

# Whey protein supplementation accelerates satellite cell proliferation during recovery from eccentric exercise

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**Abstract** Human skeletal muscle satellite cells (SCs) are essential for muscle regeneration and remodeling processes in healthy and clinical conditions involving muscle breakdown. However, the potential influence of protein supplementation on post-exercise SC regulation in human skeletal muscle has not been well investigated. In a comparative human study, we investigated the effect of hydrolyzed whey protein supplementation following eccentric exercise on fiber type-specific SC accumulation. Twenty-four young healthy subjects received either hydrolyzed whey protein + carbohydrate (whey,  $n = 12$ ) or iso-caloric carbohydrate (placebo,  $n = 12$ ) during post-exercise recovery from 150 maximal unilateral eccentric contractions. Prior to and 24, 48 and 168 h post-exercise, muscle biopsies were obtained from the exercise leg and analyzed for fiber

type-specific SC content. Maximal voluntary contraction (MVC) and serum creatine kinase (CK) were evaluated as indices of recovery from muscle damage. In type II fiber-associated SCs, the whey group increased SCs/fiber from 0.05 [0.02; 0.07] to 0.09 [0.06; 0.12] ( $p < 0.05$ ) and 0.11 [0.06; 0.16] ( $p < 0.001$ ) at 24 and 48 h, respectively, and exhibited a difference from the placebo group ( $p < 0.05$ ) at 48 h. The whey group increased SCs/myonuclei from 4% [2; 5] to 10% [4; 16] ( $p < 0.05$ ) at 48h, whereas the placebo group increased from 5% [2; 7] to 9% [3; 16] ( $p < 0.01$ ) at 168h. MVC decreased ( $p < 0.001$ ) and muscle soreness and CK increased ( $p < 0.001$ ), irrespective of supplementation. In conclusion, whey protein supplementation may accelerate SC proliferation as part of the regeneration or remodeling process after high-intensity eccentric exercise.

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## Introduction

Understanding of myocellular systems involved in muscle regeneration, growth and remodeling is fundamentally important to address and understand several clinical disorders affecting skeletal muscle. In this regard, a functional pool of skeletal muscle stem cells (satellite cells—SCs) are believed to be capable of exerting important direct and indirect effects on the myofibers and the microenvironment surrounding these. SCs comprise a heterogeneous population of stem cells situated between the basal lamina and sarcolemma (Mauro 1961; Yin et al. 2013). The SCs can be identified by their location (Mauro 1961) and/or by the expression of several specific proteins, among which the paired box transcription factor 7 (Pax7) is regarded as

a valid marker and known to be expressed during both SC quiescence and proliferation (Yin et al. 2013). Upon activation, the SC is directed from its quiescent state toward a state of proliferation and, following a number of proliferation cycles, can proceed into the myogenic lineage specification (Seale et al. 2000; Yin et al. 2013).

During regeneration from muscle injury, the SCs are essential for myofiber repair and myofiber formation in rodent models (Brack and Rando 2012; Lepper et al. 2011), which suggest that myofiber repair is negatively affected if SCs are impaired in diseased or aging muscle (Conboy et al. 2005; Cosgrove et al. 2014; Jeong et al. 2013; Sousa-Victor et al. 2014). Activation of Pax7<sup>+</sup> SCs has also been demonstrated in human skeletal muscle during post-exercise recovery from muscle damage, typically inflicted by eccentric exercise (Cramer et al. 2004, 2007; McKay et al. 2009; Mikkelsen et al. 2009). Under such circumstances, different studies have reported increased SC content from 24 and up to 30 days post-exercise (Cramer et al. 2007; McKay et al. 2009, 2010; Paulsen et al. 2012). Concomitant with such exercise-induced increase in SC number, indices of muscle damage, such as increases in muscle soreness and a loss of maximal muscle force, are observed within 24–72 h post-exercise and frequently maintained for 7–8 days post-exercise (Mackey et al. 2011; Mikkelsen et al. 2009; Proske and Allen 2005; Vissing et al. 2008). Thus, the current contention is that SCs are important for post-exercise myofiber repair and remodeling. In addition, during eccentric exercise type II fibers may be more susceptible to sarcolemma damage (Vijayan et al. 2001) compared to type I fibers. Therefore, repair processes that require SC proliferation may follow a fiber type-specific pattern as recently indicated (Cermak et al. 2012). However, as this was only examined within the first 24 h, it is not known if the fiber type-specific SC accumulation persists within the later post-exercise recovery period.

Whereas mechanical strain (e.g., during eccentric contractions) through the extracellular matrix and the SC niche is generally accepted to constitute an important mediator in directing SC activity (Tatsumi et al. 2001; Urciuolo et al. 2013), another important mediator of SC activity is the systemic environment (Conboy et al. 2005). In accordance, the often close proximity of SCs to capillaries (Christov et al. 2007) allows immediate interaction with systemically delivered cytokines (McKay et al. 2009; Serrano et al. 2008), growth factors (McKay et al. 2008) and endocrine hormones (Heinemeier et al. 2012). Moreover, delivery and uptake of glucose and essential amino acids (EAAs) may also influence SC activity (Dibble and Manning 2013; Han et al. 2008; Ito and Suda 2014; Jeong et al. 2013). As such, *in vitro* SC proliferation is increased with administration of the essential amino acid (EAA) leucine, possibly mediated

through an increased activation of the mTOR kinase complex and subsequent increased mRNA translation (Dibble and Manning 2013; Han et al. 2008). Therefore, since protein supplements high in leucine are known to directly stimulate mTOR activation, this may provide the basis for increased SC proliferation *in vivo* when ingesting EAAs during the post-exercise recovery phase. In fact, recent studies have indicated that protein supplements may affect the rate of post-exercise recovery of muscle force (Kirby et al. 2012) and muscle soreness (Jackman et al. 2010) following eccentric exercise; however, none of these have investigated whether delivery of EAAs affects the SCs during the recovery from eccentric exercise.

In the present study, we therefore aimed to investigate whether a high dosage of hydrolyzed whey protein could augment the fiber type-specific SC content and muscle fiber regeneration/recovery following eccentric exercise. We hypothesized that (1) high-intensity eccentric exercise *per se* would increase SC content and that whey protein ingestion would accelerate this increase in SCs; (2) the increased SC content, with and without protein ingestion, would be more predominant in type II fibers, and; (3) whey protein would increase the rate of recovery, as judged by indices of myofiber damage.

## Methods

### Participants

Twenty-four healthy young recreationally active men were included in the study. The sample size was determined by completing a power analysis (power = 0.8,  $\alpha$  = 0.05) based on isometric strength data from Kirby et al. (2012) and satellite cell data from Mikkelsen et al. (2009). All subjects were informed of the purpose and risks of the study and provided written informed consent in accordance with the Declaration of Helsinki and approved by the Central Denmark Region Committees on Health Research Ethics (ref. no. M-20110179). Exclusion criteria were: (1) participation in systematic resistance training or eccentrically dominated activities for lower extremity muscles within 6 months prior to participation; (2) a history of musculoskeletal lower extremity injuries; (3) vegan diet; and (4) use of dietary supplements or medication that potentially could influence muscle recovery or function (i.e., protein supplements, antioxidant supplements, NSAIDs, angiotensin-converting enzyme inhibitors). All subjects were instructed to avoid any strenuous physical activity 48 h before the exercise day and throughout the testing period. Furthermore, subjects were asked to refrain from taking any type of non-steroidal anti-inflammatory drugs or alcohol during the entire experimental protocol.

