Influence of exercise contraction mode and protein supplementation on human skeletal muscle satellite cell content and muscle fiber growth.

Jean Farup¹, Stine Klejs Rahbek¹, Simon Riis¹, Mikkel Holm Vendelbo², Frank de Paoli³, Kristian Vissing¹

¹Section of Sport Science, Department of Public Health, Aarhus University, ²Department of Internal Medicine and Endocrinology, Aarhus University Hospital, ³Department of Biomedicine, Aarhus University, and Department of Rheumatology, Aarhus University Hospital, Aarhus, Denmark.

Running head: Exercise mode modulates satellite cell content

Keywords: Hyperplasia; eccentric training; pax7; whey protein

Address correspondence to:

K.Vissing, Section of Sport Science, Department of Public Health, Aarhus University, Dalgas Avenue 4, DK-8000 Aarhus, Denmark; Tel: +45 8716 8178; Fax: +45 87150201 Email: vissing@sport.au.dk
ABSTRACT

Skeletal muscle satellite cells (SCs) are involved in remodelling and hypertrophy processes of skeletal muscle. However, little knowledge exists on extrinsic factors that influence the content of SCs in skeletal muscle. In a comparative human study, we investigated the muscle fiber type specific association between emergence of satellite cells (SCs), muscle growth and remodelling in response to 12 weeks unilateral resistance training performed as eccentric (Ecc) or concentric (Conc) resistance training ± whey protein (Whey, 19.5g protein+19.5g glucose) or placebo (Placebo, 39g glucose) supplementation. Muscle biopsies (vastus lateralis) were analysed for fiber type specific SCs, myonuclei and fiber cross-sectional area (CSA). Following training, SCs increased with Conc in both type I and type II fibers (p<0.01), and exhibited a group difference from Ecc (p<0.05), which did not increase. Myonuclei content in type I fibers increased in all groups (p<0.01), while a specific accretion of myonuclei in type II fibers was observed in the Whey-Conc (p<0.01) and Placebo-Ecc (p<0.01) groups. Similarly, whereas type I fiber CSA increased independently of intervention (p<0.001), type II fiber CSA increased exclusively with Whey-Conc (p<0.01) and type II fiber hypertrophy correlated with whole muscle hypertrophy exclusively following Conc training (p<0.01). In conclusion, isolated concentric knee extensor resistance training appears to constitute a stronger driver of SC content than eccentric resistance training while type II fiber hypertrophy was accentuated when combining concentric resistance training with whey protein supplementation.
INTRODUCTION

Skeletal muscle maintenance is essential for preservation of metabolic health and contractile function, and relies on continuous remodelling of muscle tissue initiated through both extrinsic (e.g. physical activity or dietary supplements) and intrinsic (e.g. effects of endogenous molecules released from various tissues/cells) mechanisms. An essential aspect of muscle remodelling and repair is a functional population of quiescent muscle specific stem cells, commonly referred to as satellite cells (SCs) (7, 46, 68). Since the discovery and proposed role as a resident pool of quiescent myogenic stem cells (40), the SCs have been shown to possess a critical role in animal skeletal muscle regeneration (34, 65), during which SCs are activated, proliferate and differentiate to support regeneration and new myofiber formation. In agreement, several studies on human skeletal muscle, have demonstrated a proliferative SC response during exercise-induced muscle regeneration and remodelling following high intensity eccentric exercise (13, 14, 37, 44), in which the SC cell-cycle progression is regulated by transcription factors, such as the paired box transcription factor 7 (Pax7), known to induce expression of specific myogenic regulatory factors such as MyoD (55, 68).

While the role of SCs during skeletal muscle regeneration is generally accepted, the need for SC involvement in muscle hypertrophy in response to overloading (i.e. resistance exercise) is more debated (7, 24, 27, 41, 49). In earlier rodent studies, an impaired hypertrophic capacity following irradiation was observed to be associated with limited proliferation of SCs and addition of nuclei to existing myofibers (3, 49). However, these results have been challenged by recent study reports demonstrating that 2 weeks of functional muscle overloading (synergist ablation) in rodents can promote hypertrophy despite conditional blockage of Pax7+ SCs (41). Yet, despite no immediate imperative need for SCs and accretion of nuclei to induce myofiber hypertrophy during the initial hypertrophic phase in rodents, other recent findings still render possible, that the SCs may be involved during the later phases of myofiber hypertrophy (2, 24), possibly related to maintaining a constancy in myonuclei domain size (49, 52). Furthermore, as recently shown in rodents, the additional myonuclei generated from activated SCs following resistance exercise may possess functional importance by augmenting the regain...
of muscle mass following a period of muscle atrophy (9). The latter supports the notion that SCs can be regarded as important in promoting and sustaining myofiber hypertrophy.

