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Blood flow restricted training leads to myocellular macrophage infiltration and upregulation of heat shock proteins, but no apparent muscle damage

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Key points

- Muscular contractions performed using a combination of low external loads and partial restriction of limb blood flow appear to induce substantial gains in muscle strength and muscle mass.
- This exercise regime may initially induce muscular stress and damage; however, the effects of a period of blood flow restricted training on these parameters remain largely unknown.
- The present study shows that short-term, high-frequency, low-load muscle training performed with partial blood flow restriction does not induce significant muscular damage.
- However, signs of myocellular stress and inflammation that were observed in the early phase of training and after the training intervention, respectively, may be facilitating the previously reported gains in myogenic satellite cell content and muscle hypertrophy.
- The present results improve our current knowledge about the physiological effects of low-load muscular contractions performed under blood flow restriction and may provide important information of relevance for future therapeutic treatment of muscular atrophy.

Abstract Previous studies indicate that low-load muscle contractions performed under local blood flow restriction (BFR) may initially induce muscle damage and stress. However, whether these factors are evoked with longitudinal BFR training remains unexplored at the myocellular level. Two distinct study protocols were conducted, covering 3 weeks (3 wk) or one week (1 wk). Subjects performed BFR exercise (100 mmHg, 20% 1RM) to concentric failure (BFRE) (3 wk/1 wk), while controls performed work-matched (LLE) (3 wk) or high-load (HLE; 70% 1RM) (1 wk) free-flow exercise. Muscle biopsies (3 wk) were obtained at baseline (Pre), 8 days into the intervention (Mid8), and 3 and 10 days after training cessation (Post3, Post10) to examine macrophage (M1/M2) content as well as heat shock protein (HSP27/70) and tenascin-C expression. Blood samples (1 wk) were collected before and after (0.1–24 h) the first and last training session to examine markers of muscle damage (creatine kinase), oxidative stress (total antibody capacity, glutathione) and inflammation (monocyte chemotactic protein-1, interleukin-6, tumour necrosis factor α). M1-macrophage content increased 108–165% with BFRE and LLE at Post3 ($P < 0.05$), while M2-macrophages increased (163%) with BFRE only ($P < 0.01$). Membrane and intracellular HSP27 expression increased 60–132% at Mid8 with BFRE ($P < 0.05$–0.01). No or only minor changes were observed in circulating markers of muscle damage, oxidative stress and inflammation. The amplitude, timing and localization of the above changes indicate that only limited muscle damage was evoked with BFRE. This study is the first to show that a period of high-frequency, low-load BFR training does not appear to induce general myocellular damage. However, signs of tissue inflammation and focal myocellular membrane

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stress and/or reorganization were observed that may be involved in the adaptation processes evoked by BFR muscle exercise.

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Abbreviations 1RM, 1-repetition maximum; BFR, blood flow restricted; CK, creatine kinase; DOMS, delayed onset muscle soreness; GSH, glutathione; HSP, heat shock protein; IF, immunofluorescence; IL-6, interleukin-6; MCP-1, monocyte chemotactic protein 1; MP, macrophage; MSC, myogenic satellite cell; TAC, total antioxidant capacity; TNF-α, tumour necrosis factor α; VAS, visual analogue scale; WB, Western blotting.

Introduction

Muscle exercise using concurrent blood flow restriction (BFR) and low-load [20–50% of maximal load (1-repetition maximum, 1RM)] contraction intensities has shown promising results in terms of inducing increases in maximal muscle strength and muscle mass (Takarada *et al.* 2000*b*, 2002; Nielsen *et al.* 2012), also when compared to heavy resistance exercise (65–90% of 1RM) (Takarada *et al.* 2000*b*). In consequence of the marked adaptive change in muscle mass and contractile strength despite using low external loadings, muscle exercise performed under BFR has been suggested to possess therapeutic potential in individuals who lack muscle mass and in whom training with heavy external loading may be contra-indicative.

However, although previously addressed by some researchers (Manini & Clark, 2009; Clark *et al.* 2010; Loenneke *et al.* 2011) the potential adverse side effects and safety concerns related to BFR exercise remains largely unresolved, including the potential for evoking exercise-induced muscle damage. Recently, we showed that short-term (3 weeks), high-frequency (twice daily), low-load BFR training led to marked proliferation of myogenic satellite cells (MSCs) (~250% elevated post training) (Nielsen *et al.* 2012). MSCs represent a pool of myogenic precursor cells situated in the skeletal muscles that play a key role in both myofibre growth and regeneration (Wang & Rudnicki, 2012; Blaauw & Reggiani, 2014). With high-frequency BFR training, MSC activity appears to play an important role in the high magnitude of myofibre hypertrophy observed (Nielsen *et al.* 2012). However, it is unclear whether the marked proliferation of MSCs with BFR exercise is in part stimulated by the presence of myocellular damage, as previously reported after exhaustive eccentric muscle exercise (Crameri *et al.* 2007; Mackey *et al.* 2011; Saclier *et al.* 2013).

Previous investigations have examined the potential of muscle damage with BFR exercise. A majority of studies have reported no or minor changes in circulating markers of muscle damage (i.e. creatine kinase (CK), myoglobin) (Takarada *et al.* 2000*a*; Abe *et al.* 2006; Karabulut *et al.* 2013) and inflammation (C-Reactive Protein, interleukin-6 (IL-6)) (Clark *et al.* 2010; Karabulut *et al.* 2013; Patterson *et al.* 2013) in response to acute and longitudinal BFR exercise modes. In contrast, a recent study reported large (11- to 36-fold) increases in circulating CK and myoglobin levels 2 and 4 days after an acute bout of unaccustomed BFR exercise indicating severe acute muscle damage (Sieljacks *et al.* 2016). Furthermore, Cumming and co-workers recently showed an upregulation in heat shock proteins (HSPs) (i.e. HSP70 and αB-crystallin) within myofibres along with intracellular translocation of HSPs (i.e. HSP27, HSP70 and αB-crystallin) to cytoskeletal structures after acute BFR exercise (Cumming *et al.* 2014). HSPs acts as molecular chaperones and exercise-induced increases in myocellular expression of HSP proteins have been related to myocellular stress, ranging from metabolic stress to severe structural myofibre damage (Morton *et al.* 2006, 2009; Paulsen *et al.* 2009).

Characteristic initial responses to muscle-damaging exercise may involve ultrastructural disruption, intracellular HSP accumulation at damaged structures and increased myocellular content of MSCs (Crameri *et al.* 2007; Paulsen *et al.* 2009; Mackey *et al.* 2011). After the event of initial damage the innate immune system can act to augment the myocellular trauma response, while local tissue inflammation processes also seem important for ensuring optimal regeneration (Tidball, 2011; Chazaud, 2016). In this context, macrophages (MPs) are interesting as they appear to mediate important pro- and anti-inflammatory responses in relation to muscle damage (Tidball, 2011; Chazaud, 2016; Schiaffino *et al.* 2016). Most human studies have investigated MP infiltration in injured human skeletal muscle without distinguishing between subpopulations of MPs. Differentiating between the main macrophage subpopulations; i.e. pro- (M1) and anti- (M2) inflammatory MPs appear important, as they possess distinct and contrasting functional roles. M1-MPs are mainly present in the early phase after injury, where they are involved in removal of cell debris (i.e. phagocytosis), whereas M2-MPs are mainly present in later stages of regeneration, where they attenuate the inflammatory response and promote tissue remodelling/repair (Arnold *et al.* 2007; Tidball, 2011; Chazaud, 2016; Schiaffino *et al.* 2016).

Considered overall, the literature show contrasting findings with regard to whether or not BFR exercise/training leads to muscle damage. Most studies have assessed indirect/direct markers of inflammation, stress and muscle damage after acute BFR exercise in previously unaccustomed subjects. Consequently, markers of inflammation and myocellular damage have yet to be investigated directly at the myocellular level following longitudinal (weeks to months) training.

Therefore, the aim of the present study was to investigate the effect of longitudinal BFR training on molecular markers of muscle damage and inflammation, including analysis performed at the myocellular level as well as in the circulation (plasma). Specifically, the effect of low-load BFR exercise on muscle damage and inflammation was investigated using two distinct randomized, controlled study protocols. In an initial 3 week study (3 wk) we investigated myocellular damage and inflammation in response to low-load BFR exercise and work-matched free-flow exercise (Nielsen *et al.* 2012). Subsequently, we performed a 1 week study (1 wk) to gain more insight about the changes occurring in the very early phase of low-load BFR training in comparison to a training protocol based on heavy-load resistance exercise.

It was hypothesized that longitudinal short-term high-frequency BFR training would increase the expression of molecular markers related to myocellular damage and inflammation, with the most marked changes emerging in the early phase of the intervention, while these markers would remain unaffected by work-/load-matched free-flow muscle exercise. In addition, we expected that BFR training and heavy-load free-flow resistance training would induce similar changes in circulating markers of muscle damage and inflammation, i.e. demonstrate increase following the first training session followed by a return to baseline levels during subsequent days of training.