General study design

The study was conducted in a double blinded, placebo-controlled fashion in relation to dietary supplementation. Following inclusion, subjects were randomly allocated into either a whey protein + carbohydrate group (whey,  $n = 12$ ) or iso-caloric carbohydrate placebo group (placebo,  $n = 12$ ). While a crossover study design could decrease variability in SC and damage measures, the repeated eccentric exercise on the same subjects could confound SC and damage measures (Rodgers et al. 2014; Xin et al. 2013) and was therefore not chosen. Subject anthropometrics were recorded on a separate visit to the laboratory (displayed in Table 1). During this visit, the individual settings for the isokinetic dynamometer were also determined.

A schematic overview of the study protocol is presented in Fig. 1. Fourteen days (-14) prior to the exercise day (day 0), the subjects reported to the laboratory between 8.00 and 10.00 am after an overnight fast (from 10 pm). Subjects rested in supine position for 45 min before a basal biopsy was obtained from the pre-selected non-exercise leg (randomly chosen as either preferred or non-preferred leg). On

the exercise day, subjects again reported to the laboratory at 07.30 am in a fasted state. Before commencing with the eccentric exercise protocol, muscle soreness was evaluated using a visual analog scale, a blood sample was drawn and knee extensor muscle contractile function was evaluated. After testing on day 0, the subjects initiated the exercise protocol, which lasted for approximately 30 min. Immediately after exercise, subjects ingested a group-dependent drink after which the subjects rested for 3 h. At 3 h post-exercise, a biopsy was obtained from both the exercise and the non-exercise control leg. Before leaving the laboratory, the subjects ingested the second drink (1.00 pm) and received a third drink to ingest 3 h later (4.00 pm). On days 1 and 2 (24 and 48 h following exercise, respectively), the subjects were instructed to ingest the supplement at absolute time points corresponding to day 0, with the first drink always ingested after the functional tests and biopsy sampling. Biopsy sampling from both the exercise and control leg on day 1, 2 and 7 was performed under conditions similar to the pre-exercise biopsy, with the subjects in a fasted state and following 45 min of supine resting between 8.00 and 10.00 am corresponding to 24, 48 and 168 h following exercise termination. Functional assessments (contractile function, muscle soreness and blood samples) were repeated at 24, 48, 72, 96 and 168 h between 8.00 and 10.00 am, in a standardized order and with the subjects remaining in a fasting condition.

**Table 1** Anthropometrics and pre-exercise strength

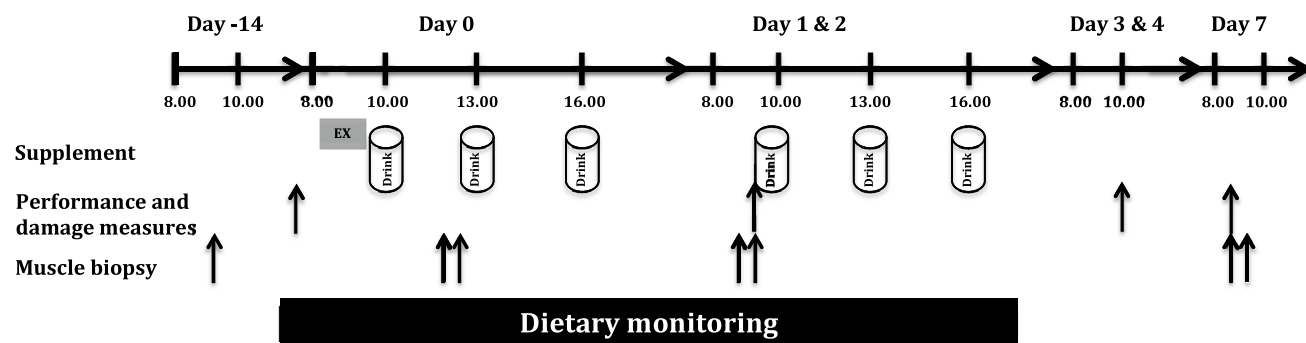
	Group			
	Whey		Placebo	
Height (cm)	181.8	[177.5; 186.1]	181.8	[178.1; 185.4]
Age (years)	22.5	[21.1; 23.9]	24	[22.3; 25.7]
Body mass (kg)	74.2	[67.7; 80.7]	76.8	[71.1; 82.5]
Body fat (%)	12.3	[9.3; 15.3]	14.5	[11.7; 17.2]
Pre-MVC (Nm)	295.2	[276; 314]	289.8	[254; 326]

Anthropometric data and pre-exercise isometric strength (MVC) for the whey and placebo groups, respectively

Data are shown as mean and 95 % confidence intervals in brackets

Exercise protocol

The exercise protocol consisted of  $15 \times 10$  repetitions of maximal isokinetic eccentric contractions for the knee extensors in an isokinetic dynamometer (Humac Norm, CSMI, Stoughton, USA) comparable to previous studies (Crameri et al. 2004, 2007). Knee joint range of motion was set at 70 dg and contraction velocity at 30 dg/s to ensure standardized conditions for all subjects and thereby



**Fig. 1** Schematic presentation of the study timeline. Performance and damage measures included maximal muscle strength, blood samples for serum creatine kinase analysis and muscle soreness. Biopsies were collected from one leg prior to exercise and from both exercise and non-exercise legs at the selected time point during post-exercise

recovery. Exercise (Ex) was conducted as  $15 \times 10$  maximal isolated isokinetic eccentric knee extensions. Supplements (drink) were ingested three times per day on days 0, 1 and 2 and contained either hydrolyzed whey protein + carbohydrate or iso-caloric carbohydrate placebo

comparability between groups in the total work during exercise. Individual dynamometer settings were identical to settings during muscle contractile function testing. During exercise, subjects received standard verbal and visual feedback and encouragement to ensure maximal effort during exercise. Exercise repetitions and sets were interspaced with 3 and 60 s recovery, respectively. Force and work data during exercise were recorded and saved for later off-line analysis.

### Muscle biopsies

Muscle biopsies were obtained under local anesthesia (10 mg/ml lidocaine) from the middle section of the vastus lateralis muscle by applying the Bergstrom needle technique as described previously (Vissing et al. 2011). The samples were dissected free of visible fat and connective tissue and a well-aligned part of the biopsy was immediately mounted in Tissue-Tek (Qiagen, Valencia, CA, USA), frozen in isopentane pre-cooled with liquid nitrogen and stored at  $-80^{\circ}\text{C}$  until further analysis. The remaining crude muscle tissue was immediately frozen and stored at  $-80^{\circ}\text{C}$  until further analysis. For all post-exercise biopsies, the sampling sites were attempted to cover a large area within the middle section of the vastus lateralis muscle to minimize any effect of an ongoing immune and satellite cell response from previous biopsies (Vissing et al. 2005). Biopsies were dispersed with at least 3 cm apart and a similar depth was attempted. By a similar procedure, biopsies corresponding to all post-exercise time points were also obtained from the non-exercise control leg to try to ensure that time effects were not simply related to the effects of previous biopsies or changes in the systemic environment (Vissing et al. 2005).

### Immunohistochemistry

All biopsies were assigned a random unique identification number, thereby blinding the investigator to subject identity and time point. Serial transverse sections (10  $\mu\text{m}$ ) were cut at  $-20^{\circ}\text{C}$  using a cryostat and placed onto Superfrost Plus glass slides (Menzel-Gläser, Braunschweig, Germany) with both control and exercise leg samples from one subject/time point on the same slide. The same person carried out all analysis of a single target to avoid interindividual variation. The immunohistochemical analyses were completed by microscopic evaluation of cryosections stained with the same batches of primary and secondary antibodies.