The importance of SCs in myofiber hypertrophy and/or remodelling is indicated in human exercise studies as well, in which the SC pool has been shown to expand acutely following traditional hypertrophy inducing exercise (i.e. within hours-days; (43, 56)) as well as following accumulated resistance exercise bouts (i.e. after weeks-months; (32, 36, 38, 50, 52, 60)). However, while some studies have reported accretion of myonuclei following resistance training in humans (48, 50, 52) others have failed to observe this accretion (32, 36, 38), which may be related to the relative magnitude of fiber hypertrophy induced by the training protocol (50, 52). Furthermore, whereas traditional resistance training is inherent of both eccentric and concentric muscle actions, the results of some studies indicate, that isolated eccentric contractions may promote greater single fiber hypertrophy than isolated concentric contractions in untrained (29) as well as trained (62) individuals. In particular the type II muscle fibers have been shown to exhibit an augmented responsiveness to the eccentric resistance training (29, 62), a finding which is also common following a period of heavy traditional resistance training (19, 21). We have recently demonstrated that eccentric and concentric resistance exercise may acutely evoke divergent responses in relation to stretch mediated signalling (64) as well as divergent regulation of atrogene/FOXO activity (57) which may collectively influence myofibrillar protein turnover. However, while exhibiting differences in the signalling responses following acute exercise (57, 64) the two muscle actions were equally capable of inducing whole muscle hypertrophy (as measured by magnetic resonance imaging) following 12 weeks resistance training (20). Importantly, it is to our knowledge unknown if the two muscle actions differently regulate satellite cell proliferation and accretion of myonuclei. In relation to this, we speculate that the strain on the extracellular matrix, myofibers and the satellite cells from eccentric muscle actions (13, 14, 35) may more profoundly influence satellite cell proliferation (59) than the stimuli from concentric muscle actions.
In addition to exercise contraction mode, protein supplementation combined with traditional resistance training is known to augment muscle growth (10). Moreover, we recently reported that whey protein supplementation augments whole muscle hypertrophy following both eccentric and concentric resistance training (20). While whey protein is known to stimulate muscle protein synthesis (6), a second mechanism through which whey protein may influence muscle growth is the proliferation of SCs. In regards to the latter speculation, the amino acids leucine is known to stimulate the kinase complex mTORC1 (16) and through this pathway leucine administration may, at least during in vitro conditions, stimulate SC proliferation (28). In vivo, protein ingestion combined with long-term traditional resistance training may also augment skeletal muscle SC content (50). We therefore speculate that protein ingestion combined with eccentric resistance training may comprise a potent regulator of satellite cell proliferation and myonuclei accretion.

The objectives of the present study were firstly; to investigate if eccentric resistance training (i.e. inherent of a high degree of mechanical stress (53) and/or strain (35)) modulates the SC pool differently than concentric resistance training (i.e. the latter being more metabolically demanding (23)). Secondly; to investigate if the SC proliferation is fiber type dependent and whether this would induce donation of nuclei to the existing myofibers. Thirdly; to investigate if intake of whey protein hydrolysate could augment the SC pool expansion, myonuclei accretion and myofiber hypertrophy.

We hypothesized; 1) that eccentric exercise resistance training would induce a greater accumulation of SCs, greater accretion of myonuclei and greater fiber CSA hypertrophy in type II muscle fibers compared with concentric resistance training and; 2) that the fiber type specific response of SCs, myonuclei and fiber CSA would be further augmented with provision of a whey protein hydrolysate supplementation.
METHODS

Ethical approval

All subjects were informed of the purposes 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 (j. no. M-20110003).

Participants

Twenty-two healthy young men were included in the study (mean±SEM; height 181.5±1.5 cm, body mass 78.1±1.8 kg, age 23.9±0.8 years, body fat 16.0±0.9%). Exclusion criteria were; 1) participation in systematic resistance or high intensity training for lower extremity muscles within 6 months prior to participation; 2) a history of musculoskeletal lower extremity injuries; 3) vegans, and; 4) use of dietary supplements or prescription medication that potentially could influence muscle size or satellite cell function.

Experimental design

This 12 weeks training study was conducted in a double-blinded fashion in relation to dietary supplementation. Following inclusion, subjects were randomly allocated into either a whey protein hydrolysate group (Whey, n=11) or an isocaloric carbohydrate placebo group (Placebo, n=11). Regardless of supplementary intake, all subjects performed eccentric (Ecc) training with one leg and concentric (Conc) training with the other (i.e. total legs n=44). Accordingly, the following four interventions were compared; Whey+Eccentric (Whey-Ecc), Placebo-Eccentric (Placebo-Ecc), Whey+Concentric (Whey-Conc) and Placebo+Concentric (Placebo-Conc). The eccentric leg was randomly chosen to be either the dominant (preferred kicking leg) or the non-dominant leg, to exclude any potential pre-training difference between the two. Throughout the study period, the subjects were instructed to maintain habitual physical activity level and dietary intake. Subjects were instructed not to engage in high intensity activities 48 hours before pre and post training tests/measurements, not to consume...
alcohol and to maintain normal habitual dietary intake (to minimize fluid shift). All tests were performed at the same time of the day, pre and post training, to control for potential effects of diurnal and circadian rhythm.

**Resistance training and supplementation**

The details of the resistance training program and peptide/amino acid profile of the hydrolyzed whey protein have been described previously (20). In brief, the subjects completed 33 resistance training sessions during the 12 weeks with each session consisting of 6-12 sets × 6-15 repetitions (with repetition loading equal to repetition maximum), with identical set and repetition numbers for the eccentric and concentric legs. The training exercise consisted of isolated knee extensions performed in a Technogym knee extensor machine (Technogym-Selection line, Technogym, Italy) with all repetitions conducted in a 75-85 dg range of motion and a 2-3 s tempo. All training sessions were closely supervised and monitored to ensure proper execution and loading. The average training load was 11.0±0.8% and 10.3±0.8% greater in the eccentric leg compared to the concentric leg for the Whey and Placebo group, respectively.