Methods

Study participants

This study consisted of two distinct intervention protocols (3 wk and 1 wk) using two separate study populations as detailed in Fig. 1. All subjects volunteered to participate in the interventions protocols. In the 3 week study (3 wk), 20 subjects were included, 12 performed BFR training (BFRE) (age: 23 ± 2 years, height: 181 ± 6 cm, body weight: 82 ± 14 kg), while 8 performed work-matched free-flow

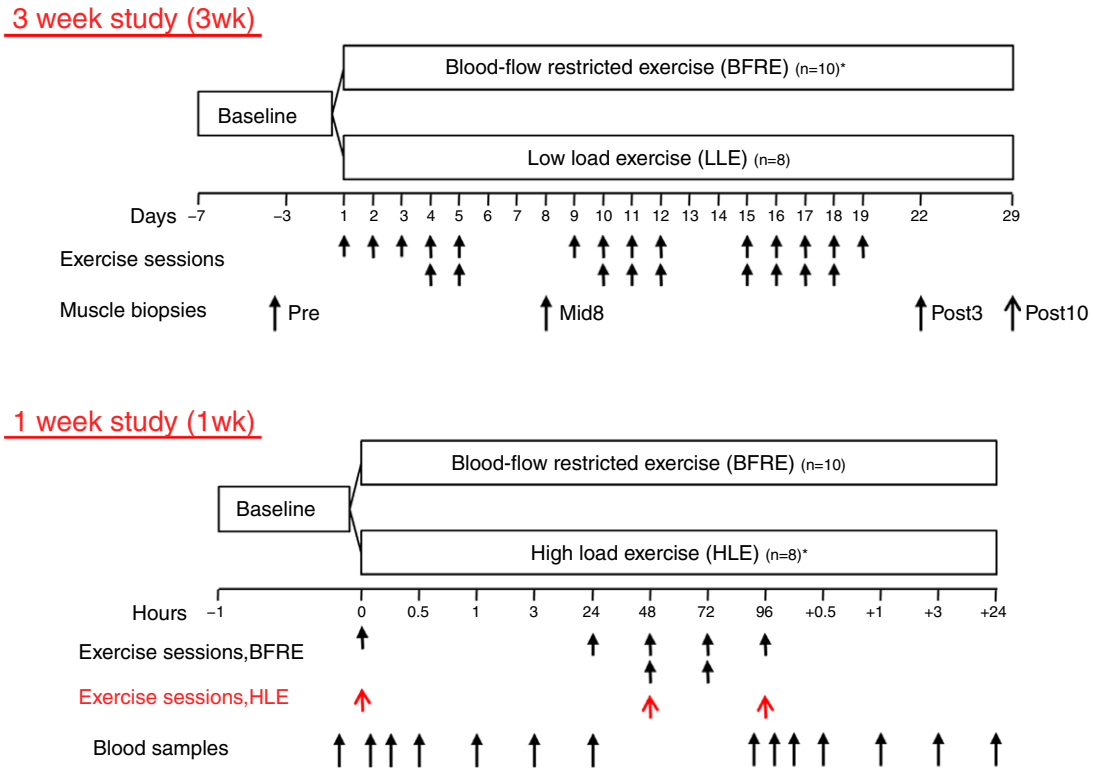

Figure 1. Schematic outline of the study protocol
Timing of exercise sessions, muscle biopsies and blood samples are represented by upward-facing arrows; 1wk study: arrows with filled/open arrowheads denote exercise sessions for the BFRE/HLE group, respectively. *initially $n = 12$ and $n = 10$ were included in BFRE (3 wk) and HLE (1 wk); 2 participants in each group left the study prematurely due to circumstances unrelated to the study intervention. [Colour figure can be viewed at wileyonlinelibrary.com]

training ('low-load exercise', LLE) (age: 22 ± 2 years, height: 181 ± 6 cm, body weight: 82 ± 14 kg). In the 1 week study (1 wk), 20 subjects were randomized to either BFRE ($n = 10$) (age: 23 ± 2 years, height: 182 ± 4 cm, body weight: 81 ± 9 kg) or heavy-load free-flow resistance training ('high-load exercise', HLE) ($n = 10$) (age: 24 ± 3 years, height: 182 ± 10 cm, body weight: 76 ± 4 kg). All participants were healthy recreationally active men aged 18–35 years, who had not participated in systematic resistance training 1 year prior to the study. Study protocols were accepted by the local Ethics Committee (Region of Southern Denmark) (S-200900070/S-20150022) in accordance with the *Declaration of Helsinki* and written informed consent was obtained prior to inclusion. Data related to the 3 week study that are not included in the present report (myofibre size, satellite cell/myonuclei content and muscle strength) have been reported elsewhere (Nielsen *et al.* 2012).

Exercise protocols

Intervention procedures, 3 wk study: the training protocol has been described in detail previously (Nielsen *et al.* 2012). In brief, participants completed a 3 week training programme consisting of 23 exercise sessions (weeks 1/2/3: 7/7/9, 1–2 daily training sessions Mon–Fri) separated by at least 4 h. BFRE and LLE subjects performed four sets of unilateral knee extensor exercise at 20% of 1RM interspersed by 30 s pause. BFRE performed repetitions to concentric failure at a concentric/eccentric phase cadence of 1.5 s/1.5 s, while LLE performed a work-matched protocol without blood flow restriction.

Intervention procedures, 1 wk study: subjects completed a 1 week supervised training programme involving seven or three exercise sessions for BFRE and HLE, respectively. BFRE performed high-frequency exercise once per day (Mon, Tue, Fri) and twice per day (Wed, Thu) while HLE trained once per day (Mon, Wed, Fri). Successive training sessions were separated by at least 4 h. BFRE and HLE subjects performed four sets of unilateral knee extensor exercise to concentric failure using a load of 20 and 70% of 1RM, respectively. Rest periods between sets were 30 and 90 s for BFR and HLE. The discrepancy in training frequency between groups enabled matching on total volume.

During all exercise sessions, BFRE subjects (1 wk/3 wk study) were subjected to blood flow restriction of the working limb achieved by placing a tourniquet cuff (13.5 cm width) (Delfi Medical, Vancouver, BC, Canada) around the proximal end of the working limb (for details, see Nielsen *et al.* 2012). Cuff inflation pressure was set at 100 mmHg and controlled by a computerized tourniquet system (A.T.S. 750, Zimmer, Warsaw, IN, USA). The cuff remained inflated until cessation of the fourth (final) exercise set.

Muscle biopsy sampling (3 wk study)

Muscle biopsy samples (~150 mg) were obtained from the m. vastus lateralis (VL) with a 5 mm Bergström biopsy needle under local anaesthesia and sterile conditions (1% lidocaine (lignocaine), Amgros 742122, Copenhagen, Denmark), as described previously (Aagaard *et al.* 2001; Nielsen *et al.* 2012). Muscle biopsies were obtained from the same region and depth of the VL in a randomized order, approximately 2–3 cm apart to avoid potential bias of multiple muscle samples. Muscle samples were aligned and mounted in Tissue-Tec (4583, Sakura Finetek, AV Alphen aan den Rijn, The Netherlands) and subsequently frozen in isopentane pre-cooled in liquid nitrogen and stored at –80°C.

Immunoblotting (3 wk study)

Muscle samples specimens were put in ice-cold homogenizing buffer containing (in mM) 300 mM sucrose, 1 mM EDTA, 10 mM sodium azide, 40 mM Tris-base (pH 7.8) and a protease inhibitor (5892791001, Sigma-Aldrich, St. Louis, MO, USA). Muscle tissue samples were homogenized in a glass Teflon homogenizer with a pestle. Samples were kept on ice during the whole procedure. Aliquots of homogenate were frozen at −180°C for later analysis.

Standardized volumes of homogenate were loaded per well (10–20 mg) and separated by SDS-PAGE (10%) (Bio-Rad (BR), Hercules, CA, USA) for 30 min at 180 V in room temperature running buffer (162–0734, BR). Subsequently, samples were transferred to polyvinylidene difluoride (PVDF) membranes (162–0177, BR) for 60–90 min at 100 V in 4°C transfer buffer (162–0734, BR). Then, membranes were blocked at room temp. for 30 min in 5% non-fat milk (170–6404, BR), after which they were incubated with primary antibodies: anti-HSP27 (SPA-803, StressGen (SG), San Diego, CA, USA; 1:1000), anti-HSP70 (SPA-810, SG; 1:1000) and GAPDH (D16H11, Cell Signalling, Danvers, MA, USA; 1:1000) at 4°C overnight. Membranes were washed in TBS/0.05% Tween (28358, Thermo Scientific (TS), Waltham, MA, USA/170–6531, BR) at room temp. for 3 × 5 min and then incubated with secondary antibody (GE Healthcare, Chicago, IL, USA; 1:5000) at room temp. for 30 min. Membranes were visualized using Super-Signal West Dura Extended Duration Substrate (34075, TS) and Bio-Rad ChemiDoc XRS+ (BR). Band intensities were evaluated with Image Lab software (BR). HSP27/70 expressions were normalized to GAPDH.

Immunofluorescence microscopy (3 wk study)

Transverse serial sections (8 μm) of the embedded muscle biopsy specimen were cut at −22°C (HM560, Microm, Germany) and were mounted on glass slides. Tissue

sections were fixed for 10 min at room temperature in a 4% formaldehyde fixation buffer containing 0.05% Triton X-100 (Sigma-Aldrich, St Louis, MO, USA). After fixation, sections were blocked (X0909, Dako) for 10 min, and subsequently primary and secondary antibodies were applied for 60 min incubation.

