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The combined effect of sprint interval training and post-exercise blood flow restriction on critical power, capillary growth and mitochondrial proteins in trained cyclists

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ABSTRACT

Sprint interval training (SIT) combined with post-exercise blood-flow restriction (BFR) is a novel method to increase maximal oxygen uptake ($\dot{V}O_{2max}$) in trained individuals, and also provides a potent acute stimulus for angiogenesis and mitochondrial biogenesis. The efficacy to enhance endurance performance has however yet to be demonstrated. 21 trained male cyclists ($\dot{V}O_{2max}$; 62.8 ± 3.7 $ml.min^{-1}.kg^{-1}$) undertook 4 weeks of SIT (repeated 30 sec maximal sprints) either alone (CON; n = 10) or with post-exercise BFR (n = 11). Before and after training $\dot{V}O_{2max}$, critical power (CP) and *W′* were determined and muscle biopsies obtained for determination of skeletal muscle capillarity and mitochondrial protein content. CP increased (*P* = 0.001) by a similar extent following CON (287 ± 39 W to 297 ± 43 W) and BFR (296 ± 40 W to 306 ± 36 W). $\dot{V}O_{2max}$ increased following BFR by 5.9% (*P* = 0.02) but was unchanged after CON (*P* = 0.56). All markers of skeletal muscle capillarity and mitochondrial protein content were unchanged following either training intervention. In conclusion, 4 weeks of SIT increased CP, however this was not enhanced further with BFR. SIT was not sufficient to elicit changes in skeletal muscle capillarity and mitochondrial protein content with or without BFR. However, we further demonstrate the potency of combining BFR with SIT to enhance $\dot{V}O_{2max}$ in trained individuals.

NEW & NOTEWORTHY

This investigation has demonstrated that 4 weeks of SIT increased critical power in trained individuals, however, post-exercise BFR did not enhance this further. SIT, with or without BFR, did not induce any changes in skeletal muscle capillarity or mitochondrial protein content in our trained population. We do however confirm previous findings that SIT combined with BFR is a potent stimulus to enhance $\dot{V}O_{2max}$.

KEYWORDS BFR; power-duration relationship; angiogenesis, mitochondrial biogenesis

INTRODUCTION

Well-trained individuals are typically accustomed to high training volumes across a broad spectrum of intensities. However, it is generally accepted that eliciting further adaptations within this population becomes challenging (30, 31). Early research has highlighted the reduced plasticity of skeletal muscle in the trained state (22, 44) and a body of literature exists demonstrating that an increase in traditional endurance training volume alone is insufficient to improve aerobic performance or associated physiological determinants in well-trained and 'physically active' individuals (12, 13). This blunting of the adaptive scope in trained individuals is also reflected at a molecular level (14, 37) as demonstrated by an attenuated acute transcriptional response as individuals become accustomed to a specific exercise bout (37). Therefore, the development of effective novel training methods is of particular relevance to this population.

We have recently demonstrated the potency of combining sprint interval training (SIT) with blood flow restriction (BFR) in enhancing the adaptive responses in well-trained individuals (48). The addition of BFR elicited an increase in $\dot{V}O_{2max}$ of ~4.5%, compared to no change with SIT alone. Alongside this we presented preliminary mechanistic evidence that SIT combined with BFR led to enhanced angiogenic signalling, suggesting the potential for a greater capillary growth with this novel intervention. Increased muscle capillarity is a critical adaptation to enhance oxygen and substrate delivery (28). Classic work in skeletal muscle has shown that the number of capillaries per fibre is proportional to the oxidative activity of that fibre (43) and subsequently demonstrates a strong correlation with $\dot{V}O_{2max}$ (44). An enhanced capillary network also facilitates the greater removal of metabolic end products, which would improve sub-maximal exercise tolerance (28). Both SIT combined with BFR and SIT alone were also potent in upregulating PGC-1α expression suggesting the potential for mitochondrial biogenesis (48). Therefore, it was surprising that despite the gains in $\dot{V}O_{2max}$

Taylor *et al.* (48) did not observe any improvements in exercise performance, measured through a 15 km time trial. This might be because the relative contribution of central and peripheral factors to performance of such a self-paced exercise is task dependent, with increased contribution of central fatigue within longer low intensity time trials (>30 min) and a greater degree of peripheral fatigue after shorter high intensity efforts (approximately 6 min) (49). Therefore, the peripheral adaptations that we have hypothesised may not be reflected in the performance measure selected but would be more relevant to shorter high intensity efforts.

