ORIGINAL ARTICLE

WILEY

Frequent blood flow restricted training not to failure and to failure induces similar gains in myonuclei and muscle mass

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Funding information
We received no funding for this study.

Abstract

The purpose of the present study was to compare the effects of short-term high-frequency failure vs non-failure blood flow–restricted resistance exercise (BFRRE) on changes in satellite cells (SCs), myonuclei, muscle size, and strength. Seventeen untrained men performed four sets of BFRRE to failure (Failure) with one leg and not to failure (Non-failure; 30-15-15-15 repetitions) with the other leg using knee-extensions at 20% of one repetition maximum (1RM). Fourteen sessions were distributed over two 5-day blocks, separated by a 10-day rest period. Muscle samples obtained before, at mid-training, and 10-day post-intervention (Post10) were analyzed for muscle fiber area (MFA), myonuclei, and SC. Muscle size and echo intensity of *m.rectus femoris* (RF) and *m.vastus lateralis* (VL) were measured by ultrasonography, and knee extension strength with 1RM and maximal isometric contraction (MVC) up until Post24. Both protocols increased myonuclear numbers in type-1 (12%–17%) and type-2 fibers (20%–23%), and SC in type-1 (92%–134%) and type-2 fibers (23%–48%) at Post10 ($p < 0.05$). RF and VL size increased by 5%–10% in both legs at Post10 to Post24, whereas the MFA of type-1 fibers in Failure was decreased at Post10 ($-10 \pm 16\%$; $p = 0.02$). Echo intensity increased by ~20% in both legs during Block1 ($p < 0.001$) and was ~8 to 11% below baseline at Post24 ($p = 0.001$–0.002). MVC and 1RM decreased by 5%–10% after Block1, but increased in both legs by 6%–11% at Post24 ($p < 0.05$). In conclusion, both short-term high-frequency failure and non-failure BFRRE induced increases in SCs, in myonuclei content, muscle size, and strength, concomitant with decreased echo intensity. Intriguingly, the responses were delayed and peaked 10–24 days after the training intervention. Our findings may shed light on the mechanisms involved in resistance exercise-induced overreaching and supercompensation.

KEYWORDS
ischemic exercise, kaatsu, muscle damage, muscle hypertrophy, myogenic stem cells, overreaching, overtraining, satellite cells

1 | INTRODUCTION

Low-load resistance training at 20%–50% of the one repetition maximum (1RM) combined with blood flow restriction (BFR) can be used to achieve hypertrophic and strength responses similar to traditional high-load non-restricted strength training.[1,2] This has applications for individuals who may not tolerate the mechanical stresses associated with higher load strength training, and/or athletes who seek to facilitate muscular development without adding substantially to the total training dose experienced.[1,3]

Blood flow–restricted resistance exercise performed with multiple sets to voluntary failure can induce high ratings of perceived pain, -exertion, and delayed onset muscle soreness (DOMS),[4] as well as other signs of muscle damage in unaccustomed individuals.[5] However, perceived pain seems to be reduced if volitional failure is not reached in the exercising sets.[6] Furthermore, reaching voluntary failure does not seem to be required to promote increases in muscle size and strength during BFRRE[6]; hence, non-failure BFRRE may be more feasible if it efficiently produces increases in muscle size and strength with reduced perceptions of exertion and discomfort. To the best of our knowledge, no previous study has investigated the influence of exertion during short-term high-frequency blocks of low-load BFRRE on the training adaptations and perceptual responses.

The precise mechanisms involved in muscle adaptations to BFRRE are still not completely elucidated. However, satellite cell (SC) activation, proliferation, and subsequent addition of new myonuclei have recently been implicated in the hypertrophic response observed with low-load BFRRE.[3,4,7] We recently investigated if a rest period could reset the responsiveness of the myofibers after an initial high-frequency (twice daily) training block similar to Nielsen et al,[7] and consequently allow the muscle fibers to respond to a second block of BFRRE by further increases in SCs, myonuclei, and hypertrophy.[4] In line with the findings by Nielsen and coworkers, we observed robust increases in SC and myonuclear numbers, along with gains in muscle size and strength.[4]

Importantly though, our results also suggested that these processes can be delayed with very strenuous high-frequency BFRRE, as the increases in SC, myonuclei, muscle size, and strength did not peak until 10–20 days after the last training session. Furthermore, we observed prolonged reductions in strength (>5 days), relatively high overall increases in circulating markers of muscle damage, and transient muscle fiber atrophy despite increases in muscle thickness after the initial week of frequent BFRRE, altogether indicating excessive myofiber stress. The discrepancies between our findings[4] and Nielsen et al.[7] may originate from a greater degree of effort and a higher training volume in our study, especially during the first week of training (80 vs 52 repetitions per session).

Therefore, the purpose of the present study was to compare the effects of a failure versus a non-failure BFRRE protocol on myonuclear and SC numbers as well as muscle size and strength, and the time courses of the changes, during two blocks with high-frequency BFRRE separated by 10 days of rest. We hypothesized that the non-failure protocol would induce larger increases in myofiber areas and in the numbers of SC and myonuclei after the first training block compared to the failure protocol.

Intriguingly, the SC, myonuclear responses and myofiber hypertrophy in Nielsen et al.[7] appeared to plateau already after one week of training (seven sessions), with no further increases after two subsequent high-frequency blocks. Therefore, we also hypothesized that the 10-day rest period would resensitize the myofibers, so that in contrast to the plateau observed after the first seven training session by Nielsen et al.,[7] further increases in whole muscle areas, myonuclear, and SC numbers would be expected to take place with the second training block.

2 | METHODS

2.1 | Participants

Eighteen healthy untrained men were recruited from the University of Agder in Kristiansand; Norway. To be included in the study, participants should not have participated in regular strength training (>1 session every week) of their thigh muscles the last 6 months or had any prior experience with BFRRE. The participants were instructed to refrain from strenuous activities and maintain their usual food intake during the intervention. Exclusion criteria were any injuries in the musculoskeletal system that could prevent the participants from training or testing, as well as any use of medication and/or supplements (eg, protein powder, vitamins, creatine, NSAIDs, etc.). The study complied with the standards set by the Declaration of Helsinki and the ethical committee of the Faculty of Health and Sport Sciences at the University of Agder, as well as the Norwegian Centre for Research Data, approved the study. The nature and goals of the study were thoroughly explained, and all participants provided a written informed consent.

2.2 | Study design

The present study was conducted as a controlled experiment with stratified randomization (stratified based on 1RM test results) between the right and left leg of the participants, to train with a failure protocol with one leg, and the other leg to a non-failure protocol. A total of 14 low-load BFRRE sessions were conducted, including seven BFRRE sessions in 5 days

during the first block, followed by 10 days of rest, and then additionally seven more BFRRE sessions in the next 5 days (Figure 1). One BFRRE session per day was performed the first 3 days of each training block, and then two sessions per day the following two days, separated by at least 4 hours.[7] Subjects received 20 g of whey protein (Proteinfabrikken AS, Stokke, Norway) after every training session to ensure sufficient protein balance and maximize the hypertrophic response to the training. One week prior to the intervention period, subjects underwent a familiarization session in which they practiced knee extensions (without BFR) and strength tests. Machine settings (seat position) were noted and standardized for each participant. Muscle biopsies were obtained from *m. vastus lateralis* (VL) from both legs at baseline, 2 hours after the first BFRRE session ("Acute1", Day1), at rest during the Rest Week ("Rest Week": five days after the 7th BFRRE session) and 10 days after the second training block ("Post10"). Participants arrived fasted in the mornings before muscle biopsies were obtained and were served a standardized breakfast consisting of oatmeal (0.16 g protein/kg bodyweight) mixed with sugar (6 g) and water 2 hours before the biopsies were sampled. Increases in the blood concentrations of creatine kinase and myoglobin and decrements in force can be used as indirect markers of myocellular stress and "overreaching".[8,9] Furthermore, increases in muscle echo intensity on ultrasound images may be an indicator of edema-related muscle swelling[10] and/or fibrosis,[11] whereas others have suggested that decreases in echo intensity reflect an improved muscle quality.[12] Consequently, blood samples and regular measurements of muscle strength and echo intensity were included in this study.

Muscle function, muscle size, and echo intensity were evaluated at baseline, during the Rest Week, and at 3, 10, 17, and 24 days after the second BFRRE block ("Post3" to "Post24", Figure 1). Muscle size and echo intensity (by

ultrasonography) were also measured every other training day before the training sessions, and muscle soreness, perceived pain, and -exertion were registered every training day and at the Rest Week. In addition, maximal voluntary isometric contraction (MVC) was evaluated one hour before and two hours after BFRRE at Acute1 (Day1) and Acute2 (Day15). Measures of body composition were performed with the subjects in a fasted state at baseline with InBody 720 (InBody body composition analyzer, Biospace, Korea). Each test was conducted by a single investigator and done in the same order during each visit to maintain consistency.

2.3 | Blood flow restricted resistance exercise

Training was conducted in a unilateral knee extension machine (G200 Knee Extension, David health solutions LTD, Helsinki, Finland) at 20% of 1RM. The non-failure exercise protocol consisted of 75 repetitions distributed over four sets (30, 15, 15, 15), while the failure protocol consisted of four sets performed to voluntary failure. The right leg was exercised before the left with a 5-minute break in between. A 145-mm-wide pressure cuff with a 12-cm bladder (9-7359-003, Delfi Medical, Vancouver BC, Canada) was placed around the proximal part of the thigh and inflated by a tourniquet system (Zimmer A.T.S. 2000, Warsaw, IN, USA) to 100 mm Hg before initiation of BFRRE (similar to Bjørnsen et al.[4] and Nielsen et al.[7]). The pressure was adjusted continuously during the exercise to remain at 100 mm Hg. We have previously observed a reasonably narrow range (54%–64%) of the pressure needed to fully occlude blood flow of a. femoralis superficialis with this same equipment and pressure in young participants.[4] Repetitions were performed with a 1 second concentric phase and a 1 second eccentric phase. Warm up consisted of 15 repetitions of 5 kg resistance performed
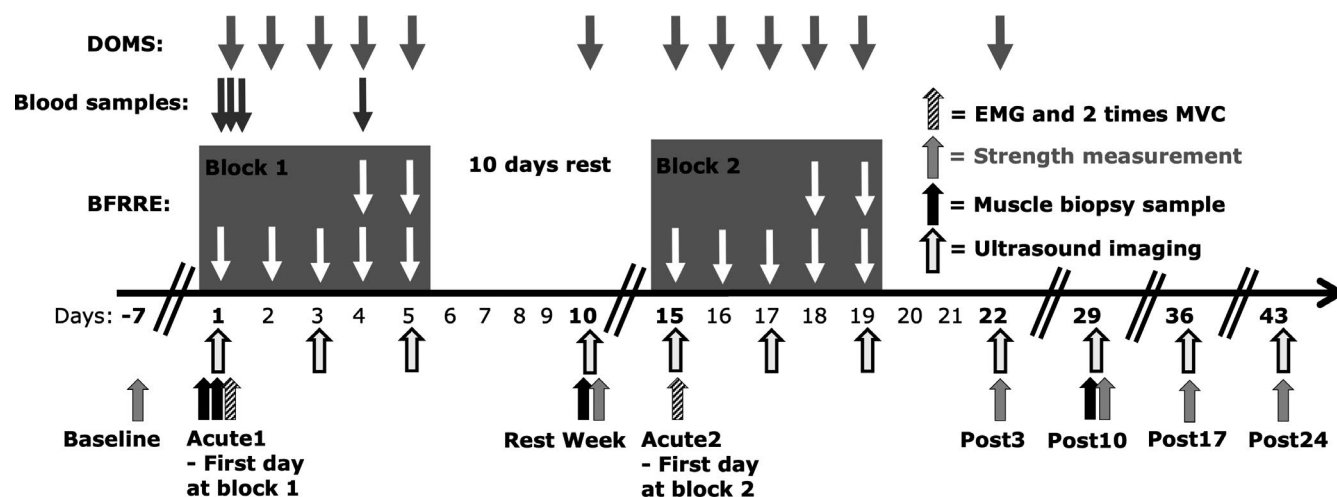

FIGURE 1 Schematic illustration of the study design. White arrows denote individual training sessions. Refer the Methods section for more details. BFRRE, blood flow restricted resistance exercise; EMG, electromyography; MVC, maximal voluntary isometric contraction

without BFR. During BFRRE, the cuff was kept inflated and released immediately upon completion of the fourth set. Range of motion was set from 100° to 10° (0° = full extension). To ensure maximal performance, all participants were given verbal motivation during each training session.