Macrophages (MPs) were visualized using antibodies for CD68 (M0814, Dako; 1:100) and CD206/MMR (AF2435, R&D systems, Minneapolis, MN, USA; 1:100), while laminin (Z0097, Dako, 1:250) was added in order for distinction of the myofibre sarcolemma. Additional stainings were performed with markers of cellular stress and damage: heat shock proteins 27 and 70 (HSP27/70) (SPA-803/SPA-810, SG; 1:1000/1:100), tenascin C (ncl-tenas-c, Novocastra, Wetzlar, Germany; 1:200) and laminin (Z0097, Dako; 1:1000). Specific secondary antibodies were used; order listed: Alexa-555 donkey-anti-mouse (A31570, TS; 1:1000), Alexa-488 donkey-anti-goat (A11055, TS; 1:1000) and Alexa-350 donkey-anti-rabbit (A10039, TS; 1:250); Alexa-555 donkey-anti-rabbit (A31572, TS; 1:1000) and Alexa-488 goat-anti-mouse (A11029, TS; 1:1000). Finally, mounting medium (H-5501, Vector, Olean, NY, USA) was added, and slides and cover glass were pasted together and stored protected from light at 5°C. Staining specificity was verified using negative controls. Immunofluorescence staining was visualized on a computer screen using a microscope (Axio Imager M1, Carl Zeiss, Germany) and a high-resolution AxioCam (Carl Zeiss). Digital images were captured using a ×10 objective and standardized exposure.

Quantification of immunofluorescence images (3 wk study)

For quantification of MPs, distinction between pro- (M1) and anti- (M2) inflammatory MPs was achieved by analysis of $CD68^+/CD206^-$ cells (M1), $CD68^+/CD206^+$ (M2) and $CD68^-/CD206^+$, respectively. In addition, MP location was noted by distinguishing between extracellular as well as intracellular sublaminal and central positions (Fig. 5). Numbers of recorded MPs were normalized to number of myofibres and total area analysed, respectively. Myofibre regeneration was defined as myofibres with infiltration of >1 MP; the number of involved myofibres and total area were noted. Furthermore, non-MP nuclei with a central myofibre position were counted, as an additional indicator of myofibre regeneration (Folker & Baylies, 2013). These analyses were performed in 519 ± 272 fibres.

HSP27 and HSP70 immunofluorescence intensity was measured within myofibres (intracellular). In addition, HSP27 revealed specific membrane expression (cf. Fig. 5), therefore separate intensity analysis of the myofibre membrane was performed. Intensity analyses were performed in 142.9 ± 35.3 (HSP27) and 113.8 ± 15.6 (HSP70) myofibres. All immunofluorescence intensity measures were background corrected for an average of three separate measures and subsequently corrected to the intensity level of negative fibres (average of 25 fibres). A ratio index was calculated to evaluate the degree of staining intensity for individual fibres, i.e. myofibre intensity divided by the average intensity of negative fibres. Accordingly, myofibres and membranes with an intensity index of 0–2, 3–5 and ≥6 were classified as negative, medium or highly positive, respectively. The above image analyses were performed using AxioVision 4.6 (Carl Zeiss).

Quantification of tenascin C calculated as the immunoreactive area expressed relative to the total area in ≥4 random images for each time point (Mackey *et al.* 2011). Care was taken to exclude any immunoreactive areas that were not related to the analysis or were typically unequally distributed between biopsy tissue sections (auto-fluorescence, large vessels, etc.). This analysis was performed in ImageJ 1.8 (NIH; Bethesda, MD, USA) using the built-in threshold function. All quantifications of immunofluorescence images were performed manually with the investigator blinded for group, subject-ID and time point.

Blood sampling (1 wk study)

Blood samples were obtained from the antecubital vein in a supine position. Resting blood samples were drawn ∼5 min before the first (A) and last (B) exercise session, while additional samples were taken at 5, 15, 60 and 180 min (P5, P15, P60, P180) and at 24 h (P24h) after cessation of the first and last training session. All blood samples were obtained in EDTA vials, centrifuged at 4000 r.p.m. and 5°C for 8 min, after which plasma was isolated and stored at 80°C for later analysis. Blood sampling was obtained in a fasted state (≥12 h) and conducted at the same time of the day (≤60 min) to avoid any influence from nutrient intake and diurnal variations. One exception from fasting was P24h after the last training session, as an optimal energy level in the evaluation of functional performance was prioritized here. Subjects were asked to refrain from any additional moderate to strenuous exercise as well as ingestion of caffeine and alcohol 48, 12 and 24 h before blood sampling, respectively.

Blood analysis (1 wk study)

Creatine kinase (CK) activity was analysed in selected blood samples (Pre, P60, P180 and P24h of first and last training session), while all other analyses were performed on all blood samples. Quantification of CK (muscle damage marker) as well as glutathione

(GSH) and total antioxidant capacity (TAC) (markers of redox reactions) were performed with commercial kits (MAK116, CS0260, CS0790, Sigma-Aldrich, USA) in accordance with manufacturer's instructions. Analyses of markers related to inflammation: monocyte chemotactic protein 1 (MCP-1), interleukin-6 (IL-6) and tumour necrosis factor α (TNF-α) were performed using AlphaLISA Kits in accordance with manufacturer's instructions (AL244C, AL223C, AL208C; Perkin Elmer, USA).

Perceived pain and soreness (1 wk study)

Subjects' perceived pain (visual analogue scale, VAS) and delayed onset muscle soreness (DOMS) were evaluated in the resting condition and during activity (10 step stair climb) immediately before, and 24 and 48 h after the first and last training session in each group. Evaluation of VAS and DOMS were performed using a 100 mm scale; zero was designated as 'no pain/soreness' while 100 mm denoted 'worst imaginable pain/soreness'.

Statistical analysis (1 wk + 3 wk study)

Assumptions of homogeneity of variance and a Gaussian distribution of the data were verified. A mixed linear model was used to evaluate interactions, main effect and pre-to-post changes for all variables with subject-ID defined as a random effect and time and group as fixed effects (except for ordinal data sets (DOMS, VAS)). Data with a non-Gaussian distribution were transformed. Group data sets with one time point (training data) were compared using unpaired Student's *t*-tests. Ordinal data sets and data sets that did not meet the criteria of Gaussian distribution (HSP27 intensity index) after transformation (IL-6, TNF-1α) were assessed using Wilcoxon signed-rank testing (within-group assessment from baseline). Outliers were defined as mean ± 2.5 SD (Leys *et al.* 2013) and accordingly two outliers were excluded from the 1 wk study dataset (one from TNF-1α (BFRE) and one from IL-6 (BFRE)); this approach did not change the statistical results. All statistical analyses were performed using STATA 14.1 (StataCorp, College Station, TX, USA). The α level was set at 0.05 for all tests using two-tailed comparison. Values are presented as means ± SD, unless otherwise stated.

Results

Training data (1 wk + 3 wk study)

Training data from the 3 wk study have been reported previously (Nielsen *et al.* 2012). For the 1 wk study, no difference between total absolute (total repetitions × 1RM load) (BFRE/HLE: 3102.2 ± 904.3/3462.9 ± 550.3 kg) or relative (total repetitions × %1RM) training volume was observed (BFRE/HLE: 81.3 ± 17.2/92.2 ± 23.0%) between BFRE and HLE, although the numbers of weekly training sessions were different (BFRE/HLE: 7/3). When comparing total training volume per set, HLE demonstrated a 34–600% larger volume per set in all four sets performed (BFRE/HLE; set 1: 295.6 ± 71.1 *vs.* 398.2 ± 58.7 kg; set 2: 76.4 ± 29.8 *vs.* 290.5 ± 34.7 kg; set 3: 39.6 ± 23.3 *vs.* 245.3 ± 50.7 kg; set 4: 31.6 ± 27.0 *vs.* 220.3 ± 53.8 kg) ($P < 0.01$).

Macrophage content (3 wk study)

$CD68^+/CD206^-$ MP (M1) content expressed per square millimetre of fibre cross-sectional area increased in BFR subjects from Pre (3.37 ± 2.85) and Mid8 (3.93 ± 2.66) to Post3 (6.03 ± 4.03) ($P \leq 0.001$; $P < 0.05$), corresponding to relative changes of 121 and 72%. In LLE, $CD68^+/CD206^-$ MPs increased 107% and 76% from Pre (2.85 ± 1.58) and Mid8 (3.77 ± 1.85) to Post3 (6.67 ± 3.71) ($P < 0.01$, $P < 0.05$) (Fig. 2). For $CD68^+/CD206^+$ MPs (M2), a tendency towards a group × time interaction was observed at Post3 ($P = 0.056$). Further analysis revealed increases of 109% and 82% from Pre (12.23 ± 4.56) and Mid8 (13.52 ± 7.90) to Post3 (23.62 ± 11.99) ($P < 0.001$) in BFRE subjects ($P < 0.001$), while an increase from Pre to Post10 was observed as well ($P < 0.05$) (Fig. 2). For $CD68^-/CD206^+$ cells an increase (120%) and a tendency to an increase was observed in BFR subjects from Pre to Mid8 and Post3, respectively ($P < 0.05$, $P = 0.067$), while an increase of 120% was observed between Pre and Post3 in LLE ($P < 0.05$) (Fig. 2).