Since its introduction by Monod and Scherrer (33) the critical power (CP) concept has been used to describe the relationship between the tolerable duration that high intensity exercise can be maintained at a given power output for whole body exercise, (i.e. the power-duration relationship) (39). This hyperbolic relationship can be described by two constants; the asymptote CP, which is considered to represent the greatest rate of oxidative metabolism that can be maintained in the absence of a progressive loss of muscle metabolic homeostasis; and the curvature constant (W') representing the capacity for work performed above critical power (27, 34, 50). Accordingly, performance within the severe intensity domain is a function of CP and W', which makes them important determinants of performance (27, 52). CP is enhanced under conditions of increased oxygen delivery and is linked to the ability to maintain a metabolic steady state (38, 50), therefore CP could be hypothesised to be sensitive to changes in skeletal muscle capillarity. Indeed, we recently demonstrated a strong positive association between CP and skeletal muscle capillarity in well-trained individuals (32). Furthermore, since CP represents the highest sustainable rate of oxidative metabolism, it is likely to be closely related to mitochondrial content and could therefore be sensitive to an increase in the content of mitochondrial enzymes.

Therefore, the present investigation assessed the potency of SIT combined with BFR in enhancing CP. Furthermore, the angiogenic and mitochondrial biogenesis potential of SIT combined with BFR was also assessed. It was hypothesised that SIT combined with BFR would result in a greater increase in CP, which would be associated with a greater increase in skeletal muscle capillarity and mitochondrial protein content, compared to SIT alone.

METHODS

Participants

21 healthy males (age 23 ± 5 yr, height, 179.5 ± 6.4 cm, body mass, 75.5 ± 7.9 kg) volunteered to take part in the study. Participants were competitive cyclists or triathletes and had to achieve the inclusion criteria of $\dot{V}O_{2max}$ ≥60 $ml.min^{-1}kg^{-1}$. All completed health and biopsy screening questionnaires prior to participation to mitigate for contraindications to maximal exercise, muscle biopsy procedures and blood flow restriction. Participants did not have a history of neuromuscular, haematological or musculoskeletal abnormalities and were not using pharmacological treatments during the study period. All experimental procedures were approved by the Loughborough University Ethics Approvals (Human Participants) Sub-Committee and conformed in all respects with the Declaration of Helsinki. Participants were fully informed of the risks and discomforts associated with all experimental trials before providing written, informed consent.

Experimental protocol

The study used an independent-groups design whereby participants were assigned to one of two groups to perform four weeks of SIT either on its own (CON, n = 10) or combined with post-exercise blood flow restriction (BFR, n = 11). Participants were pair matched between groups based upon initial $\dot{V}O_{2max}$, maximal aerobic power (MAP) and critical power (CP). Participants were initially familiarized to the testing and training procedures during preliminary visits. Pre-training outcome measures were assessed over a period of 10 days. $\dot{V}O_{2max}$ was tested initially to ensure participants attained the appropriate inclusion criteria. After approximately two days the pre-training muscle biopsy was then obtained. After a further two days the power-duration relationship for determination of CP and W' was assessed, with a minimum of 24 hours separating each test. Participants then embarked on the

four-week supervised training programme. After a maximum of four days following the final training session the post-training outcome measures were assessed in the same order and over a similar time period

All performance tests were conducted on an electronically braked cycle ergometer (Lode Excalibur Sport, Lode B.V. Gronigen, The Netherlands). Ergometer saddle and handle bar dimensions were recorded for each participant during preliminary testing and remained standardised for the rest of the study. Participants were instructed to maintain a normal diet during the pre-training testing and replicate that diet during the post-training measures. Participants were instructed to refrain from ingesting alcohol and caffeine during the 48 hours preceding testing. Exercise trials were undertaken at approximately the same time each day (± 2 hours). Laboratory conditions during pre- and post-training exercise measurements remained constant (19-21°C, 40-50% humidity).

Pre and post training outcome measures

VO_{2max} and MAP

Participants performed an incremental test to exhaustion to establish $\dot{V}O_{2max}$ and MAP. Participants began cycling, at a freely chosen, constant pedal cadence for 1 min at 50 W, after which power increased 25 W every 60 sec until volitional exhaustion or when cadence fell 10% below the freely chosen cadence for more than 5 sec, despite strong verbal encouragement. Pulmonary gas exchange was measured continuously throughout exercise (Cortex MetaLyzer 3B, Leipzig, Germany). $\dot{V}O_{2max}$ and MAP were defined as the highest $\dot{V}O_2$ and power output achieved for a 30 and 60 sec period during the test, respectively.