2.4 | Strength testing

Muscle function was evaluated by testing MVC and 1RM in the same unilateral knee extension machine during BFRRE. General warm-up consisted of 5 minutes cycling on a stationary ergometer bike (Monark Ergomedic 839E, Monark Exercise AB, Varberg, Sweden) with a standardized load (100 watt at 80-90RPM), followed by a specific warm-up for MVC by four submaximal isometric contractions (aimed at 50%, 60%, 80%, and 90% of a maximal contraction). The MVC test was conducted with a knee angle of 90 degrees, with three maximal isometric contractions each lasting for 5 seconds, 2 minutes rest between attempts and 60 seconds rest between legs. The trial with the highest torque was selected for further analysis. After MVC tests, specific warm-up to the 1RM test was conducted and consisted of four sets in total, with 10 repetitions at 50% of the estimated 1RM, six repetitions at 70% of 1RM, three repetitions at 80% of 1RM and one repetition at 90% of 1RM, respectively. After this, single repetitions with increasingly heavier load were performed until the 1RM load was found (minimum 1.25 kg increments), that is, the highest load that could be lifted throughout the range of motion. Two-minute rests were given between each 1RM attempt and at least 60 seconds rest between each leg. The right leg was always tested first. The lift was approved if the knee joint reached an angle of 10° (0° = full extension). Participants were secured to the chair by a seat belt around the waist and their hands placed on handles on the side of the chair at both the MVC and 1RM measurements. Strong verbal motivation was given during the tests.

2.5 | Ultrasonography

Muscle size and echo intensity were assessed using a brightness mode (B-mode) ultrasonography apparatus (LogicScan 128 XEXT-1Z kit, Telemed, LT), with linear probe of 4 mm width and an excitation frequency of 9 MHz. Ultrasound settings of focus, depth, dynamic range, power, gain, and frequency were optimized to identify the muscle aponeurosis and then kept constant for all measurement time points. Ultrasound measurements of muscle thickness in *m. rectus femoris* (RF), *m. vastus lateralis* (VL), and the anterior portion of *m. vastus intermedius* (VI), as well as the cross-sectional area (CSA) of RF were obtained at a distance equal

to 40% (distally) of the femur length from the lateral epicondyle of the knee to the great trochanter major. Images were analyzed blindly in a random order by one examiner using the software ImageJ (Wayne Rasband, National Institutes of Health, Bethesda, MD, USA). Test–retest measurements of muscle size between the familiarization and baseline measurements revealed coefficients of variation of 0.5%–2% for muscle thickness and 2.9% for RF CSA.

Echo intensity was assessed by computer-aided gray-scale (0 and 256 arbitrary units [A.U.], black = 0; white = 256) analysis using the histogram function in ImageJ. The region of interest (ROI) was selected using the polygon function including the largest ROI possible. The average of three pictures per measurement site was used for further analysis. Test–retest measurements between the familiarization and baseline measurements revealed a coefficient of variation of 6.2% for echo intensity in VL and 5.6% for echo intensity in RF.

2.6 | Muscle biopsy sampling

Muscle biopsies (~200–300 mg) were obtained from *m. vastus lateralis* using a 6-mm sterile Bergström needle (Pelomi, 6 mm, Albertslund, Denmark) under local anesthesia (Xylocain-adrenaline, 10 mg/mL +5 μg/mL, AstraZeneca, Södertälje, Sweden). The incisions were taken ~3 cm apart to minimize the repeated biopsy effect, as it is demonstrated no result in an increased satellite cell content or pronounced changes in other markers of regeneration when sampling sites are separated by 3 cm and adhere to a somewhat similar time course as the present study (four biopsies from *m.vastus lateralis* within 30 days).[13] Visible connective tissue and fat were dissected away before a bundle of fibers for later immunohistochemical analyses was mounted in OCT Embedding Matrix (Tissue-tek, O.C.T. compound, Sakura, USA) and immediately frozen in isopentane, which was pre-cooled (−140°C) with liquid nitrogen and stored at −80°C for later analysis.

2.7 | Immunohistochemical staining

Muscle biopsies were cut to 8-μm-thick cross-sections at −20°C using a cryostat (CM 3050, Leica Biosystems GmbH, Wetzlar, Germany), mounted on microscope slides (Superfrost Plus, Menzel-Gläser, Braunschweig, Germany) air-dried at room temperature, and stored at −80°C. Before immunostaining, frozen sections were air-dried and subsequently permeabilized with 0.1% Tween 20 in PBS for 3 × 10 minutes and blocked with serum-free protein blocker (Dako, 10082504, Glostrup, Denmark) and PBS-t (PBS with 0.05% Tween 20) solution (QC213624, Thermo Fisher

Scientific, Carlsbad, CA, USA) for 10 minutes at room temperature. Sections were incubated with primary antibodies for 60 minutes (room temperature) in a serum-free protein blocker (Dako, 10082504, Glostrup, Denmark), and PBS-t (PBS with 0.05% Tween 20) solution (QC213624, Thermo Fisher Scientific, Carlsbad, CA, USA), and incubated with secondary antibodies for 60 minutes at room temperature. SCs were visualized with antibodies against Neural Cell Adhesion Molecule (NCAM, ABCam, 153377-1, Cambridge Science Park, Cambridge, UK, 1:200) together with laminin (Dako, 20025756, Glostrup, Denmark, 1:400) and DAPI-stains (for nuclear staining) (Invitrogen, 1266174, Carlsbad, CA, USA). Neighboring sections were stained for MHC-II (SC-71c, DSHB, Iowa City, Iowa, USA, 1:1000) and dystrophin (Abcam, GR42138-1, Cambridge, UK, 1:500) for identification of type II myofibers[14] and delineation of the myofiber border, respectively, together with DAPI-stains. Specific secondary antibodies (Alexa-488 goat anti-mouse [Invitrogen, 1008801, Carlsbad, CA, USA, 1:200] and CF-594 goat anti-rabbit [Invitrogen, 1008648, Carlsbad, CA, USA, 1:200]) were applied after each primary antibody. Sections were mounted with a fluorescent anti-fade containing DAPI solution. The sections were visualized on a computer screen using a light microscope (Olympus BX61, Tokyo, Japan) connected to a fluorescent light (X-Cite 120PCQ; EXFO Photonic Solutions Inc., Mississauga, Ontario, Canada). The microscope was connected to a digital camera (Olympus DP72, Tokyo, Japan), and pictures were taken at 20× (SCs and myonuclei) and 4× (muscle fiber areas and fiber type) magnification. All morphometric analyses were performed in Cell-F (Olympus, Tokyo, Japan), TEMA (ChekVision, Hadsund, Denmark), and ImageJ. The criteria for identifying SCs were that they had to stain positive for NCAM, be placed within the basal lamina, and have a nucleus with a subordinate placement (Figure 2). The analyses of SCs and MFA were conducted with an average of 545 ± 241 muscle fibers, and with a minimum of 150 fibers. Myonuclei had to be stained for DAPI and be placed within the dystrophin staining, and 50 fibers were counted from each fiber type (50 fibers with and without MHC-II/SC-71c staining). Furthermore, nuclei with a central or internal position were counted; as an

additional indicator of myofiber regeneration.[15] Myonuclei and nuclei with an internal position in the myofiber were only counted in muscle fibers with intact staining around the whole fiber.

2.8 | Myoelectric activity during BFRRE

Myoelectric activity was measured with surface electromyography (EMG, Trigno™ 103 Wireless Systems and Smart Sensors, Delsys, Boston, MA, USA) on VL and RF. After a skin preparation (shaving and alcohol rinse), two electrodes (Blue sensor M, Ambu, Ballerup, Denmark; diameter: 31 mm, interelectrode distance 10 mm) were placed on the distal parts of VL and RF, according to recommendations for sensor locations by SENIAM.[16] EMG was recorded at 2000 Hz (bandwidth 20–450 Hz) and rectified and smoothed (100 ms moving average) with the root-mean-square (RMS) algorithm (hardware and software from Delsys, Boston, MA, USA, version 4.2). The peak EMG values during the concentric phases from the average of the three first, middle, and last repetitions in each set of BFRRE were expressed relative to the peak EMG values during MVC measurements obtained 1 hours before the BFRRE sessions at Acute 1 and 2, with the EMG electrodes still in place.

2.9 | Delayed-onset muscle soreness, perceived exertion and –pain

Delayed-onset muscle soreness (DOMS) was registered on a 100-mm visual analogue scale[17] before every BFRRE bout. Subjects were asked to quantify their DOMS by palpating their quadriceps with two fingers on five different locations on both legs: distal, middle, and proximal parts of the VL, then distal and middle parts of *m. vastus medialis* (VM). The measurement locations were recorded on transparent, acetate paper. Palpations were done in a standing position and participants were familiarized to apply palpation pressures two times before the baseline measurements. The average of the five positions was used for further analysis. Subjects were
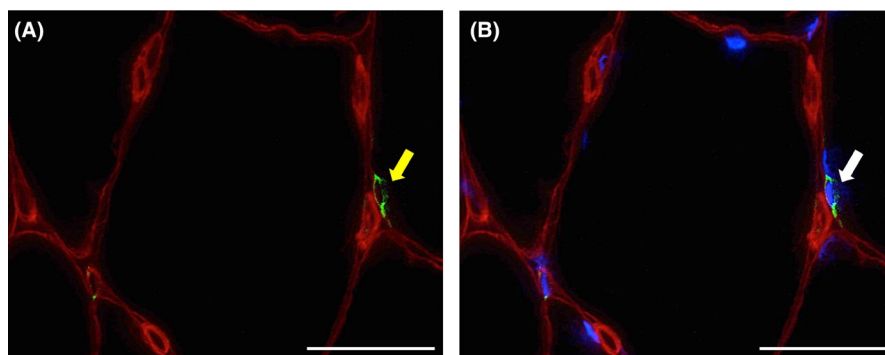

