significance accepted at $P \leq 0.025$). A Pearson's product correlation was applied to assess the relationship between SC, muscle protein synthesis, and fibre capillarization with significance accepted at $P < 0.05$. Statistics were performed using GraphPad Prism V6.02 software (GraphPad Software Inc., La Jolla, CA, USA). Data presented as means ± SD.

Results

Fibre cross-sectional area

Type I and type II muscle fibre area in SR + EX was ~12% ($P < 0.05$) and ~29% ($P < 0.01$) greater, respectively, than in SR (*Figure* 1A). There was no difference in type I fibre area between COM and SR ($P = 0.25$) and SR + EX ($P = 0.74$). Whereas type II fibre area was ~34% greater in COM compared with SR ($P < 0.01$; *Figure* 1B), there was no difference between SR + EX and COM ($P = 0.71$; *Figure* 1B).

Myonuclei

Myonuclei per fibre was similar across all conditions for both type I and type II muscle fibres (all $P \geq 0.16$; *Table* 1). There was no difference between conditions for myonuclear domain size for type I fibres ($P \geq 0.18$). In contrast, the similar myonuclei per type II fibre for all conditions resulted in a greater type II myonuclear domain for COM relative to SR ($P < 0.01$) but not relative to SR + EX ($P = 0.21$). There was no difference in myonuclear domain between SR + EX and SR ($P = 0.11$).

Figure 1 Cross-sectional area of type I (A) and type II (B) muscle fibres during step reduction (SR; *N* = 14), step reduction with low-load resistance exercise (SR + EX; *N* = 14), and in healthy ambulatory comparators (COM; *N* = 7). Data presented as mean ('+'), median (line), interquartile range (box), and minimum and maximum (whiskers). *Significantly different, $P < 0.05$. **Significantly different, $P < 0.01$.

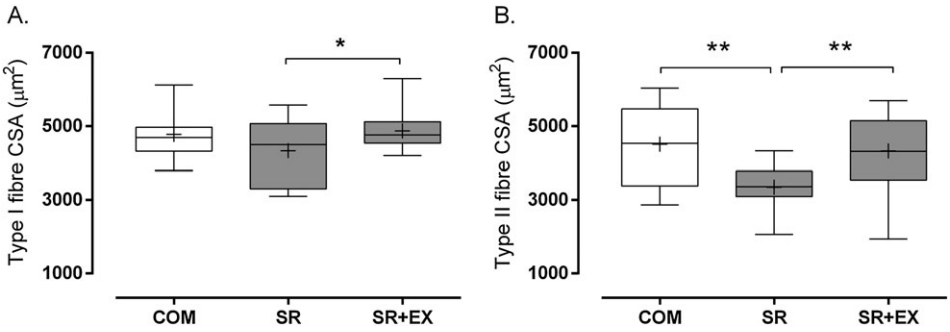


Table 1 Myonuclei per fibre and myonuclear domain

		SR	SR + EX	COM
Myonuclei per fibre	Type I	3.0 ± 0.4	3.1 ± 0.3	2.9 ± 0.3
	Type II	2.9 ± 0.5	3.0 ± 0.4	2.7 ± 0.2
Myonuclear domain (μm^2)	Type I	1480 ± 270	1570 ± 220	1660 ± 250
	Type II	1190 ± 300[a]	1440 ± 330[b]	1640 ± 350[b]

N = 14 for step reduction (SR) and step reduction with low-load resistance exercise (SR + EX) and *N* = 7 for comparator (COM). Conditions with different superscripts are significantly different, $P < 0.01$. Means ± SD.