CP and W'

Participants performed a series of 3-5 constant-load tests that were continued until the limit of tolerance at between 70-100% of MAP, the sequence of which was randomised. These were designed to elicit exhaustion within 2 to 15 min (39). Time to exhaustion (t) was recorded to the nearest second and was taken as either volitional exhaustion or when cadence fell 10% below the freely chosen cadence for more than 5 sec, despite strong verbal encouragement. No feedback regarding the power output or times achieved were provided, however participants were permitted to view pedal cadence throughout. To enhance the accuracy of parameter estimates, when the standard error of CP was >5% and W' >10% an additional test was performed.

The parameters of the power-duration relationship, CP and W', were calculated using the inverse linear relationship (equation 1), the linear work-time model (equation 2) and the hyperbolic relationship (equation 3). The equation associated with the lowest combined standard error was selected and used for all further analysis.

$$P = W' \cdot (1/t) + CP \quad (1)$$

$$W = CP \cdot t + W' \quad (2)$$

$$t = W' / (P - CP) \quad (3)$$

Muscle sampling and analysis

16 participants consented to provide muscle biopsy samples (CON, n = 7; BFR, n = 9). Muscle biopsies were obtained, at rest, from the lateral portion of the vastus lateralis muscle under local anaesthesia (1% lidocaine) using the percutaneous needle biopsy technique with suction. Pre- and post-training samples were obtained through separate incisions 2 cm apart on the same leg. Muscle samples were split into two portions. One portion was immediately embedded in mounting medium (Tissue-Tek OCT Compound, Sakura Finetek Europe, The

Netherlands) and immediately frozen in liquid nitrogen-cooled isopentane. The other portion was snap-frozen in liquid nitrogen. All samples were then stored at -80°C until analysis.

Immunohistochemistry

Transverse serial sections (8 μm) were obtained using a cryotome and placed onto poly L-lysine coated glass slides. Sections were fixed for 10 min in 3.7% formaldehyde at room temperature and blocked with phosphate buffered saline (PBS) containing 2% bovine serum albumin (BSA) and 5% goat serum for 1 h at room temperature. Serial muscle sections were then incubated with either primary antibody CD-31 (ab119339, abcam, Cambridge, UK) diluted 1:100 and MHC II (ab91506, abcam, Cambridge, UK) diluted 1:1000 in PBS-2% BSA or MHC I (A4.951, DSHB, Iowa, USA) diluted 1:500 in PBS-2% BSA for 1 h at room temperature. A separate slide was also incubated in CD-31 as described above and was subsequently incubated with Ki-67 (ab92742, abcam, Cambridge, UK) diluted 1:250 in PBS-2% BSA overnight at room temperature. Sections were then incubated for 2 h at room temperature with the appropriate secondary antibodies; goat anti-mouse Alexa Fluor 488, (CD-31, MHC I) and goat anti-rabbit Alexa Fluor 594 (MHC II, Ki-67) diluted 1:500 in PBS-2% BSA. Following incubation cover slips were mounted with fluoromount aqueous mounting medium (F4680, Sigma-Aldrich, Dorset, UK). Specificity of staining was assessed with no primary antibody negative controls.

Images were captured with a fluorescence microscope (Leica DM2500) at 20x magnification. Images were taken across the entire CSA of the sample to avoid bias toward smaller fibres. Camera exposure time and gain were adjusted and kept consistent for all images captured for each participant. An average of 117 ± 43 type I and 90 ± 49 type II fibres were analysed per sample. Capillarity was expressed as; capillary to fibre ratio (C:F), capillary density (CD) and number of capillary contacts around type I (CC type I) and type II fibres (CC type II). Ki-67

positive nuclei co-localised within capillaries were expressed per fibre (all Ki-67 data are CON n = 6 and BFR n = 9, with one participant missing due to insufficient tissue sample size). Only transverse fibres were included in the analysis, which was assessed primarily by the presumption of circularity. Any fibres that were clearly oblique or not transverse to the long axis of the fibre were excluded from analysis. Cross sectional area (CSA) of fibres was assessed by manually drawing the perimeter of each muscle fibre with the image analysis software Fiji (ImageJ). Although absolute fibre size may be overestimated because of fibre swelling during thawing of frozen sections, this should be consistent between all samples. The investigator was blinded to the exercise training status and condition of samples for all analysis.

