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Whey protein with potassium bicarbonate supplement attenuates the reduction in muscle oxidative capacity during 19 days bed rest

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Running head: Whey protein sustains muscle oxidative capacity in bed rest.

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Abstract

The effectiveness of whey protein plus potassium bicarbonate enriched-diet (WP+$KHCO_3$) to mitigate disuse-induced changes in muscle fibre oxidative capacity and capillarization was investigated in a 21-day crossover design bed rest study. Ten healthy men (31±6 years) once received WP+$KHCO_3$ and once received a standardized isocaloric diet. Muscle biopsies were taken two days before and during the 19th day of bed rest (BR) from the soleus (SOL) and vastus lateralis (VL) muscle. Whole body aerobic power (VO_{2max}), muscle fatigue and isometric strength of knee extensor and plantar flexor muscles were monitored. Muscle fiber types and capillaries were identified by immunohistochemistry. Fiber oxidative capacity was determined as the optical density (OD) at 660 nm of succinate dehydrogenase (SDH)-stained sections. The product of fiber cross-sectional area and SDH-OD (integrated SDH) indicated the maximal oxygen consumption of that fiber. The maximal oxygen consumption supported by a capillary was calculated as the integrated SDH in its supply area. BR reduced isometric strength of knee extensor muscles (P<0.05), and the fiber oxidative capacity (P<0.001) and VO_{2max} (P=0.042), but had no significant impact on muscle capillarization or fatigue resistance of thigh muscles. The maximal oxygen consumption supported by a capillary was reduced by 24% in SOL and 16% in VL (P<0.001). WP+$KHCO_3$ attenuated the disuse-induced reduction in fiber oxidative capacity in both muscles (P<0.01). In conclusion, following 19 days bed rest, the decrement in fiber oxidative capacity is proportionally larger than the loss of capillaries. WP+$KHCO_3$ appears to attenuate disuse-induced reductions in fiber oxidative capacity.

Key words: bed rest, oxidative capacity, capillarization, whey protein, muscle atrophy, microgravity, $KHCO_3$, maximal voluntary contraction, muscle fatigue.

New and noteworthy: Reduced muscle oxidative capacity and capillary rarefaction may be critical factors in disuse-induced muscle weakness in space flight or bed-rest. Here we show that 19 days bed rest induced a reduction in the fiber oxidative capacity, irrespective of muscle (soleus and vastus lateralis muscle) or fiber type, without significant capillary loss, that was in part attenuated by a whey protein plus potassium bicarbonate enriched diet.

Abbreviations. BDC, before bed rest; BR, bed rest; BSA, bovine serum albumin; C:F, capillary to fiber ratio; CD, capillary density; CFD, capillary fiber density; DLR, Deutsches Zentrum für Luft- und Raumfahrt; ECG, electrocardiography; ESA, European Space Agency; FCSA, fiber cross-sectional area; HDT, head-down-tilt; HRP, horseradish peroxidise; $KHCO_3$, potassium bicarbonate; LCFR, local capillary to fiber ratio; $\log_D SD$, standard deviation of the logarithm of domain areas; LTBR, long-term bed rest; MatLab, Matrix Laboratory; MO_{2max}, maximal oxygen consumption supported by a capillary; MTBR/MEP, Medium-Term Bed Rest Whey protein; MyHC, myosin heavy chain; MVC, maximal voluntary contraction; NOS3, nitric oxide synthase 3; ns, not statistically significant; O.C.T., optimum cutting temperature; OD, optical density; PBS, phosphate buffered saline; PGC-1α, peroxisome proliferator-activated receptor gamma coactivator 1-alpha; SDH, succinate dehydrogenase; SOL, soleus; VL, vastus lateralis; VO_{2max}, maximal oxygen uptake.

Introduction

Skeletal muscle disuse, such as occurs during prolonged immobilization, bed rest and spaceflight, is associated with muscle wasting, weakness and reduced fatigue resistance (17). As muscular forces are important to maintain bone density, the reduction in muscle mechanical forces may lead to an increased risk of falls and bone injury (24). In astronauts, microgravity-induced changes in the musculoskeletal system may lead to muscle or bone injury during activity and may limit their ability to perform their mission and daily tasks, and presents a potential risk to their safety and health (1). There is therefore considerable interest to develop effective nutritional and exercise interventions to attenuate the muscle wasting following prolonged space missions.

Besides muscle atrophy, disuse also causes arterial structural remodeling and reductions of blood flow to the active muscles (54). Mechanical signals and endothelial cell shear stress are crucial for capillary maintenance and angiogenesis (31), and a reduced blood flow during muscle disuse may result in capillary rarefaction. An adequate capillary supply is crucial not only for delivery of oxygen but also for the delivery of nutrients and removal of heat and waste products and hence for tissue remodeling and repair. As fatigue resistance correlates positively with capillarization and fiber oxidative capacity of the muscle (20), capillary rarefaction in disuse or in microgravity, combined with a reduced oxidative capacity, may lead to a lower muscle fatigue resistance (17), or even exacerbate tissue damage.

Although it has been well documented that gravitational unloading during short-term spaceflight is associated with muscle atrophy and a reduced oxidative capacity in humans (4,23) and rodents (4,50), there are still limited data on changes in muscle capillarization and fiber oxidative capacity during prolonged microgravity in humans. Microgravity-induced muscle weakness, reduced fiber cross-sectional area and a slow-to-fast fiber type transition (4) are more pronounced and occur earlier in oxidative and antigravity muscles (such as the soleus) than in non-postural mixed muscles (i.e. the vastus lateralis) (17). One might therefore expect that also the reduction in oxidative capacity and loss of capillaries are more pronounced in the more oxidative weight-bearing muscles, but this has hitherto not been investigated systematically.

It has been shown that high protein intake and essential amino acid supplements have anti-catabolic, anti-inflammatory and anti-oxidant effects, where in particular whey protein (WP) appears effective in overcoming protein wasting during short-term bed rest (3,52). Although WP has been reported to enhance the gain in lower body strength and VO_{2max} and mitochondrial enzyme activities in combination with resistance- (39) or aerobic- (see review: 42) training, we (8) and others (in review: 52) did not see any significant effect on muscle fiber size of a WP-enriched diet during prolonged period of bed rest (8,52). It remains to be seen, however, whether WP could attenuate any disuse-induced reductions in muscle fiber oxidative capacity in bed rest.

A potential limitation of a daily high protein intake is the introduction of an acid-load, caused by the endogenous oxidation of cationic and sulfur-containing amino acids, which during bed rest will add to the acidogenic load resulting from the amino acids derived from broken down muscle proteins. If the acidogenic effect of high protein intake is not compensated by an alkaline agent a chronic low-grade metabolic acidosis may cause further activation of muscle proteolysis (57), bone demineralization (24) and potentially also inhibit aerobic energy metabolism, resulting in an earlier onset of muscle fatigue (36). The supplementation of alkaline mineral salts, such as potassium bicarbonate ($KHCO_3$), has been shown to effectively reduce muscle wasting in the setting of acidogenic or high vitamin D diets and in chronic metabolic acidosis in human (10) and animal models (14). It was therefore expected that the addition of the alkaline salt $KHCO_3$ supports the action of whey protein and helps to sustain muscle fiber aerobic capacity during prolonged periods of disuse.

In the present study, we investigated the potential of whey protein supplementation plus $KHCO_3$ to counteract the effects of 19 days 6° head down-tilt bed rest (21 days of medium-term bed rest, MTBR/MEP study) on muscle fiber capillarization and oxidative capacity. Our principal hypothesis was that bed rest-induced reductions in fiber oxidative capacity and capillary rarefaction are more pronounced in the soleus than the vastus lateralis muscle, which can all be prevented by alkaline whey protein enriched diet.