FIGURE 2 Representative immunohistochemical staining. White arrows denote NCAM+ satellite cell, yellow arrows denote NCAM+/Dapi+ satellite cell (A) NCAM and Laminin staining, (B) NCAM, Dapi and Laminin merged. The scale bar is 50 μm
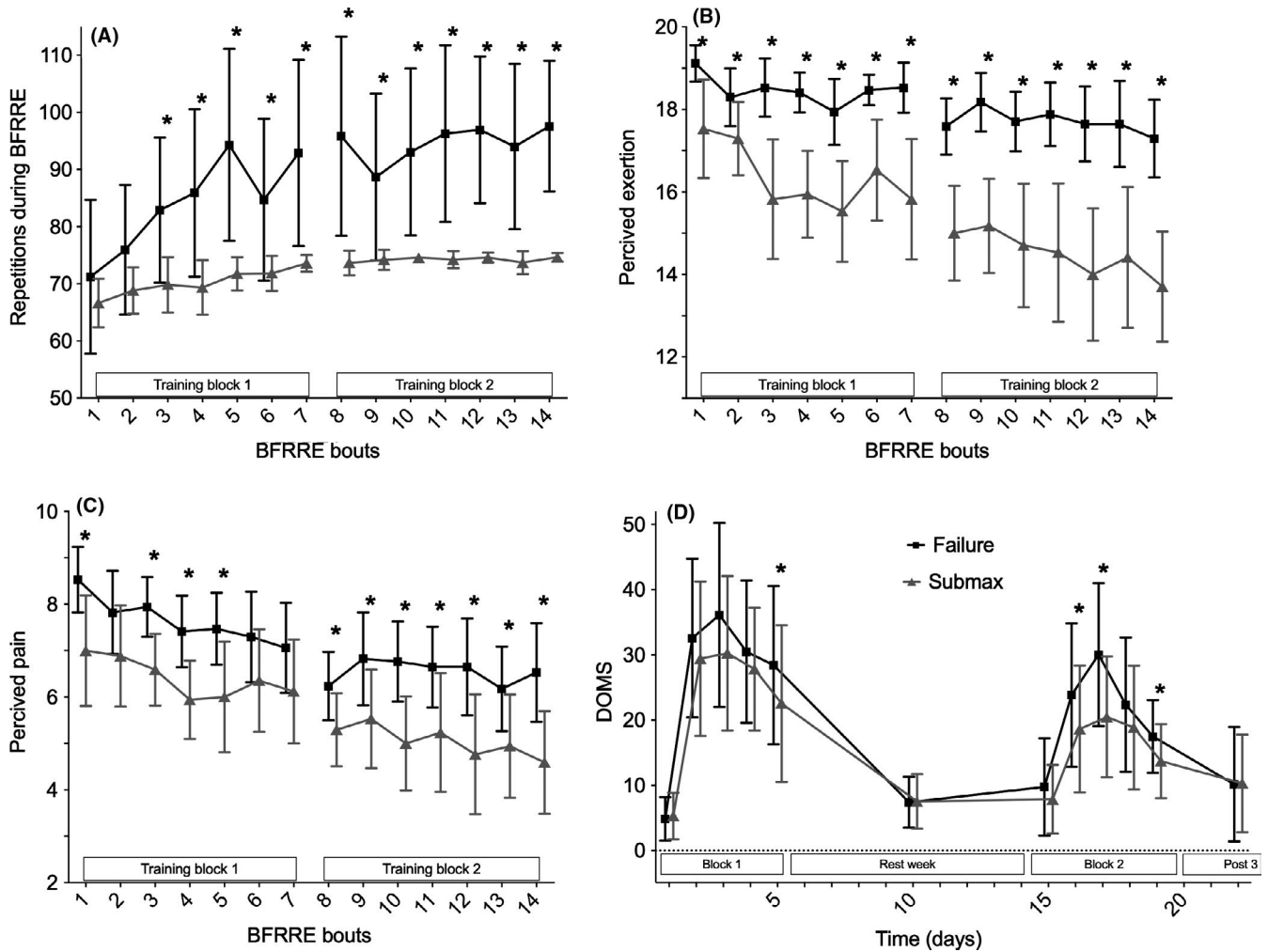
FIGURE 3 Number of repetitions, DOMS, perceived exertion and -pain. Number of repetitions (A) and perceived exertion (B) and -pain (C) per blood flow restricted resistance exercise (BFRRE) bout, as well as delayed muscle onset soreness (DOMS) (D), expressed on a timeline from the first day of BFRRE until 3 days after the intervention. * represents significant differences between legs ($p < 0.05$). Data are expressed as means with 95% confidence intervals

instructed to not include soreness or pain from the area that biopsies were obtained in the assessment. Test–retest DOMS measurements acquired before the intervention demonstrated limits of agreements (mean difference ±1.96 standard deviations SD of the difference) of between 0.1–0.7 ± 1.9–3.6 mm at all measurement locations. Participants were asked to report perceived pain (Borg CR10) and -exertion (Borg-RPE scale) immediately after every BFRRE bout, as they perceived it during the exercise efforts.

2.10 | Blood samples

Changes in blood concentration of creatine kinase and myoglobin were assessed to investigate indirect markers of myocellular stress and "overreaching" across legs in each participant.[8,9] Blood samples were obtained in a fasted state (≥12 hours) from resting individuals at baseline, the fourth day of BFRRE (Day

4), as well as 2- and 4-hour post-BFRRE (not fasted) the first day of the first training block. Blood was drawn from an antecubital vein into 8-mL serum vacutainer tubes. After 30 minutes, the tubes were centrifuged at 2100 g for 10 minutes at 4°C and serum was transferred to 1-mL blood collection tubes. Serum were immediately stored at −80°C until analysis. A Modular Analyzer P800 (Roche Diagnostics, Mannheim, Germany) was used to analyze creatine kinase and a Modular Analyzer E170 (Roche Diagnostics, Mannheim, Germany) was used to assess myoglobin levels. Test–retest measurements revealed a coefficient of variation of 5% creatine kinase and 6% for myoglobin analysis.

2.11 | Statistical analysis

With a minimum of 16 participants in each group, we had 80% power to detect a mean group difference between the two

groups of 10% in the number of myonuclei with an expected SD of 15%[3] (alpha: 0.05). We considered such difference to be well within the physiological meaningful range.[3,4,7] For secondary outcomes, such as muscle size, muscle strength (1RM), and the number of SCs, we estimated our statistical power to be 80% to detect a true change of 11%. Statistical analyses were performed using IBM SPSS (Statistical Product and Service Solutions, version 22, IBM, Chicago, IL, USA) and Graph Pad Prism Software (GraphPad Software Inc., La Jolla, CA). A linear mixed model was used to assess group differences between the failure and non-failure protocol and differences from baseline within each group. Group and time were included as fixed effects, while subject-ID was included as a random effect and baseline values were added as a covariate. Sidak posthoc test was used to assess the group differences at each time point. Pearson r was used to assess correlations. Descriptive data are presented as mean ± SD, whereas results are presented as mean with 95% confidence intervals. Statistical significance level was set to 5%.

3 | RESULTS

3.1 | Subjects

One participant dropped out due to circumstances unrelated to the study, leaving seventeen participants (age 25 ± 6 years old; height 181 ± 12 cm, body mass 80 ± 13 kg, lean body mass 64 ± 10, fat 15 ± 6%) who completed all 14 BFRRE sessions with both legs.

3.2 | Blood flow–restricted resistance exercise

The training load (20% 1RM) of the failure leg (15.1 ± 3.2) and non-failure leg (15.1 ± 2.6 kg) remained unchanged during the BFRRE intervention. On average, participants performed more repetitions per bout with the failure leg than the non-failure leg during training Block1 (84 [95% CI: 71, 97] vs 70 [67, 73] repetitions, $p = 0.02$, respectively) and Block2 (95 [82, 108] vs 74 [73, 75] repetitions, $p = 0.003$) (Figure 3A). However, the failure leg only performed more repetitions than the non-failure leg in set 1 (Block1: 52 [47, 58] vs 30, $p < 0.001$; Block2: 60 [54, 65] vs 30, $p < 0.001$, respectively), whereas the non-failure leg performed more repetitions than the failure leg in set three (Block1: 14 [14, 15] vs 9 [7, 12], $p < 0.001$; Block2: 15 [15, 15] vs 11 [8, 14], $p = 0.005$, respectively) and four (Block1: 11 [8, 14] vs 8 [5, 12], $p = 0.03$; Block2: 14 [13, 16] vs 10 [7, 13], $p = 0.003$, respectively). All participants reached the target repetitions during the two first sets of BFRRE in the non-failure leg. However, several participants failed to complete

15 repetitions during the fourth set of non-failure BFRRE during the first training block (10 and 4 participants in the first and seventh BFRRE session, respectively), as well as one to three participants during the second block. Total training volume (repetitions * kg) and repetitions per session increased proportionally from training Block1 to Block2 in both legs ($p < 0.01$).

3.3 | Myonuclei and myonuclear domain

The myonuclear data at baseline is presented as the number of myonuclei per muscle fiber, whereas change values are presented as percentage change from baseline. No differences were observed between legs in changes of myonuclear number per muscle fiber or myonuclear domain (Figure 4, myonuclei: A and B; myonuclear domain: G and H). The number of myonuclei in type I fibers increased in both legs from baseline (Failure: 2.2 [2.0, 2.4]; Non-failure: 2.1 [1.9, 2.3]) to Post10 (Failure: 11.7 [3.6, 19.8]%, $p = 0.01$; Non-failure: 17.0 [8.9, 25.1]%, $p < 0.01$). The non-failure leg also increased myonuclear number in type I fibers from baseline to the Rest Week (11.1 [4.8, 17.3]%, $p < 0.01$), whereas a tendency was observed in the failure leg (7.5 [−1.2, 16.3]%, $p = 0.09$). The number of myonuclei in type II muscle fibers increased from baseline (Failure: 2.4 [2.2, 2.5]; Non-failure: 2.3 [2.1, 2.5]) to the Rest Week (Failure: 13.3 [5.3, 21.3]%; Non-failure: 18.8 [11.6, 26.0]%) and Post10 (Failure: 19.9 [11.2, 28.7]%; Non-failure: 22.9 [16.0, 29.7]%; all $p < 0.01$).

The number of internally placed nuclei are presented as absolute numbers per 50 muscle fibers for both baseline and all subsequent time points. The number of internally placed nuclei in type I fibers was not significantly different between the legs at baseline (Failure: 1.9 [1.5, 2.2], vs non-Failure: 2.4 [1.7, 3.0], $p = 0.18$), but increased more from baseline to the Rest Week in the failure leg (3.4 [2.5, 4.3]) compared to the non-failure leg (2.6 [2.0, 3.2], $p = 0.05$). With no differences between protocols, the number of internally placed nuclei in type I fibers further increased to the Post10 biopsy (failure leg 3.6 [2.7, 4.4], $p = 0.001$) and the non-failure leg 3.3 [2.5, 4.1], $p = 0.04$). In type II muscle fibers, the numbers of internally placed nuclei increased in both legs from baseline (Failure: 2.8 [2.3, 3.4]; Non-failure: 2.9 [2.3, 3.6]) to the Rest Week (Failure: 4.8 [4.0, 5.7], $p < 0.001$; Non-failure: 4.3 [3.3, 5.2], $p = 0.03$) and Post10 (Failure: 4.8 [4.1, 5.4], $p < 0.001$; Non-failure: 4.3 [3.4, 5.2], $p = 0.01$), again with no significant differences between the protocols.