Western blot analysis

Muscle tissue was homogenised in cold lysis buffer containing PBS-0.2% Triton X-100 and protease and phosphatase inhibitor cocktail (Fisher Scientific, Loughborough, UK). Samples were blitzed using a tissue lyser (Qiagen, UK) twice for 2 min at 20 Hz and centrifuged at 12000 g for 10 min to pellet insoluble material. The supernatant was transferred to a fresh Eppendorf tube and protein concentrations were determined by Pierce 660 protein assay according to the manufacturer's instructions (Fisher Scientific, Loughborough, UK). Samples were mixed with dH_2O, 4x LDS sample buffer (Invitrogen, Loughborough, UK) and 0.1% β-mercaptoethanol (Sigma, Dorset, UK) to a concentration of 1.5 μg.μl. 15 μg of protein was loaded on to 4-20% TGX polyacrylamide gels (Bio-Rad, Herts, UK) and separated by electrophoresis at 100 V for 80 minutes. All samples were run in duplicate to establish coefficients of variation. Proteins were transferred onto PVDF membrane at 30 V for 90 minutes (Bio-Rad, Herts, UK) and washed for 5 min in Tris buffered saline with tween (TBST) before being blocked in 5% blotting grade milk (Bio-Rad, UK) for 1 h at room temperature. Membranes were washed three times for 5 min in TBST and were incubated

overnight with the primary antibodies; citrate synthase (CS) (ab129095, abcam, Cambridge, UK), cytochrome c oxidase (COX) subunit II (ab110258, abcam, Cambridge, UK) and COX IV (ab33985, abcam, Cambridge, UK) diluted 1:1000 in 3% blotting grade milk at 4°C. Membranes were then washed three times for 5 min in TBST and incubated with the appropriate secondary antibody; anti-mouse horseradish peroxidase-conjugated secondary antibody (Dako, Stockport, UK) or anti-rabbit horseradish peroxidase-conjugated secondary antibody (Bio-Rad, Herts, UK) diluted 1:10,000 in 3% blotting grade milk for 1 h at room temperature. Following three 5 min washes in TBST, membranes were incubated with enhanced chemiluminescence substrate (ClarityMax, Bio-Rad, Herts, UK) for 5 min. Membranes were visualised using image analysis (ChemiDocTM XRS+, BioRad, Herts, UK) and band densities determined using image analysis software (Quality One 1-D analysis software v 4.6.8, Bio-Rad, Herts, UK). GAPDH was used as a loading control and protein content was expressed in arbitrary units relative to GAPDH. All protein content data are CON n = 7 and BFR n = 7, with two participants missing due to insufficient tissue sample size. The coefficients of variation for CS, COX II and COX IV were 5.1 ± 3.9%, 6.8 ± 5.8% and 4.3 ± 3.2% respectively.

Exercise training

Participants completed a four-week supervised sprint interval training (SIT) programme (2 sessions per week) each session being separated by a minimum of 48 h. Participants were encouraged to maintain their regular training regime with the exception of performing any form of interval training. This was to ensure a substantial reduction in training volume was avoided. Each training session consisted of repeated 30 sec maximal sprints performed on a mechanically braked cycle ergometer (SE-780 50, Monark, Stockholm, Sweden) against a manually applied resistance equivalent to 0.075 kg/kg body mass. The training was progressive whereby all participants performed a total of 4, 5, 6 and 7 maximal 30 sec sprints

in weeks 1, 2, 3 and 4, respectively. Each sprint was separated by a 4.5 min recovery period, during which participants immediately dismounted the cycle ergometer and lay in a semi-supine position upon a couch. In BFR participants were subjected to blood flow restriction (applied within 25 sec of each sprint). This was achieved by rapidly applying pneumatic pressure cuffs (Hokanson SC12L) as high up as possible on the proximal portion of each thigh, which were inflated (E20 Rapid Cuff Inflator and AG101 Cuff Inflator Air Source, Hokanson, WA) to a pressure of ~ 120 mmHg for 2 min (this pressure was kept constant throughout the four-week training period). The cuffs were then rapidly deflated and participants remained in the supine position until 30 sec prior to the next sprint where they re-mounted the ergometer in time for the subsequent sprint which began precisely 4.5 min after the previous sprint ended. In CON participants remained in the semi-supine position before re-mounting the ergometer in time for the subsequent sprint. Pre- and post-training measurements of peak power output (PPO) and mean power output (MPO) were obtained (Monark software) from the best sprint within the first and last training sessions respectively. Total work completed throughout the training period was calculated as a sum of the product of the MPO from each sprint and sprint duration.