Materials and Methods

Bed rest study

The 21-day 6° head-down-tilt (HDT) **Me**dium-**T**erm **B**ed **R**est Whey protein (MTBR/MEP) study was performed at the German Aerospace Center (DLR) in Cologne, Germany, in accordance with the European Space Agency (ESA) bed rest standardization plan. The design of the study was described previously (11). Briefly, the study was a controlled randomized crossover design performed in two campaigns, separated by a 125-day wash-out period. Each campaign comprised a 7-day adaptation, a 21-day bed rest (intervention period) and a 6-day recovery phase. The caloric intake was controlled throughout the study and was during the 7-day adaptation and 6-day recovery phases around 2700 kCal·d^{-1} and reduced to around 2030 kCal·d^{-1} during bed rest (for details see 11). For the first campaign (September and October 2011), five healthy participants were randomly assigned to a bed rest-only (BR), and another five healthy participants to a bed rest plus whey protein + $KHCO_3$ intervention (NUTR). For the second campaign (February and March 2012), the participants were assigned the other way around (Fig.1). The crossover design minimized any potential bias from carry-over and seasonal effects (possible differences in the habitual activity levels during the summer and mid-winter) on the structure and function of skeletal muscle. Table 1 shows the participant characteristics.

The recruited subjects (ten healthy men aged between 23 to 43 years, an age typical for astronauts) successfully completed all medical, physical and psychological screenings (11). Exclusion criteria included presence of muscle/cartilage/joint diseases, herniated disc, chronic back pain, chronic hypertension, diabetes, obesity, arthritis, hyperlipidemia, any infectious and hepatic disease, disorders of calcium or bone metabolism, history of orthostatic intolerance or vestibular disorders (11). Negative results of a thrombophilia screening panel (Antithrombin III, Protein C and S, Factor-V-Leiden, Pro- thrombin muteins, Lupus- Partial Thromboplastin Time) were mandatory for final inclusion in the study (11).

The study was conducted in compliance with the protocol (and its subsequent amendments) for the MEP bed rest study, as approved by the independent ethics committee of the Ärztekammer Nordrhein, Düsseldorf, Germany. During the study the rights, safety and well-being of subjects were protected according to the Declaration of

Helsinki. All subjects participated after providing signed informed consent. More detailed data on exclusion criteria, anthropometric characteristic, energy intake and baseline data of the MTBR/MEP study are reported in (www.clinical.trials.gov, Identifier: NCT01655979; 8,11).

Nutritional intervention

The nutritional intervention (NUTR) was a combination of whey protein (0.6 g whey protein·kg body mass^{-1}·day^{-1}; Diaprotein®, Dr. Steudle Inc, Krueger GmbH) plus potassium bicarbonate (90 mmol $KHCO_3$·day^{-1}), that isocalorically replaced fat and carbohydrates in the daily diet in a 1:1 ratio (11). During the control bed rest condition (BR) the participants received a basic protein diet of 1.2 g protein·kg^{-1}·day^{-1}. This intake was higher than the current recommended daily intake (0.8 g protein·kg^{-1}·day^{-1}) and was moderately acidifying (potential renal acid load of the diet: 13±1 mEq·day^{-1}). During the NUTR condition, the alkaline urine content confirmed an alkali over acid production, suggesting that there was no acidification in this group. More detailed data are reported in (11).

Maximal Oxygen Uptake (VO_{2max})

Maximal oxygen uptake was assessed using a graded exercise protocol on an electronically-braked cycle ergometer (Model Excalibur Sport, LODE B.V, The Netherlands). The oxygen uptake throughout the test was measured with a Metalyzer (Spirometer: Cortex Metalyzer, CORTEX Biophysik GmbH, Germany), before (BCD-7) and post (R+1) bed rest. Heart rate, ECG and blood pressure were monitored continuously during the test (Finometer, TNO, The Netherlands, Biopac systems inc. USA). Participants were considered to have reached VO_{2max} if they fulfilled at least two of the following three criteria: they could not maintain the cadence of 60 revolutions per minute due to voluntary exhaustion, reached the predicted maximal heart and/or had a respiratory exchange ratio > 1.1.

Isometric maximal voluntary contraction (MVC)

The torque during maximal voluntary isometric contractions (MVC) was determined for the knee extensors and the plantar flexors before (BCD-7) and post (BR+0) bed rest, using a dynamometer (Biodex Medical Systems, Inc., Shirley, NY) as described previously

(38). The highest torque (Nm) was considered the subject's maximum. If a subject continued to improve at the third trial contraction, testing was continued until no further improvement was observed.

Muscle fatigue resistance

Muscle fatigue resistance was determined before (BCD-3) and post (BR+0) bed rest, in the knee extensors. Muscle fatigue resistance was given as the time to failure during a sustained contraction at 50% of the actual MVC (38).

Muscle Biopsies

Muscle biopsies were obtained two days before bed rest (BDC-2) and during the 19th day of bed rest (BR+19) from the vastus lateralis (VL) and soleus (SOL) muscles of the right leg. Biopsies of the vastus lateralis were taken at 40% of the length between the knee joint cleft (0% being the knee joint cleft) and the anterior superior iliac spine. Soleus biopsies were obtained via a lateral approach, at least 2 cm below the distal end of the lateral gastrocnemius muscle. In both muscles, sequential biopsies were at least 2 cm apart. To minimize any bias due to regional differences in muscle morphology, sequences (distal *vs.* proximal) of biopsy localization were permutated between subjects. In the second campaign, two of the subjects provided no biopsies (one for medical reasons and one withdrew from the study for personal reasons during the second campaign and did not provide a post-bed rest biopsy). There were no adverse events or side effects in the MEP study, associated with neither the bed rest nor the biopsies. However, one subject developed petechiae during the orthostatic tests that were performed after bed rest in both campaigns, as previously reported (25). The samples were subdivided into a piece for histological analysis and other tissue pieces (approx. 20 mg each) for biochemical and molecular analysis, as described (8). The histology piece was embedded in a 3-mm silicone tube filled with Optimum Cutting Temperature (O.C.T.) compound (Scigen® Gardena) to facilitate cross-sectional orientation. All samples were immediately frozen in liquid nitrogen and stored at -80°C until analysis.

Histological staining for muscle capillarization and fiber typing

Muscle cross-sections were prepared as previously described (8). Briefly, from all biopsies, serial 8-µm cross-sections were cut in a cryotome at -20°C (CM 1860, LEICA Microsystems). The sections were mounted on polarized glass slides (SuperFrost® Plus, 631-0108, VWR International) and stored at –80°C until use. Capillaries and type I fibers were stained in the same section using a combined immunostaining (Fig. 2). The cross-sections were dried at room temperature for 30 min and then fixed for 15 min in ice-cold acetone (100%). The sections were then washed twice for 5 min in phosphate buffered saline (PBS) at pH 7.6 and blocked for 1 h in 0.1% bovine serum albumin (BSA) in PBS. The sections were then washed twice in PBS for 5 min and the endogenous peroxidases blocked by incubation in 3% H_2O_2 and 10% Triton X-100 in PBS for 30 min at room temperature. The anti-mouse myosin heavy chain type I (MyHC I, 1:100; Novocastra, Leica Biosystems, UK) and biotinylated *Ulex europaeus* agglutinin I (50 $\mu L \cdot mL^{-1}$ in 1% BSA in HEPES; Vector Laboratories, USA) were used to visualize type I fibers and capillaries, respectively. Unlike previously reported (8), further sub-classification of type II fibers and of hybrid fibers (co-expressing both MyHC types I/II) was not performed here. The effect of bed rest and the WP-enriched diet on fiber cross-sectional area (FCSA) and myosin heavy chain composition have been published previously (8). After two 5-min washes in PBS the sections were incubated with the VECTASTAIN® Elite ABC System (Vector Laboratories, USA), as described by the manufacturer. After a further 2x5-min washes the sections were incubated 30 min with a secondary goat anti-mouse horseradish peroxidase (HRP) labelled antibody (1:200; Dako, UK) and then stained using the Vector® VIP HRP substrate kit (Vector Laboratories, USA), as described by the manufacturer. After the staining, the sections were washed in distilled water, mounted in glycerol-gelatin and stored at 4°C.