A similar pattern was observed when MPs were expressed relative to myofibre number. Thus, a 165% and 84% increase in $CD68^+/CD206^-$ MPs (M1) per 100 myofibres was observed after BFR training from baseline (1.39 ± 1.33) and Mid8 (2.00 ± 1.35) to Post3 (3.69 ± 2.71) ($P \leq 0.001$, $P < 0.05$). In LLE, $CD68^+/CD206^-$ MPs increased 108% from Pre (1.66 ± 0.60) to Post3 (3.46 ± 1.87) ($P < 0.05$) (Fig. 2). For $CD68^+/CD206^+$ MPs (M2) a group × time interaction was observed at Post3 ($P < 0.05$). Further analysis revealed increases of 163% and 84% in BFR subjects from Pre (6.05 ± 2.11) and Mid8 (8.62 ± 4.71) to Post3 (15.92 ± 9.92), respectively ($P \leq 0.001$) (Fig. 2). Increases in $CD68^-/CD206^+$ cells were observed with BFR training from baseline to Mid8 and Post3 ($P < 0.01$, $P < 0.05$), respectively, corresponding to relative changes of 177% and 134%, respectively. In LLE, $CD68^-/CD206^+$ cells tended to increase and showed an increase (129%) from baseline to Mid8 and Post3, respectively ($P = 0.054$; $P < 0.05$) (Fig. 2). Overall no changes in M1/M2 MP ratio were observed, and no signs of severe myofibre regeneration/necrosis (i.e. myofibres with >1 MP) were observed.

Central nuclei (3 wk study)

For type II fibres increases in centrally placed nuclei were observed with BFR training from baseline (2.60 ± 1.26) to Mid8 (6.32 ± 4.50), Post3 (6.70 ± 2.59) and Post10 (5.19 ± 5.23), corresponding to relative changes of 112%, 158% and 100% ($P < 0.05$–0.01), respectively. In addition, a tendency to increases in type I myofibres from baseline (2.20 ± 1.75) to Mid8 (4.56 ± 2.92) and Post3 (4.09 ± 2.69) ($P = 0.054$–0.055) emerged in BFRE. No changes were noted in LLE.

Heat shock protein (3 wk study)

HSP70 expression evaluated with Western blotting (WB) analysis showed a tendency towards an increase at Post3 ($P = 0.06$) in LLE, while no changes were observed in BFRE (Fig. 3). Similarly, HSP70 evaluated with immunofluorescence (IF) analysis showed an increase of 58% Post3 in LLE ($P < 0.05$) with no changes in BFRE. As the HSP70 myofibre intensity index remained fairly uniform between baseline and later time points, no further analysis was performed.

HSP27 expression (WB) increased 82% from baseline to Mid8 in BFRE ($P < 0.05$), while an increase of 83% was seen from baseline to Post3 in LLE ($P < 0.05$) (Fig. 3). HSP27 expression evaluated with IF revealed 60% and 132% increases in BFRE subjects between baseline and Mid8 for the within fibre and membrane analysis, respectively ($P < 0.05$), while no changes were observed in LLE (Fig. 4 and 5). A significant group × time interaction was observed for the membrane fraction ($P < 0.05$) at Mid8. Although a HSP27 pattern towards more light and highly positive myofibres and membranes showed especially at Mid8 (Fig. 4 and 5), these changes seldom reached statistical significance due to high levels of within-group variation. However, an increase in medium positive membranes was noted between baseline (10.9 ± 11.7%) and Mid8 (23.8 ± 16.7%) in subjects exposed to BFRE.

Tenascin C (3 wk study)

No changes in tenascin C expression were observed in either group. Immunoreactivity was <1.8% in all samples.

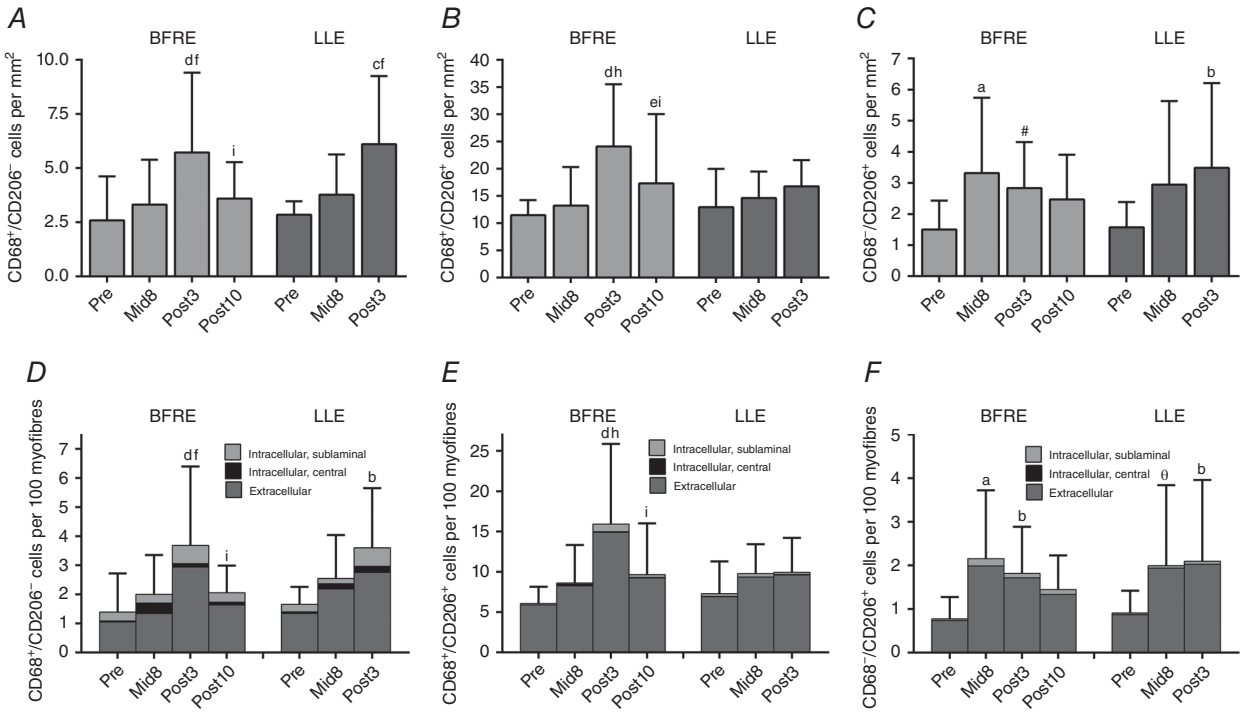

Figure 2. CD68$^+$/CD206$^-$, CD68$^+$/CD206$^+$ and CD68$^-$/CD206$^+$ cells at baseline (Pre), 8 days into the training intervention (Mid8), and 3 and 10 days after cessation of training (Post3 and Post10) (3 wk study)

A–C, CD68$^+$/CD206$^-$/M1-macrophages (*A*), CD68$^+$/CD206$^+$/M2-macrophages (*B*) and CD68$^-$/CD206$^+$ cells (*C*) per mm^2. *D–F*, CD68$^+$/CD206$^-$/M1-macrophages (*D*), CD68$^+$/CD206$^+$/M2-macrophages (*E*) and CD68$^-$/CD206$^+$ cells (*F*) per 100 myofibres (light grey, black and medium grey bars denote intracellular sublaminal and central as well as extracellular MP position). Pre to Mid8: $^aP < 0.05$, $^\theta P = 0.054$; Pre to Post3 difference: $^bP < 0.05$, $^cP < 0.01$, $^dP < 0.001$, $^\#P = 0.067$; Pre to Post10 difference: $^eP < 0.05$; Mid8 to Post3 difference: $^fP < 0.05$, $^gP < 0.01$, $^hP < 0.001$; Post3 to Post10 difference: $^iP < 0.05$. Values are means ± SD; BFRE: $n = 10$ at Mid8 $n = 9$; LLE: $n = 7$.

Creatine kinase (1 wk study)

Plasma CK showed no change from baseline to the first or last training session, respectively (Fig. 6). In contrast, CK increases were observed from Pre (104.8 ± 44.9 units l^{-1}) to Post180 (131.9 ± 54.0 units l^{-1}) and Post24 (145.1 ± 50.0 units l^{-1}) after the initial training session in HLE ($P < 0.01$). In addition, tendencies towards increased CK values were observed from Pre (142.7 ± 49.5 units l^{-1}) and Post60 (140.5 ± 38.2 units l^{-1}) to Post180 (170.7 ± 70.3 units l^{-1}) following the last training session in HLE ($P = 0.069$–0.091). No differences were observed between baseline of the first and last training session in either training group.

Plasma markers of inflammation (1 wk study)

After the first training session, a tendency to a reduction (10%) in plasma MCP-1 emerged between Pre and Post180 after BFRE ($P = 0.067$), while a decline was observed from Pre to Post24 (27%) ($P < 0.05$). In addition, an increase (33%) and a tendency to an increase (28%) were seen between baseline at the first training session compared to baseline at the last training session with BFRE and HLE training, respectively ($P = 0.017$; $P = 0.073$).