Statistics

Training data were analysed using an unpaired *t*-test. Two-factor repeated-measures ANOVA, with one within factor (time; pre vs post) and one between factor (condition; CON vs BFR) were utilised to undertake all subsequent analysis. Where significant interaction effects were observed Bonferroni-corrected *post hoc* paired *t*-tests were used to locate differences. Data are presented as mean ± SD. Significance was accepted at $P \leq 0.05$.

RESULTS

Performance measures

There were no differences in physiological and performance measures prior to training between groups (Table 1). Participants completed 99% of the assigned training sessions without any complications (one participant missed one training session). The total amount of work done throughout the training was not different (interaction; $P = 0.75$) between CON (815 ± 88 kJ) and BFR (830 ± 129 kJ).

Physiological and performance variables measured before and after CON and BFR are presented in Table 1. There were significant interactions for absolute and relative $\dot{V}O_{2max}$. Subsequent *post hoc* tests revealed both absolute (Fig 1A) and relative $\dot{V}O_{2max}$ increased following BFR (absolute $P = 0.02$; relative $P = 0.01$) but not in CON (absolute $P = 0.56$; relative $P = 0.88$). Absolute MAP (Fig 1B) was unchanged with training in either group. Relative MAP increased with training (main effect for time; $P = 0.03$), however there was no difference between CON and BFR.

The inverse linear relationship produced the lowest combined standard error for CP (CON, Pre; 1.8 ± 1.0%, Post; 1.1 ± 0.7%, BFR, Pre; 1.7 ± 0.8%, Post; 1.9 ± 1.1%) and W' (CON, Pre; 7.4 ± 4.4%, Post; 4.9 ± 3.5%, BFR, Pre; 8.4 ± 5.7%, Post; 9.1 ± 5.9%) and therefore this equation was used to calculate parameter estimates. CP (Fig 1C) increased with training, however there was no difference between CON and BFR. W' (Fig 1D) was unchanged with training in either group.

Absolute (Fig 1E) and relative PPO and absolute (Fig 1F) and relative MPO increased with training, however there were no differences between CON and BFR.

Capillarisation and muscle morphology

All measures of capillarisation (Fig 2) were unchanged with training in either group (Table 2). The number of Ki-67 proliferating cells co-localised with capillaries was not significantly changed (interaction; $P = 0.06$) with training in either group (Fig 3). CSA of type I and type II fibres were unchanged with training in either group (Table 2).

Mitochondrial enzyme protein content

Protein content of CS, COX II and COX IV (Fig 4) were all unchanged with training in either group.

DISCUSSION

This study has demonstrated that 4 weeks of SIT increased CP in trained individuals, however the addition of BFR did not enhance this further. Furthermore, SIT, with or without BFR, did not induce any changes in skeletal muscle capillarity or mitochondrial protein content in our trained population. The study has, however, confirmed the potency of SIT combined with BFR in increasing $\dot{V}O_{2max}$ to a greater extent than SIT alone.

We had hypothesised that the increase in CP following SIT would be further enhanced with BFR. In contrast to this hypothesis, whilst there was an increase in CP of 3.6% with SIT, there was no greater enhancement with BFR which had a similar magnitude of increase of 3.3%. The improvement in CP is in line with previous research which has demonstrated increases in CP following high intensity interval training (15, 40). The increases of 10-15% in the aforementioned studies are notably greater than that of the present study; however, considering our shorter training period and well-trained participants this is not surprising. Nevertheless, it is important to note that the gains of ~3.5% are greater than the smallest worthwhile change in power for well-trained individuals of 1% (36) and therefore represents a meaningful observation.