### Satellite cells, fiber type and myonuclei

Satellite cells were stained as described in detail previously (Mackey et al. 2010). Sections were fixed in Histofix (Histolab, Gothenborg, Sweden) and endogenous peroxidase

was quenched in 0.3 %  $\text{H}_2\text{O}_2$  and 2.5 % horse serum (ImmPRESS, cat. no. MP-7402, Vector Laboratories, Burlingame, CA, USA). This was followed by a 1 h blocking in blocking buffer (0.01 % Triton, 1 % BSA, 1 % skimmed milk, 0.1 % sodium azide). The sections were incubated overnight at  $4^{\circ}\text{C}$  with primary antibody for Pax7 (1:500; cat. no. MO15020, Neuromics, Edina, MN, USA), followed by a biotinylated goat anti-mouse secondary antibody for 45 min (1:200; cat. no. E0433, Dako Norden, A/S, Glostrup Denmark) and Vectastain ABC reagent (cat. no. PK6100; Vector Laboratories). The visualization of the primary antibody binding was completed using diaminobenzidine (DAB) substrate kit for peroxidase (ImmPACT, cat. no. SK-4105, Vector Laboratories). Following this the sections were incubated with primary antibodies for Type I myosin (1:100; cat. no. A4.951, Developmental Studies Hybridoma Bank (DSHB), IA, USA) and laminin (1:100; cat. no. Z0097, Dako Norden) for 2 h and secondary Alexa Flour 568 goat anti-mouse red and Alexa Flour 488 goat anti-rabbit green (Molecular Probes, cat. no. A11031 and cat. no. A11034, Invitrogen A/S, Taastrup, Denmark) antibodies for 45 min. Finally, a mounting media containing 4',6-diamidino-2-phenylindole (DAPI) was utilized to visualize nuclei (Molecular Probes Prolog Gold anti-fade reagent, cat. no. P36935, Invitrogen A/S) and samples were stored at  $-20^{\circ}\text{C}$  until final analyses.

The total number of Pax7 cells was expressed relative to the total number of fibers counted. Furthermore, the number of Pax7 cells associated with type I ( $\text{A4.951}^+$ ) or type II ( $\text{A4.951}^-$ ) fibers was quantified separately and expressed relatively to the total number of type I or II fibers. Additionally SCs were normalized to myonuclei content (SC per 100 myonuclei). The total number of fibers included in the SC analysis is displayed in Table 2.

### Muscle fiber remodeling

Remodeling was assessed by staining of sections for embryonic myosin heavy chain, eMHC (F1.652, DSHB), or neonatal myosin heavy chain, nMHC, (NCL-MHCn, Novocastra, Newcastle upon Tyne, UK) combined with laminin (Dako Norden). The sections were fixed in Histofix (eMHC stain only), followed by 1.5 h in blocking buffer (0.2 % Triton, 2 % BSA, 5 % FBS, 2 % goat serum and 0.1 % sodium azide) and incubated in primary antibody overnight at  $4^{\circ}\text{C}$  (F1.652 + laminin) or for 2 h at room temperature (NCL-MHCn + laminin), followed by secondary Alexa Flour 568 goat anti-rabbit and Alexa Flour 488 goat anti-mouse (Molecular Probes, cat. no. A11011 and cat. no. A11001) antibodies for 1.5 h. The sections were then fixed in Histofix (nMHC stain only) and mounted in media containing DAPI to visualize nuclei. The number of fibers containing one or more central nuclei, as well as fibers positive for embryonic or neonatal

**Table 2** The number of fibers included in the biopsy analyses

	Pax7 analysis		eMHC, nMHC, CLN analyses Mixed fiber	Fiber-type distribution (%)	
	Type I fiber	Type II fiber		Type I fiber	Type II fiber
<b>Whey</b>					
Pre	291 [136; 445]	438 [243; 632]	912 [670; 1,153]	40 [35; 44]	61 [56; 65]
24 h	297 [173; 421]	308 [199; 419]	1,032 [820; 1,244]	49 [39; 59]	51 [41; 61]
48 h	313 [179; 446]	339 [222; 455]	903 [727; 1,078]	47 [37; 58]	53 [42; 64]
168 h	239 [139; 338]	237 [164; 310]	722 [593; 852]	49 [40; 57]	51 [43; 60]
<b>Placebo</b>					
Pre	400 [178; 622]	454 [175; 731]	1,127 [800; 1,454]	47 [39; 56]	53 [44; 61]
24 h	257 [123; 389]	254 [108; 399]	809 [656; 962]	51 [41; 61]	49 [39; 59]
48 h	325 [220; 430]	374 [301; 446]	801 [661; 941]	46 [40; 53]	48 [36; 60]
168 h	374 [253; 495]	359 [205; 512]	818 [596; 1,041]	53 [42; 64]	47 [36; 58]
<b>Control</b>					
Pre	359 [220; 498]	448 [277; 618]	1,019 [827; 1,212]	44 [39; 50]	56 [50; 61]
24 h	262 [198; 324]	262 [198; 324]	824 [654; 994]	52 [46; 57]	48 [43; 54]
48 h	327 [243; 423]	337 [250; 423]	1,041 [829; 1,252]	49 [42; 56]	51 [44; 58]
168 h	360 [258; 462]	367 [277; 457]	881 [749; 1,013]	49 [42; 54]	51 [46; 57]

Displayed is the number of type I (type I) and type II (type II) fibers included in the assessment of SC (Pax7/fiber) content. Since no distinction between fiber types was made for eMHC, nMHC and central nuclei (CLN) analyses, the number includes both fiber types (i.e., mixed fibers). Finally, the time- and group-specific fiber-type distribution (%) is shown. All analyses were performed on cross sections of vastus lateralis muscle biopsies pre and post (24, 48, 168 h) for the eccentric exercise leg in the whey and placebo groups, respectively, and for the non-exercise control leg (collapsed for supplementation). Values are means and 95 % confidence intervals in brackets

myosin heavy chain, was expressed relative to the total mixed fiber number from the sections. The total number of fibers included in the eMHC, nMHC and central nuclei analyses are shown in Table 2.

#### Diet and supplementation

Subjects recorded all energy-containing food and drinks on days 0, 1 and 2 after exercise, while maintaining normal habitual food intake during the recording period. Subjects received a login to an online food registration software program (Madlog.dk Aps, Kolding, DK) and received verbal and written information on the usage before day 0. During the later off-line analysis, the dietary intake was analyzed for macronutrient distribution and total energy intake.

Supplementary drinks contained 952 kJ in an 8 % solution consisting of 28 g whey protein hydrolysate high in leucine (4 %) + 28 g of carbohydrate (4 %) or 56 g of carbohydrate (8 %). All drinks were identically flavored with a non-caloric flavoring product. The carbohydrate source was a standard monosaccharide glucose product. The hydrolyzed whey protein (Arla Foods Ingredients Group P/S, Viby J., Denmark) contained 27.7 % BCAA (leucine 14.2 %, isoleucine 6.6 %, valine 6.9 %) and 53.3 % essential amino acids [amino acid/peptide profile reported previously (Farup et al. 2013)]. The total additional supplementary protein ingestion was 84 g/day and the BCAA ingestion was 23.0 g/day for the whey group.