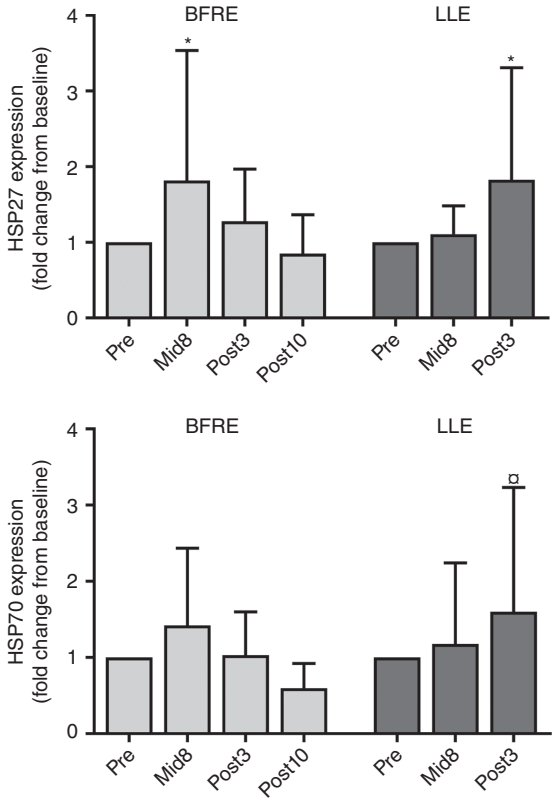

Figure 3. HSP27 and HSP70 expression (WB) at baseline (Pre), 8 days into the training intervention (Mid8), and 3 and 10 days after cessation of training (Post3 and Post10) (3 wk study)
Values are means ± SD; BFRE: $n = 10$ at Mid8 $n = 9$; LLE: $n = 7$.

Plasma IL-6 remained unchanged after the first BFR training session, while a decline of 18% was observed between Pre and Post180 ($P < 0.05$) in the last BFR training session. In contrast, an increase of 69% was seen between baseline and Post24 after the first HLE training session, while no changes were observed in relation to the last training session. No differences were observed between baseline of the first and last training session in either group.

No changes were observed for plasma TNF-α after the first or last BFRE sessions, respectively. After the first session of HLE, reductions from baseline to Post180 and Post24 were observed (11–16%) ($P < 0.05$). No differences were observed between baseline of the first and last training session in either group.

Plasma markers of oxidative capacity (1 wk study)

TAC remained unchanged after the first training session in BFR subjects, while increasing from baseline to Post15 and Post60 in subjects performing HLE ($P < 0.05$) (Fig. 7). In relation to the last training session no changes were observed in either intervention group. Similarly, no differences were observed between baseline of the first and last training session in either group.

GSH content remained unchanged at all time points in subjects exposed to BFR exercise. In contrast, a 2-fold increase was observed between baseline and Post5 after the first exercise session ($P < 0.05$) in HLE subjects (Fig. 7). Similarly, increases (2- to 3-fold) were observed between baseline and Post5 and Post180 after the last exercise session, respectively ($P < 0.05$, $P < 0.01$).

Delayed onset muscle soreness and perceived pain perception (VAS) (1 wk study)

As DOMS and VAS overall showed the same trends, with VAS showing approximately half of the mean value of DOMS on all time points, it was decided to test DOMS data only for statistical significance (Table 1). Overall, BFRE and HLE showed small, but significant increases in DOMS evaluated at rest and during activity 24 and 48 h after the first exercise session ($P < 0.05$). When evaluated after the last exercise session, increases emerged for DOMS 24 h after both BFR and HLE exercise in the resting condition ($P < 0.05$), while only increasing during activity after BFR training ($P < 0.05$). In addition, DOMS was increased at 48 h after the last exercise session when evaluated in the resting state in HLE subjects.

Discussion

The present study is the first to investigate the effect of longitudinal short-term low-load resistance training with concurrent blood flow restriction on myocellular damage, inflammation and stress. Firstly, it was

demonstrated that myocellular infiltration of pro- and anti-inflammatory (M1/M2) MPs increased 3 days (Post3) following 19 days of high-frequency BFR training. A similar pattern emerged for work-matched free-flow training, but involving pro-inflammatory MPs only (3 wk). Secondly, HSP27 expression was upregulated after 1 week of BFR training, while both HSP27 and HSP70 were upregulated in the early recovery phase after cessation of training in work-matched controls (3 wk). Thirdly, low-load BFR training and high-load free-flow strength training demonstrated highly similar changes in circulating markers of myocellular damage, inflammation and oxidative stress in response to acute and 1 week of exercise training, and whenever within-group increases emerged this was exclusively in response to high-load free-flow exercise (1 wk).

Macrophage activation

A consistent and gradual increase in $CD68^+$-macrophage (MP) content was observed in BFRE and LLE from baseline to Post3 ($CD68^+$ cells mm^{-2}, BFRE/LLE:

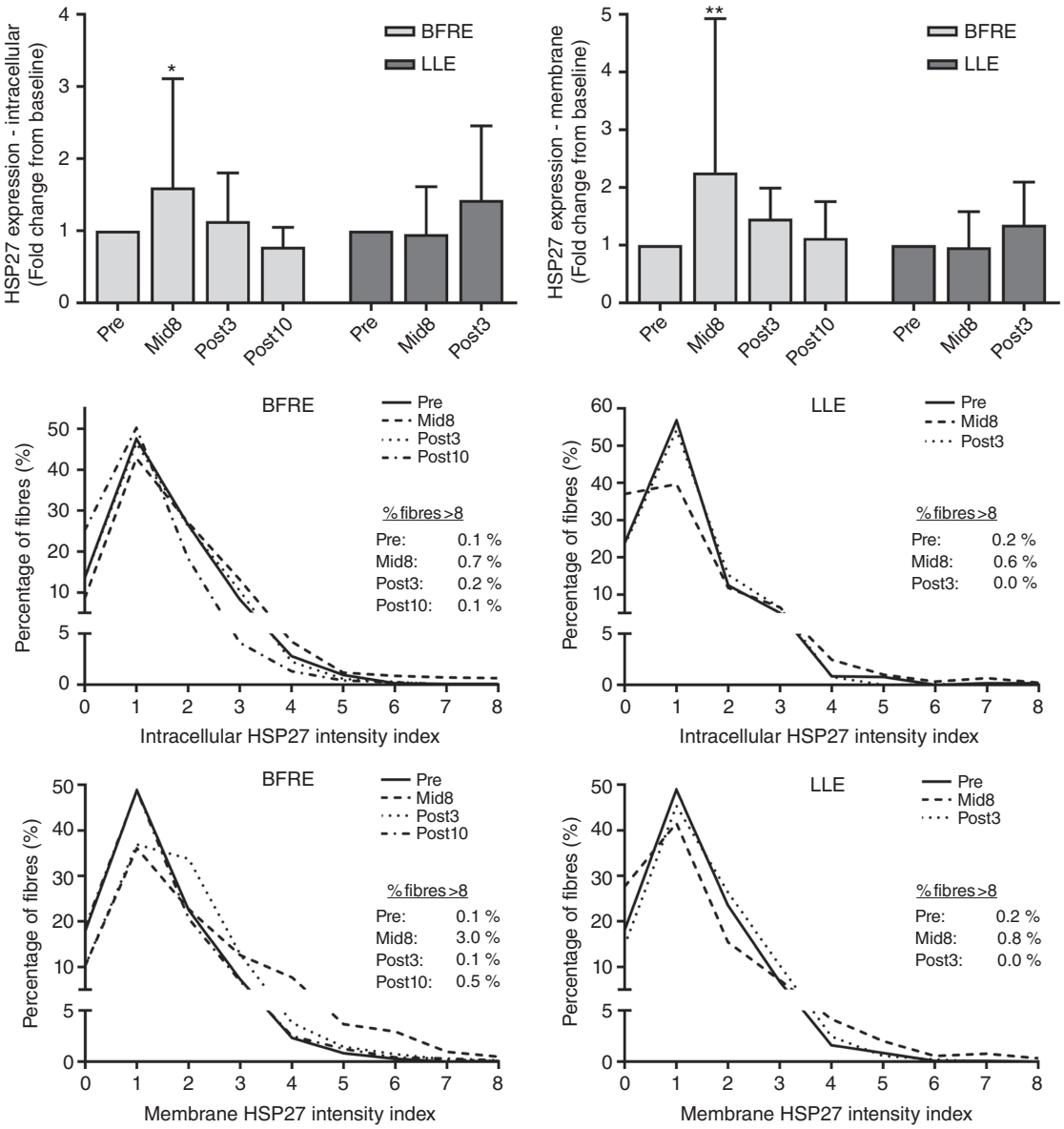

Figure 4. Intracellular/membrane specific HSP27 expression (IF) at baseline (Pre), 8 days into the training intervention (Mid8), and 3 and 10 days after cessation of training (Post3 and Post10) (3 wk study)
Upper right/left: HSP27 expression intracellular/at membrane. Middle right/left: intracellular HSP27 intensity index myofibre distribution in BFRE/LLE. Lower right/left: membrane HSP27 intensity index myofibre distribution in BFRE/LLE. Myofibres with an intensity index above 8 are omitted from the figure and shown as a mean. Pre to Post3 difference: $^*P < 0.05$, $^{**}P < 0.01$. Values are means ± SD; BFRE: $n = 10$ at Mid8 $n = 9$; LLE: $n = 7$.