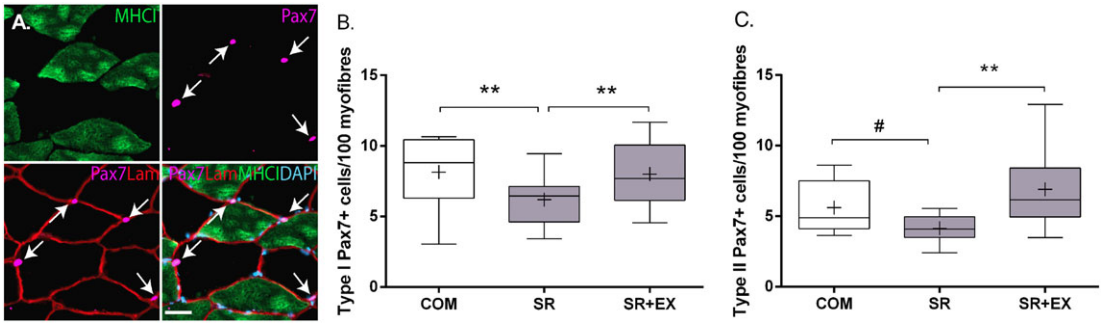
Satellite cells

Type I fibre-associated PAX7$^+$ cells were lower in SR compared with SR + EX ($P < 0.01$) and COM ($P < 0.01$; *Figure* 2A). There was no difference between SR + EX and COM for type I-associated PAX7$^+$ cells ($P = 0.39$). Type II fibre-associated PAX7$^+$ cells were lower in SR compared with SR + EX ($P < 0.01$) with a trend ($P = 0.027$) towards a difference with COM (*Figure* 2B). In contrast, PAX7$^+$ cells were not different between SR + EX and CON ($P = 0.26$).

Capillarization

A total of 153 ± 12, 139 ± 11, and 114 ± 7 fibres were analysed for SR, SR + EX, and COM, respectively. Capillary density was similar between conditions (SR = 244 ± 68 μm^2/capillary; SR + EX = 248 ± 81 μm^2/capillary; COM = 234 ± 36 μm^2/capillary, $P \geq 0.67$). Global C:F was greater in SR + EX compared with SR (1.24 ± 0.19 vs. 1.12 ± 0.15, respectively; $P < 0.05$). There was a trend for a difference in global C:F between COM (1.45 ± 0.32) and SR + EX ($P = 0.07$) that reached

Figure 2 Representative images (A) of single channel stains for myosin heavy chain (MHC)1, and Pax7 and merged stain of Pax7/laminin (LAM) and Pax7/LAM/MHCI/DAPI of a muscle cross section. Scale bar = 50 μm. Quantification of the number of satellite cells (Pax7$^+$ myonuclei/fibre) in type I (B) and type II (C) muscle fibres during step reduction (SR; *N* = 14), step reduction with low-load resistance exercise (SR + EX; N = 14), and in healthy ambulatory comparators (COM; *N* = 7). Data presented as mean ('+'), median (line), interquartile range (box), and minimum and maximum (whiskers). **Significantly different, $P < 0.01$. #Trend for a difference, $P = 0.027$.



significance with SR ($P < 0.01$). There were trends towards a greater C:Fi between SR + EX compared with SR for type I (~12%; $P = 0.06$) and type II (~13%; $P = 0.09$) fibres (*Figure* 3A, 3B and 3C respectively). Sensitivity analysis revealed that the difference between SR and SR + EX for C:Fi became significant ($P < 0.01$) after removal of an outlier, as defined by being greater than 2 SD from the mean fold difference between conditions. There was no difference in C:Fi between COM and SR or SR + EX for type I ($P \geq 0.27$) or type II ($P \geq 0.14$) muscle fibres.

Correlations

There were moderate to large ($r = 0.45$–0.62; all $P \leq 0.016$) correlations between average fibre area, average SC content, and fasted myofibrillar protein synthesis (*Figure* 4A–C) when collapsed across SR and SR + EX. There was a moderate ($r = 0.41$; $P < 0.05$) correlation between C:Fi and post-prandial myofibrillar protein synthesis when collapsed across SR and SR + EX (data not shown). Correlations between C:Fi and SC expression revealed no difference in type I fibres ($r = 0.06$; $P = 0.77$) but a potential trend in type II fibres ($r = 0.32$; $P = 0.09$) in SR and SR + EX.

Discussion

Muscle disuse results in the loss of lean mass across the lifespan[30,31] and can have profound health and mobility consequences particularly in older persons.[8] We previously demonstrated that a reduction in daily steps, as a model of inactivity and partial muscle disuse, leads to the development of anabolic resistance of muscle protein synthesis (i.e. a relative refractoriness of muscle protein synthesis to hyperaminoacidaemia) and the loss of skeletal muscle mass in older adults.[9,10] Our results here extend those of our previous observations by demonstrating that LLRE (with high effort) not only attenuates inactivity-induced muscle loss in older adults[10] but also is associated with a greater muscle fibre cross-sectional area, particularly in type II fibres. Moreover, type I and II fibre area in the SR + EX leg was similar in size to healthy age-matched fully ambulatory older adults (COM). In contrast, type II fibre area in SR was significantly less than ambulatory controls, demonstrating that LLRE may be an effective countermeasure to mitigate type II muscle fibre atrophy associated with inactivity. Our results are fundamentally aligned with, for example, the ability of neuromuscular electrical stimulation to attenuate muscle and fibre

Figure 3 Representative images (A) of single channel stains for myosin heavy chain (MHC)1, CD31, and laminin (LAM) and a merged stain of MHC1/CD31/LAM/DAPI of a muscle cross section. Scale bar = 50 μm. Capillary contacts per fibre (C:Fi) in type I (B) and type II (C) muscle fibres during step reduction (SR; $N = 14$), step reduction with low-load resistance exercise (SR + EX; $N = 14$), and in healthy ambulatory comparators (COM; $N = 7$). Data presented as mean ('+'), median (line), interquartile range (box), and minimum and maximum (whiskers). #Trend for a difference, $P = 0.06$–0.09.