Given the established relationship between capillarity and high intensity exercise performance (24), particularly CP (32), together with the potency of the acute angiogenic stimulus our novel training intervention provides (48), it was surprising that we did not observe an increase in any measure of capillarity in either training group. The lack of increase in capillarity could be due to the volume of training undertaken, given that, in comparison to rodents where angiogenesis can occur within a week (53), in humans it is generally considered that angiogenesis manifests later than other training adaptions, typically occurring after 4-5 weeks of training (3,26). Whilst the current training protocol was 4 weeks in

duration it involved only 8 low volume training sessions. Although SIT has been shown to increase skeletal muscle capillarity, this was in untrained individuals ($\dot{V}O_{2max}$ of 41.9 ± 1.8 $ml.min^{-1}.kg^{-1}$) and was after 18 sessions over a longer period of 6 weeks (11), resulting in a training volume more than double that of the present study. Volume of training seems to play a greater role than intensity in stimulating angiogenesis (17). For example, high intensity interval training has been shown to produce a lower acute angiogenic response in interstitial fluid than moderate intensity training (20) and an increase in training intensity at the expense of the volume of training in trained individuals has been demonstrated to reduce skeletal muscle VEGF protein content (18) and stunt increases in capillarisation (20). Therefore, in spite of our hypothesis, the 8 sessions may still have been an insufficient stimulus to induce an increase in capillarisation. This is further exacerbated by the trained nature of our participants in which the baseline capillary to fibre ratio of 2.9 is around double that previously reported in untrained participants of ~1.4 (3, 26).

Nevertheless, the angiogenic potential of the present training intervention was further explored by investigating the presence of proliferating endothelial cells. The antibody Ki-67 detects a proliferation-associated nuclear antigen whereby its co-localisation within endothelial cells in skeletal muscle allows the identification of proliferating endothelial cells and thus location of growing capillaries (21, 26) and is therefore a measure that would precede increases in skeletal muscle capillarity. There was no significant change in Ki-67 positive endothelial cells following training in either group, although the interaction effect was $P = 0.06$ and there was an approximate 100% increase in Ki-67 positive endothelial cells following BFR, which was not present after CON. Indeed, there was a greater presence of the number of Ki-67 positive endothelial cells in 7 out of 9 participants of the BFR group compared to only one participant demonstrating any evidence of increased EC proliferation in CON and a calculation of Cohen's d revealed a medium effect size of 0.63. These

observations suggest the potential of post-exercise BFR to enhance angiogenesis is worth further exploration and to induce measurable further adaptation in this already well adapted population may require a greater training period or a higher training volume.

There were no changes in the protein content of mitochondrial enzymes CS, COX II and COX IV following either training group. This contrasts with previous findings which have consistently demonstrated increases in markers of mitochondrial biogenesis following SIT, including increased activity of citrate synthase and COX (8, 9, 10, 16), increased protein content of COX II and COX IV (7, 16) and increased maximal mitochondrial respiration (19). These previous investigations have, however, only been undertaken on untrained or recreationally active populations; therefore, it seems likely that the lack of effect in the present study is attributable to our already well adapted trained population.

In the absence of any observable angiogenesis and mitochondrial biogenesis the improvement in CP in both groups could be attributable to multiple factors related to the so-called anaerobic capacity of skeletal muscle. For example, increases in skeletal muscle buffering capacity (54) and monocarboxylate transporter protein (5) have been demonstrated following high intensity interval training in trained individuals. These adaptations, which would enhance the ability for the removal of fatigue inducing metabolites and thus be expected to increase the power at which the loss of metabolic homeostasis occurs, could be hypothesised to increase CP. Such adaptations may also be expected to increase W' which, as the second parameter of the power-duration relationship, has classically been considered to represent an anaerobic component (34). W' was, however, unchanged in the present study, as has typically been reported in many high-intensity training studies that have reported an increase in CP (15, 40, 51). It is important to consider the interrelated nature of CP and W' which have regularly been reported to change in opposite directions in response to multiple interventions (38) and as such the observed increase in CP may have offset any increases in W'.