#### Muscle contractile performance measures

Subsequent to a standardized warm-up consisting of 3 min of low-intensity exercise on a stationary ergometer cycle (Monark, Varberg, Sweden), the subjects were seated in an isokinetic dynamometer (Humac Norm, CSMI, Stoughton, USA) as previously described (Farup et al. 2012). Isometric maximal voluntary contraction (MVC) was measured at 70° knee flexion (0° equals full extension). Subjects were allowed four trials (however, if a subject continued to improve, additional trials were provided) and all contractions were interspaced with 1-min recovery. Before each trial, a verbal instruction to contract as “fast and forcefully as possible” was given. Subjects were not allowed to use a counter-movement (stretch–shortening cycle movement) before exerting a maximal knee extension. All trials were sampled at 1,500 Hz. The off-line analyses were performed in custom-made software (Labview 2011, National Instruments Corporation, TX, USA). MVC was determined as the highest peak torque from the best trial and this was used for further analysis.

#### Muscle soreness

Muscle soreness was evaluated for both the exercise and control leg in a standardized fashion before the exercise bout and 24, 48, 72, 96 and 168 h following exercise and was always conducted as the first test. Subjects were asked

to rise from a seated chair position and slowly lower back onto the chair while only using the leg to be evaluated. Subjects evaluated knee extensor muscle soreness on a visual analog scale (VAS) of 100 mm going from no pain at all (0 mm) to worst possible pain (100 mm), which has been previously described as a method for identification of pain (Bijur et al. 2001). All VAS scores were later analyzed by a blinded investigator.

#### Serum CK

Blood samples were collected before exercise and 24, 48, 72, 96 and 168 h following exercise. Blood was obtained from the antecubital vein into a 10 ml blood collection tube and allowed to clot at room temperature. The whole blood was centrifuged at 1,500g for 10 min at 5 °C, after which serum was divided into Eppendorf tubes and stored at −80 °C. Serum samples were analyzed for creatine kinase content by use of a commercial kit applied in a multi-analyzer system (Cobas c 311/501, Rotkreuz, Switzerland).

#### Date presentation and statistical analysis

Following check for normality of distribution and tests of equal variance, data were expressed as mean  $\pm$  SEM in figures or mean with 95 % confidence intervals in bracket in text and tables or as individual values and median bars for non-parametric data (eMHC and nMHC). CK data were log-transformed for statistical analysis and presented as geometric mean  $\pm$  back transformed SEM or 95 % confidence intervals. Accumulated changes in MVC were calculated by integrating the relative change with respect to time, and differences between groups were evaluated by Student's *t* test.

The effects of time, group and their interactions on dependent variables were assessed using a mixed-effect two-way ANOVA with repeated measures for time. When interactions were observed, linear comparison analysis was used to evaluate differences within and between individual conditions. Since eMHC and nMHC showed a non-parametric distribution, we analyzed the overall effect of time and leg using a Kruskal–Wallis test on ranks and a Wilcoxon–Mann–Whitney test to examine individual differences from pre to post on ranks. As no effect of group or group  $\times$  time was observed for eMHC, nMHC and central nuclei, the data were collapsed for group (supplementation). Significance was set at an alpha level  $\leq 0.05$ . All statistical analysis was performed using Stata (Stata v 12.0, StataCorp LP, TX, USA) and all graphs were designed in GraphPad Prism (Version 6.0, San Diego, CA, USA).

## Results

### Exercise performance and dietary monitoring

The total work performed during exercise was 33.6 [30.5; 36.6] KJ and 31.0 [29.2; 32.9] KJ for the whey and placebo group, respectively. Work performance decreased as the number of exercise sets progressed ( $p < 0.01$ ), with no group differences. The dietary analysis (not including provided supplements) revealed no group difference in the total energy intake and macronutrients' distribution (protein 86.3 [78.9; 93.7] g/day (18 % [17; 19]), carbohydrate 258.3 [237.9; 278.7] g/day (51 % [50; 53]), fat 72.3 [63.5; 81.0] g/day (31 % [29; 33]), energy 8,532.7 [7,869.3; 9,196.0] KJ/day). Protein intake per kg body mass (BM) revealed no group differences (1.16 [1.05; 1.26] g/BM/day).

### SC content

A representative IHC image of fiber type-specific SCs is shown in Fig. 2 and SC content changes in whey and placebo groups are displayed in Fig. 3.

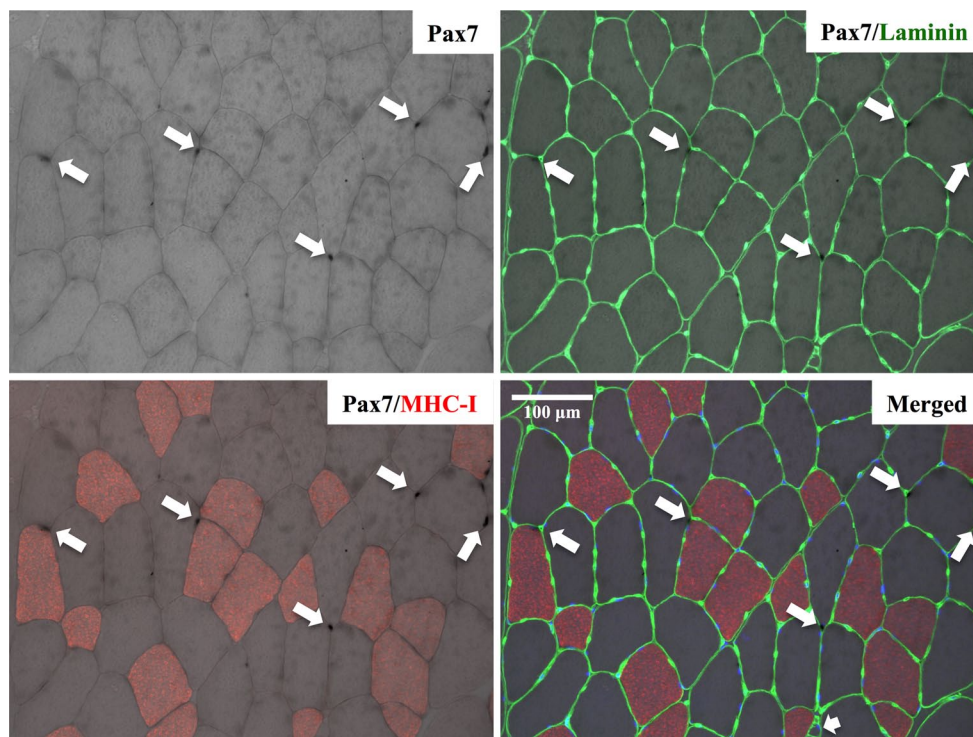
Mixed fiber SC/fiber content increased in the whey group from 0.05 [0.03; 0.08] to 0.10 [0.05; 0.14] ( $p < 0.01$ ) at 48 h, corresponding to a relative increase of 100 %, which was greater than the placebo group ( $p < 0.05$ ) wherein no changes were observed (Fig. 3a). For SC/myonuclei, a similar pattern was observed with an increase in the whey group from 4 % [2; 6] to 9 % [4; 14] ( $p < 0.05$ ) at 48 h, whereas the placebo group showed increase from 5 % [2; 8] to 8 % [4; 12] at 168 h (Fig. 3b).