Analysis of muscle capillarization and fiber type composition

The capillarization of a muscle has traditionally been described by the overall indices of capillary density (CD) and capillary to fiber ratio (C:F). Here, in addition to conventional measures of muscle capillarization, we used the method of capillary domains, as described previously (9), where the capillary domain is the area around a capillary delineated by equidistant boundaries from adjacent capillaries. The capillary domain provides an estimation of the capillary supply area (2). The capillary domain method also gives information about the distribution of capillaries within the tissue, considers fibers that

lack direct contact with a capillary and allows the analysis of the capillary supply to individual fibers (9).

The data processing was performed on photomicrographs of stained muscle cross-sections containing at least 70 complete fibers. The coordinates of the outlines of the fibers and capillary coordinates were collected using a digitizing tablet (Model MMII 1201, Summagraphics Digitizers, Austin, Texas, USA). These data were then fed into a computer program (AnaTis, BaLoH Software, http://www.baloh.nl) that calculates capillary domains (9) and parameters related to muscle fiber size and composition (). For each muscle biopsy, the fiber cross-sectional area (FCSA) and the numerical and areal fiber type composition were calculated (55). In addition, the % connective tissue was given as the % area of the region of interest not covered by contractile material. The number of capillaries supplying a fiber, or the local capillary to fiber ratio (LCFR) for a given fiber, was determined by the sum of the domain fractions overlapping that fiber (9). Note that the LCFR of a fiber takes into account remote capillaries, thus allowing the determination of the capillary supply to a fiber even when it lacks direct capillary contacts. The capillary fiber density (CFD) was calculated as the LCFR divided by the fiber cross-sectional area and was expressed as the number of capillaries per mm^2. To get information about the capillary contacts per fiber, reflecting the oxygen exchange area per fiber (28), the LCFR per fiber perimeter (LCFR/perimeter) was also calculated. Finally, the standard deviation of log transformed domain areas (log_DSD) was used as an index for the heterogeneity of capillary spacing.

Succinate Dehydrogenase and maximal oxygen consumption

The succinate dehydrogenase (SDH) activity in individual muscle cells was determined in histological sections (Fig, 2B), as described previously (9,55). Briefly, a section adjacent to the capillary-stained section was incubated at 37°C in the dark for 20 min in 37 mM sodium phosphate buffer pH 7.6 with 74 mM sodium succinate and 0.4 mM tetra-nitroblue-tetrazolium. After 20 min of incubation, the reaction was stopped with 0.01 N HCl (5 s) and after washing with water mounted in glycerol gelatin (9,55). Photomicrographs of stained cross-sections were then captured and the SDH optical density (OD) of a fiber was determined by measuring the absorbance of the final reaction product using an interference filter at 660 nm (9,55). Absorbance was converted to the rate

of staining quantified by a calibration curve specific for each individual section created with a set of filters with known OD (ImageJ software) to minimize bias related to differences in lighting. The OD of the SDH stain was determined in fibers also identified in the serial section stained for myosin type I and capillaries (Fig. 2B). The OD of the SDH stain is a measure of the mass-specific fiber maximal oxygen consumption. For each of those fibers the product of FCSA and OD SDH gives the integrated SDH, a reflection of the maximal oxygen consumption of that fiber when oxygen is not rate limiting (55). The maximal oxygen consumption supported by a given capillary was calculated as the sum of the overlap areas times the SDH OD of that overlap area of a given domain (9), using Matrix Laboratory (MatLab).

Statistics

All analyses were done on the data of individual fibers. During the design of the study we hoped that all participants completed both trials, and thereby make full use of the power of such a design allowing paired observations (and hence no 'between-factor' analysis). However, not all participants completed both campaigns and to be able to include all data nevertheless, we decided to treat all observations as non-paired observations. Appropriateness of the wash-out period in the MEP/MTBR crossover-designed study has been reported previously (11). Here we tested for possible differences between the baseline data for each of the analyzed factors between the campaigns, with a 3-way ANOVA, with as factors muscle, fiber type and campaign, and as random variable subject. This showed that baseline data did not differ significantly between the two campaigns. To assess the effects of the intervention, the baseline data were pooled and a 3-way ANOVA performed with as factors condition (baseline, BR and NUTR), muscle (VL and SOL) and fiber type (I vs II), with subjects again as random factor. Three way interactions and interactions with subject were excluded. The differences between baseline data, 19 days bed rest (BR) and 19 days bed rest plus diet (NUTR) on %CT, numerical and areal fiber type composition, domain area, domain radius, C:F, CD, and $\log_D$SD were tested with a repeated-measures ANOVA, with muscle as within-factor and condition (BL, BR, NUTR) as between-factor. Regression analysis (SPSSX 19.0) of individual data was performed to analyze relationships between selected variables. Differences and relationships were considered significant at $P < 0.05$. All P-values were Bonferroni corrected to adjust for multiple comparisons.

Results

Maximal voluntary force (MVC), fatigue resistance and fiber type composition

Knee extensor MVC was significantly reduced after BR (P = 0.021; Fig. 3A), but no significant changes were seen in plantar flexor MVC. There were no significant differences between NUTR and BR for either knee extensor or plantar flexor MVC (Fig. 3A), or muscle fatigue resistance of thigh muscles (Fig. 3B). The impact of BR or NUTR on myosin heavy chain composition and fiber size (FCSA) has been presented previously (8). Here, we show that the % connective tissue did not differ significantly between the SOL and VL and was not significantly affected by BR or NUTR (Table 2). The SOL contained a larger number % and areal % of type I fibers than the VL, irrespective of condition (Table 2; P < 0.001). Neither BR nor NUTR induced a significant change in the fiber type proportions.

Oxidative capacity

To investigate whether the BR and whey protein + $KHCO_3$ intervention (NUTR) may affect fiber oxidative capacity, we quantified the succinate dehydrogenase (SDH) activity of muscle fibers (Fig. 4). The specific SDH activity (reflected by the OD) was higher in type I than type II fibers (Fig. 4A; P < 0.001) in both SOL and VL. In addition, the integrated SDH, reflecting the maximal oxygen consumption of a fiber, was higher in fibers of the SOL than the VL (P = 0.046). BR did result in a reduced fiber oxidative capacity in type I and type II fibers in both muscles, both in terms of specific SDH activity (Fig. 4A) and integrated SDH activity (Fig. 4B; P < 0.01). WP + $KHCO_3$ attenuated the BR-induced reduction in specific SDH activity in both VL and SOL, as reflected by higher SDH activities in the NUTR than the BR condition (Fig. 4A; P < 0.01). This was also reflected by an attenuated reduction in integrated SDH in the SOL (P < 0.01), but not in the VL, of the NUTR than the BR condition (Fig. 4B). These changes in integrated SDH activity in the VL were mirrored by the bed rest-induced reductions in whole body VO_{2max} (P = 0.042) that was not attenuated by the nutritional intervention (Fig. 3C).