Muscle fiber area per nucleus (myonuclear domain) decreased in type I fibers from baseline (2221 [1988, 2454] μm^2) to the Rest Week (−11.0 [−18.9, −3.1]%, $p < 0.01$) and Post10 (−18.3 [−26.3, −10.3]%, $p < 0.01$) in the failure leg,

FIGURE 4 Immunohistochemistry. Myonuclei number per muscle fiber type I (A) and II (B). NCAM+ myogenic stem cells per muscle fiber type I (C) and II (D). Muscle fiber cross-sectional area, type I (E) and type II (F). Muscle fiber cross-sectional area (MFA) per nucleus, type I (G) and II (H). The relative changes are from baseline to after the first block of training (Rest Week) and 10 days after cessation of training (Post10). * represents $p < 0.05$. Data are expressed as means with 95% confidence intervals
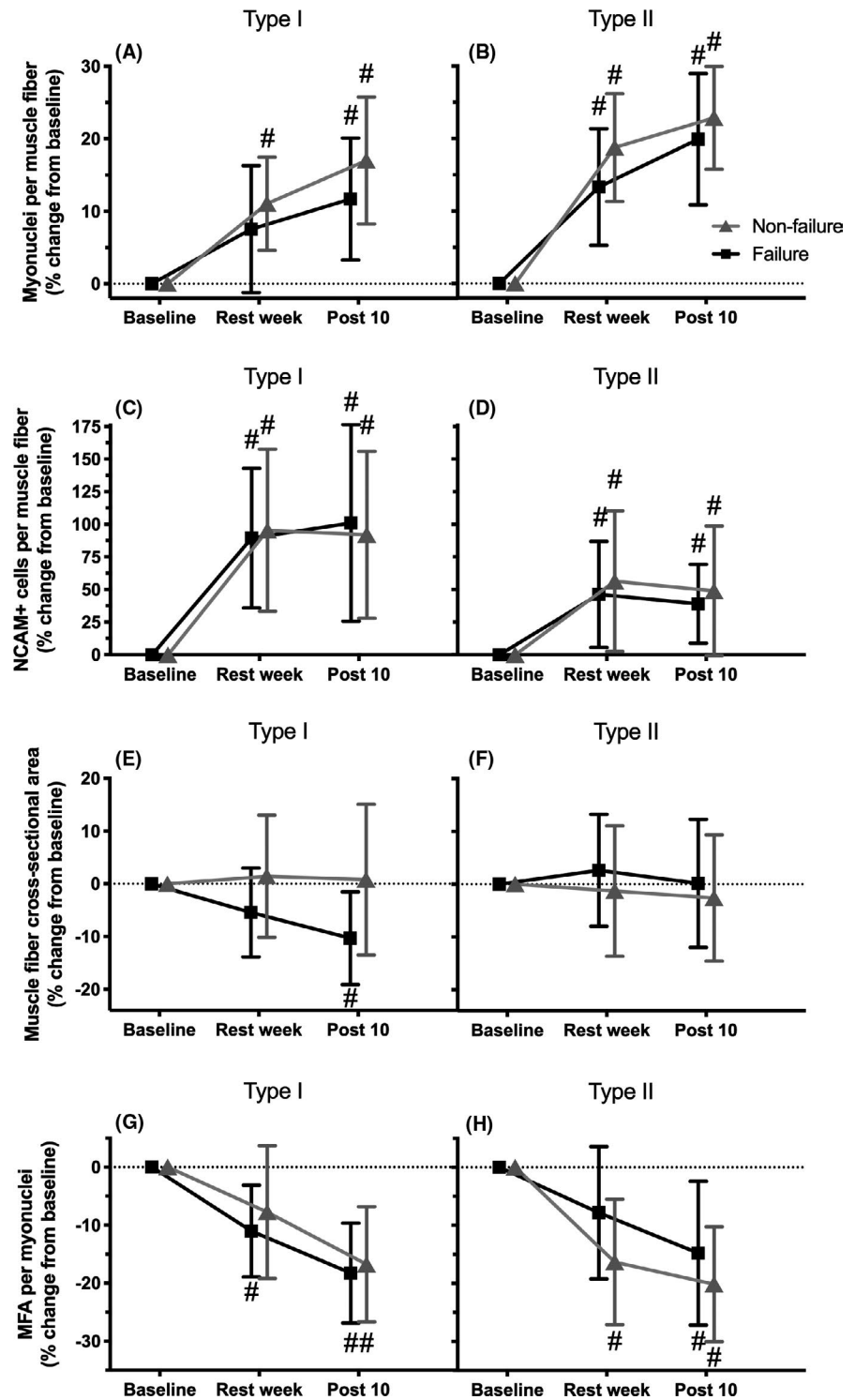
but only from baseline (2319 [2083, 2555] μm^2) to Post10 (-16.8 [-26.0, -7.5]%, $p < 0.01$) in the non-failure leg. In both legs, MFA per nucleus in type II fibers decreased from baseline (Failure: 2437 [2101, 2771] μm^2; Non-failure: 2621 [2242, 3000] μm^2) to Post10 (Failure: -14.8 [-26.3, -3.2]%, $p = 0.02$; Non-failure: -20.1 [-29.7, -10.6]%, $p < 0.01$), but only the non-failure leg decreased significantly to the Rest Week (-16.3 [-26.8, 5.9]%, $p < 0.01$).

3.4 | Satellite cells

No differences between legs were observed in the increases of NCAM positive cells (Figure 4C and D). Both the failure and non-failure leg increased NCAM positive cells around type I muscle fibers from baseline (Failure: 4.0 [2.9, 5.1]; Non-failure: 3.8 [3.1, 4.6], NCAM positive cells per 100 muscle fibers) to the Rest Week (89.4 [35.7, 143.0]%,
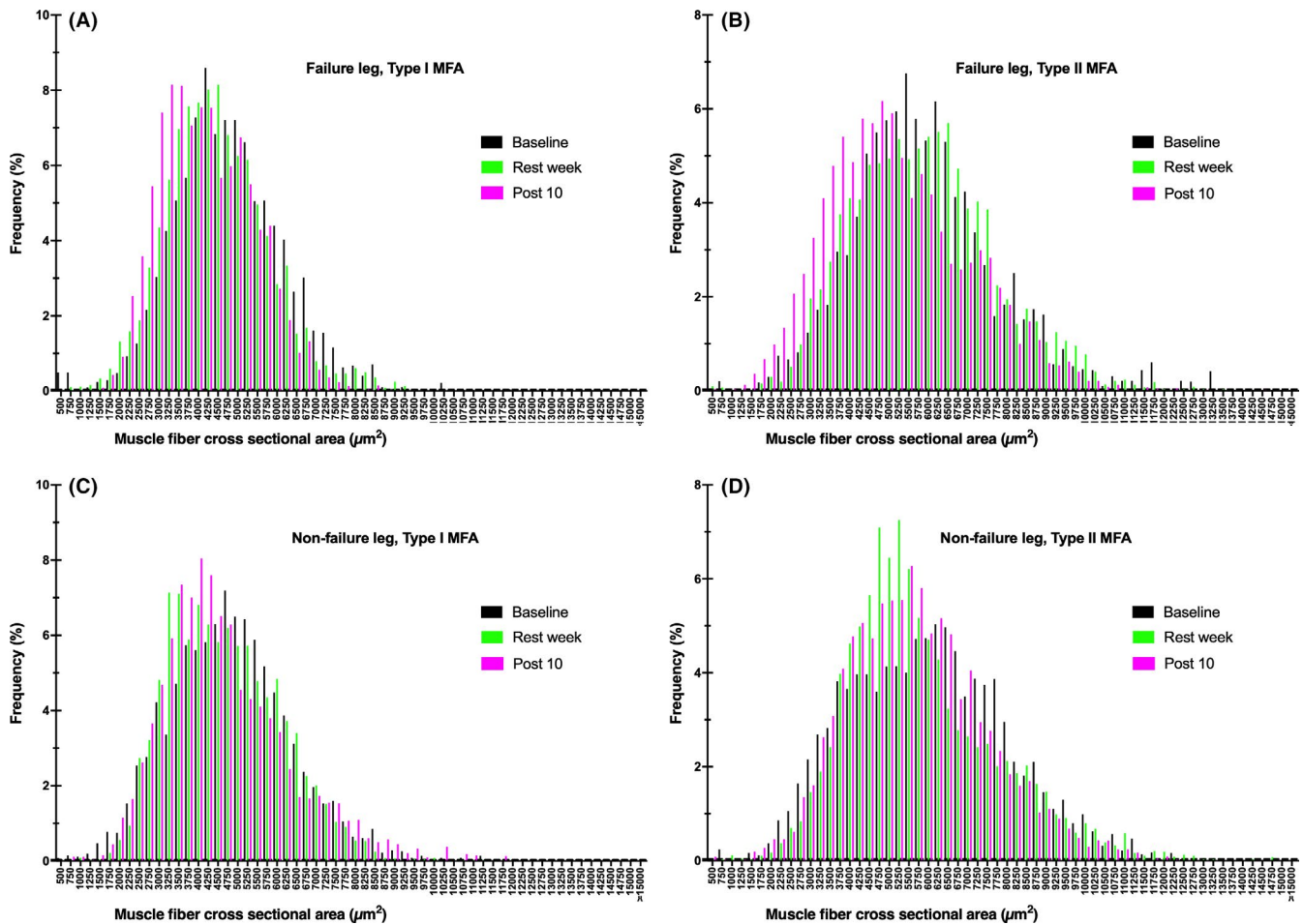
FIGURE 5 Histogram of muscle fiber size. Frequency of muscle fiber areas in type I muscle fibers of the Failure leg (A), type I muscle fibers of the Non-failure leg (B), type II muscle fibers of the Failure leg (C), and type II muscle fibers of the Non-failure leg (D). Black bars denote baseline muscle fiber areas, green denote Rest Week data, and purple denote Post10 data

$p = 0.01$, and 95.4 [35.4, 155.4]%, $p = 0.01$, respectively) and Post10 (100.9 [28.1, 173.7]%, $p = 0.01$, and 91.9 [30.1, 153.6]%, $p = 0.01$, respectively). Similarly, NCAM positive cells around type II muscle fibers increased from baseline (Failure: 4.8 [3.3, 6.3]; Non-failure: 4.7 [3.3, 6.1]) to the Rest Week (Failure: 46.2 [5.6, 86.8]%, $p = 0.03$; Non-failure: 56.4 [4.4, 108.3]%, $p = 0.04$) and Post10 (Failure: 39.0 [9.8, 68.2]%, $p = 0.02$; Non-failure: 49.0 [0.9, 97.0]%, $p = 0.05$) in both legs.

3.5 | Muscle fiber cross-sectional areas

No differences between legs were observed in MFA changes. However, type I MFA decreased from baseline (Failure: 4680 [4337, 5022] μm^2; Non-failure: 4727 [4255, 5199] μm^2) to Post10 in the failure leg (−10.3 [−18.5, −2.1]%, $p = 0.03$), while no change was observed in the non-failure leg (−0.8 [−13.0, −13.8]%, $p > 0.05$). (Figure 4E and F). The type II MFA did not change from baseline (Failure: 5092 [4926, 6344] μm^2; Non-failure: 5979 [5187, 6772] μm^2) to Post10 in

either leg (Failure: 0.2 [−11.1, −11.4]%, Non-failure: −2.6 [−14.2, −11.5]%, $p > 0.05$). A histogram of muscle fiber sizes is presented in Figure 5.

3.6 | Fiber type distribution

The percentage distribution between the type I and type II fibers for the failure leg was 39/61 at baseline, 40/60 at 2 hours after the first session, 38/62 at Rest week, and 39/61 at Post10. The percentage distribution between the type I and type II fibers for the non-failure leg was 40/60 at baseline, 40/60 at 2 hours after the first session, 39/61 at Rest week, and 38/62 at Post10. No significant changes were observed in the fiber type distribution. However, in a few of the images used for fiber type and area analyses, some clusters of small diameter myofibers were apparent in samples from the Rest Week and also from Post10 displayed only weak SC-71c staining (1–2 subjects each for the failure and the non-failure leg for each of these time points). Given that type IIA and type IIX fibers show strong

FIGURE 6 Representative examples of areas with strongly NCAM + regenerating muscle fibers. The images were slightly overexposed to highlight the thicker basal lamina in the NCAM + fibers. Laminin staining in red and NCAM staining in green
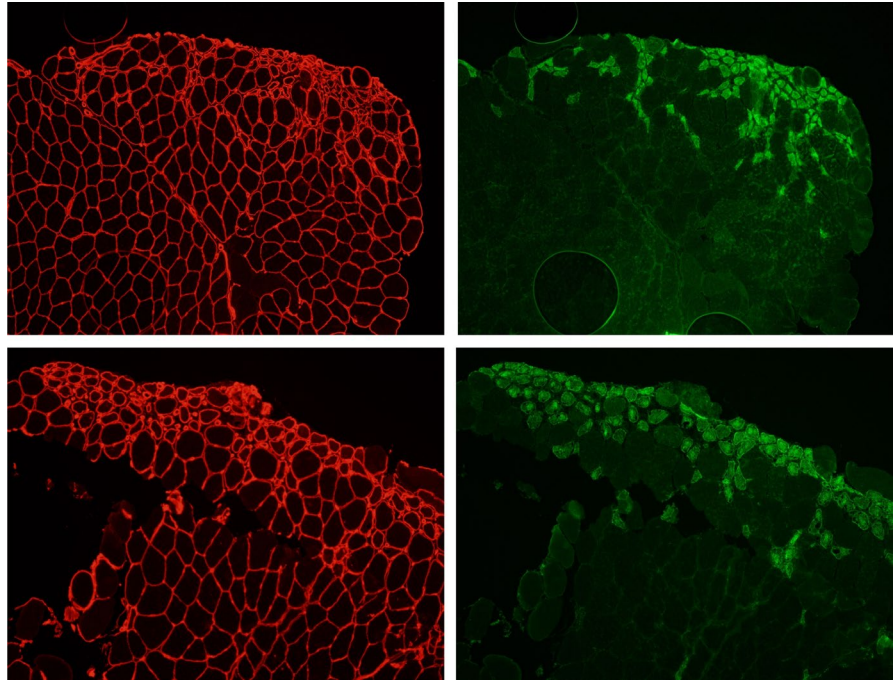
and moderate staining, respectively, while type I fibers do not stain positively for SC-71,[14,18] these small fibers were excluded from MFA analyses because they could not be identified with certainty as either type I or II myofibers. Interestingly, the same small fibers were typically strongly positive for NCAM on nearby sections, and these fibers were often found in the periphery of the fascicles. These observations prompted us to take new images covering the whole sections in order to search specifically for NCAM-positive muscle fibers.