For fiber type-specific SC evaluation, no group  $\times$  time interactions emerged for SCs associated with type I fiber number (Fig. 3c, d). However, we did note a tendency ( $p = 0.08$ ) toward an interaction when normalizing SC to myonuclei.

In contrast, for SCs associated with type II fibers, a group  $\times$  time interaction ( $p < 0.05$ ) emerged when normalizing to fiber number as well as to the number of myonuclei (Fig. 3e, f). SCs/fiber in the whey group increased from 0.05 [0.02; 0.07] to 0.09 [0.06; 0.12] ( $p < 0.05$ ) and 0.11 [0.06; 0.16] ( $p < 0.001$ ) at 24 and 48 h, corresponding to relative increases of 80 and 120 %, respectively. At 48 h the increase in the whey group was greater than in the placebo group ( $p < 0.05$ ), wherein no changes were observed. SCs/myonuclei revealed a similar pattern with the whey group increasing from 4 % [2; 5] to 10 % [4; 16] ( $p < 0.05$ ) at 48 h, whereas the placebo group increased from 5 % [2; 7] to 9 % [3; 16] ( $p < 0.01$ ) at 168 h.

No interactions or time effects were observed for SCs in the non-exercise control leg and therefore the data are presented as collapsed by group (Table 3).

**Fig. 2** Triple immunohistochemical staining to detect fiber type-specific SC content. Representative image of a muscle cross section stained for Pax7, MCH-I, laminin and nuclei (DAPI). *Arrows* indicate the location of the Pax7<sup>+</sup> SCs, one associated with an MHC-I<sup>+</sup> fiber and four with MHC-I<sup>-</sup> fibers. A very small, possibly regenerating fiber is indicated with a *smaller arrow*



### Remodeling and regenerating fibers

To explore fiber remodeling and/or regeneration, we quantified the presence of fibers expressing either embryonic MHC (eMHC) or neonatal MHC (nMHC) and fibers with one or more centrally located nuclei. No effects of supplement were observed and data were therefore collapsed to investigate only exercise versus control situation.

Prior to exercise, the content of central nuclei was  $1.1 \pm 0.2\%$  at and did not change during post-exercise recovery (Fig. 4a). Only one biopsy contained fibers positive for eMHC prior to exercise and, while the number of positive eMHC fibers did numerically increase at 168 h post-exercise, this was not significant (Fig. 4b). For nMHC fibers, an increase was observed at 24 ( $p < 0.01$ ) and 168 ( $p < 0.01$ ) h in the control leg only and to a greater extent than in the exercise leg at 168 h ( $p < 0.05$ , Fig. 4c). Representative images of eMHC and nMHC positive fibers are shown in Fig. 4d, e, respectively.

### Muscle function

MVC decreased by  $23 \pm 4\%$  and  $27 \pm 3\%$  at 24 h following exercise ( $p < 0.001$ ) for whey and placebo groups, respectively, with gradual return to baseline during the remainder of the observed time course and remained depressed at 168 h ( $p < 0.001$ , Fig. 5a). There were no group  $\times$  time interactions. The accumulated decrease in MVC (integration of relative decrease in MVC with

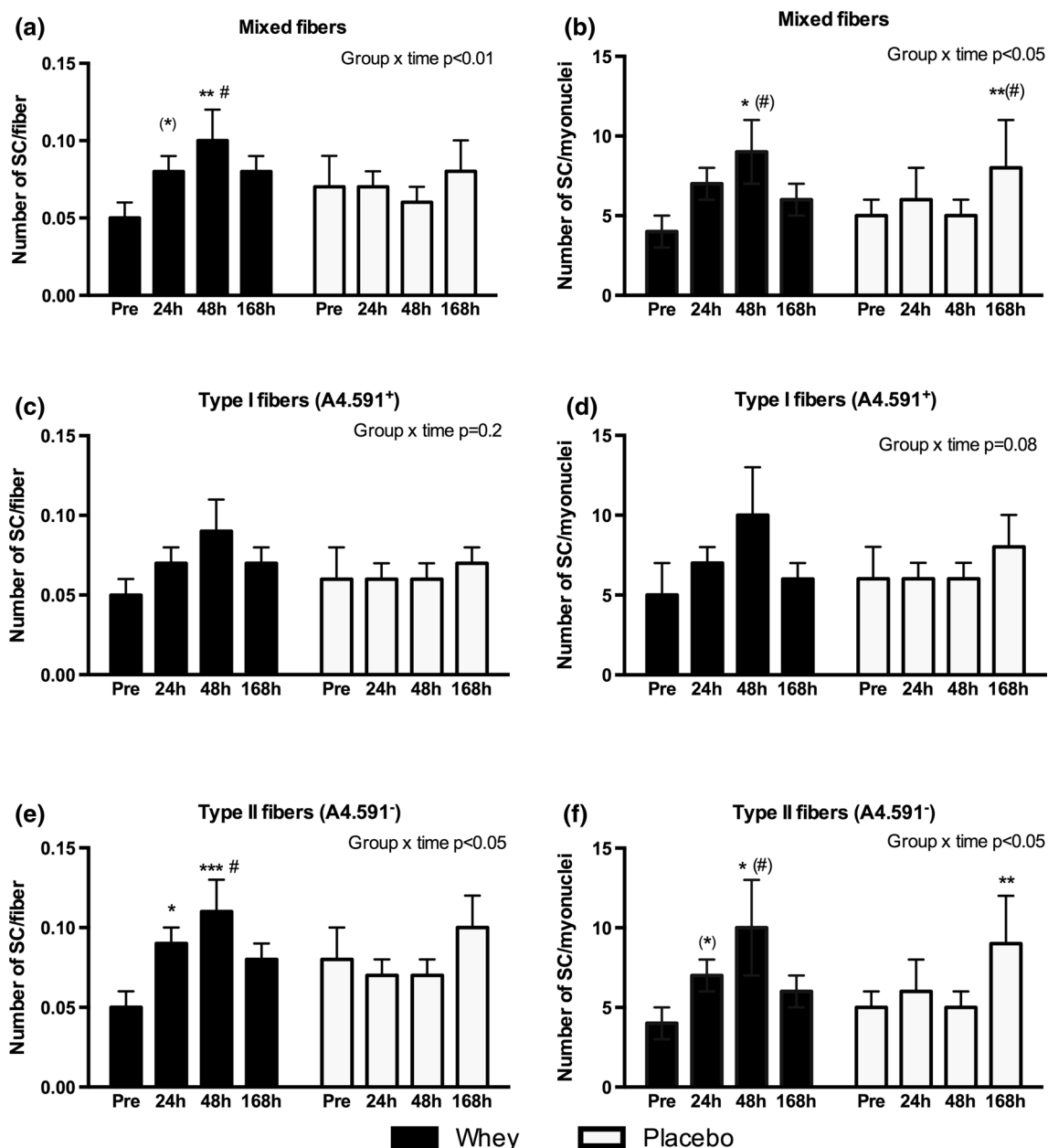
respect to time) was  $-2,272 \pm 588$  [ $-3,424$ ;  $-1,120$ ] and  $-3,212 \pm 645$  [ $-4,476$ ;  $-1,948$ ] (% h) for whey and placebo, respectively, with no group differences observed.