112/55%) indicating a progressive accumulation of myocellular inflammation. On the other hand, these changes appear modest compared to previous investigations reporting much larger increases (≥240%) in $CD68^+$-MP content from 0.5 h to 7 days after muscle damage induced by excessive eccentric exercise (Stupka *et al.* 2001; Paulsen *et al.* 2010; Mackey *et al.* 2011). Interestingly, no signs of severe myofibre regeneration/necrosis (i.e. intracellular myofibre accumulation of >1 MP) were observed in the present study participants exposed to either BFRE or LLE training in contrast to previous observations following muscle damaging exercise (Paulsen *et al.* 2010; Mackey *et al.* 2011). MPs located in central and sublaminal myofibre positions were observed both at baseline (<0.55 per 100 fibres) and during/after the training intervention (<0.85 per 100 fibres), but these changes failed to reach statistical significance and indicate a low degree of myofibre regeneration. On the other hand, marked increases in centrally positioned nuclei were identified exclusively in subjects exposed to BFR training (86–158%). Centrally placed nuclei have been associated with tissue regeneration in adult skeletal muscle (Folker & Baylies, 2013), consequently suggesting a higher degree of regeneration with BFR training. However, myonuclei appear to centralize upon fusion (Cadot *et al.* 2012), so although not apparently described in the literature, centrally positioned nuclei could reflect increased myonuclei addition induced by marked proliferation of myogenic satellite cells (MSCs) noted during the period of BFR training (Nielsen *et al.* 2012).

Interestingly, MP tissue infiltration was detected only at a single time point, namely 3 days post training (Post3) in BFRE and LLE. The apparent peak in MP tissue infiltration at Post3 is intriguing because we expected the primary inflammatory response to occur in the initial phase of training intervention (Mid8), where subjects were unaccustomed to the training stress and consequently were expected to be more vulnerable to exercise-induced muscle damage. Altogether, the overall relative change, time course and lack of pronounced intramyocellular MP

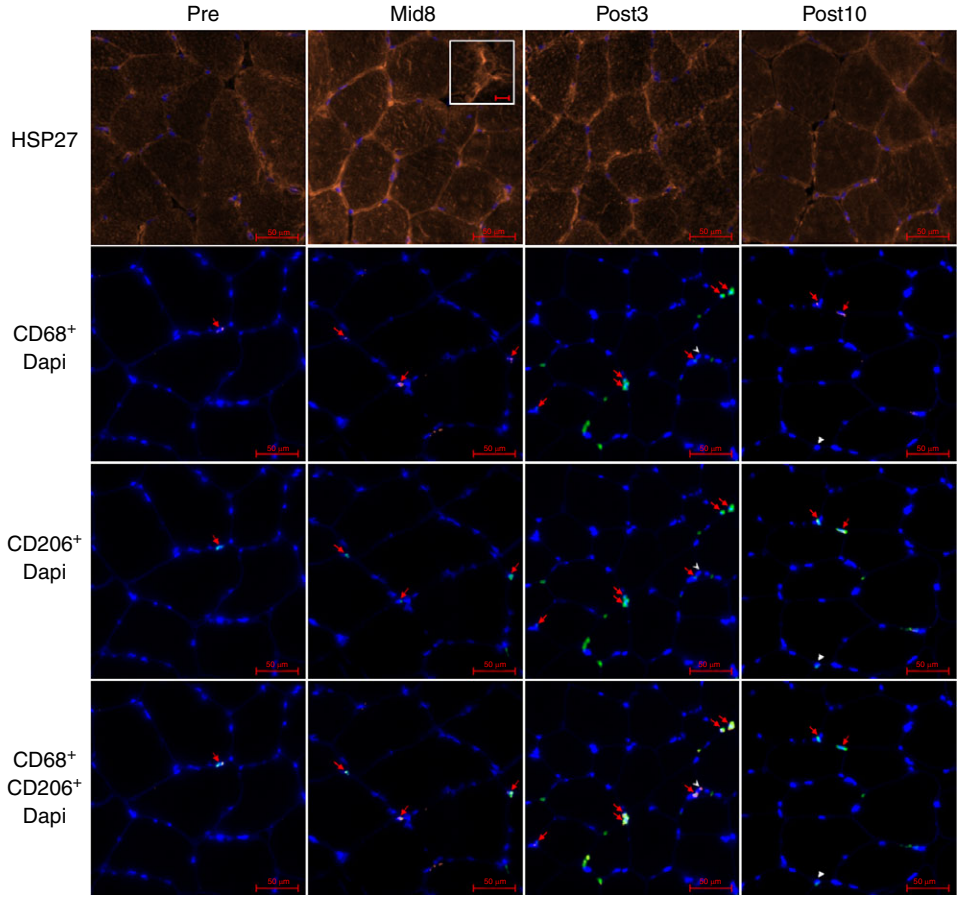

Figure 5. Representative skeletal muscle cross sections displaying immunoreactivity for HSP27 (red), CD68/CD206 (red/green), laminin (blue) and DAPI (blue, nuclear stain) (3 wk study)
Samples are from BFRE at baseline (Pre) and 8 days into the intervention (Mid8) as well as 3 and 10 days after the intervention (Post3 and Post10). Arrows, and open and filled arrowheads denote $CD68^+/CD206^+$, $CD68^+/CD206^-$ and $CD68^-/CD206^+$ cells, respectively. Note the distinct immunoreactivity within myofibres and at the membrane for HSP27 at Mid8. [Colour figure can be viewed at wileyonlinelibrary.com]

infiltration suggest that BFRE and LLE did not induce substantial amounts of muscle damage. In support of this, the larger proportion of MPs both at baseline and Post3 comprised anti-inflammatory M2-MPs, which suggests a predominant anti-inflammatory myocellular milieu.

The present findings of induced MP infiltration followed a different time course from that observed for myogenic satellite cell (MSC) expansion as previously reported for the BFR exercise group (Mid8: +275%, Post3: +250%, Post10 +150%) (Nielsen *et al.* 2012). This mismatch is interesting given that the pronounced concurrent increase (∼275%) in MSC content observed following the first week of BFR training (cf. Nielsen *et al.* their Fig. 4) could have been ascribed to muscle damage/inflammation, including M1-MP infiltration (Tidball, 2011; Saclier *et al.* 2013). However, the different time courses as well as the apparent lack of significant muscle damage collectively indicate that the marked degree of MSC proliferation observed in the early phase of BFR training (i.e. Mid8) primarily was stimulated by physiological stimuli other than ultrastructural muscle damage/inflammation involving M1 macrophage activation. This notion is further supported by the present increase in M1-MPs following LLE training (cf. Fig. 2) in parallel with a general lack of MSC proliferation in this intervention group using free-flow low-load resistance exercise (Nielsen *et al.* 2012). These observations suggest that the previously proposed stimulatory interaction between MPs and MSCs during early and late muscle regeneration (i.e. M1-MPs stimulating MSC proliferation and M2-MPs promoting MSC differentiation) (Tidball, 2011; Saclier *et al.* 2013) was probably not strongly involved during the present BFR training intervention.

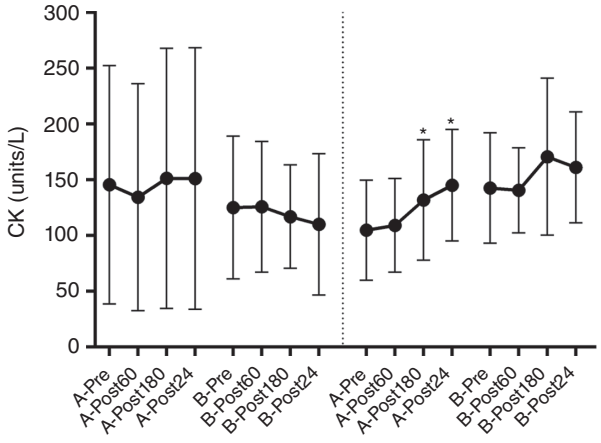

Figure 6. Plasma creatine kinase content at baseline (Pre), 60 and 180 min and 24 h after the first (A) and last (B) exercise session (1 wk study)
Pre to Post3 difference: $^*P < 0.05$. Values are means ± SD; BFRE: $n = 10$; HLE: $n = 8$.