3.7 | NCAM-positive muscle fibers

In the muscle samples from the Rest Week, clusters or groupings of strongly NCAM-positive muscle fibers were observed in seven subjects in the failure leg, and in four subjects in the non-failure leg. In two subjects, clusters were found in samples from both legs. In the samples from the Post10, clusters of strongly NCAM-positive muscle fibers were observed in one subject in the failure leg, and in two subjects in the non-failure leg. The percentage of NCAM-positive fibers in these subjects ranged between 1% and 25%, with the exception of one individual who displayed ~50% positive fibers in the failure leg at Rest Week. No small or large groupings of strongly NCAM-positive fibers were observed in any subject in the Pre and 2 hours samples. Two representative examples of clusters of NCAM-positive muscle fibers are shown in Figure 6. A more detailed analysis of the nature and extent of these fibers is planned for a separate publication.

3.8 | Whole muscle size

No differences in CSA or muscle thickness were observed between the failure and non-failure leg at any time-point or over the time course of the study (Figure 7). The CSA of RF (Failure: 7.3 [6.2, 8.4] cm^2; Non-failure: 6.7 [5.7, 7.6] cm^2, at baseline), thickness of RF and VL (Failure: RF, 18.6 [16.8, 20.5] mm and VL, 25.6 [23.8, 27.5] mm; Non-failure: RF, 18.0 [16.5, 19.6] mm and VL, 25.5 [23.6, 27.3] mm, at baseline) and thickness of VI (Failure: 17.2 [14.7, 19.6] mm; Non-failure: 17.7 [15.5, 19.9] mm, at baseline) all showed a biphasic pattern with significant increases during the two training blocks (4%–13%) and transient reductions in the Rest Week in the failure and non-failure leg. After the last training block, muscle CSA and thickness stabilized at a level 4%–10% above baseline levels for RF and VL (all $p < 0.01$), but thickness of VI was not significantly different from baseline at the post-training measurements, except for a 4% gain in the failure leg at Post3 ($p < 0.05$). The gains in muscle thickness were greater in RF than VI in both legs at all post-training time points ($p = 0.001$–0.03). Muscle thickness of RF also increased more than that of VL in both legs at Post3 to Post24 ($p = 0.01$–0.05), except for the failure leg at Post10 ($p = 0.14$) and the non-failure leg at Post24 ($p = 0.14$). VL muscle thickness increased more than VI in the non-failure leg at Post3 ($p = 0.03$) and also tended to increase more than VI in the same leg at Post17 ($p = 0.06$). In addition, the gain in muscle thickness tended to be greater in VL than in VI in the failure leg at Post24 ($p = 0.10$). Increases in CSA of RF correlated with the increases in muscle thickness of RF (eg, Failure: $r = 0.68$, Non-failure: $r = 0.67$, both $p < 0.05$, at Post24).
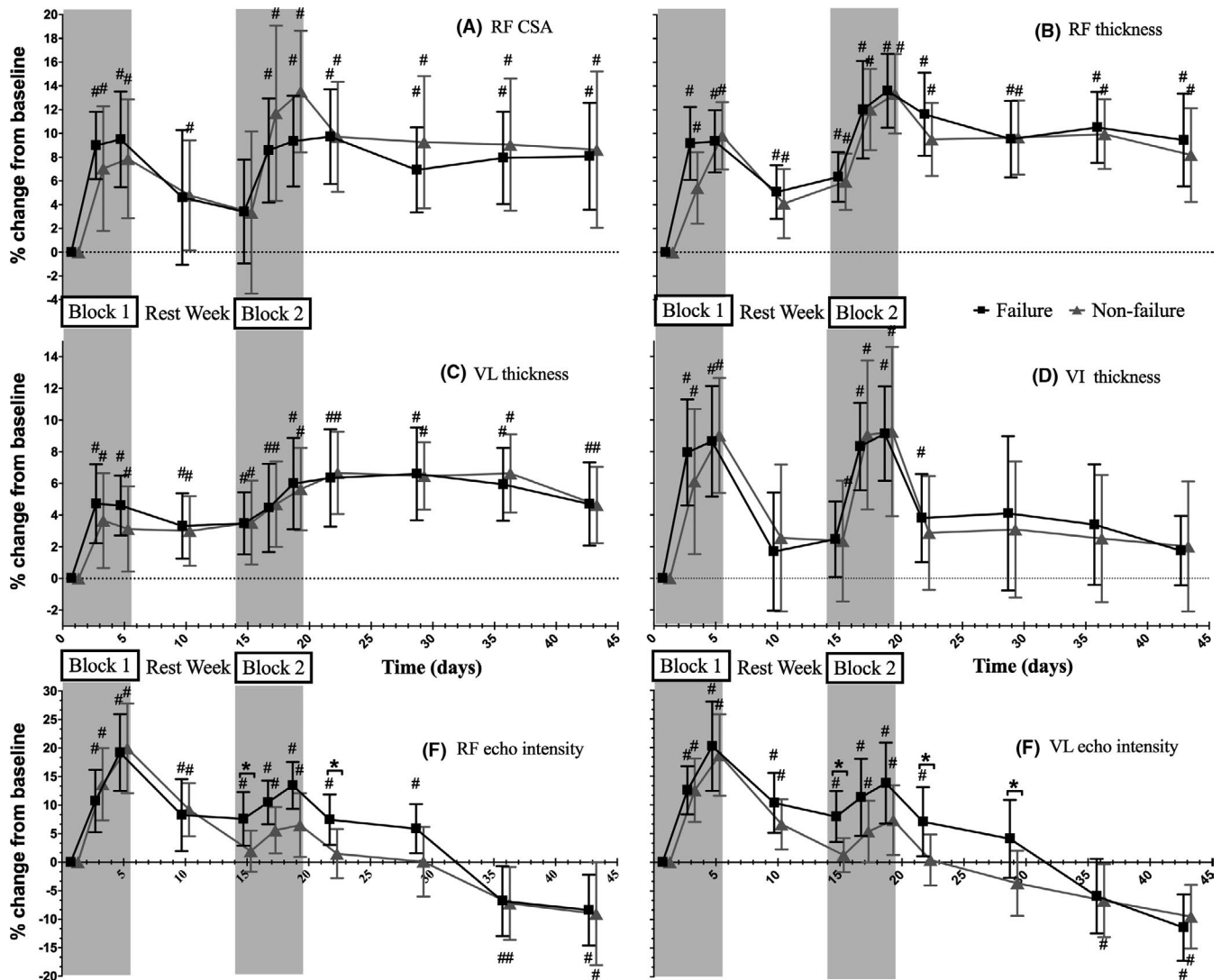
FIGURE 7 Muscle cross sectional area, thickness and echo intensity. Relative increases in the (A) cross-sectional area (CSA) of m. rectus femoris (RF) and muscle thickness of (B) RF, (C) m. vastus lateralis (VL) and (D) m.vastus intermedius, as well as echo intensity of (E) RF and (F) VL, expressed on a timeline from the first day of BFRRE until the last measurements at day 43. * represents significant differences between legs ($p < 0.05$), # represents significant within-leg changes compared to baseline ($p < 0.05$). Data are expressed as means with 95% confidence intervals

3.9 | Echo intensity

Changes in echo intensities of VL and RF are presented in Figure 7E and F. VL echo intensity increased in both legs from baseline (Failure: 47.2 [40.5, 53.9] arbitrary units [AU]; Non-failure: 44.9 [40.3, 49.5] AU) to Day3 (~13%) and Day5 (~20%) during Block1 ($p < 0.001$). However, echo intensity of VL normalized after the Rest Week only in the non-failure leg (Day15: Failure: 8.0% vs Non-failure: 1.2%, $p = 0.003$). VL echo intensity of the failure leg tended to increase more than the non-failure leg during Block2 (Failure: 11%–14% vs Non-failure: 5%–7%, $p = 0.08$), and was significantly higher than in the non-failure leg at Post3 (Failure: 7.1% vs Non-failure: 0.4%, $p = 0.04$) and at Post10 (Failure: 4.1% vs Non-failure: −3.7%, $p = 0.03$). At Post17, echo intensity of VL

was decreased compared with baseline in the non-failure leg ($p = 0.04$) and tended to decrease in the failure leg ($p = 0.07$), with no differences between legs (−6% to 7% in both). At Post24, echo intensity was significantly decreased in both legs compared with baseline (−11% to 10%, $p \leq 0.002$).

RF echo intensity was increased after both protocols from baseline (Failure: 53.3 [47.4, 59.2] AU; Non-failure: 52.8 [48.8, 56.9] AU) to Day3 (11%–14%) and Day5 (19%–20%) during the first block of BFRRE ($p \leq 0.001$), but was normalized after the Rest Week only in the non-failure leg (Day15: Failure: 7.6% vs Non-failure: 1.9%, $p = 0.03$). The failure leg tended to increase more in echo intensity than the non-failure leg during the second block of BFRRE (Failure: 11%–14% vs Non-failure: 6%–7%, $p = 0.06$). Echo intensity was also increased more in the failure leg at Post3 (Failure: 7.5% vs
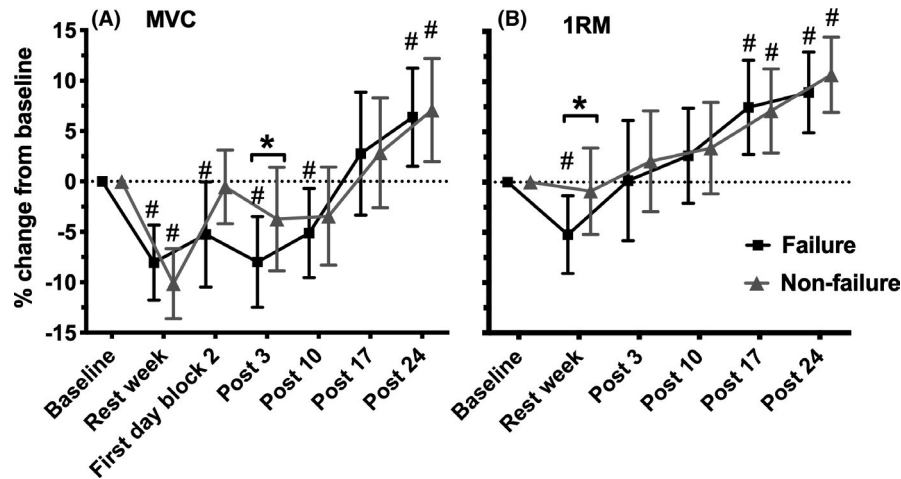
FIGURE 8 Maximal strength. Relative change in (A) maximal voluntary isometric contraction (MVC) and (B) 1-repetition maximum during the Rest Week, first day of Block2 (only MVC), as well as 3- (Post3), 10- (Post10), 17- (Post17), and 24 days (Post24) after cessation of training, * represents significant differences between legs ($p < 0.05$), # represents significant within-leg changes compared to baseline ($p < 0.05$). Data are expressed as means with 95% confidence intervals

Non-failure: 1.5%, $p = 0.02$) and tended to increase more at Post10 (Failure: 5.9% vs Non-failure: 0.1%, $p = 0.09$). With no differences between legs, echo intensity of RF was decreased after both protocols at Post17 (~7%) and Post24 (~9%, both $p < 0.05$).