### Muscle soreness

With regard to muscle soreness in the exercise leg, a group  $\times$  time interaction ( $p < 0.05$ ) was noted. While both groups showed a peak in muscle soreness at 24–48 h ( $p < 0.001$ ) and this remained elevated at 96 h ( $p < 0.05$ ), a higher VAS score emerged in whey compared to placebo ( $p < 0.05$ , Fig. 5b) groups at 96 h. Complete return to baseline was observed at 168 h for both groups. In the non-exercise control leg, a time effect ( $p < 0.001$ ) was also noted, with a time course similar to the exercise leg and with no differences between dietary supplementation types (Fig. 5c). At all time points (24–96 h), the magnitude of soreness was greater in the eccentric than the control leg ( $p < 0.001$ ).

### Serum CK

Pre-level serum CK levels were  $135.8$  [ $83.9$ ;  $219.9$ ] IU/L and  $105.2$  [ $74.4$ ;  $148.7$ ] IU/L for the whey and placebo groups, respectively. At 24 h, CK levels were equally elevated by  $477.7$  [ $318.7$ ;  $716.0$ ] IU/L and  $502.5$  [ $318.7$ ;  $880$ ] IU/L for whey and placebo ( $p < 0.001$ , Fig. 5d) groups, respectively, and remained elevated for all post-testing time points ( $p < 0.001$ ), however, with no group  $\times$  time interaction.



**Fig. 3** Fiber type-specific SC content following exercise-induced muscle damage. Satellite cells (SCs) in mixed (a, b) type I (c, d) and type II (e, f) fibers are shown as expressed per fiber (a, c, e) or per 100 myonuclei (b, d, f) in biopsies obtained prior to and at 24, 48 and 168 h following eccentric exercise with either hydrolyzed whey protein + carbohydrate (whey) or iso-caloric carbohydrate (placebo)

supplementation. Data are shown as mean  $\pm$  SEM. Overall interactions are displayed in the upper right corner. Significant differences from pre are denoted by \*\* ( $p < 0.05$ ), \*\*\* ( $p < 0.01$ ) or \*\*\*\* ( $p < 0.001$ ) and tendencies by \* ( $p < 0.1$ ). Group differences within time are denoted by # ( $p < 0.05$ ) and tendencies by # ( $p < 0.1$ )

## Discussion

The primary myogenic stem cells, i.e., the SCs, are regarded important for skeletal muscle regeneration, remodeling and hypertrophy processes, wherefore a functional pool of SCs may be vital for muscle health and function (Fry et al. 2014; Parise 2013; Yin et al. 2013). The potential impact of ergogenic aids to reinforce SC

proliferation and muscle regeneration processes is therefore of great interest in settings of impaired regeneration and remodeling during aging and disease (Conboy et al. 2005; He et al. 2013). The present study is the first to investigate the effects of protein supplementation on the SC response to high-intensity eccentric exercise. Our main finding indicates that supplementation with whey protein, high in leucine, during post-exercise recovery accelerates

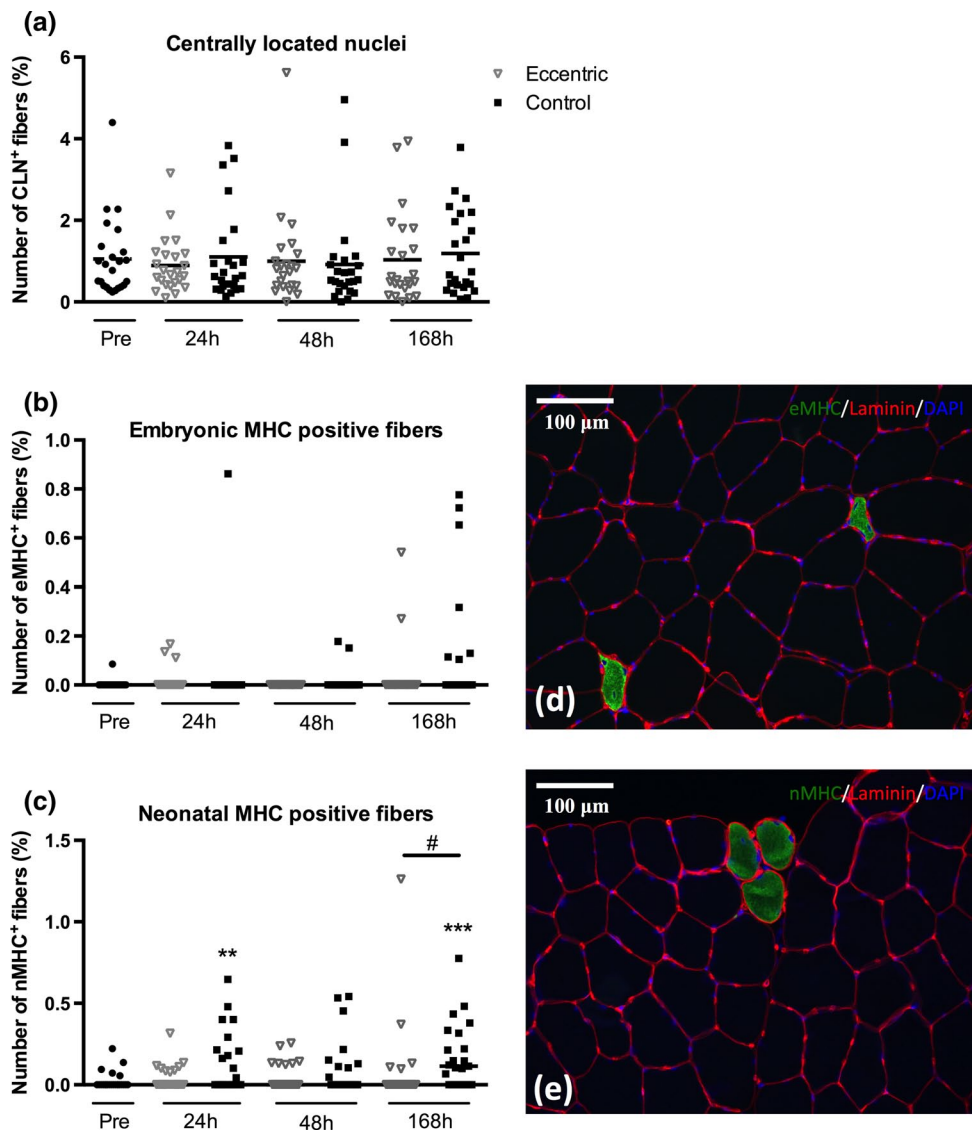


**Table 3** SC content in the non-exercise control leg

	Satellite cell/fiber		Satellite cell/myonuclei	
	Type I fiber	Type II fiber	Type I fiber	Type II fiber
Pre	0.06 [0.04; 0.08]	0.07 [0.05; 0.10]	5 [3; 7]	5 [3; 6]
24 h	0.06 [0.05; 0.07]	0.08 [0.06; 0.10]	6 [4; 7]	7 [5; 8]
48 h	0.07 [0.05; 0.09]	0.09 [0.05; 0.13]	8 [5; 10]	8 [4; 11]
168 h	0.08 [0.05; 0.11]	0.09 [0.06; 0.12]	7 [5; 10]	8 [5; 10]