Overall capillarization

The CD (Table 2) and C:F (Table 2) were higher in the SOL than the VL (P < 0.01). The capillary domain area was smaller in the SOL than the VL (Table 2; P < 0.001), but there was no significant difference in the heterogeneity of capillary spacing (Los_DSD) between muscles (Table 2). Neither BR nor NUTR did significantly affect the CD, C:F,

367 Los_DSD or domain area (Table 2). Noteworthy, not only the maximal fiber oxygen
368 consumption, indicated by a reduced integrated SDH in fibers of both muscles after BR
369 (Fig. 4B), but also the maximal oxygen consumption supported by a capillary (MO_{2max}),
370 (Table 2, BR vs BL; $P < 0.001$), was attenuated by NUTR intervention (Table 2; $P <$
371 0.001). There was a non-significant trend (P=0.057) for a difference between the MO_{2max} at
372 baseline between the two campaigns, suggesting a possible carry-over effect of bed rest
373 or nutritional intervention, or a seasonal effect on MO_{2max}.
374

375 ***Fiber specific capillary supply***

376 The local capillary to fiber ratio (LCFR; Fig. 5A) and the capillary fiber density (CFD;
377 Fig. 5B), were higher in SOL than in the VL ($P < 0.01$). The LCFR of type II was higher
378 than that of type I fibers in both muscles ($P < 0.001$), while type I fibers had a higher CFD
379 than type II fibers ($P < 0.001$). The LCFR/perimeter ratio was larger in type I than type II
380 fibers (P = 0.012), and it was larger for fibers in the SOL than the VL ($P < 0.001$).
381 Irrespective of fiber type, NUTR, but not BR, was associated with a reduction in LCFR in
382 the SOL muscle ($P < 0.001$; Fig. 5A). BR did induce an increase in CFD in both muscles
383 ($P < 0.001$). We found that the fibers became less circular during BR, as indicated by an
384 increased perimeter:FCSA ratio (Fig. 6; $P < 0.001$) and this was even more pronounced in
385 the SOL, but not in the VL after NUTR (Fig. 6; $P < 0.001$). The LCFR/perimeter ratio was
386 lower in BL than in BR and NUTR ($P < 0.001$; Fig. 5C).
387

388 **Discussion**

389 The main observations of the present study are that 19 days of bed rest significantly
390 reduced the fiber oxidative capacity, irrespective of fiber type, in both the soleus and
391 vastus lateralis muscle. This was associated with a reduction in the whole body maximal
392 oxygen uptake (VO_{2max}). There was no significant loss of capillaries, resulting in a denser
393 capillary network than expected for the fiber size and fiber oxidative capacity, suggesting a
394 superfluous capillarization. The reduction in fiber oxidative capacity was to some extent
395 prevented by a WP + $KHCO_3$-enriched diet.
396

397 Bed rest has been widely used as a model to mimic the effects of microgravity and
398 unloading, and to test the efficacy of exercise, nutritional and pharmacological
399 interventions to prevent or attenuate unloading-induced muscle wasting and weakness

(43). Previously, our group showed that after 19 days of bed rest there was no marked atrophy in either the SOL or VL muscle nor a significant change in myosin heavy chain composition (8), corresponding with the absence of significant changes in fiber type composition observed here (Table 2). The reduction in maximal voluntary isometric force (MVC) of the knee extensor muscles we observed (Fig.3) can thus not be attributable to atrophy after 19 days, but may be mainly due, as suggested by others, to a decreased ability to activate motor units (7, 33) and/or to a disproportionate loss of thin filaments (46).

The effect of bed rest on skeletal muscle morphology

Capillarization

During unloading and bed rest, there is little contractile activity and few, if any, periods of elevated muscle blood flow. Since both mechanical strains and shear stress are important for angiogenesis and the maintenance of the capillary bed (31), and there is reportedly, a close correlation between the fiber oxidative capacity of a fiber and its capillary supply (5), one might expect that bed rest is associated with capillary rarefaction. In line with this, it has been observed that the capillary to fiber ratio, was reduced in the human soleus, but not in the vastus lateralis muscle, after 90 days bed rest and was maintained by exercise during bed rest (47). We, however, did not observe reductions in the number of capillaries per fiber (Table 2) or capillary density (Table 2) after 19 days bed rest in the soleus or vastus lateralis muscle. Others also found no atrophy or changes in capillary density in the vastus lateralis muscle after 5 weeks bed rest (34). In another study with 6 weeks bed rest, the decrease in FCSA in the VL was associated with a maintained capillary density (22), suggesting that in the long-term capillary loss may occur during bed rest that is proportional to the decrease in fiber size. Importantly, in our study, bed rest did not significantly affect the capillary spacing within the muscle (Table 2), a factor that can have a significant impact on local tissue oxygenation (18,26).

Oxidative capacity

The bed rest-induced reduction in the oxidative capacity of the fibers, indicative for a decreased mitochondrial volume density, was independent of muscle or fiber type (Fig. 4) and was accompanied by a reduction in whole body VO_{2max}. A reduction in mitochondrial volume density and mitochondrial enzyme activities has also been observed in the vastus lateralis muscle after 37 days bed rest (22), indicating that even after 37 days

434 the loss of mitochondria is proportionally larger than the atrophy. In denervated rat soleus
435 muscles something similar was observed, where initially the loss of mitochondria was
436 disproportionally more than fiber atrophy (19). Our observations were also consistent with
437 an earlier report on the effects of 4 weeks unilateral lower limb suspension (7), where
438 unloading did reduce work and oxidative capacity of skeletal muscle without changes in
439 capillary to fiber ratio, fiber type composition or FCSA of the vastus lateralis muscle. Part
440 of the impairment of peripheral gas exchange (O_2 transfer and/or utilization) and maximal
441 oxygen consumption (VO_{2max}) after medium- and long-term bed rest may thus not only be
442 attributable to cardiovascular "deconditioning" and muscle atrophy (13,22), but also to a
443 reduced capacity for oxidative metabolism of the disused muscles (32).
444
445 Because of the unaltered morphology of the capillary network and the reduction of
446 the fiber oxidative capacity, the maximal oxygen consumption supported by a capillary
447 (Table 2) was significantly reduced after bed rest. Thus, in terms of oxidative capacity, the
448 muscle has an 'excessive' capillary supply; something also observed in old rat muscles
449 without significant fiber atrophy (27) and in atrophied denervated muscles (19). A similar
450 situation occurs after cessation of a training program where the decrease in muscle
451 oxidative capacity develops faster than the decrease in muscle capillarization and whole-
452 body VO_{2max} (28). These observations suggest that reductions in mitochondrial volume
453 may precede capillary rarefaction and thus might represent one of the early hallmarks of
454 muscle adaptation to disuse.
455
456 Previously we suggested that the increased ability of older people to sustain a 50%
457 MVC (37) is more a reflection of their slower contractile properties or fiber type
458 composition than changes in oxidative capacity, where more economical type I fibers (53)
459 are better able to sustain a prolonged isometric contraction than type II fibers. Similarly,
460 the absence of a significant change in fatigue resistance observed in our study in the face
461 of reductions in fiber oxidative capacity, could thus be explicable by the absence of
462 significant changes in fiber type composition.
463
464 It remains unclear how unloading would result in a reduction in mitochondrial
465 content. It is possible that a disuse-induced increase in the generation of reactive oxygen
466 species (ROS) contributes to impaired mitochondrial homeostasis and biogenesis (45). In
467 spaceflight or bed rest, the transition from the standing weight-bearing position to