On training days the subjects received an 8% solution (663 Kj) drink containing either 19.5 g hydrolysed whey protein + 19.5 g of carbohydrate (Whey group) or 39 g of carbohydrate (Placebo group). Half of the supplement was ingested immediately before exercise and the remaining half immediately after exercise. The hydrolysed 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.

**Sample collection and preparation**

Pre and post training muscle biopsies were obtained under local anesthesia (10 mg/ml lidocaine) from the middle lateral part of the vastus lateralis muscle by applying the Bergstrom needle technique as described previously (63). At pre training, one biopsy was obtained representing the basal level from both legs (randomly distributed between the Ecc and Conc leg), while at post training a biopsy from each leg was extracted. All pre and post training muscle biopsies were obtained between 8am and 12pm following an overnight fast and post
training biopsies were collected three to six days post the final training session. The samples were dissected free of visible fat and connective tissue. A well-aligned portion of the biopsy was immediately mounted in Tissue-Tek (Qiagen, Valencia, CA, USA), frozen in isopentane precooled with liquid nitrogen and stored at -80°C until further analysis.

All biopsies were assigned a random unique identification number, thereby blinding the investigator to subject identity and time-point. Serial transverse sections (10 μm) were cut at -20°C using a cryostat and placed onto Superfrost Plus glass slides (Menzel-Gläser, Braunschweig, Germany) with both pre and post training samples from one subject on the same slide.

Satellite cell and myonuclei analysis

Muscle biopsy sections were fixed in Histofix (Histolab, Gothenborg, Sweden) followed by a 1.5 hour in blocking buffer (0.2% Triton-X, 2% BSA, 5% FBS, 2% goat serum and 0.1% sodium azide). The sections were incubated overnight at 4°C with primary antibody for Pax7 (1:500; cat. no MO15020, Neuromics, Edina, MN, USA), followed by 1.5 hour in secondary Alexa Fluor 568 goat anti-mouse antibody (Molecular Probes, cat no. A11034, Invitrogen A/S, Taastrup, Denmark). Following this, the sections were incubated with primary antibodies for Type I myosin (1:500; cat. no. A4.951, Developmental Studies Hybridoma Bank (DSHB), IA, USA) and laminin (1:500; cat. no. Z0097, Dako Norden) for 2 hours and secondary Alexa Fluor 488 goat anti-mouse green and Alexa Fluor 488 goat anti-rabbit green (Molecular Probes, cat no. A11031 and cat no. A11034, Invitrogen A/S, Taastrup, Denmark) antibodies for 1 hour. 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°C until final analyses. Staining was verified using appropriate negative controls to ensure specificity.

Images were obtained at 20x magnification using a Leica DM2000 microscope (Leica, Stockholm, Sweden) and a Leica Hi-resolution Color DFC camera (Leica, Stockholm, Sweden). The number of Pax7 positive (Pax7+) cells (SCs) associated with type I (A4.951+) or type II (A4.951-) fibers was quantified separately and expressed
relatively to the total number of type I or II fibers and fiber area (SC/mm²). To ensure reliable numbers of SCs, in accordance with Mackey et al. (39), we counted a mean of 246±3 fibers.

Finally, in accordance with Bruusgaard et al. (8), we utilized these sections to quantify total sublaminar nuclei (assumed to be myonuclei) by only counting Pax7 negative nuclei with a visible geometric center within the basal lamina to ensure that non-myonuclei were not counted. The myonuclei content from total type I and type II fibers were enumerated and the SC content was additionally normalized to these.

**Single fiber area and phenotype**

Sections were stained as described above with MHC-I (A4.951, DSHB) and laminin (Dako Norden) to display fiber basal lamina and enable categorization into type I (A4.951⁺) or type II (A4.951⁻) fibers. The sections were visualized and analyzed using a Leica DM2000 microscope and a Leica Hi-resolution Color DFC camera combined with image-analysis software (Leica Qwin ver. 3.2, Leica, Stockholm, Sweden) with the investigator blinded to pre/post samples and subject information. Only fibers cut perpendicularly to their longitudinal axis were used in the determination of fiber size in accordance with Andersen & Aagaard (4). The mean number of fibers used for the area analysis was 280±10.

**Muscle fiber regeneration/remodelling**

Regeneration/remodelling was assessed by staining of biopsy sections for embryonic myosin heavy chain (F1.652, Developmental Studies Hybridoma Bank) or neonatal myosin heavy chain (NCL-MHCn, Novocastra, Newcastle upon Tyne, UK) combined with laminin (Dako Norden). Following 1.5 hour in blocking buffer (0.2% Triton, 2% BSA, 5% FBS, 2% goat serum and 0.1% sodium azide), the sections were incubated in primary antibody overnight at 4°C (F1.652 + laminin) or for 2 hours at room temperature (NCL-MHCn + laminin), followed by secondary Alexa Fluor 568 goat anti-rabbit and Alexa Fluor 488 goat anti-mouse (Molecular Probes, cat no. A11011 and cat no. A11001) antibodies for 1.5 hour and were finally mounted in
media containing DAPI to visualize nuclei. Again staining was verified using negative controls to ensure specificity. The number of fibers positive for embryonic or neonatal myosin heavy chain was expressed relative to total fiber number from the sections.