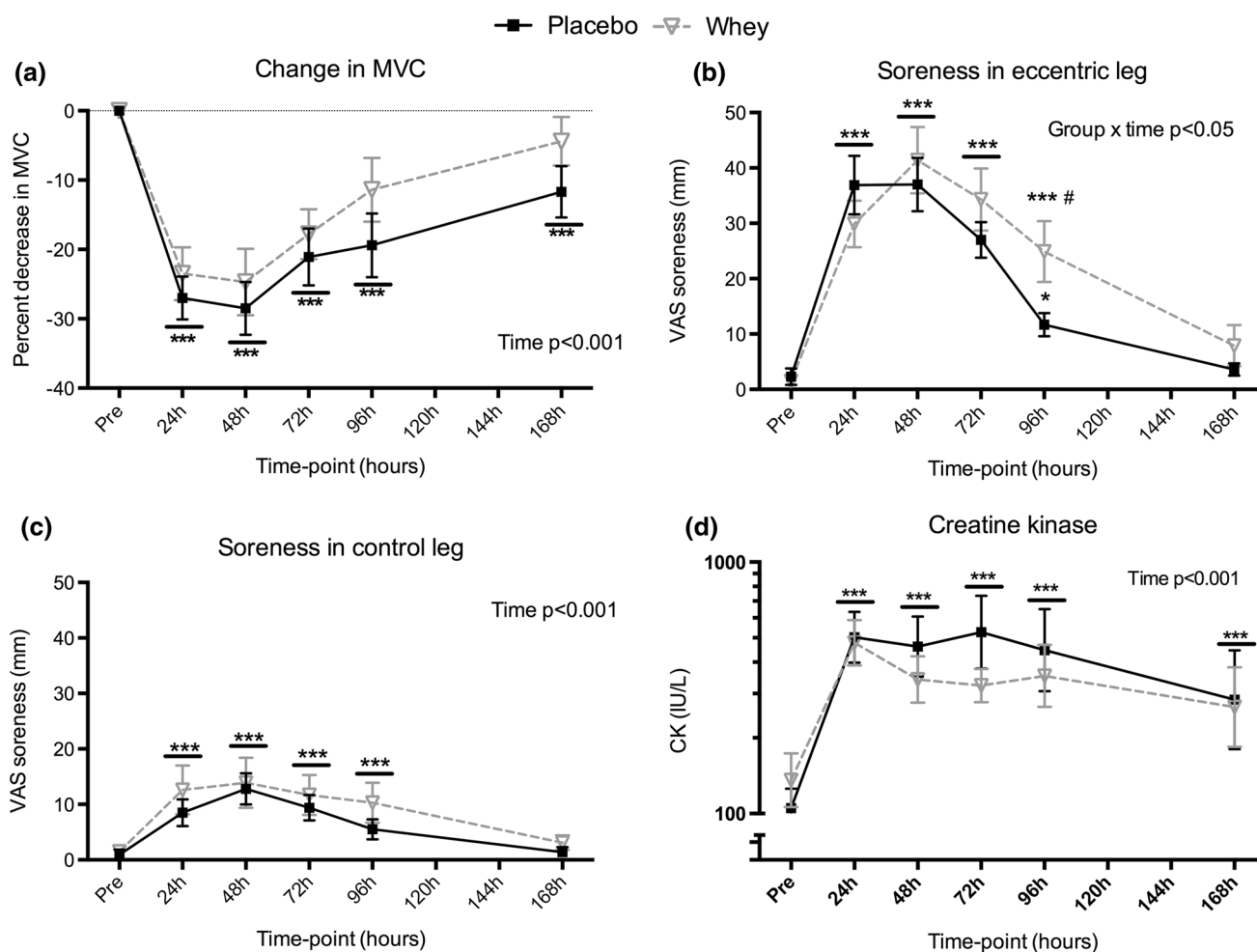
SC content in the non-exercise control leg (collapsed by supplementation groups) normalized to either fiber number or myonuclei  
 Data are presented as mean and 95 % confidence intervals in brackets. No significant changes were observed

**Fig. 4** Muscle fiber regeneration. Fibers containing one or more central nuclei (a), expressing embryonic myosin heavy chain (eMHC, b) or neonatal myosin heavy chain (nMHC, c) relative to fiber number (%) in biopsies obtained prior to and at 24, 48 and 168 h following eccentric exercise (eccentric) or non-exercise control (control) leg combined with ingestion of either hydrolyzed whey protein + carbohydrate or isocaloric carbohydrate supplementation. No supplementation effect was observed and data were collapsed for groups. Data are shown as individual changes and mean (a) or as individual changes and median (b, c). Significant difference from pre are denoted by \*\* ( $p < 0.01$ ) or \*\*\* ( $p < 0.001$ ). Leg differences are denoted by # ( $p < 0.05$ ). Representative images of eMHC and nMHC positive fibers combined with laminin and nuclei (DAPI) are displayed in d, e, respectively



the expansion of the SC pool, when compared with an isocaloric placebo supplement. This difference was driven by increases in the SCs associated with type II fibers, supporting that repair and remodeling requirements primarily adhere to type II fibers with this type of exercise.

Furthermore, no changes in SCs were observed in a contralateral non-exercise control leg, which rules out the effect of non-exercise stressors related to the biopsy procedures, as well as a direct supplementation effect without prior exercise.



**Fig. 5** Muscle strength, soreness and serum CK following eccentric exercise. Relative change are shown for exercise leg maximal voluntary contraction (MVC) (a), muscle soreness (visual analog scale from 0 to 100 mm [VAS (0 = no pain at all)]) from exercise (b) and control leg (c) and serum creatine kinase (CK) (d) at time points 24, 48, 72, 96 and 168 h following eccentric exercise with either hydrolyzed whey protein + carbohydrate (whey) or iso-caloric carbohydrate (placebo) supplementation. Data are shown as mean  $\pm$  SEM

on a linear scale for MVC and VAS and geometric mean  $\pm$  back-transformed SEM on a  $\log_{10}$  scale for CK. The overall effects of time are shown in upper or lower right corner. Significant differences from pre-exercise are denoted by \* ( $p < 0.05$ ), \*\* ( $p < 0.01$ ) or \*\*\* ( $p < 0.001$ ) and bars indicate similar time effects for both groups. Significant group differences are denoted by # ( $p < 0.05$ )

#### Effect of whey protein supplementation on fiber type-specific SCs during recovery from eccentric exercise

Expansion of the SC pool following severe skeletal muscle injury has been demonstrated in several animal models (Brack and Rando 2012; Lepper et al. 2011; Yin et al. 2013), but also in humans a substantial increase in SC content has been observed subsequent to eccentric exercise-induced regeneration/remodeling (Mackey et al. 2010, 2011; Paulsen et al. 2012). Recently, results from Cermak et al. (2012) suggested that the increase in SCs after eccentric exercise was predominantly related to SCs associated with type II fibers, which may be explained by type II fiber susceptibility to sarcolemma damage induced by eccentric exercise (Vijayan et al. 2001). The results of the present

study support these findings, by showing that SC accumulation was primarily evident in type II fibers in both the whey group and the placebo group. A numerical increase was observed in SCs associated with type I fibers, but this did not reach statistical significance. One possible explanation for the fiber type-specific regulation of SCs following eccentric exercise could adhere to a selective recruitment of high threshold motor units (i.e., type IIa and IIx fibers) during maximal eccentric contractions. In relation to this hypothesis, mechanical strain has been suggested, from both in vitro and in vivo animal studies (Tatsumi et al. 2001; Urciuolo et al. 2013), to activate and regulate SC proliferation. Moreover, the greater magnitude of sarcolemma damage in type II fibers (Vijayan et al. 2001) could constitute an additional signal for the SCs in the type II fibers

to break quiescence and promote migration (Bischoff 1990; Montarras et al. 2013).