468 microgravity or a supine position may affect the cell tensegrity, as several *in vitro* and *in*
469 *vivo* (murine) studies indicated that gravitational changes caused cytoskeleton
470 disarrangement (15) that in turn may be responsible for aberrant mitochondrial distribution
471 and impair respiratory function (41). This has been confirmed in other models of disuse-
472 induced muscle atrophy, such as denervation-induced atrophy, where changes in inter-
473 myofibrillar mitochondrial content or in mitochondrial distribution are paralleled by
474 increased generation of ROS during active respiration, altered fiber metabolism and
475 impaired muscle cell survival (6). Disarrangement of the cytoskeleton may also contribute
476 to the increase in the 'perimeter:FCSA' ratio, as we observed in bed rest (Fig. 6), indicating
477 that the fibers became more angular. The changes in cytoskeletal components, such as
478 microtubules, may therefore explain the effects of the lack of weight-bearing on the
479 distribution of mitochondria, shape of the fiber and other cellular functions (56).
480
481 ***The effects of whey protein and $KHCO_3$ on oxidative capacity***
482 Dietary amino-acids and protein supplements have been suggested to attenuate the
483 loss of muscle mass after space flight, aging and bed rest, possibly by stimulating anabolic
484 signaling pathways and reducing proteolysis (3,52). To date, there is little information on
485 the effectiveness of alkaline whey protein-enriched diet to attenuate the bed rest-induced
486 reduction in muscle oxidative capacity. Here we found that a whey protein +$KHCO_3$-
487 enriched diet attenuated the bed rest-induced reduction in fiber oxidative capacity (Fig. 4),
488 irrespective of muscle or fiber type.
489 It has been reported that whey protein supplementation improved mitochondrial
490 activity in mouse brain and liver by reducing oxidative stress and stimulating mitochondrial
491 biogenesis *via* transcriptional activation of the peroxisome proliferator-activated receptor
492 gamma coactivator 1-alpha (PGC-1α) (51). A similar action of whey proteins on
493 mitochondria may occur in muscle, as a reduced expression of PGC-1α plays a major role
494 in disuse atrophy, while its overexpression prevents activation of catabolic systems and
495 disuse atrophy (12). It is likely that the attenuated bed rest-induced reduction in muscle
496 fiber oxidative capacity by alkaline whey protein was due to an increased expression of
497 PGC-1α or other proteins involved in mitochondrial biogenesis.
498
499 While, the whey protein-enriched diet attenuated the bed rest-induced reduction in
500 fiber oxidative capacity (in terms of oxidative capacity per gram of muscle), it did not result
501 in an attenuated reduction of whole body VO_{2max} (Fig. 3B). Something similar was also

found in a 60-day bed rest study in women (35,48), where the protein-intervention without exercise proved ineffective to attenuate the bed rest-induced reduction in VO_{2max} (48). The discrepancy between the attenuated reduction in fiber oxidative capacity and no such effect of whey protein-enriched diet on whole body VO_{2max} may be explained by the fact that VO_{2max} is primarily determined by the cardiovascular system rather than by the oxidative capacity of the working muscles (22, 49).

We cannot exclude that $KHCO_3$ itself may have contributed to the attenuated loss of fiber oxidative capacity during bed rest. Bicarbonate salts have been demonstrated to improve muscle strength and endurance, primarily by increasing the buffering capacity of the extracellular fluid and hydrogen ion efflux from muscle cells (16). Extracellular acidosis slows down proton efflux from mitochondria, which may affect fiber oxidative capacity (16,30). Thus, one would expect that by removing intracellular proton excess, $KHCO_3$ may have contributed to improved fiber oxidative capacity during bed rest. However, we are lacking specific information whether oral whey protein and $KHCO_3$ intake do change proton concentrations in muscle tissue. Finally, it is important to consider that during bed rest the moderate acidogenic dietary load may have acted synergistically with disuse to negatively impact on mitochondrial function and content, as observed in the kidney (40).

Perspective

There is a large interest to develop nutritional interventions to attenuate bed rest-induced muscle wasting and reduction in muscle oxidative capacity in the clinical setting. This is particularly relevant for older adults or sarcopenic individuals as they may have slower recovery to the pre-inactivity muscle condition than young adults (44). Our data suggest that a whey protein plus KHCO3-enriched diet attenuates the decrements in muscle oxidative capacity and may well enhance the benefits of integrated physical therapy to counteract the loss of muscle oxidative capacity during hospitalization not only in the young (35,48), but also in the older (21) patient.

Conclusion

In conclusion, medium-term bed rest, even without overt muscle fiber atrophy, induces a reduction in the fiber oxidative capacity of the soleus and vastus lateralis muscle. As the capillary bed was not significantly affected, there was an excessive

capillary supply to the muscle during bed rest. Part of the reduction in bed rest-induced oxidative capacity was prevented by supplementation with whey protein plus $KHCO_3$.

Author Contributions: Experiments and data analysis were done at the Manchester Metropolitan University, Manchester, UK. Preparations of muscle cryosections were done at the Charité Center of Space Medicine Berlin (ZWMB), Berlin, Germany. MTBR/MEP bed rest study was performed at the Institute of Aerospace Medicine, German Aerospace Center DLR, Cologne, Germany. H.D.: conceived and designed the experiments. A.B. performed the experiments. H.D. and A.B. analyzed and interpreted the data. A.B. wrote the first draft of the manuscript. M.S. and D.B. prepared the muscle cryosections and helped in muscle sampling. J.B. conducted the organization of the MTBR/MEP study. E.M. collected the torque data, the body VO_{2max} and its related parameters. J.R. and B.G. took the muscle biopsies and over-saw the medical care of the volunteers. M.H.Y. set MatLab programming. A.B. and H.D. wrote the final version of the manuscript. All authors discussed the results, gave input to writing of manuscript, revising it critically and approved the final version of the manuscript.

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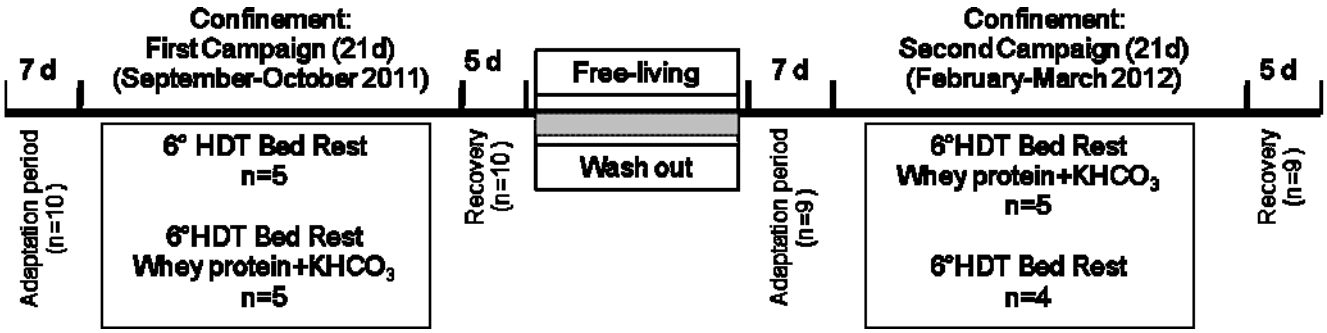
739 **Figures and Figure legends:**