The spatial distribution of M2-MPs and $CD68^-/CD206^+$ cells remained unaltered throughout the time course of intervention, while a trend towards more M1-MPs located in central myocellular positions was observed following BFR training (cf. Fig. 2). Notably, the latter observation was highly influenced by a single individual showing a pronounced increase in central positioned MPs post BFR training. A predominant portion of MPs were located in the extracellular compartment (M2 + $CD68^-/CD206^+$: >95%; M1: >75%). The functional role of these extracellular positioned MPs remains unknown. M2-MPs are known to play an essential functional role in promoting muscle regeneration and remodelling (Arnold *et al.* 2007; Tidball, 2011; Chazaud, 2016; Schiaffino *et al.* 2016), exemplified by 50% attenuation of compensatory muscle hypertrophy in mice subjected to macrophage depletion (DiPasquale *et al.* 2007). Thus, we speculate that the increase in extracellular positioned M2-MPs observed exclusively after BFR training (cf. Fig. 2) may have contributed to extracellular matrix (ECM) remodelling to accommodate the extensive magnitude of myofibre growth (∼35%) observed in this intervention group (Nielsen *et al.* 2012). The present lack of elevated tenascin C (TNC) expression seems to contradict this theory, since TNC expression has been implicated to promote ECM de-adhesion and remodelling after eccentric exercise (Mackey *et al.* 2011; Hyldahl *et al.* 2015). On the other hand, TNC involvement in de-adhesion of the ECM microenvironment seems to be related primarily to remodelling processes involved in myocellular regeneration (Järvinen *et al.* 2003; Hyldahl *et al.* 2014), and since the present BFRE protocol did not appear to involve any major extent of muscle damage, a combination of enhanced ECM remodelling and lack of TNC expression may be possible. Additionally, the elevated pool of extracellularly placed M2-MPs could also potentially contribute to the formation of new blood vessels, as this MP subpopulation has been shown to promote angiogenesis (Jetten *et al.* 2014; Szade *et al.* 2015). The potential for BFR exercise- induced angiogenesis seems to be present, as angiogenesis can be evoked by hypoxia, shear vascular stress and/or metabolic stress (Hoier & Hellsten, 2014), which are all factors that have been associated with BFR exercise (Iida *et al.* 2007; Larkin *et al.* 2012; Takada *et al.* 2012).

Activation of heat shock proteins

In the present study, an increase in HSP27 expression was detected in muscle homogenate (82%) as well as in myofibre cross-sections (60%) and at the membrane level (132%) obtained in BFRE subjects in the early phase of the intervention (Mid8). HSP27 acts as a molecular chaperone during cellular stress, preventing protein denaturation and aggregation (Noble *et al.* 2008), and

consequently HSP27 accumulation typically has been observed at stressed/damaged myocellular structures in response to muscle-damaging exercise (Koh & Escobedo, 2004; Paulsen *et al.* 2009). The observation of an early increase in HSP27 expression with BFR training is in line with our initial hypothesis (early BFRE-induced myocellular stress), and probably reflects the fact that significant amounts of myocellular stress were present during the first week of BFR training. Yet, the present relative increases in intracellular HSP27 (60%) and on the whole myocellular level (82%) (WB) appear modest compared to the increases (severalfold) previously reported in response to muscle-damaging exercise (Thompson *et al.* 2001; Paulsen *et al.* 2009). In the present study, we only rarely observed HSP27-positive fibres or granulated intramyocellular staining patterns characterizing accumulation at stressed cytoskeletal structures, which have previously been observed with muscle damage and myofibrillar disruptions (Paulsen *et al.* 2009). In contrast, distinct HSP27 expression was observed at the myofibre membrane in BFRE subjects (mean change +132%), suggesting HSP27 accumulation and that membrane-specific stress responses may have occurred in this intervention group. The membrane specificity of our staining was similar to previous HSP25 (rat HSP27-analogue) staining performed in rat EDL muscle exposed to ischaemia (Golenhofen *et al.* 2004). The link between HSP27 expression and ischaemia-related phenomena such as oxidative stress is well described in skeletal and heart muscle (Escobedo *et al.* 2004; Jain *et al.* 2014).

Interestingly, the early HSP27 membrane response and subsequent later downregulation in BFRE subjects coincided with a similar pattern for the change in

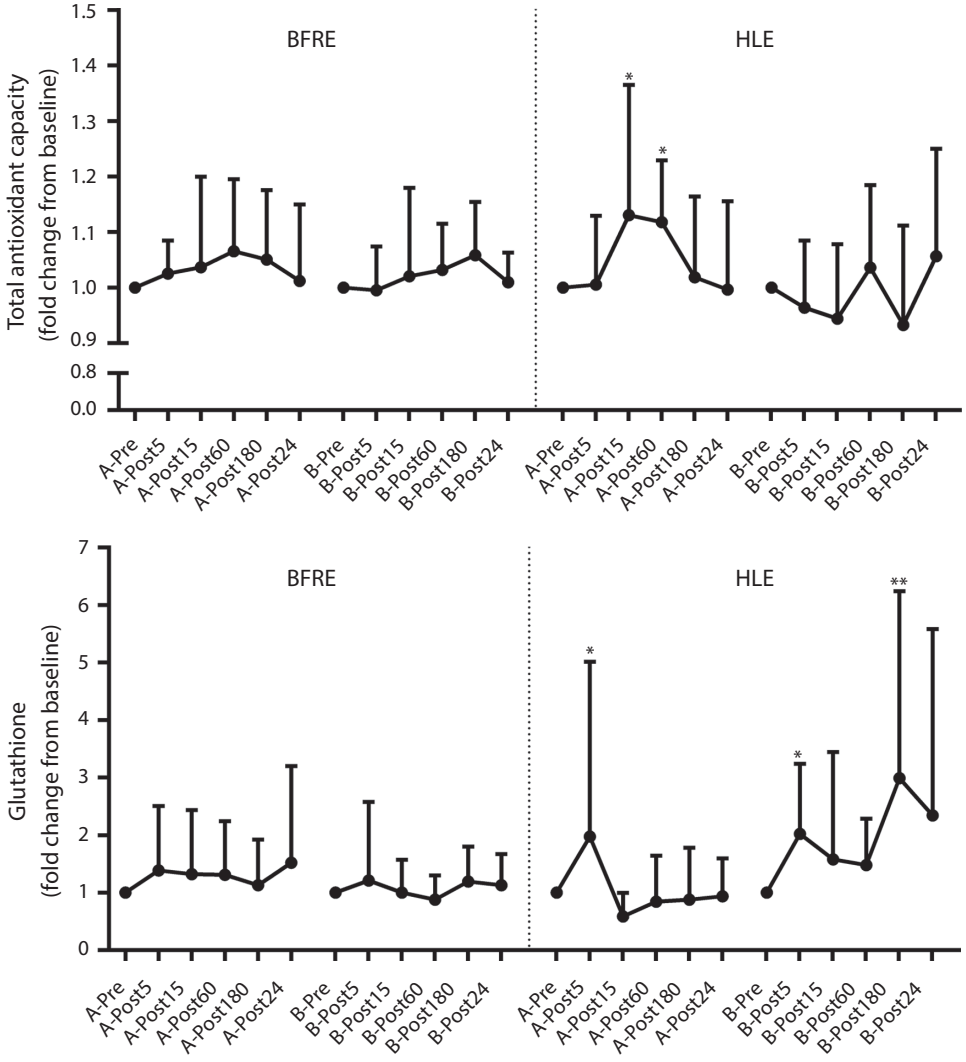

Figure 7. Plasma antioxidant (TAC, GSH) content at baseline (Pre), 5, 15, 60 and 180 min and 24 h after the first (A) and last (B) exercise session (1 wk study)
Pre to Post3 difference: $^{*}P < 0.05$, $^{**}P < 0.01$. Values are means ± SD; BFRE: $n = 10$; HLE: $n = 8$.

Table 1. Time course of exercise-induced changes in self-perceived muscle soreness (1 wk study)

	Delayed onset muscle soreness (DOMS)					
	First exercise session			Last exercise session		
	Pre	Post24	Post48	Pre	Post24	Post48
DOMS-rest						
BFRE	0.0 (0.0–0.0)	9.0 (3.0–12.5)**	8.0 (4.8–9.8)*	0.0 (0.0–1.8)	3.0 (2.0–3.8)*	0.0 (0.0–0.0)
HLE	0.0 (0.0–0.0)	3.5 (0.8–4.3)*	5.0 (3.0–8.3)*	0.0 (0.0–0.3)	1.5 (1.0–3.0)*	2.5 (0.8–3.0)†
DOMS-activity						
BFRE	0.0 (0.0–0.0)	10.5 (6.8–19.3)**	14.5 (8.0–19.8)**	0.0 (0.0–0.3)	3.0 (2.0–3.8)*	1.0 (0.0–4.5)
HLE	0.0 (0.0–0.3)	3.0 (0.8–9.3)*	9.0 (5.3–20.3)*	0.0 (0.0–2.8)	2.0 (1.5–2.3)	4.5 (2.8–5.5)

Pre-to-post differences: *$P < 0.05$, **$P < 0.01$, †$P = 0.065$. Values af medians (25th–75th percentiles). BFRE $n = 10$, HLE $n = 8$.

myogenic satellite cell (MSC) content. A mechanism initiating these cellular events could potentially be transient (i.e. non-chronic) micro-focal alterations in plasma membrane permeability, as previously indicated after acute BFR exercise (Wernbom *et al.* 2012). Such transient changes in plasma membrane permeability could be a consequence of short duration micro-ruptures in the myofibre membrane (Boucher & Mandato, 2015) or dysfunction in normal membrane transport mechanisms (Gissel, 2005). A transient increase in plasma membrane permeability would allow increased ion influx (e.g. Ca^{2+}), which could initiate a cellular stress response (i.e. leading to elevated HSP27 expression) in close proximity to the membrane. In addition, disturbance of normal membrane permeability could potentially *per se* initiate MSC activation and proliferation directly by triggering release of growth factors (e.g. hepatocyte/fibroblast growth factor (HGF/FGF)) (Allen & Boxhorn, 1989; Tatsumi *et al.* 2009) or indirectly by modifying the MSC niche (Thomas *et al.* 2015). Thus, the pronounced degree of MSC proliferation observed following the present regime of BFR exercise (Nielsen *et al.* 2012) may rely on specific molecular or cellular responses to micro-damage at the membrane level, without involving severe myocellular damage or inflammation.