The observation that whey protein supplementation can accelerate the exercise-induced increases specifically in type II fiber SC content is both interesting and logical. Very little investigation has been conducted on the acute effects of protein ingestion on SC responses in human muscle. In one study from Snijders and co-workers (2013), it was observed that a single bout of traditional resistance exercise (i.e., not an eccentric only exercise protocol) produced no difference in SC pool expansion between a very low (0.1 g/kg BW/day) and a normal (1.2 g/kg BW/day) dietary protein condition. In this regard, it should be noticed that all subjects in the present study ingested adequate amounts of protein in their habitual diet (Burke and Deakin 2000), and that energy and macronutrients inherent in the supplements were added to this habitual intake. The extra supplementary energy intake amounted to 2,856 kJ/day in both groups; however, because of the fasting conditions (from 22.00 to 8–10.00 i.e., 8–10 h) on days 0, 1 and 2, the total energy intake was still within the range of a normal diet.

The increased type II fiber-associated SC content in the whey group may relate to the fact that our supplement was rich in BCAAs and in particular leucine known to directly stimulate mTORC1 and the downstream signaling proteins associated with this kinase complex (Dibble and Manning 2013; Dickinson et al. 2011). In this regard, it is interesting that during quiescence the SC metabolism is characterized by an anaerobic energy production and associated with a low mTOR activity (Montarras et al. 2013). In contrast, during proliferation a shift toward a more aerobic metabolism takes place which is associated with an increased mTOR activity to initiate mRNA translation and de novo nucleotide synthesis (Dibble and Manning 2013). We therefore speculate whether the high leucine content may have served to stimulate mTOR activation in the SCs. This speculation is in accordance with in vitro findings where leucine promoted proliferation of SCs (Han et al. 2008). In addition to stimulating proliferation, leucine availability may also direct SCs toward the myogenic lineage, since leucine restriction, in vitro, is shown to regulate the expression of MyoD (Averous et al. 2012).

In the placebo group, our eccentric exercise protocol did not produce increases in SCs/fiber, although a numerical increase was noted. In contrast, an increase was observed in SC/myonuclei in type II fibers at 168 h (7 days) in the placebo group, which was not evident in the whey group. This was somewhat surprising, given that such increases in SCs 24–48 h post-exercise have previously been observed without supplementation (McKay et al. 2009, 2010). However, in other previous studies, which did not include protein supplementation, SCs have been observed to accumulate later in the recovery phase [i.e., 4–8 days after eccentric

exercise (Cramer et al. 2004, 2007)]. A few subjects in the placebo group had high type II fiber SC content prior to exercise; however, they responded identically to other subjects in the placebo group and maintained the high SC content throughout the study period and were therefore not considered outliers.

Another source of error we cannot exclude is that our findings are partly related to random variation between biopsies. On the other hand, in an effort to reliably enumerate SC content, SCs were counted from a high number of fibers (i.e., >450 at all time points), which has previously been reported to be of importance (Mackey et al. 2009).

In an effort to explore the extent of myofiber regeneration and remodeling at the fiber level, we quantified fibers expressing eMHC, nMHC and centrally located nuclei, often considered hallmarks of regeneration. We found no changes in either intervention group or leg (i.e., exercise versus control leg) for centrally located nuclei. This is in accordance with previous human exercise trials (Mackey et al. 2007; Mikkelsen et al. 2009) and could be related to the relatively mild muscle damage inflicted from voluntary eccentric contractions (Cramer et al. 2007) compared to involuntary, electrically stimulated eccentric contractions (Cramer et al. 2007). For nMHC we observed a time effect in the control leg, indicating a potential repeated biopsy effect. In support of this speculation, performing repeated biopsies in the same muscle within a short time frame has been shown to induce the expression of cell cycle-related transcription factors as well as IL6 and IL8 mRNA (Moller et al. 2013; Vissing et al. 2005), collectively supporting the importance of introducing a non-exercise control group/leg when performing repeated biopsies. Alternatively, since very few positive fibers expressing eMHC and nMHC were found, these somewhat minor increases may also be related to random variation in the biopsy samples.

#### Effect of whey protein on indices of muscle recovery following eccentric exercise

Eccentric exercise has been consistently shown to induce muscle soreness, increased serum CK levels and decreased MVC in the days following eccentric exercise (Mikkelsen et al. 2009; Newham et al. 1987; Proske and Allen 2005; Vissing et al. 2008). This muscle soreness and loss of muscle force often persist for several days or even sometimes weeks (Proske and Allen 2005), depending on the extent of muscle damage (Paulsen et al. 2012). Therefore, medical or ergogenic substances to accelerate recovery rate is of great interest (Michailidis et al. 2013; Pasiakos et al. 2014). Protein supplementation can be speculated to constitute one such strategy to accelerate recovery rate, e.g., by modulating the activity (e.g., chemotaxis and phagocytosis) and cytokine expression from leukocytes (e.g.,

neutrophils) (Rusu et al. 2009). Interestingly, these seem to be influenced by the protein content of  $\beta$ -lactoglobulin and  $\alpha$ -lactalbumin (Rusu et al. 2009) which are often found high in whey protein products. Moreover, whey protein ingestion is known to increase muscle protein synthesis both immediately (1–5 h) and 24 h post-resistance exercise (Atherton and Smith 2012; Burd et al. 2011), which could support the remodeling processes following eccentric exercise (Yu et al. 2004) and provide another mechanism for an increased recovery rate. However, as for the latter, it is difficult to comprehend if an increased protein synthesis could augment muscle recovery rate during the early recovery phase [as observed by some (Buckley et al. 2010)], but potentially in the later phase of muscle recovery (i.e., 7 days post-exercise) this could influence MVC. In the present study, while changes and MVC and serum CK favored the contention that myofiber membrane damage had occurred with our eccentric exercise protocol, no differences in the rate of restoration were evident between supplement types. However, in relation to the above-stated potential mechanisms influenced by protein ingestion, it should be emphasized that these indirect measures do not allow for any conclusions regarding myofibrillar/cytoskeletal damage or regeneration. Our findings are in agreement with Jackman et al. (2010), who found no difference between a BCAA and a non-caloric placebo intervention on electrically stimulated MVC. In contrast, Kirby et al. (2012) did observe an effect of leucine supplementation on the regain of MVC. However, this latter effect may be trivial as no differences were observed for functional muscle performance measures (Kirby et al. 2012). It should be acknowledged, though, that the placebo in the present study was iso-caloric, in contrast to previous studies using a non-caloric placebo (Jackman et al. 2010; Kirby et al. 2012), which may limit direct comparison.

In contrast, we did observe a supplement effect on rate of attenuation of muscle soreness. However, unexpectedly, less muscle soreness was observed with placebo compared to whey at 96 h. This observation is in opposition to the study by Jackman et al. (2010) but in agreement with the study by Kirby et al. (2012). As the muscle soreness evaluation is based solely on subjectivity, the exact interpretation of our results may be difficult and these aspects of whey protein or BCAA formulas warrant further investigation.

In summary, we provide evidence that hydrolyzed whey protein may accelerate the exercise-induced increase in SCs during early post-exercise recovery. Furthermore, we demonstrate that this increase is predominantly related to SCs associated with type II muscle fibers. On the contrary, even with the whey protein supplementation accelerating SC proliferation, this was not observed to influence several indices of muscle damage. The present results may have important clinical perspectives in populations where

cues for SC activation and proliferation may be impaired. Therefore, further investigation into the clinical aspects of protein supplement and the effects on SCs are warranted.

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