740 **Fig. 1**

741

742

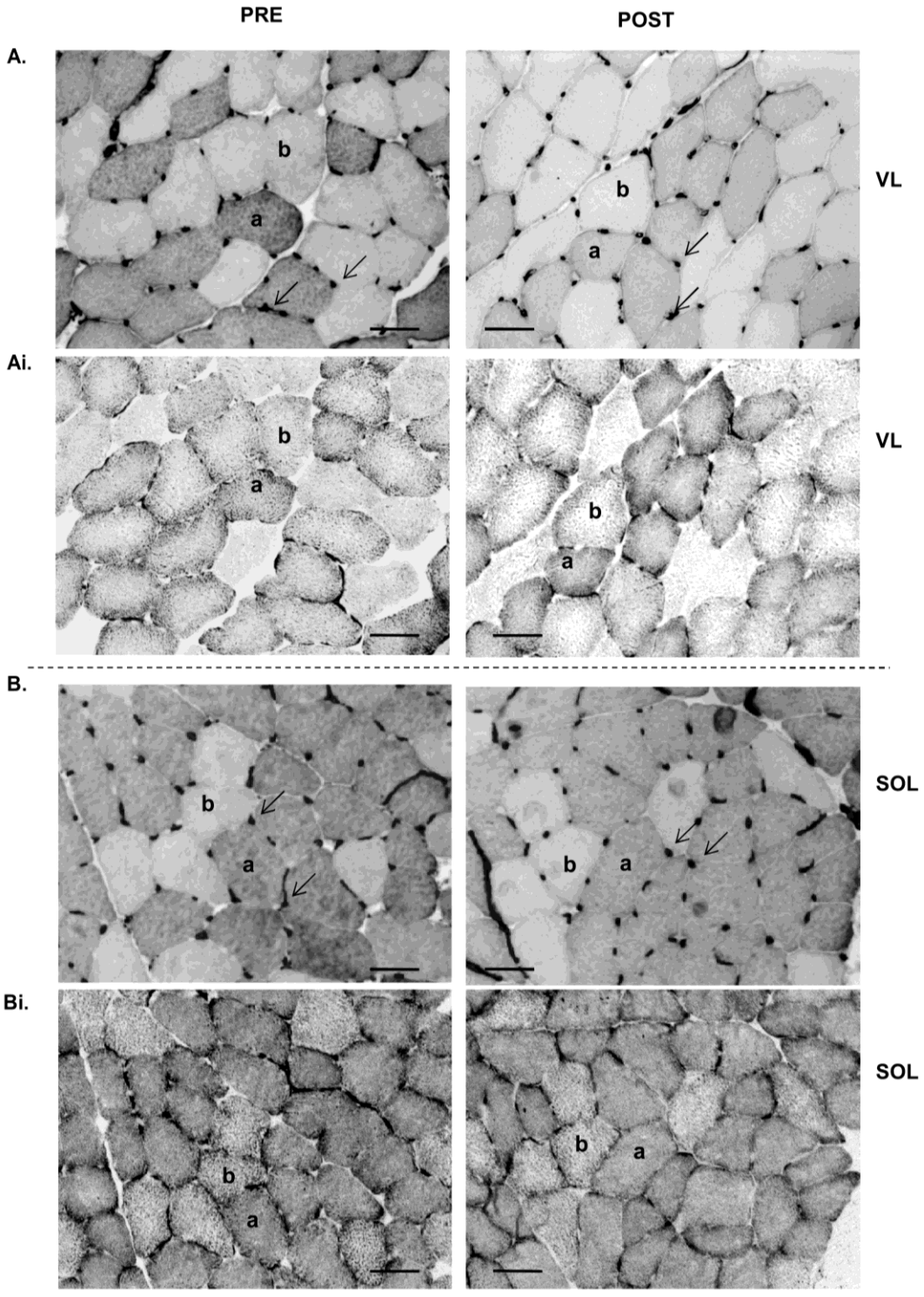


743

744 **Fig.1** Schematic diagram showing the crossover study design of the bed rest study. HDT,
745 head down tilt bed rest.

746

747 **Fig. 2**



748

749 **Fig. 2**. Representative micrographs showing immunohistochemical co-staining with anti-
750 myosin type I and lectin to identify type I (darker stained; example indicated by ***a***) and type
751 II fibers (indicated by ***b***) and to visualize capillaries (some indicated by arrows) in frozen

muscle cross-sections of vastus lateralis (VL; **A**) and soleus (SOL; **B**) muscles, before (PRE) and after (POST) 19 days of bed rest. **Ai** and **Bi**: Representative micrographs showing enzyme histochemical staining for succinate dehydrogenase (SDH) activity in the VL (**Ai**) and in SOL (**Bi**) of the same participants before (PRE) and after (POST) 19 days of bed rest. Scale Bar, 50 µm.

Fig. 3.