**Magnetic Resonance Imaging**

The details of this method have been reported previously (20). In brief imaging was performed with a 1.5-T scanner (Philips Achieva, Best, Netherlands) in which the subjects were placed in supine position with the feet entering the scanner first. After an initial frontal survey scan, 50 transversal slices were acquired, with the first slice located 70 mm proximal the femur condyles and the other slices acquired proximally. A T1-weighted, fast spin echo sequence with the following parameters was used: scan matrix = 576x576, field of view = 46x46 cm, number of slices = 50, slice thickness = 7 mm, slice gap = 3 mm, repetition time = 2 seconds, echo train length = 18, number of signal averages = 2, TR = 500 ms, TE = 6.2 ms and pixel size = 0.8x0.8 mm. The later offline analysis was conducted using a free software program (Osirix 4.1.1, Osirix Foundation, Geneva, Switzerland).

Knee extensor muscle CSA was manually outlined at ½ of the femur length corresponding to the area from which the biopsies were obtained and indicative of the overall change in whole muscle size (20).

**Muscle contractile performance**

Subsequent to a standardized warm-up consisting of 5 minutes light aerobic exercise (100 W) on a stationary bicycle (Monark, Varberg, Sweden), the subjects were seated in an isokinetic dynamometer (Humac Norm, CSMI, Stoughton, USA) with 90 dg hip flexion and restraining straps crossing the torso and non-testing leg. The transverse axis of the subject’s knee was aligned with the axis of the dynamometer. Subjects were instructed to grab the chair handles. The dynamometer was adjusted individually so the contact point between the subjects’ leg and the dynamometer arm was 3 cm proximal to the malleolus medialis. Eccentric and concentric muscle strength was evaluated using a knee joint angular speed of 30 dg/s (5-90 dg range of motion, 0 equals full knee extension) and isometric strength was evaluated at 70 dg. All recorded peak moments were
gravity corrected by measuring the passive moment exerted by the gravitational pull on lower leg around the knee joint. The mean peak torque obtained at three trials within each condition was used for further analysis. All trials were sampled at 1500 Hz.

**Data presentation and statistical analysis**

The sample size in the present study was, in part, based on the ability to detect a difference between interventions of 20-40% in myofiber hypertrophy and SC content (alpha=0.05, power=0.8). Utilizing these estimates and standard deviations from previous studies (38, 48, 50), a sample size of 9-12 subjects was sufficient to detect a difference. Following check for normality of distribution and tests of equal variance, data were expressed as mean ± SEM or individual plots and median bars for eMHC and nMHC data. The effect of time (Pre vs. Post), supplementation (Whey vs. Placebo) and contraction mode (Ecc vs. Conc) and their interactions on dependent variables were assessed using a mixed-effect linear model with repeated measures for time and contraction mode by using subject id as a random effect variable. The mixed-effect analyses for all biopsy derived parameters was conducted on calculated pre-post differences. When interactions were observed, linear comparison analysis, extracted from the mixed-effect linear model, was used to evaluate differences between conditions and paired t-test was used to evaluate time effects within conditions. Since eMHC and nMHC data showed a non-parametric distribution we performed a Kruskal Wallis test to examine pre-post differences between groups and Wilcoxon-Mann-Whitney test to examine for differences across time. To describe the association between whole muscle and single fiber cross-sectional area changes, a Pearson product-moment correlation analysis was employed. Alpha level was set to $p \leq 0.05$. All statistical analyses were 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

Fiber type specific SCs

The SC response to the different exercise training modalities and supplementation regimes were quantified from the Pax7 (SCs)/MHC-I/Laminin/DAPI immunofluorescent staining (exemplified in Fig 1). Conc training elicited increases in type I fiber SCs of 132±21% and 78±30% (p<0.001) in Whey and Placebo group (Fig 2-A+C), respectively. This was greater than with Ecc training (p<0.05), where tendencies (p=0.08) towards increases were observed (110±62% and 24±32% in Whey and Placebo group, respectively). For SCs associated with type II fibers, Conc training increased the SC content by 78±26% and 81±43% (p<0.01) in the Whey and Placebo group, respectively, which was greater than Ecc training (p<0.05), which did not evoke any changes (Fig 2-B+D).

The number of SCs per mm$^2$ type I fiber area increased in the Conc leg by 90±16% and 56±23% (p<0.001) in the Whey and Placebo groups, respectively, which was greater than the Ecc legs (p<0.05, Fig 2-E), where no changes were observed. Furthermore, a greater overall increase in the Whey group compared to the Placebo group (p<0.05) was noted. In contrast, no between-group differences were observed in SCs per mm$^2$ type II fiber area, in which we only found an overall training effect (p<0.01) with Conc training increasing by 39±14% and 72±42% and Ecc training increasing by 14±23% and 35±16%, in Whey and Placebo group, respectively (Fig 2-F).