Although CP increased in both groups, $\dot{V}O_{2max}$ increased in the BFR group only. This supports our previous work (48) which initially demonstrated the potency of this novel training intervention, with an increase in $\dot{V}O_{2max}$ of ~4.5%, and agrees with further studies which have demonstrated an increase in $\dot{V}O_{2max}$ with the addition of BFR to low intensity (~40% of $\dot{V}O_{2max}$) exercise (1, 35). Improvements in $\dot{V}O_{2max}$ have been demonstrated to precede increases in skeletal muscle capillaries (3) and while submaximal thresholds are predominantly determined by peripheral mechanisms (28), central components, in particular maximal cardiac output, are considered to be the principal limiting factors of $\dot{V}O_{2max}$, at least within whole body exercise such as cycling (4, 28). Therefore, on the basis we have not observed any increase in capillarity, the increase in $\dot{V}O_{2max}$ following BFR is perhaps more likely to be attributable to central adaptations, i.e. increased cardiac output, with SIT alone not presenting a sufficient challenge within our trained population. The addition of BFR during and after exercise poses a significant challenge to the central cardiovascular system through the induction of the exercise pressor reflex (2, 45) that results in an increase in HR and systolic blood pressure (6, 25, 41, 42, 46). Central cardiovascular adaptations have previously been reported with BFR exercise whereby Park *et al*. (35) demonstrated that 2 weeks of walk training combined with BFR increased stroke volume by 21.4%. Although no assessment of stroke volume was made in the control group of that study, so the effect of exercise alone is unknown, it seems plausible that the pressor reflex related stimulus of BFR could induce a central adaptive response that contributes to an increase in maximal cardiac output and thus $\dot{V}O_{2max}$.

The present study is not without its limitations. Whilst the use of trained individuals clearly enhances the validity of the application of the study findings to elite training practice, this resulted in a reduced sample size, in particular of participants who consented to muscle biopsies. Although typical of the current literature, the sample size must therefore be

considered when interpreting the results, which is likely to have reduced the chances of correctly accepting or rejecting the null hypotheses. This is perhaps reflected in the present study with several parameters which displayed *P* values close to the critical value. An absolute BFR cuff pressure of 120 mmHg was also utilised for all participants. It is known that there is a variation between individuals in the level of blood flow restriction imposed by a standard absolute external cuff pressure (23) which will affect the level of muscle oxygenation and muscle metabolite accumulation (29, 47). Indeed, unpublished work in our laboratory using the same standard cuff pressure suggests that the decrease in muscle oxygenation imposed by the post-exercise BFR in the present training protocol varies between 11 and 43% (Mitchell EA, Bailey SJ & Ferguson RA, unpublished observations). It is therefore possible that the physiological signals imposed with the addition of post-exercise BFR were not consistent between individuals and may have impacted the extent of any adaptations. Furthermore, the present study only focused on the peripheral adaptations to the current intervention. As discussed above it seems likely that the observed increase in $\dot{V}O_{2max}$ could be attributable to central adaptations, such as increased cardiac output. The central adaptive responses to BFR exercise clearly require further investigation.

In conclusion, the addition of post-exercise BFR did not enhance the increase in CP oberserved after 4 weeks of SIT in trained individuals. SIT with or without BFR did not induce any changes in skeletal muscle capillarity or mitochondrial protein content. The study has, however, confirmed previous findings of the potency of combining post exercise BFR during SIT in enhancing $\dot{V}O_{2max}$ in trained individuals.

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GRANTS

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

E.A.M., C.W.T., and R.A.F. conceived and designed research; E.A.M., and R.A.F. performed experiments; E.A.M. analyzed data; E.A.M., N.R.W.M., M.C.T., and R.A.F. interpreted results of experiments; E.A.M. prepared figures; E.A.M., and R.A.F. drafted manuscript; E.A.M., N.R.W.M., M.C.T., C.W.T., and R.A.F. edited and revised manuscript; E.A.M., N.R.W.M., M.C.T., C.W.T., and R.A.F. approved final version of manuscript.

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FIGURE LEGENDS

Figure 1. Individual responses of $\dot{V}O_{2max}$ (A), MAP (B), CP (C), W' (D), PPO (E) and MPO (F) before and after control (CON) and blood flow restriction (BFR) training interventions.

Figure 2. Muscle capillaries before and after control (CON) and blood-flow restriction (BFR) training interventions. A) Representative images of muscle capillaries stained with CD-31 (green) and type II fibres (blue). Scale Bar = 50 μm. B) Individual responses of capillary density and capillary to fibre ratio. Data are CON n = 7; BFR n = 9.

Figure 3. Presence of proliferating endothelial cells before and after control (CON) and blood-flow restriction (BFR) training interventions. A) Representative image of Ki-67 (red) positive endothelial cell (green). Scale Bar =50 μm. B) Individual responses of Ki-67 positive endothelial cells per fibre. Data are CON n = 6; BFR n = 9. One bar for BFR is hidden as data are pre =0.00 and post = 0.00.