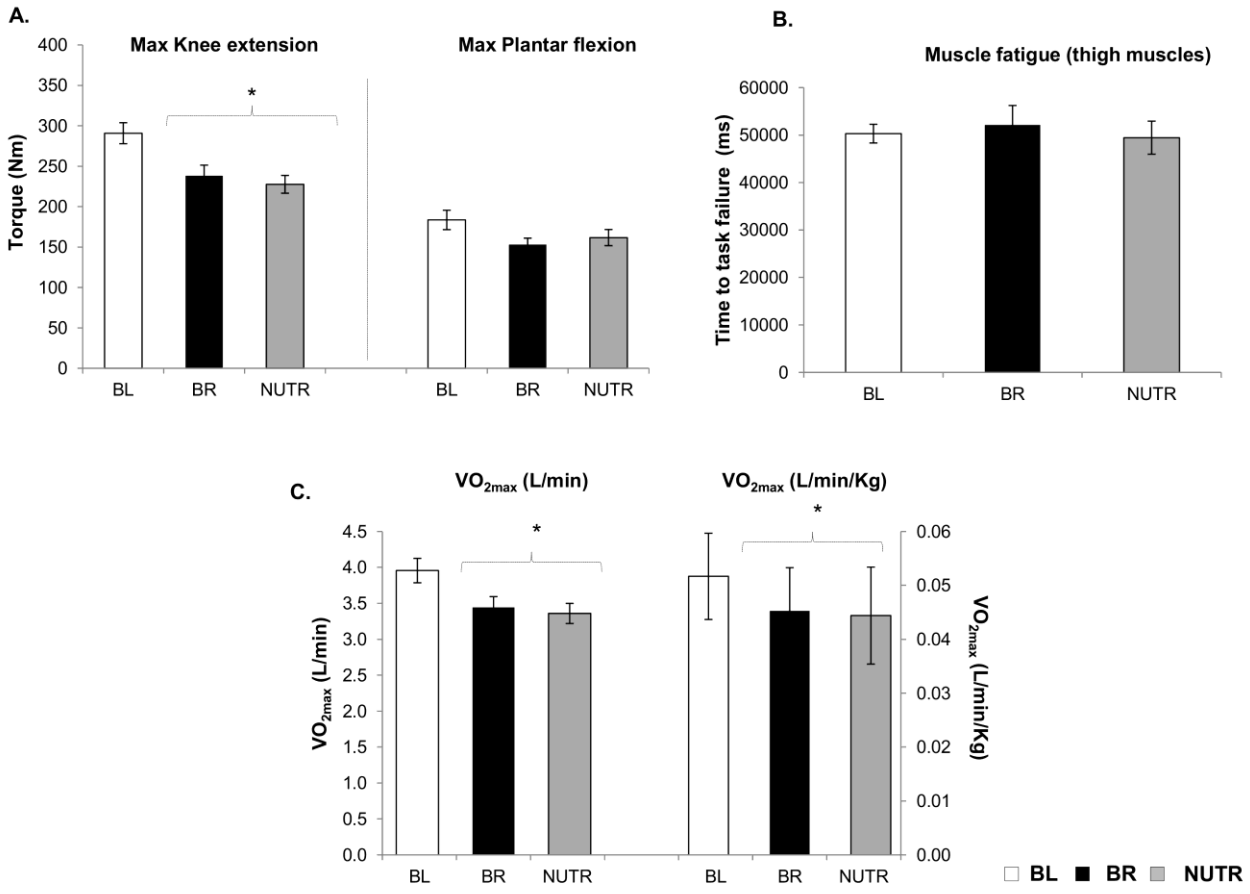


Fig. 3. The effect of 19 days of bed rest with or without WP+$KHCO_3$ supplementation on (**A**) maximal voluntary contraction of knee extensors and plantar flexors of the left leg, (**B**) muscle fatigue of thigh muscles and (**C**) whole body peak oxygen uptake (Vo_{2max}). In **C**, secondary axis: peak oxygen uptake normalized per body mass. BL: baseline; BR: bed-rest plus standardized diet; NUTR: bed-rest plus WP+$KHCO_3$-enriched diet. Data are expressed as mean ± SEM.

In **A**.: *Significantly different from the corresponding value before bed rest (P = 0.021); in **C**.: *Significantly different from the corresponding value before bed rest (P = 0.042).

Fig. 4.

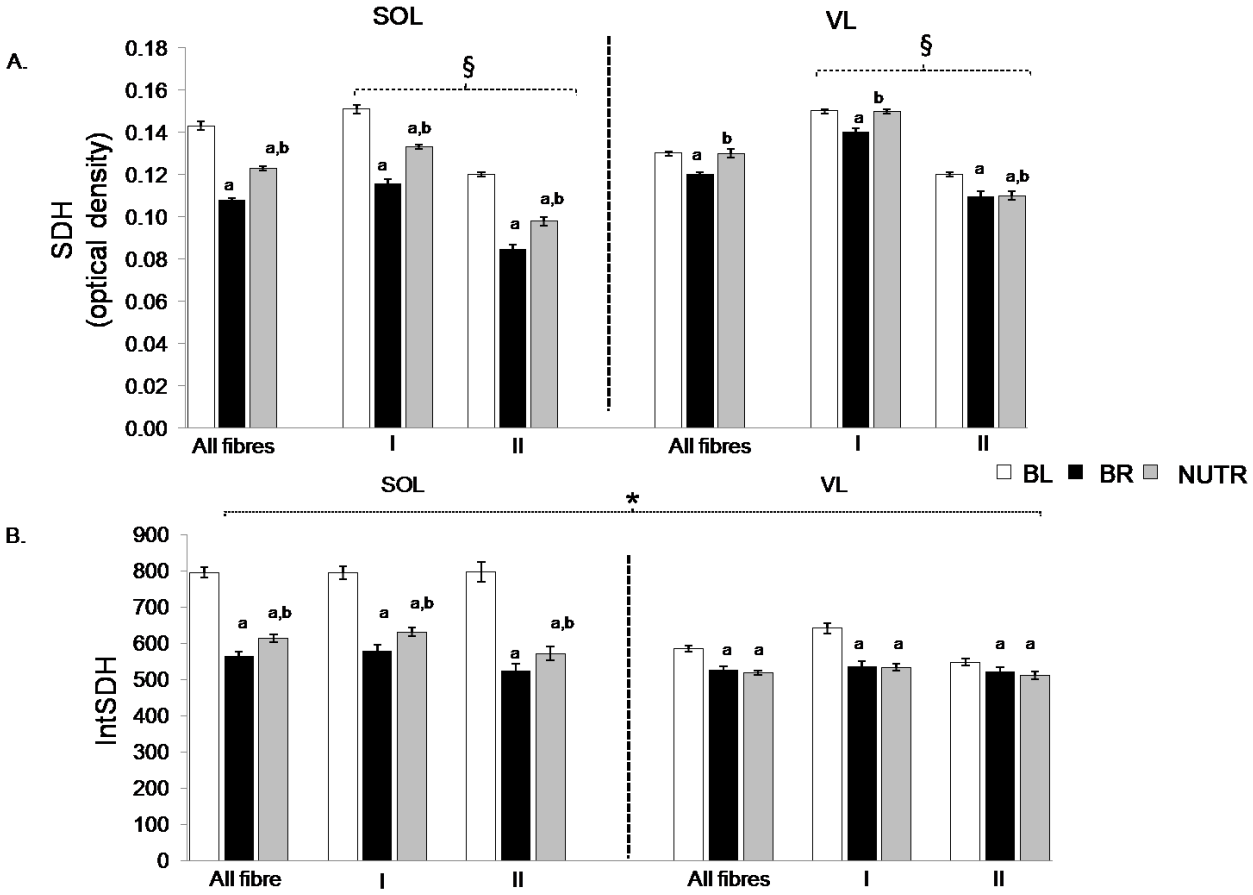


Fig. 4. The effect of 19 days of bed rest with or without WP+$KHCO_3$ supplementation on (**A**) specific succinate dehydrogenase (SDH) and (**B**) integrated SDH activity in the soleus (SOL) and vastus lateralis (VL) muscle. BL: baseline; BR: bed-rest plus standardized diet; NUTR: bed-rest plus WP+$KHCO_3$ supplement. Data are expressed as mean ± SEM. *: significant difference between muscles at $P = 0.046$; §: significant difference between fiber types at $P < 0.001$. a: different from BL; b: different from BR at $P < 0.01$.

Fig. 5.