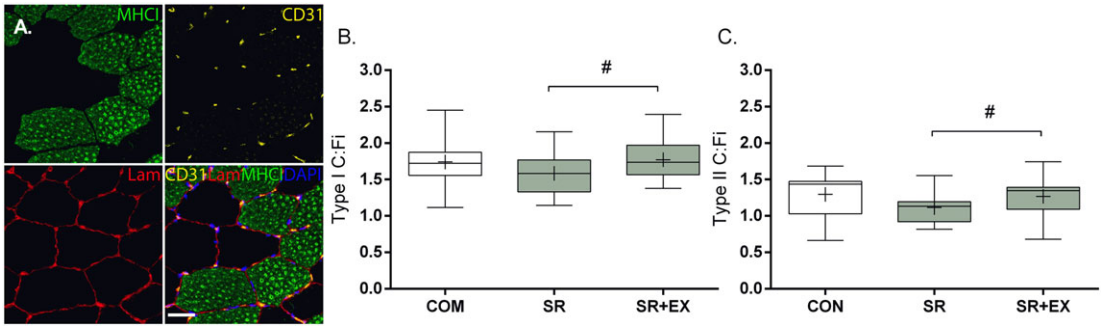
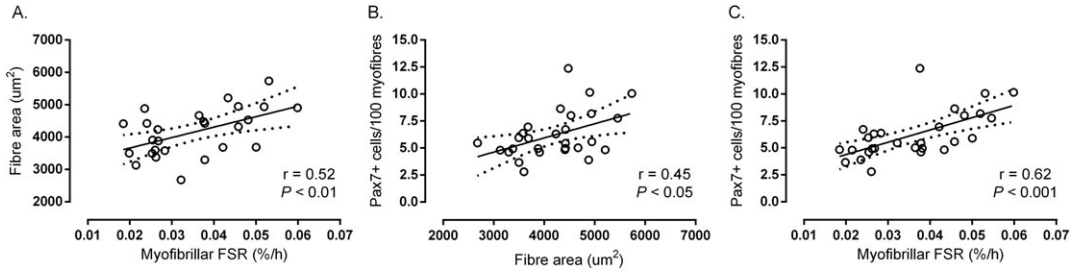


Figure 4 Correlation between average fibre cross-sectional area and fasted myofibrillar protein synthesis rates (A), average PAX7$^+$ cells/100 fibres and average fibre cross-sectional area (B), and average PAX7$^+$ cells/100 fibres and fasted myofibrillar protein synthesis (C) collapsed across step reduction and step reduction with low-load resistance exercise ($N = 28$ for all correlations). Fasted myofibrillar protein synthesis determined previously.[10]



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atrophy during bed rest.[32] The apparent preservation of type II fibre area with unilateral LLRE in the present study is notable given that preferential atrophy of these high force-generating and power-generating muscle fibres is a hallmark of age-related muscle loss.[16,17] Moreover, periods of inactivity have been suggested to accelerate the typical loss of muscle mass with age given that older adults generally experience an attenuated recovery of inactivity-induced muscle loss.[6–8] Thus, our results and those of others[32] collectively highlight the importance of some consistent contractile stimulus during even brief periods of inactivity, as with complete disuse, to prevent muscle atrophy in otherwise healthy older adults. As a countermeasure, LLRE may, over time, mitigate or delay the characteristic loss of muscle with age.