As for SCs/myonuclei (data not shown), a leg x time (P<0.05) and a group x time (p<0.01) interaction was observed in I fibers. Accordingly, the Whey group increased by 109±23% and 113±55% (p<0.001) in the Conc and Ecc legs, respectively, whereas no changes were observed in the Placebo group. Post-hoc analysis revealed a greater overall increase in the Whey compared to the Placebo group (p<0.01) and a greater overall increase in Conc compared to Ecc legs (p<0.05). As for type II fibers, only a tendency towards a leg x time interaction (p=0.06) was observed.
Fiber type specific myonuclei

From the immunofluorescent SC staining we determined the number of myonuclei in a fiber type dependent manner. In accordance with the changes in type I fiber area, we observed an overall accretion of myonuclei in type I fibers (p<0.01) with no differences between groups (Fig 3-A+B). In contrast, in the Whey group, an accretion of myonuclei in type II fibers was observed (Fig 3-C+D) with Conc training (28±9%, p<0.01), while Ecc training did not influence myonuclei content. As a consequence, the increase with Conc training was greater than with Ecc training (p<0.01). Additionally, in the Placebo group, an accretion of myonuclei was observed in type II fibers with Ecc training only (29±8%, p<0.01).

Mean fiber and fiber area frequency distribution

To describe the fiber cross-sectional area (CSA) changes in detail, we quantified fiber type specific mean fiber area and area frequency distribution. As depicted in Fig 4, type I fibers increased similarly with Conc training; 22±6% and 12±5% as well as Ecc training; 14±6% and 16±8% (p<0.001) in the Whey and Placebo group, respectively. In contrast type II fiber CSA increased exclusively in Whey-Conc by 25±7% (p<0.01) and this was significantly greater than both Whey-Ecc (p<0.01) and Placebo-Conc (p<0.05).

Using area frequency plots (Fig 5-A+B), a greater proportion of larger type I fibers were noted as reflected by a right shift of the mean area-frequency curve, in all groups from pre to post training. However, as for type II fibers in the whey group, the Conc post training leg contained a relatively higher quantity of large fibers, which resulted in a right shift of the area-frequency curve compared to the Ecc post training leg (Fig 5-C+D). To examine if these observations differed significantly between the different exercise modes, we quantified the percentage of large (>8000 μm²) and small (fibers >1000 μm² and < 5000 μm²) fibers. As shown in Fig 5-E, the frequency of large type II fibers increased selectively with Whey-Conc from 5±2% to 20±4% (p<0.01), which was greater than with Whey-Ecc (p<0.01), where no changes were observed. In contrast, the proportion of small fibers (Fig 5-F) remained relatively constant with Whey-Ecc (pre 45±9% versus post 45±6%) while a decrease
was noted in the Whey-Conc (pre 45±9% and post 21±4%, p<0.05). Hence the proportion of small fibers was
greater in the Whey-Ecc than the Whey-Conc at post training (p<0.001).

Changes in single fiber CSA versus whole muscle CSA

The changes in whole muscle CSA have been reported by our group previously (20). To investigate whether
single fiber hypertrophy correlated with whole muscle hypertrophy following Conc and Ecc resistance training,
we correlated the relative change in mean and type II fiber CSA with the relative change in whole muscle CSA
(quantified by MRI scans). As shown in Fig 6-A+C, significant correlations were observed in the Conc leg for
both mean fiber (r=0.45, p<0.05) and type II fiber (r=0.56, p<0.01), while for the Ecc leg (Fig 6-B+D), no
association was observed with mean fiber (r=-0.03, p=0.90) or type II fibers (r=0.04, p=0.87).

Muscle fiber regeneration/remodelling

To evaluate muscle fiber regeneration, remodelling and/or de Novo fiber formation, we quantified the content
of embryonic (F1.652) and neonatal (NCL-MHCn) MHC positive fibers (Fig 7-D+E).

Quantification of eMHC⁺ or nMHC⁺ fibers revealed no statistical difference between the four groups and
therefore the groups were collapsed graphically (Fig 7-A+B). An overall tendency towards an increase in
eMHC⁺ fibers (p=0.06) and a significant increase in nMHC⁺ fibers (p<0.05) from 0.0% at pre training to 0.2%
(median) post training was observed.

Dynamic and isometric muscle strength

Maximum torque (Nm) obtained during eccentric, isometric and concentric contractions from each group are
shown in Table 1. Eccentric and isometric strength increased from pre to post training by 13±3% (p<0.001) and
16±4% (p<0.001), respectively, with no effect of contraction mode or supplementation. Concentric strength
showed a group x time interactions (p<0.05) and increased by 13±4% with Conc (p<0.01), whereas no changes
were observed with Ecc.
DISCUSSION

An important aspect of skeletal muscle growth and remodelling processes is related to the content and function of the SCs. Therefore, identification of extrinsic factors, which can modulate SC proliferation as well as SC differentiation, is essential. Furthermore, as much of the current understanding on SCs is founded on in vitro and animal models, these important questions should also be pursued in vivo in human skeletal muscle.

In the present human study, we report on the effects of two extrinsic variables potentially affecting SCs, i.e. exercise contraction mode and dietary supplementation type. Here we report that Conc resistance exercise conducted during a prolonged period represent a potent modulator of skeletal muscle SC content. Accordingly, one main finding was the observation that Conc resistance training constitutes a stronger stimulator than Ecc resistance training for increasing the SC pool in both type I and II fibers. Another main finding was the specific increase in type II fiber CSA when Conc resistance exercise was combined with whey protein supplementation, which was accompanied by accretion of myonuclei. Finally, in opposition with our initial research hypothesis, in which we expected the Ecc resistance training to constitute a superior inducer of SC proliferation and type II fiber hypertrophy (compared to Conc resistance training), Ecc resistance training did not increase SC content or fiber CSA in type II fibers.