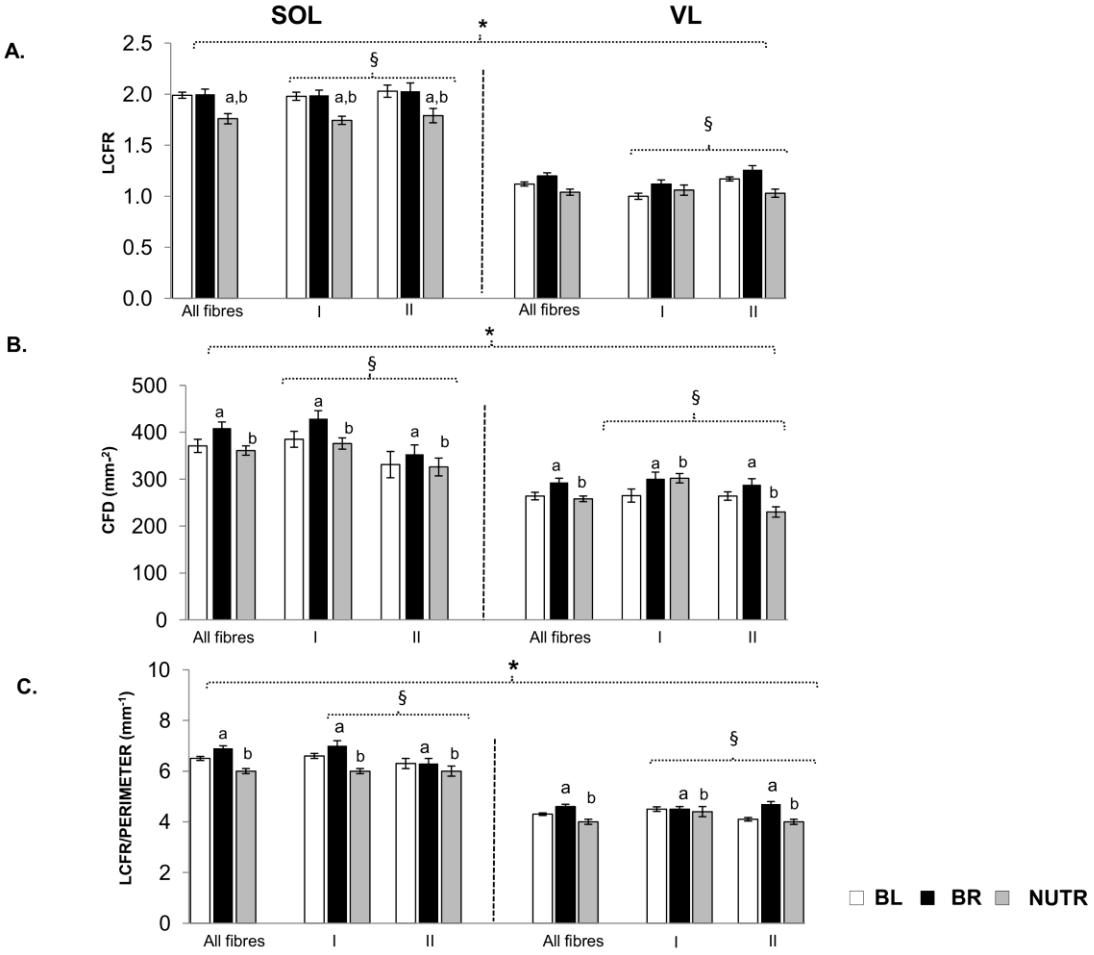


Fig. 5. The effect of 19 days of bed rest with or without WP+$KHCO_3$ supplementation on the (**A**) local capillary to fiber ratio (LCFR; sum of domain fractions overlapping a fiber); (**B**) capillary fiber density (CFD) and (**C**) LCFR/perimeter ratio in the soleus (SOL and vastus lateralis (VL) muscle. BL: baseline; BR: bed-rest plus standardized diet; NUTR: bed rest plus WP+$KHCO_3$. In **A** and **B**: *: significant difference between muscles at $P < 0.001$; §: significant difference between fiber types at $P < 0.001$. In **C**: *: significant difference between muscles at $P < 0.001$; §: significant difference between fiber types at $P = 0.012$. In all panels: a: different from BL at $P < 0.001$. b: different from BR at $P < 0.001$. There were no significant interactions. Data are expressed as mean ± SEM.

Fig. 6.

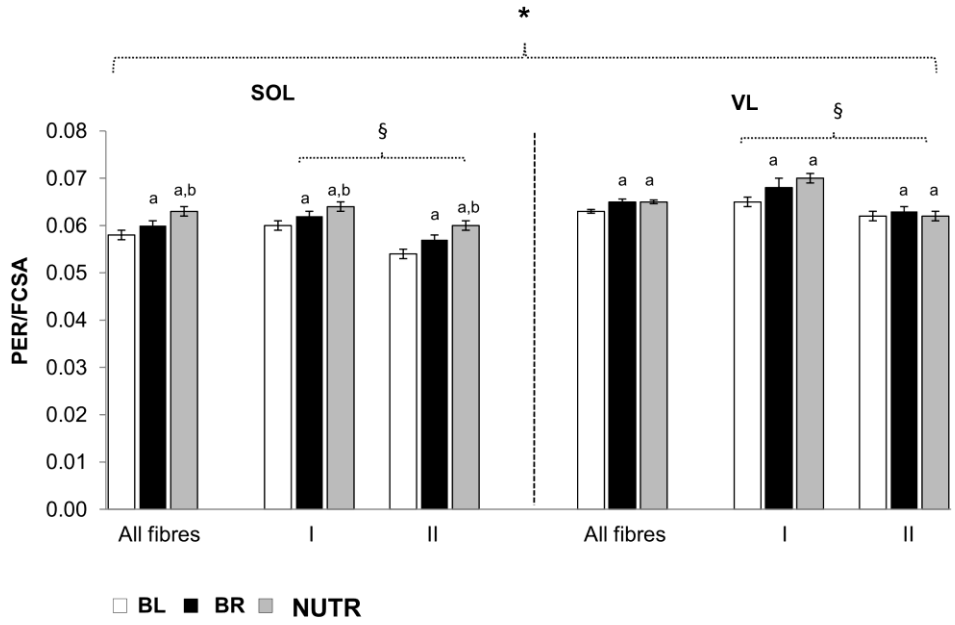


Fig. 6. The effect of 19 days of bed rest with or without WP+$KHCO_3$ supplementation on the perimeter:FCSA ratio. BL: baseline; BR: bed-rest plus standardized diet; NUTR: bed rest plus WP+$KHCO_3$. *: significant difference between the two muscles at $P < 0.001$; §: significant difference between fiber types at $P < 0.001$; a: different from BL at $P < 0.001$. b: different from BR at $P < 0.001$. Data are expressed as mean ± SEM.

Tables:

Table 1. Anthropometric characteristics of participants

Participants	1st campaign n = 10	2nd campaign n = 9
Age (years)	31.6 ± 6.2	31.5 ± 6.2
Height (m)	1.80 ± 0.05	1.80 ± 0.06
Mass (kg)	76.1 ± 5.4	77.7 ± 4.8
BMI ($kg \cdot m^{-2}$)	23.4 ± 1.6	24.0 ± 1.5

Cross-over design: BMI: Body Mass Index; more details see www.clinical.trials.gov Identifier NCT01655979 (See also 10,13).

Table 2: Skeletal muscle morphometric parameters and capillary oxygen supply areas.

		% CT	% n. type I	% n. type II	% Area type I	% Area type II	Capillary Domain Area (μm^2)	Capillary Domain Radius (μm)	CD (mm^{-2})	Log_DSD	C:F	MO_{2max} ($pL \cdot mm^{-1} \cdot min^{-1}$)
SOL	**BL**	7.4±0.6	75±4	25±4	71±5	29±5	2912±166	30±1	352±21	0.187±0.007	2.50±0.23	213±3
	BR	6.4±0.7	75±6	25±6	71±7	29±7	2603±204	29±1	378±27	0.175±0.007	2.25±0.19	163±3
	NUTR	9.1±1.1	70±5	30±5	71±6	29±6	2859±146	30±1	349±18	0.194±0.009	2.13±0.19	192±3
VL	**BL**	10.8±1.5	36±4	64±4	31±4	69±4	3818±178	35±1	261±13	0.188±0.009	1.18±0.12	273±4
	BR	11.8±1.0	40±3	60±3	37±4	63±4	3655±212	34±1	264±18	0.195±0.013	1.26±0.14	237±6
	NUTR	11.2±1.6	35±3	63±3	28±5	72±5	4271±323	36±1	236±19	0.215±0.014	1.10±0.19	293±6
Muscle		ns	P <0.001	P <0.001	ns	ns	P < 0.001	P < 0.001	P < 0.01	ns	P < 0.01	P < 0.01
Condition		ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	P < 0.001
Interaction		ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	P < 0.001

Table 2: Skeletal muscle morphometric parameters and global capillarisation parameters. The table shows the numerical (%n) and areal (%Area) fiber type composition, connective tissue content (CT%), oxygen supply area (capillary domain area and capillary domain radius), the numerical capillary density (CD), capillary to fiber ratio (C:F), the heterogeneity of capillary spacing (Log_DSD; logarithmic standard deviation of the domain area) and the maximal oxygen consumption supported by a capillary (MO_{2max}) in the soleus (SOL) and vastus lateralis (VL) muscles, at baseline (BL) and after 19 days bed rest without (BR) or with (NUTR) WP+$KHCO_3$ enriched diet. Data are expressed as mean ± SEM.