Ageing is associated with a general reduction in SC content in skeletal muscle, especially SC associated with type II fibres.[33,34] Coincident with a lower muscle fibre area, we also observed a greater type I and II fibre SC content in the SR + RE compared with the SR leg after step reduction. Moreover, whereas SC content was similar in SR + EX compared with COM, there was a decreased SC pool size associated with type II fibres in the SR leg. This reduced type II fibre-associated SC content in the SR leg compared with healthy controls suggests that inactivity contributed to a contraction of the SC pool. Whether immobilization results in a contraction of the SC pool in younger adults is debatable[35,36]; however, it appears that older skeletal muscle may be susceptible to inactivity-induced loss of SC.[32] Mechanistically, the factors responsible for the loss of SC with inactivity are not defined but may be related to cell apoptosis. For example, SC isolated from aged muscle display greater rates of apoptosis concomitant with rises in caspase family members and cell death genes.[37] In contrast, resistance exercise can induce an expansion of the SC pool in older skeletal muscle despite a delayed type II fibre-associated SC response.[14,15] Thus, a greater SC content in the present study may be a residual effect of the prior bout of exercise and not necessarily a new basal steady state expansion of the SC pool. Nevertheless, the difference between SC pool sizes and muscle fibre areas in the SR and SR + EX legs is consistent with the observation that SC are decreased in non-exercising older adults,[14,15,19] which correlates with total muscle mass in this population.[38] Thus, while our retrospective design precludes our ability to assess whether there was an inactivity-induced contraction of the SC pool and/or an acute/chronic exercise-induced expansion, our data clearly demonstrate that LLRE is associated with a greater SC content associated with both type I and II muscle fibres compared with a leg that is inactive.

The loss of muscle mass with ageing and/or inactivity is underpinned in part by changes in muscle protein metabolism, the most consistent feature of which is an attenuated protein synthetic response in older adults.[30,31] Given the myofibrillar protein pool represents ~65% of total skeletal muscle protein, the relationship between fasted myofibrillar protein synthesis and muscle fibre cross-sectional area during inactivity in the SR and SR + RE legs in the present study is perhaps not altogether surprising.[30] Nonetheless, evidence is accumulating that SC depletion and/or dysregulation is involved in muscle loss during ageing[14,38] and inactivity.[32] This notion is consistent with our observation of a relationship between myofibre size and SC content in the present study. Our observation that fasted myofibrillar protein synthesis was correlated with SC content in the SR and SR + RE legs suggest, we propose, these processes may be responding to contractile stimuli in a coordinated manner.[39] Thus, given that changes in muscle protein synthesis are an important variable influencing muscle mass in otherwise healthy humans,[40] the relationship between myofibrillar protein synthesis and SC content in the present study is aligned with previous observations that SC content is a modest predictor of muscle mass with ageing.[38]

Muscle tissue microcirculation is important for metabolic health because it can influence the delivery of hormones and nutrients to skeletal muscle.[23,41,42] We observed a trend (P = 0.06 and 0.09 for type I and II fibres, respectively) for a greater capillary contact per muscle fibre in the SR + RE compared with the SR leg during step reduction. Immobilization may decrease[43] whereas resistance exercise can increase muscle capillarization,[29] which collectively may function to maintain a constant capillary density.[44,45] Thus, the difference between the SR and SR + RE legs may be related to the angiogenic nature of the LLRE stimulus in the SR + RE leg and/or an inactivity-induced contraction of the capillary network in the SR leg. The slightly greater capillarization in SR + RE compared with SR leg is notable given that the apparent difference is of similar magnitude as the age-induced changes over ~12 years.[21] The greater capillary contacts in the SR + RE leg may result in greater delivery of circulating nutrients and hormones, which would be consistent with previous observations of a greater insulin sensitivity being associated with muscle capillarization in older adults.[23,41] In the present study, there was a correlation between average fibre capillary contacts and previously determined mixed meal myofibrillar protein synthetic response,[10] which highlights the importance of an adequate microcirculation and nutrient delivery to support muscle mass. However, capillaries are also an important contributor to the SC niche[18] and can influence their post-exercise activation.[19] Moreover, a greater number of capillary contacts per fibre are associated with greater training-induced fibre hypertrophy in older muscle.[20] In as much as there was a contraction in both the SC pool and capillary density in the present study, these inactivity-induced changes may have been mediated in part through apoptosis given the potentially greater susceptibility of SC and endothelial cells to regulated cell death with age.[46] Thus, our results support the use of LLRE to maintain or enhance skeletal muscle morphology during periods of inactivity in older adults.

In addition to maintaining muscle mass and anabolic sensitivity,[10] we demonstrate that LLRE is associated with greater muscle fibre area, SC content, and capillarization in older men after 2 weeks of inactivity. Importantly, the observed differences in muscle morphology occurred in response to only six sessions of LLRE, which is supportive of exercise countermeasures to prevent and/or recover from inactivity/disuse in older populations.[12] The congruence between muscle fibre size, SC content, and estimates of basal myofibrillar protein synthesis in the present study provides further evidence of a coordinated response between muscle protein metabolism and muscle stem cell activity, both of which have been independently shown to be an important regulator of muscle mass.

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Conflicts of interest

The authors report no conflict of interest.

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