Resistance training contraction mode may modulate fiber type specific SC content.

The observation that isolated Conc resistance training can act as a potent inducer of skeletal muscle SC content suggests that eccentric contractions per se are not necessary for increasing the skeletal muscle SC content. This finding is supported by a recent study in which training, characterized by concentric contractions (i.e. high intensity cycling), increased SC proliferation, in particular in fibers co-expressing MHC-I and MHC-II (30). Combined with the results from the present study, this indicates that fiber growth and/or fiber remodelling in response to Conc contractions likely involve the SCs (30). The greater increase in SC content with Conc compared to Ecc resistance training is interesting and may adhere to several mechanisms; one such could be the greater metabolic requirement with concentric compared to eccentric exercise (1) leading to a greater lactate
accumulation during concentric exercise (17), which has been shown to influence SC proliferation in vitro (67).

Alternatively, expression of growth factors, known to influence SC proliferation (e.g. IGF-I (26, 28, 42)), may diverge between muscle actions. In this regard, we have previously shown that transcription of IGF-I (specifically IGF-1Ec), a growth factor associated with SC proliferation and increases in myogenic regulatory factors (42), is more profoundly upregulated following concentric compared to eccentric resistance exercise (64).

When normalising the SC content to fiber area (SC/mm²), Conc resistance training displayed a greater increase in SC/mm² in type I fibers compared to Ecc resistance training, whereas no group differences were observed in type II fibers. The interpretation of this fiber type specific SC regulation is not straightforward. One explanation may relate to different SC subpopulations (e.g. more proliferating SCs at the time of biopsy sampling) for type I compared to type II fiber SCs. Alternatively, it could be speculated that a greater aerobic metabolism associated with type I fibers may increase reactive oxygen species (ROS) production, which again may influence satellite cell activity as shown in vitro (67). As concentric exercise does require more ATP production and glycogen utilization compared to eccentric exercise (23), the former may be more prone to increasing the ROS production. However, these speculations will require more detailed investigation in vivo in humans in order to understand the mechanisms underlying the present study findings.

With regards to Ecc resistance training, the lack of SC pool expansion was in opposition to our initial hypothesis. Accordingly, eccentric contractions in human skeletal muscle are known to induce a mild-to-moderate muscle injury (13, 51) or even no injury (14), which in either scenario is associated with an increased proliferation of SCs in the days post exercise (13, 14, 37, 44, 45). The exercise-induced myofiber damage has been observed to be more prevalent in type II fibers in rodents (61), supported by an increased proliferation of SCs associated with type II fibers in humans 24 hours post eccentric exercise (11). Whether such acute changes can be extrapolated to chronic adaptations with prolonged Ecc training is, to our knowledge, unknown. However, the current results with Ecc resistance training do not seem to support this notion. Although we did not obtain biopsies during the early stages of the training period, with reference to the literature, we can
reasonably assume that the SCs in the Ecc leg have been activated and undergone proliferation during the very early phase of the training period. Furthermore, the accretion of myonuclei in type II fibers, observed in the Placebo-Ecc group, which was accompanied by a tendency (p=0.11) towards an increase in fiber CSA, also supports this notion. Surprisingly, we did not observe any changes in myonuclei or SC content in type II fibers in the Whey-Ecc group. The lack of increase in both myonuclei and SC content in the Whey-Ecc group is, however, in accordance with the lack of hypertrophy of type II fibers in the Whey-Ecc group. Notably, when normalising the SC content to type II fiber area, all groups increased equally, indicating that fiber size may be a significant determinant of the SC content, and which is in accordance with previous findings (58).

The mechanism underlying the lack of increase in SC/fiber following eccentric resistance training, combined with the increase in SC/mm², might relate to an enhanced ability of eccentric exercise to effectively promote SC differentiation by allowing the SCs to enter the myogenic differentiation program (68) and fuse with other myoblasts to form new myofibers. However, it is difficult to provide strong evidence to back this speculation based on an in vivo human model.

Whey protein supplementation may accentuate fiber CSA and SC content in a fiber type specific manner.

The results from our CSA analysis suggest, that while type I fibers exhibit an increase in CSA following resistance training in general, the type II fiber CSA is exclusively increased when Conc resistance training is combined with whey protein supplementation. The differentiated fiber type response is interesting, although somewhat in contrast to the observation that traditional resistance training without protein supplementation predominantly increases type II fiber CSA (21). In this regard, whey protein supplementation has previously been shown to augment muscle hypertrophy induced by traditional resistance training (5, 6, 10, 18, 20).

However, to our knowledge, it is unknown whether type II fibers are more influenced by amino acid availability immediately post exercise compared to type I fibers. Previous studies have indicated that limited amino acid availability in the hours before and after resistance exercise may limit myofiber growth (5, 18). Moreover, a
recent observation in rodents suggests that protein synthesis is more decreased in the faster fiber types compared to the slower fiber types in response to food deprivation (25). Thus, in relation to the results from the present study, we speculate that type I fibers are less dependent on immediate supply of amino acids following resistance exercise, compared to type II fibers.