In the present study, no changes in HSP70 expression emerged in response to BFR exercise, while an increase was observed in the early recovery phase following load/work-matched training in free-flow conditions. The lack of change in HSP content with BFR exercise is in contrast to earlier findings (Cumming *et al.* 2014) showing increases in cytosolic and cytoskeletal fractions as well as on the whole myofibre level (>100%) after acute BFR exercise. Interestingly, Cumming and co-workers reported that HSP70 expression increased selectively in type I 24 and 48 h after acute BFR exercise and that positive HSP70 expression was correlated with low intramuscular glycogen (Cumming *et al.* 2014). These observations indicate that the acute HSP70 response may primarily be related to the metabolic demands/conditions of the exercise performed. It is well known that myocellular metabolic pathways are able to adapt quickly upon changes in demands (Cadefau *et al.* 1994), and substantial increases in muscle glycogen content (+40%) have been reported after 8 weeks of BFR training (Burgomaster *et al.* 2003). Thus, longitudinal metabolic exercise adaptations and/or lack of muscle damage may explain the stability in HSP70 expression with BFRE in the present study.

Changes in plasma creatine kinase

Elevated plasma CK values (26–38%) were observed 3 h and 24 h after the first training session in subjects performing HLE, while remaining unchanged 1–24 h after BFRE. These observations are in stark contrast to the marked CK increases (~36-fold) recently reported 48 and 96 h after acute BFR exercise (Sieljacks *et al.* 2016). However, other studies have failed to detect CK changes after acute (≤24 h) or longitudinal BFR exercise (Takarada *et al.* 2000*a*; Abe *et al.* 2006; Karabulut *et al.* 2013). These diverging observations could potentially rely on different time courses of blood sampling (i.e. only CK changes ≥48 h). However, the blood sample obtained before the last exercise session in the 1 wk study (i.e. ~96 h after the first exercise session, cf. Fig. 1) did not reveal any measurable changes in plasma CK levels, which we expected had the first BFR training session induced significant muscle damage.

Interestingly, the marked increases in plasma CK values reported previously were paralleled by fairly high DOMS scores (~5 out of 10) (Sieljacks *et al.* 2016), which is higher than DOMS reported at rest and during muscle activity in the present study (median: 4.6–5.6 and 7.4–10.3 out of 100). Thus, overall there seem to be contrasting muscle damage responses between studies. This distinct discrepancy may rely on a more strenuous exercise protocol employed in the Sieljacks study, i.e. use of a higher external load (30% 1RM) and number of sets (5 sets). Notably, in line with the results of the present study, Sieljacks *et al.* did not find any changes in CK levels after

a second bout of BFRE suggesting that the critical time point for inducing muscle damage with BFR exercise may be the initial BFR exercise session.

Changes in circulating cytokines

In line with the present CK findings, only minor changes in circulating cytokines (MCP-1, IL-6, TNF-α) were observed, except for an increase in IL-6 (69%) observed at 24 h after the first HLE session. The overall lack of acute increases in BFRE and HLE were unexpected as the above cytokines have been shown to increase after acute aerobic exercise and high-load resistance exercise (Ostrowski *et al.* 1999; Peake *et al.* 2005; Della Gatta *et al.* 2014) as well as in response to single bouts of acute muscle damage (Toft *et al.* 2002; Peake *et al.* 2005). In comparison, previous studies have reported significant increases (11–300%) in plasma IL-6 after acute BFR exercise in young and old male adults (Takarada *et al.* 2000*a*; Patterson *et al.* 2013).

The cytokines presently investigated have previously been related to MP activation and polarization (Hubal *et al.* 2008; Tidball, 2011; Della Gatta *et al.* 2014), and the lack of change in plasma cytokine levels with BFRE and LLE is in line with the lack of change in MP density and polarization (cf. unchanged M1/M2 distribution) in the early phase after training initiation (Mid8). However, recruitment of circulating monocytes and/or proliferation of resident MPs are likely to be regulated mainly by intracrine/autocrine factors; thus we cannot dismiss that such chemoattractive factors may have been secreted locally without any measurable release to circulation.

Oxidative stress responses

It has been previously speculated that BFRE can induce myocellular oxidative stress, as this training modality involves cellular hypoxia and reperfusion effects that exceed that induced by free-flow exercise (Iida *et al.* 2007). Yet, the present data suggest that the degree of oxidative stress with BFRE was not of a sufficient magnitude to induce changes in systemic antioxidant capacity, as changes in total antioxidant capacity and glutathione (GSH) were absent with BFRE, while conversely increased after HLE. These observations are supported by previous reports of increased protein carbonyls and glutathione ratios 0–15 min after acute high-load training and resting blood flow occlusion, whereas no changes were observed in response to acute low-load BFR exercise (Goldfarb *et al.* 2008). The major source of oxidative stress during exercise reside within the active skeletal musculature in the form of free radical formation (Jackson *et al.* 2016), and oxidative stress appears to increase in proportion to the magnitude of contractile work performed by skeletal muscles (Nyberg *et al.* 2014) and to the degree of hypoxia (Korthuis *et al.* 1985), respectively. Thus, the present unaltered levels of oxidative stress markers (TAC, GSH) in response to BFRE, which involve a high level of both muscle hypoxia and contractile work (Iida *et al.* 2007; Larkin *et al.* 2012), remains puzzling and warrants closer experimental scrutiny.

Methodological considerations

A potential limitation of the present study was the use of a uniform absolute cuff pressure across all BFRE subjects. A recent study suggests that parameters such as blood pressure and limb circumference may influence the degree of arterial inflow during cuff-induced blood flow restriction (Hunt *et al.* 2016). Consequently, we cannot be certain that the utilized cuff pressure (100 mmHg) led to uniform blood flow restriction across all subjects. However, the potential systematic influence from this approach might have been minor due to the low inter-individual (BFRE group) variations observed in blood pressure (119.4 $\pm$ 9.2/68.9 $\pm$ 9.6 mmHg (diastole/systole)) and limb circumference (56.7 $\pm$ 5.1 cm). Another potential limitation of the present study may be the large difference in training frequency between BFRE and HLE groups engaged in the 1 wk study. The BFRE training protocol of this study was designed to imitate the first week of the 3 wk study (7 sessions in 5 days), thus enabling valid comparison between the two studies. However, the use of high-frequency (7 sessions) HLE training bouts at 70% 1RM in 5 days was deemed contraindicative in this recreationally active cohort, and consequently the groups were matched on total contractile training volume instead. Finally, the present results were obtained in male subjects, and the study conclusions may therefore not be readily generalizable to females.

Conclusion

The present study investigated for the first time the effect of longitudinal short-term, low-load, blood flow restricted exercise on myocellular markers of stress and inflammation. Although signs of myocellular stress and inflammation (i.e. increases in HSP27 and in M1/M2 macrophages) were observed in response to 3 weeks of high-frequency (daily) BFR training, the amplitude, time course and spatial macrophage distribution suggested that no or only minor muscle damage was present. Thus, the present data indicate that short-duration high-frequency BFR training does not induce muscle damage in recreationally trained healthy young males. Micro-focal signs of myocellular membrane stress were observed; however, this may be involved in the adaptation processes evoked by BFR muscle exercise.

The present findings may have useful clinical implications. Short-term high-frequency BFRE training

appears to elicit substantial gains in muscle mass and mechanical muscle function, without inducing muscle damage. These observations are important since short-term training regimes involving low levels of joint and muscle loading have strong therapeutic relevance in the clinical setting (Loenneke *et al.* 2012; Hughes *et al.* 2017), not least as a means to accelerate recovery rate. In addition, from a physiological perspective it is noteworthy that highly marked myogenic satellite cell activation can be stimulated without eliciting major amounts of myocellular damage, which suggests that the physiological mechanisms stimulated by blood flow restricted training (i.e. metabolic myocellular stress, hypoxia and reperfusion) may sensitize the myogenic stem cell niche. Hence, the current data expand our understanding of *in vivo* human skeletal muscle plasticity and may provide important information of relevance for the future treatment of secondary muscular atrophy and age-related sarcopenia.

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Additional information

Competing interests

None declared.

Author contributions

The contributions of the authors were as follows: conception and design of the study: J.L.N., P.Aa. and U.F.; collection, analysis and interpretation of data: J.L.N., P.Aa., T.A.P., T.N., R.D.B., C.S. and U.F; drafting of the manuscript: J.L.N., P.Aa. and U.F.; and revising it critically for important intellectual content: J.L.N., P.Aa., T.A.P., T.N., R.D.B., C.S. and U.F. All authors were involved in revisions and accepted the final, submitted version of the manuscript. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed. All data collection and analysis were carried out at the Department of Sports Science and Clinical Biomechanics, University of Southern Denmark, Odense, Denmark.

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