Increases in echo intensity were correlated with the increases in CSA of RF and thickness of VL during Block1 (summed average of Day3 and Day5) in the failure leg ($r = 0.40$ and $r = 0.71$, respectively) and the non-failure leg ($r = 0.44$ and $r = 0.69$, all $p < 0.05$).

3.10 | Muscle strength

At baseline, the two measures of knee extension strength (MVC and 1RM) were very similar between the two legs (MVC: Failure: 224 [203, 244] Nm; Non-failure: 224 [204, 244] Nm. 1RM: Failure: 74.8 kg [66.8, 82.7]; Non-failure: 73.2 kg [66.4, 79.9]). No differences were observed between the failure and non-failure leg in 1RM or MVC over the entire time course of the study, except for Post3, where the failure leg displayed greater reductions in MVC than the non-failure leg (-8% vs -4%, $p = 0.03$, respectively, Figure 8A). Only the failure leg reduced MVC from baseline to the first day of Block2 and to Post10 (~-5%, $p < 0.05$). Both legs showed decreases in MVC from baseline to the Rest Week (-8% to 10%, $p < 0.01$). After Post3, MVC showed an overall upward trend and at Post24 MVC was increased in both legs compared to pre-training values (~7%, $p = 0.01$).

Maximal isometric contraction of both legs was reduced 2 hours after the first BFRRE session of Block1 (-14% to 17%, $p < 0.001$) and Block2 (-7% to 12%, $p < 0.001$),

with no differences between legs. The decrease in MVC was larger 2 hours after the first BFRRE bout of Block1 compared to Block2 in the non-failure leg ($p = 0.01$), and tended to be greater in Block1 versus Block2 also in the failure leg ($p = 0.09$).

The failure leg decreased in 1RM from baseline to the Rest Week compared to the Non-failure leg (-5.2% [-9.1, -1.4] vs -0.9% [-5.2, 3.4], $p = 0.03$, respectively), but both legs increased 1RM from baseline to Post17 (7%–8%, $p < 0.01$) and Post24 (9%–11%, $p < 0.01$, Figure 8B).

3.11 | Delayed-onset muscle soreness, perceived exertion, and –pain

Changes in DOMS, perceived exertion and -pain are presented in Figure 3B–D. On average, participants reported higher DOMS in the failure leg than in the submaximal leg during Block1 (Failure: 32 [21, 43] mm vs Non-failure: 27 [17, 37] mm, $p = 0.01$) and Block2 (Failure: 24 [15, 34] mm vs Non-failure: 19 [10, 27] mm, $p < 0.01$). Reported DOMS levels were significantly higher in Block1 versus Block2 in both the failure leg ($p = 0.05$) and the non-failure leg ($p < 0.01$).

Perceived pain data are presented as Borg CR10 score (range 0–10). Participants reported lower perceived pain in the non-failure leg during Block2 (Failure: 6.6 [5.9, 7.2] vs Non-failure: 5.1 [4.1, 6.1], $p = 0.04$) and tended to report lower pain ratings in the non-failure leg also in Block1 (Failure: 7.7 [7.0, 8.3] vs Non-failure: 6.4 [5.6, 7.3], $p = 0.06$). Similarly, perceived exertion was consistently lower in the non-failure leg during both training periods (Block1, Failure: 18.5 [18.1, 18.9] vs Non-failure: 16.4

[15.5, 17.2], $p < 0.001$; Block2, Failure: 17.7 [17.0, 18.4] vs Non-failure: 14.5 [13.2, 15.8], $p < 0.001$). Perceived pain and -exertion levels were significantly higher in Block1 versus Block2 in both the failure leg ($p \leq 0.001$) and the non-failure leg ($p < 0.001$).

3.12 | Myoelectric activity during BFRRE

Electromyography amplitudes in VL and RF during the first BFRRE bout of Block1 and Block2 are presented in Figure 9. VL RMS was higher in the failure leg compared to the non-failure leg during set 1 in the last three repetitions (Block1, $p = 0.001$; Block2, $p < 0.001$) and middle repetitions (Block1, $p = 0.05$; Block2, $p = 0.03$). VL RMS was also higher in the failure leg compared to the non-failure leg during set 2 in the first three (Block1, $p = 0.006$; Block2, $p < 0.001$), middle (Block1, $p = 0.008$; Block2, $p < 0.001$) and last three repetitions (Block2: $p < 0.001$; tendency in Block1, $p = 0.08$), as well as during set 3 in the first three (Block1, $p = 0.03$; Block2: $p < 0.001$) and middle repetitions (Block1, $p = 0.05$; Block2, $p = 0.02$). No differences between legs were observed in VL RMS in the first three repetitions in set 1 and in set 4 (besides the three first repetitions during Block2: $p = 0.01$).

RF RMS was higher in the failure leg than the non-failure leg in the last three (Block1, $p = 0.007$; Block2, $p = 0.03$) and middle (only in Block1: $p = 0.003$) repetitions during set 1, in the first (Block1, $p = 0.001$; Block2, $p = 0.004$), and middle (Block1, $p = 0.03$; Block2, $p = 0.04$) repetitions during set 2, and the first three (Block1, $p = 0.02$; Block2, $p = 0.05$) during set 3. No differences between legs were observed in RF RMS for the first three repetitions in set 1, and in set 4, respectively.

3.13 | Creatine kinase and myoglobin

Creatine kinase (Figure 10) increased from 168 ± 130 U/L (mean ± SD) at baseline to 190 ± 117 U/L 2 hours post BFRRE ($p = 0.006$), to 218 ± 115 U/L 4 hours post BFRRE ($p < 0.001$), and to 407 ± 312 U/L on Day4 ($p = 0.04$). Three individuals increased CK from ~150 UL at baseline to 800–1250 UL at Day4, the remaining 14 participants increased with less than 400 UL from baseline to Day4.

Myoglobin increased from 34 ± 24 µg/L at baseline to 69 ± 35 µg/L ($p < 0.001$) 2 hours post BFRRE, to 86 ± 53 µg/L ($p = 0.01$) 4 hours post-BFRRE, and to 52 ± 35 µg/L ($p = 0.04$) at Day4.
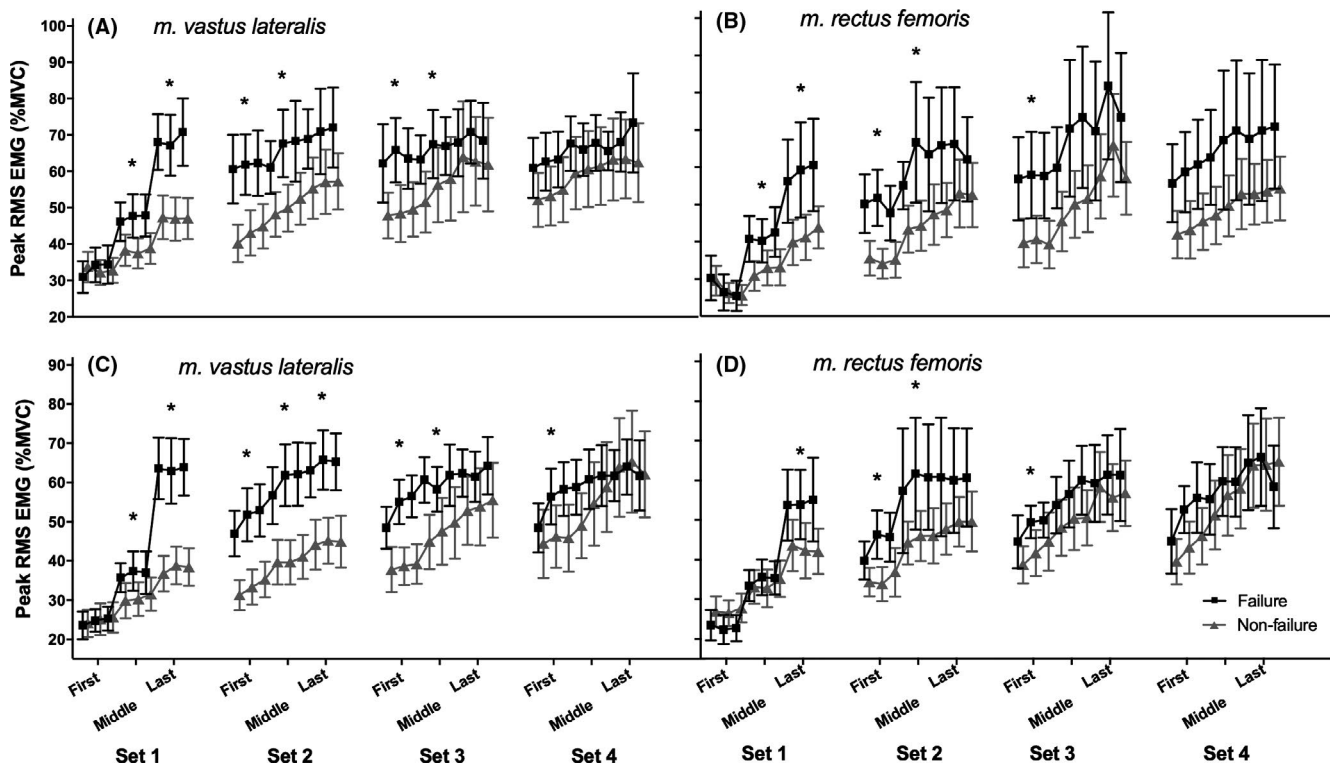

FIGURE 9 Myoelectric activity during BFRRE. Myoelectric activity of m. vastus lateralis (VL) and m. rectus femoris (RF) during the three first, middle, and last repetitions in each of the four sets of BFRRE at the first (Figure 9A in VL and -B in RF) and second training blocks (Figure 9C in VL and -D in RF). * represents significant differences between legs ($p < 0.05$). Data are expressed as means with 95% confidence intervals. RMS, root mean square
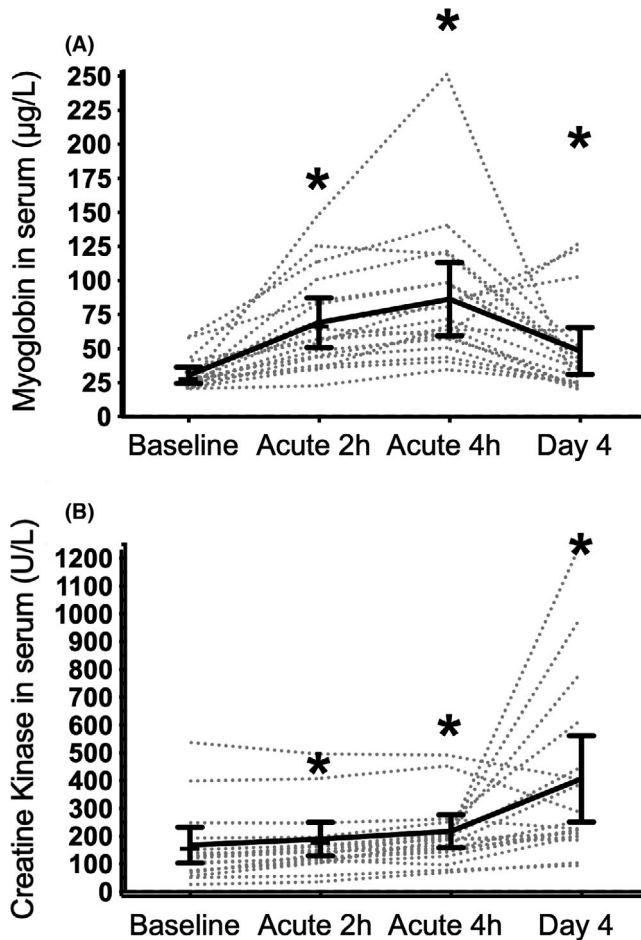
FIGURE 10 Creatine kinase and myoglobin. Increases in the (A) myoglobin and (B) creatine kinase levels at baseline, 2- and 4 h after the first BFRRE session, as well as at rest the fourth day of BFRRE. Dotted lines denote individual responses. * represents significant differences from baseline ($p < 0.05$). Data are expressed as means with 95% confidence intervals