While whey protein combined with Conc resistance training was observed to increase type II fiber CSA, this was not the case when whey protein was combined with Ecc resistance training in contrast to some earlier studies (29, 62). We have previously shown that prolonged Conc and Ecc resistance training are equally capable of promoting whole muscle hypertrophy when combined with protein supplementation (20), indicating a discrepancy between the findings on whole muscle hypertrophy versus single fiber hypertrophy. The observed changes in type II fiber CSA were reflected in the fiber area frequency analysis, in which the Whey-Conc group displayed an increase in the percentage of larger type II fibers compared to the Whey-Ecc group, wherein the frequency of smaller fibers was maintained. Moreover, the correlation analysis displayed a significant association between myofiber and whole muscle hypertrophy following Conc training, which was not present following Ecc training. A similar finding was recently reported in a human training study on elderly subjects (47) in which whole muscle hypertrophy (evaluated by DXA-scan) was positively associated with single fiber hypertrophy following traditional resistance training, while no association was present following eccentric ergometer training. Based on these findings, it was proposed that intense training, and perhaps in particular eccentric training may promote whole muscle growth by sarcomere addition or hyperplasia, whereas traditional resistance training may primarily increase the fiber CSA (47). Hyperplasia has been demonstrated in rodent and avian skeletal muscle following overload or chronic long-term stretch (33). However, direct evidence for the hypothesis in human skeletal muscle is very limited, since only reports on indirect measures, such as fascicle length or correlation between myofiber and whole muscle hypertrophy, is currently available from in vivo human studies (15, 22, 47, 54).
In an effort to provide information on the extent of regeneration, remodelling and/or de Novo fiber synthesis we quantified fibers expressing eMHC and nMHC, since extensive injury or de Novo synthesis in rodent skeletal muscle is associated with increased expression of these MHC isoforms (12, 31, 66). Prior to initiation of training, very few eMHC and nMHC positive fibers were found and while we did observe an increase with training, these changes were not dependent on the type of intervention. However, as this study only provides data before and after 12 weeks training, this may limit the ability to detect changes in eMHC or nMHC positive fibers. Furthermore, even when positive for eMHC or nMHC, we cannot specifically determine if this is related to regeneration, remodelling or de Novo fiber synthesis.

As for added effects of supplementation on SC content, a greater overall increase in SCs/mm² and SCs/total myonuclei were observed with Whey compared to Placebo supplementation in type I fibers, but not in type II fibers. Currently, the literature on the effects of protein ingestion in combination with resistance training on SCs is very scarce. In one of the few studies, Olsen, et al. (50) observed an increased SC content (no discrimination between fiber types) when combining resistance exercise with protein supplementation compared to resistance exercise alone. However, whether this increase was related to a direct effect of the amino acids on the SCs or indirectly related to the greater fiber hypertrophy from the protein supplementation is not entirely clear. Conversely, Snijders, et al. (56) observed no difference in SC proliferation between a very low and a normal protein intake group 12 to 72 hours following traditional resistance exercise, however, such acute observations are not necessarily indicative of the long-term effects.

In conclusion, unilateral isolated concentric resistance training displayed a greater capacity for increasing the SC pool in both type I and type II fibers compared to eccentric resistance training, independent of supplementary protocol. Conversely, type II fiber hypertrophy was exclusively observed when concentric resistance training was combined with whey protein supplementation, suggesting that both contraction mode and whey supplementation influence type II fiber growth. Finally, while type II fiber hypertrophy appeared to
be influenced by exercise contraction mode and whey supplementation, type I fiber CSA and myonuclei content increased independent of intervention type.
REFERENCES


We thank the participant for their effort in the project. Steffen Ringgard is thanked for assistance with magnetic resonance imaging and Janni Mosgaard Jensen is thanked for assistance in biopsy preparation. Associate professor Bo Martin Bibby is thanked for statistical guidance. The F1.652 and the A4.591 monoclonal antibodies developed by Helen M. Blau were obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by The University of Iowa, Department of Biological Sciences, Iowa City, IA, USA.

GRANTS: The study was funded by Arla Foods Ingredients Group P/S, DK

DISCLOSURES: No conflicts of interest, financial or otherwise, are declared by the authors
Figure legends

Figure 1
Representative image of a muscle cross-section stained for Pax7 (red), MCH-I (green), laminin (green) and nuclei (DAPI; blue). Boxed area is magnified in the bottom panels displaying two Pax7⁺ nuclei (SCs) localized to a type I fiber and type II fiber respectively. Arrows indicates the location of the SCs.

Figure 2
Satellite cells (SCs) associated with type I or II fibers expressed per fiber (A+B), as individual and mean relative change per fiber (C+D) or mm² fiber area (E+F) evaluated from biopsies obtained pre and post 12 weeks of eccentric (Ecc) or concentric (Conc) training combined with either whey protein (Whey) or iso-caloric carbohydrate (Placebo) supplementation. Overall effects are displayed in upper right corner and overall differences between contraction modes (Ecc<Conc) or supplementation types (Whey>Placebo) are shown with “>” or “<” symbols. Significant difference from pre training are denoted by ** (p<0.01) or *** (p<0.001) and tendencies by (*) (p<0.1).

Figure 3
Myonuclei per fiber in type I (A+B) and type II (C+D) evaluated from biopsies obtained pre and post 12 weeks of eccentric (Ecc) or concentric (Conc) training combined with either whey protein (Whey) or iso-caloric carbohydrate (Placebo) supplementation. Data are presented as mean±SEM (A+C) or relative individual change and mean (B+D). Overall effects of time are displayed in upper right corner. Significant difference from pre training are denoted by ** (p<0.01) and significant difference between groups are denoted by ## (p<0.01).

Figure 4
Fiber area (μm²) in type I (A) and type II (B) fibers evaluated from biopsies obtained pre and post 12 weeks of eccentric (Ecc) or concentric (Conc) training combined with either whey protein (Whey) or iso-caloric
carbohydrate (Placebo) supplementation. Data are presented as mean±SEM. Overall effects of time are displayed in upper right corner. Significant difference from pre training are denoted by ** (p<0.01) or *** (p<0.001) and significant difference between groups are denoted by # (p<0.05) or ### (p<0.001).

**Figure 5**

Fiber area frequency distribution shown as mean curves in type I (A+B) and type II (C+D) fibers or as percentage of “large” (> 8000 μm²) type II fibers (E) or “small” (>1000<5000 μm²) type II fibers (F) evaluated from biopsies obtained pre and post 12 weeks of eccentric (Ecc) or concentric (Conc) training combined with either whey protein (Whey) or iso-caloric carbohydrate (Placebo) supplementation. A-D are presented as mean curves and E+F are presented as mean±SEM. Overall effects of time are displayed in upper right corner. Significant difference from pre training are denoted by * (p<0.05) or ** (p<0.01) and tendencies by (*) (p<0.1). Significant difference between groups are denoted by ## (p<0.01) or ### (p<0.001).

**Figure 6**

Correlations and scatter plots of relative changes in whole muscle cross-sectional area (CSA) and relative changes in mixed (A+B) or type II (C+D) fiber CSA evaluated from magnetic resonance imaging and muscle biopsies obtained pre and post 12 weeks of Eccentric (B+D) or Concentric (A+C) training combined with either whey protein or iso-caloric carbohydrate supplementation (data are merged for supplementation). Dashed line denotes the 95% confidence intervals. Pearson correlation coefficients and significance are shown for each correlation.
Figure 7

Fibers positive for embryonic myosin heavy chain (eMHC-A+C) or neonatal myosin heavy chain (nMHC–B+D) relative to total fibers counted (%) evaluated from biopsies obtained pre and post 12 weeks of eccentric or concentric training combined with either whey protein or iso-caloric carbohydrate supplementation. Since no group or leg interactions were observed data are collapsed for group and leg. Data are presented as individual values and median bars. Overall effects of time (or tendencies) are displayed in upper right corner. Images of eMHC (C, green) and nMHC (D, green) positive fibers are obtained from one subject's eccentric post training leg containing high amounts of these fibers.
Table legend

Table 1

Maximum peak torque (Nm) during eccentric (-30 deg/s), isometric (0 deg/s) or concentric (30 deg/s) contractions evaluated as a mean of three trials in an isokinetic dynamometer pre and post 12 weeks of eccentric (Ecc) or concentric (Conc) training combined with either whey protein (Whey) or iso-caloric energetic carbohydrate (Placebo) supplementation. Significant difference from pre training are denoted by ** (p<0.01) or *** (p<0.001).
Figure 1
Figure 5

(A) Whey group type I fibers
(B) Placebo group type I fibers
(C) Whey group type II fibers
(D) Placebo group type II fibers

Type II fibers > 8000 μm²
Leg x group x time p<0.05

Type II fibers >1000 < 5000 μm²
Leg x group x time p<0.05
Figure 6

Concentric leg

Mean fiber

$r=0.45\ p<0.05$

Type II fiber

$r=0.56\ p<0.01$

Eccentric leg

Mean fiber

$r=0.03\ p=0.90$

Type II fiber

$r=0.04\ p=0.87$
Figure 7

(A) eMHC positive fibers

(B) nMHC positive fibers

- Time p=0.07
- Time p<0.05
<table>
<thead>
<tr>
<th></th>
<th>Whey</th>
<th></th>
<th>Placebo</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ecc</td>
<td>Conc</td>
<td>Ecc</td>
<td>Conc</td>
</tr>
<tr>
<td></td>
<td>Pre</td>
<td>Post</td>
<td>Pre</td>
<td>Post</td>
</tr>
<tr>
<td>Concentric (Nm) 30 deg/s</td>
<td>247±10 253±13</td>
<td>247±13 264±15**</td>
<td>257±16 268±18</td>
<td>243±19 291±17**</td>
</tr>
<tr>
<td>Isometric (Nm) 0 deg/s</td>
<td>282±15 299±17***</td>
<td>282±16 330±16***</td>
<td>298±17 329±18***</td>
<td>282±19 341±18***</td>
</tr>
<tr>
<td>Eccentric (Nm) -30 deg/s</td>
<td>349±19 384±20***</td>
<td>347±17 376±21***</td>
<td>364±25 394±25***</td>
<td>310±32 370±32***</td>
</tr>
</tbody>
</table>