4 | DISCUSSION

There were several notable findings in the present study. First, we demonstrate that both non-failure and failure high-frequency BFRRE can induce marked early increases in SCs, with no differences between protocols. Second, we corroborate and extend our previous finding that the increase in the number of myonuclei may occur gradually as opposed to only early in the time-course with both a non-failure and failure protocol during short-term frequent BFRRE. Third, echo intensity of VL and RF was increased over baseline in both legs during the first block of BFRRE (~20%), but decreased during the Rest Week and was generally lower in the non-failure than in the failure leg during and after the second training block. Interestingly, however, echo intensity was lower than baseline in both legs at 17 (~7%) and 24 days (~11%) post-training. Finally, we demonstrate that both

non-failure and failure protocols decreased muscle strength after the first week of strenuous high-frequency BFRRE, but more so after the failure protocol, followed by a very late supercompensation in strength ≥3 weeks after the last training session. Collectively, these findings indicate a temporary overtraining effect after both BFRRE protocols, especially during the first block of BFRRE.

4.1 | Training data

The knee extension exercise was used by Nielsen et al.[7] and by us at the same relative intensity (20% of 1RM). However, the range of repetitions differed between our respective studies. The participants in Nielsen et al.[7,19] performed only 45 repetitions per session in the first three days and on average ~52 repetitions in total per session during the first week, whereas the training volume in the present study and in our previous[4] was similar, around 80–84 repetitions per session during the first week in the legs that trained to failure in all sets. In the non-failure leg of the present study, the mean total training volume increased from 67 to 74 repetitions during Block1. Even these latter figures are considerably higher than the ~52 repetitions reported by Nielsen et al.[7,19] As discussed previously,[4] these discrepancies may be due to differences in cadence and possibly also in load, and probably also due to that we "pushed" our participants harder before they reached the criteria of voluntary failure.

4.2 | Satellite cell and myonuclear responses

The increases in the number of SCs (type I fibers: ~90% to 100%; type II fibers: ~45% to 55%) and myonuclei (~20%) during the short-term BFRRE in both the failure and non-failure leg were of similar or even greater magnitudes compared to responses seen previously in young men after longer periods (eg, 16 weeks) of heavy-load strength training (SCs: 30%–50%, and myonuclei number: ~20%).[20,21] However, in contrast to the BFRRE study of Nielsen et al.,[7] myofiber areas did not increase after the first block in the present study, and both our protocols induced merely one-fourth of the increases in SC numbers reported by Nielsen et al.[7] Moreover, while the peak increases in myonuclear number differed slightly in the present study compared to Nielsen et al.[7] and Bjørnsen et al.[4] (~20% vs 30% and 30%, respectively), the time courses of responses were very different. In Nielsen et al.,[7] the myonuclei and SC numbers peaked after the first week and SCs were then decreased from these peak values 10 days after the intervention, whereas we in the present study and in Bjørnsen et al.[4] observed gradual increases and peak values 10 days after the last training session. It could be speculated that this delayed peak in SCs was at least in part related to a regeneration process,
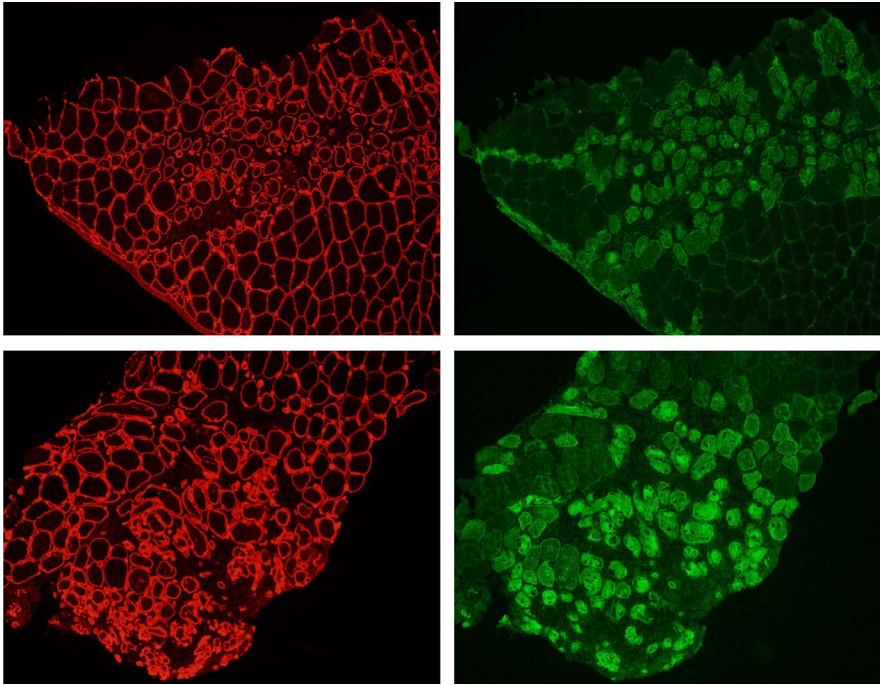
as previously observed after exercise-induced muscle damage (eg, [22]). In support of this hypothesis, we observed marked increases in internally located nuclei (95%–160%) in both fiber types, but more so in type I fibers of the failure leg after the first training block. Nuclei with an internal myofiber position are strongly associated with tissue regeneration in skeletal muscle.[15] Further evidence for some type of degeneration–regeneration response, such as NCAM-positive fibers, is discussed below.

Nevertheless, our finding in the present study of increased numbers of myonuclei in both type I and type II fibers after just seven sessions of BFRRE is in agreement with that of Nielsen et al,[7] and thus corroborates that myonuclear addition can occur already within 8–10 days of high-frequency BFRRE.

4.3 | Changes in whole muscle size and echo intensity during high-frequency BFRRE

Muscle CSA in the RF as well as muscle thickness in the VL and RF increased gradually during the training blocks and were elevated by 6%–10% after the BFRRE intervention, whereas the increase for VI was non-significant (~2%). Interestingly, the increases in echo intensity in the current study were prolonged especially in the failure leg, lasting for at least 10 days after both training blocks, but the non-failure leg also displayed prolonged (~3 to 10 days) elevations in echogenicity.

Normal muscle has a low echo intensity and is, therefore, relatively black on ultrasound pictures. The speckled appearance of muscle in pictures taken in the transverse plane is caused by reflections of perimysial connective tissue, which is moderately echogenic.[23] Notably, isotonic saline injection into muscle causes immediate increases in echo intensity,[24] which strongly suggests that fluid accumulation in the perimysial space (ie, between fascicles) is sufficient to cause increases in echogenicity. In addition, changes in connective tissue and/or adipose tissue can also increase muscle echo intensity,[24] and increased amounts of interstitial fibrous tissue are strongly correlated with elevations in echo intensity.[11]

The ~20% increases in echo intensity that we observed on Day5 in the first training block in both legs, together with the prolonged elevations, are thus probably indicative of some type of muscle damage and/or remodeling, and this notion is further supported by the fact that increases of this magnitude have been reported 2–5 days after acute maximal eccentric exercise.[25] We, therefore, suggest that the most plausible explanation for our results on echo intensity is prolonged remodeling of the extracellular matrix (ECM) and associated swelling of the perimysium and other interstitial spaces, related to muscle damage and/or stress and inflammation. In support of this suggestion, the strongly NCAM-positive fibers encountered in the samples from the Rest Week almost invariably displayed thickened laminin staining (Figure 6), thus providing direct evidence of extracellular matrix remodeling in these areas. In addition, the interstitial spaces were visibly increased, especially in the most damaged areas, in which there was apparent severe myofiber damage and/or atrophy and possibly even loss of some muscle fibers (Figure 11). Finally, we observed increased immunostaining of markers of ECM remodeling as well as muscle fibers

positive for other regeneration markers in addition to NCAM in muscle samples obtained at Rest Week and Post10 (unpublished data). The delayed peak in SCs could also in part be related to a potential remodeling of the extracellular matrix (eg, [26]). It should be noted that the muscle biopsies obtained in the Rest Week were taken 5 days after the last session in Week 1 of training, and that the echo intensity was elevated by ~20% in the VL in both legs just before the last two sessions in Week 1, as compared to the ~10% increase at Rest Week in the failure leg. Therefore, we probably did not capture the peak extent of ECM remodeling and interstitial edema in our muscle samples even in the failure leg.

Previous studies investigating the effects of high-frequency (twice daily) low-load BFRRE on increases in whole muscle size[27,28] have observed increases in quadriceps muscle CSA of 3.5% and 8% with one and two weeks training, respectively, corresponding to 0.5%–0.6% in CSA per calendar day. In the present investigation, the rates were somewhat lower, about 0.3%–0.5% in muscle thickness per day in VL and RF, and only about 0.1% per day in the VI. Our estimates are based on Post3, when echo intensity had essentially returned to baseline levels in the non-failure leg. It is possible that there were less muscle damage or stress-related remodeling in the studies by Fujita et al.[27] and Abe et al.,[28] which combined with a higher training frequency (12 sessions per week) in turn may have enabled an earlier and faster hypertrophic response.

However, it should be noted that neither of these studies included any assessments of sub-acute signal changes (eg, T2 relaxation times), and the post-training MRI measurements were performed at 48 hours after the last training session. Interestingly, Nielsen et al.[7] observed marked but transient increases (~27%) in myofiber areas in the group which performed low-load training without BFR, and suggested that early increases in MFA in the BFRRE group were similarly driven in part by transient swelling, as observed acutely after low-load BFRRE (eg,[29]). Therefore, it cannot be ruled out that some of the rapid gains in muscle CSA in the studies of Abe et al.[28] and Fujita et al.[27] consisted of interstitial and/or intracellular swelling, in addition to increased contractile protein content. This is also indicated by the data on estimated thigh muscle-bone area in Abe et al.,[28] which revealed a nearly 8% increase in just the first 6 days of training, but a subsequent ~2% loss during the 2 days of rest between the first and second training weeks.

In the present study, the increases in muscle thickness of RF and VL during the first block were correlated with the increases in echo intensity ($r \sim 0.42$ and $r \sim 0.70$, respectively) in both legs, and RF CSA and VI thickness decreased by ~5% and 7% in the 5 days between the last BFRRE session in Block1 and Day10 in the Rest Week. These findings strongly indicate that the increases in muscle size during the training blocks were in part driven by edema. Nevertheless, it is worth noting that the RF in the non-failure leg gained a further ~6%

in CSA over baseline from Day15 to Day22 (Post3), while echo intensity was almost unchanged from pre-training at the same time points. This suggests the possibility that a rapid hypertrophic response took place in the RF with Block2 in the non-failure leg, which is further supported by the relatively stable RF CSA from Post3 to Post24, although echo intensity decreased below baseline during the same period, as discussed below.

In sharp contrast, the VI muscle thickness decreased with a remarkable ~6% to 7% in both legs in just 3–5 days after both Block1 and Block2. It is of note that the VI appears to be the most active muscle in knee extension exercise at low loads.[30] We, therefore, suggest that the VI is overall the most stressed muscle with low-load BFR knee extensions. Unfortunately, we were unable to reliably assess echo intensity for the VI, because the ultrasound settings were optimized for the RF in the same pictures. Nevertheless, excessive stress of the VI would explain why this muscle showed both the most drastic decreases in muscle thickness during the Rest Week and the least hypertrophy post-training of the investigated muscles.

Most notably, while muscle thickness in the RF and VL and CSA in the RF did not change significantly between Post3 and Post24, there were marked decreases in echo intensity during the same period, ending up at ~10% below the baseline values in both legs and both RF and VL. Muscle echo intensity is increasingly used as a marker of muscle quality[23] and has been found to be inversely correlated with strength and power.[31] Furthermore, some studies have reported decreases in echo intensity after a period of strength training in both untrained[32] and trained participants.[12] Thus, the decreases in echo intensity of the present study concomitant with the delayed increases in strength occurring between 10 and 24 days after BFRRE indicate the intriguing possibility that remodeling was still going between these time points. A decreased echo intensity may have been caused by decreased perimysial spaces. The possibility of decreased interstitial spaces and thus increased packing of muscle fibers and fascicles has been suggested by Goldspink.[33]

4.4 | Changes in muscle fiber size during high-frequency BFRRE

In contrast to muscle thickness in *m. vastus lateralis*, the MFA did not increase and was even reduced in type I fibers of the failure leg at 10 days after the intervention in the present study. In further support of some type of degeneration–regeneration response, we observed small diameter myofibers that were strongly positive for NCAM in several biopsies obtained during the Rest Week and to a somewhat lesser extent also at Post10 (unpublished data).

The discrepancy between changes in MFA versus the increase in whole muscle CSA and -thickness is intriguing.

At least in part, this may be due to muscle fiber atrophy and/or some type of degeneration–regeneration induced by the very strenuous training regime. NCAM is markedly upregulated in regenerating human muscle fibers but purportedly not in necrotic or degenerating fibers.[34] On nearby sections, these fibers displayed for the most part intact but weak dystrophin staining. We therefore interpret these fibers as undergoing regeneration after some type of damage and/or stress and we are currently investigating the nature and the numbers of these NCAM + fibers in more detail, including other markers for degeneration–regeneration processes (unpublished data). We observed an increased interstitial space in some of our participants around the small NCAM-positive fibers (Figure 11). As discussed previously, we propose that an enlarged interstitial space occurred, presumably caused by extracellular swelling and remodeling of the extracellular matrix, and that this would in part explain both the increased echo intensity and the discrepancy between myofiber and whole muscle changes. Furthermore, given the results of our previous BFRRE study which showed late increases in myofiber areas,[4] it seems reasonable to speculate that the muscle fibers eventually recovered and perhaps even increased in size at Post17 and Post24. It also deserves to be noted that fiber hypertrophy and whole muscle hypertrophy can differ rather substantially after periods of resistance training, and that muscle fibers from a single level of a muscle are not necessarily representative of the whole muscle. For discussion and references, the reader is referred to Lexell et al.[35] and D'Antona et al.[36]

In contrast to our previous study,[4] we could not detect any significant type II fiber atrophy in this investigation, despite very similar training protocols and overall training volumes. On the other hand, we found a decrease in type I fiber areas which persisted at Post10 in the failure leg, in contrast to the 18% increase in type I fiber area at Post10 that we reported in our previous study. These discrepancies are puzzling and warrant some discussion.

In our previous study,[4] the subjects were generally better trained at the start of the training intervention than the subjects in this study. Another difference from our previous investigation is that the subjects in this study received 20 g of whey protein after every training session. Interestingly, Farup et al[37] showed that 20 g of whey protein preferentially enhanced type II fiber hypertrophy after concentric resistance training. It is thus possible that there was a similar preferential effect on type II fibers in this study, that is, the whey protein supplement largely protected type II fibers but not type I fibers from atrophy due to excessive stress. Another possibility, not mutually exclusive to those above, is that branching and perhaps also new fiber formation may be a regenerative response to severe stress which could serve to decrease the diffusion distance for oxygen

in the muscle fibers (for further discussion, refer Nygaard & Nielsen[38]). If the branching and new fiber formation is extensive enough, the whole muscle may hypertrophy even though the mean fiber area would seem to decrease, and branching might allow for cell survival until the microcirculation has adapted sufficiently to the imposed exercise-induced ischemia.

Regardless, the increases in internally located nuclei, the small muscle fibers strongly positive for NCAM in some of the subjects, and the increased echo intensity are all consistent with the proposal that some type of regeneration and remodeling was still ongoing as late as 10 days after the last BFRRE session in the failure leg.

4.5 | Changes in strength during high-frequency BFRRE

As already noted, the changes in strength were seemingly inversely paralleled by the changes observed in muscle echo intensity. This relationship was particularly striking between Post3 and Post24 in the failure leg, during which time the echo intensity in both RF and VL decreased markedly while MVC and 1RM increased, and the CSA and thickness in these muscles stayed elevated at an almost constant level. Interestingly, Nielsen et al[19] did not observe increases in isokinetic strength at slow velocity until 12 days after BFRRE, whereas fast velocity strength decreased at 5 days post-training, further supporting a delayed effect on force-generating capacity after high-frequency strenuous BFRRE. Very delayed strength increases have previously been observed after 4 weeks of detraining during high-frequency electrical stimulation.[8] However, to the best of our knowledge, we are the first to observe a delayed supercompensation in strength occurring as long as 24 days after BFRRE. The very late increases in strength observed with BFRRE in the present study may be explained by the prolonged time course of the physiological adaptations taking place at the muscle fiber level and/or in the surrounding extracellular matrix.

4.6 | Possible role of muscle damage in delayed effects of high-frequency BFRRE

It has been suggested that performing low-load BFRRE causes little or no muscle damage.[39] However, several recent studies have shown that performing acute high-volume BFRRE to voluntary failure can increase markers of muscle damage and cellular stress.[5,34,40–43] In line with this, we recently observed increases in markers of muscle damage with an initial block of frequent low-load BFRRE to voluntary failure.[4] The prolonged reductions and subsequent delayed increases in strength, as well as the DOMS, elevated levels of

CK and myoglobin, decreased type I fiber areas and marked increases in muscle echogenicity in the present study are also in line with some degree of muscle damage and stress.

An important point when comparing our results to previous studies on frequent low-load BFRRE concerns the progression of the training during the first sessions. For example, Abe et al.[28] gradually increased the BFR pressure during the first 10 days of training. Similarly, and as noted earlier, the participants in the investigation of Nielsen et al.[7,19] performed only 45 repetitions per session in the first three training bouts. In contrast, the present study involved BFRRE to failure in one leg and near-maximal efforts also in the non-failure leg BFRRE in the early stages of training, as several participants failed to complete the target repetitions in the non-failure leg in the first three BFRRE bouts. The EMG results generally support high levels of muscle activation also in the non-failure leg in the last 1–2 sets. Thus, a gradual familiarization to BFRRE may be necessary to protect against excessive and potentially counterproductive muscular stress at the initiation of training, which in turn could explain many of the discrepancies between our findings and those of Nielsen et al.[7]

Finally, the attenuated responses in echo intensity, DOMS, and perceived pain, as well as in acute decrements in force with the second block compared with the first block of BFRRE, are in line with the repeated-bout effect after strenuous acute BFRRE reported by Sieljacks et al,[5] which support a repeated bout effect reminiscent that seen after damaging eccentric exercise.[5]

4.7 | Strengths of the study

We employed strength tests that were both standardized and designed to minimize any "learning effects", thus allowing valid assessments of quadriceps muscle function. In parallel with these, we investigated the trophic state and adaptations of the muscles at both cellular and whole muscle levels and at many time points, particularly with reference to the post-training period. Collectively, our results may have important implications for the interpretations and the choice of time points of muscle size and strength measurements not only after BFRRE but also with conventional heavy resistance exercise. Our findings may also shed light on some of the mechanisms involved in the phenomena of exercise-induced overreaching and recovery and subsequent improvements of performance.

4.8 | Limitations

First of all, several participants did not reach the repetition goal in the non-failure leg in the first few sessions of Block1; hence, some sets were performed to volitional failure also in the non-failure leg. Second, we used an identical absolute cuff pressure across all participants. However, the same cuff pressures were always applied to both the failure and non-failure leg of each participant. Furthermore, based on the small variations in arterial occlusion pressure (AOP) in our recent study,[4] we argue that we achieved reasonably similar degrees of BFR in our subjects. Third, while within-subject designs have several advantages,[44] positive cross-transfer effects of lower-body BFRRE on the trophic state of upper limb muscles trained with low loads but without BFR have been reported.[45] A positive cross-transfer effect could potentially cause more uniform results between the failure versus non-failure training protocols. Conversely, other investigations have observed detrimental effects of strenuous unilateral exercise on the muscles of the resting contralateral limb (eg, [46]). Thus, we cannot exclude possible "cross-transfer" effects, both positive and negative, from the failure leg to the non-failure leg (and vice versa). In addition, the failure versus non failure protocol was randomized between the right and left leg, but having a fixed order of left and right leg during exercise and testing could still have induced systemic bias on capacity for volitional central neural activation between the two legs. Finally, our counting criteria for NCAM positive staining seems to be more restrictive than what is commonly used, causing the average number of SCs per muscle fiber at baseline to be lower in the present study compared to the majority of reports (~0.05 at in the present study versus ~0.10 in eg, [7, 13, 20]). This may have reduced the sensitivity of our SC analysis.

In conclusion, we report that both a non-failure and failure high-frequency short-term BFRRE protocol induced pronounced responses in SC proliferation, but delayed myonuclear addition and increases in whole muscle size, concomitantly with very delayed increases in strength. The initial strength decrements followed by delayed hypertrophy and strength increases are reminiscent of the supercompensation observed after periods of resistance exercise "overreaching".[8,9] While the gains in SC and myonuclear numbers as well as muscle size and strength were similar between the legs, perceptions of exertion, pain, and DOMS were lower in the non-failure leg. Hence, non-failure BFRRE may be a more feasible approach.

4.9 | Perspectives

It seems that very strenuous protocols with a high overall training volume can be counterproductive at least in the initial stages with high-frequency BFRRE. Our findings may also shed light on some of the mechanisms involved in the phenomena of exercise-induced overreaching and subsequent recovery, and improvements of performance. In addition, our results may have important implications for the interpretations and the choice of time points of muscle size

and strength measurements, not only after BFRRE but also with conventional heavy resistance exercise. In any case, our findings demonstrate that a gradual familiarization to BFRRE may be necessary and that it seems important that BFRRE is introduced carefully and gradually, to allow the repeated-bout effect to take place and to avoid excessive muscular stress and damage at the initiation of training. Future research should investigate if gradual familiarization can precondition against muscle damage at initiation of frequent strenuous BFRRE and thereby achieve faster and greater gains in muscle size and strength.

AUTHOR CONTRIBUTIONS

The contributions of the authors are as follows: conception and design of the study: TB, MW, GP, and TR. Collection, analysis, and interpretation of data: TB, MW, GP, SB, RB, HS, JS, and TR. Drafted the manuscript: TB. Critically evaluated and contributed to the manuscript: TB, MW, GP, SB, RB, HS, JS, and TR. All authors have approved the final version of the manuscript.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

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