Figure 4. Content of CS (A), COX II (B) and COX IV (C) protein before and after control (CON) and blood flow restriction (BFR) training interventions. Values are expressed as fold changes relative to pre-training values. Bars represent the mean and lines represent individual responses. Data are CON n = 7; BFR n = 7.

TABLE LEGENDS

Table 1. Physiological and performance variables before and after control (CON) and blood-flow restriction (BFR) training interventions.

Table 2. Capillarisation and muscle morphology before and after control (CON) and blood-flow restriction (BFR) training interventions.

627 **TABLE 1**

Parameter	CON			BFR			ANOVA interaction *P* value	ANOVA main effect of time *P* value	ANOVA main effect of condition P value
	Pre	Post	Percentage change	Pre	Post	Percentage change			
Body Mass (kg)	74.6 ± 7.7	74.3 ± 8.5	-0.3	76.3 ± 8.3	75.6 ± 7.7	-0.8	0.59	0.24	0.67
$\dot{V}O_{2max}$ (l.min^{-1})	4.59 ± 0.46	4.56 ± 0.48	-0.8	4.70 ± 0.63	4.98 ± 0.72 *	5.9	0.02	0.08	0.45
$\dot{V}O_{2max}$ (ml.min^{-1}.kg^{-1})	63.2 ± 4.4	63.0 ± 5.9	-0.3	62.2 ± 3.4	65.2 ± 4.4 *	4.9	0.04	0.06	0.79
MAP (W)	393 ± 47	397 ± 51	1.0	402 ± 51	412 ± 41	2.6	0.44	0.09	0.56
MAP (W.kg^{-1})	5.3 ± 0.5	5.4 ± 0.6	1.5	5.3 ± 0.4	5.5 ± 0.4	3.5	0.40	0.03	0.81
CP (W)	287 ± 39	297 ± 43	3.6	296 ± 40	306 ± 36	3.3	0.93	0.001	0.58
***W′* (kJ)**	18.1 ± 5.1	16.2 ± 4.3	-10.4	17.9 ± 6.5	17.4 ± 5.1	-3.0	0.53	0.26	0.80
PPO (W)	1057 ± 174	1112 ± 179	5.2	1065 ± 245	1142 ± 262	7.2	0.62	0.008	0.85
PPO (W.kg^{-1})	14.3 ± 2.3	15.0 ± 2.2	5.4	13.8 ± 2.7	14.9 ± 2.8	8.3	0.53	0.004	0.80
MPO (W)	686 ± 73	692 ± 62	0.8	701 ± 113	722 ± 112	3.1	0.23	0.05	0.60
MPO (W.kg^{-1})	9.2 ± 0.6	9.3 ± 0.6	1.4	9.1 ± 1.0	9.5 ± 1.1	4.2	0.22	0.01	0.99

Values are mean ± SD. Abbreviations: CP, critical power; MAP, maximal aerobic power; MPO, mean power output during 30 s sprint; PPO, peak power output during 30 s sprint; VO_{2max}, maximal oxygen uptake and *W′*, curvature constant. All data are CON n = 10 and BFR n = 11, except for $\dot{V}O_{2max}$ where n = 8 and PPO and MPO where n = 10. * Significantly different to Pre-training (Bonferroni-corrected *post hoc* paired *t*-test; $P < 0.05$).

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631 **TABLE 2**

Parameter	CON			BFR			ANOVA interaction *P* value	ANOVA main effect of time *P* value	ANOVA main effect of condition P value
	Pre	Post	Percentage change	Pre	Post	Percentage change			
CD (mm^{-2})	405 ± 65	386 ± 27	-4.6	420 ± 30	415 ± 40	-1.3	0.49	0.22	0.27
C:F	2.97 ± 0.63	2.98 ± 0.71	0.4	2.87 ± 0.64	2.97 ± 0.75	3.5	0.68	0.60	0.88
CC Type I	7.03 ± 1.21	6.92 ± 1.54	-1.6	6.99 ± 1.25	7.19 ± 1.43	2.7	0.44	0.85	0.86
CC Type II	6.45 ± 1.09	6.63 ± 1.40	2.8	6.01 ± 1.06	6.32 ± 1.53	5.1	0.73	0.20	0.56
CSA Type I (μm^{-2})	6456 ± 1651	6379 ± 1764	-1.2	6146 ± 1242	6375 ± 1472	3.7	0.62	0.81	0.83
CSA Type II (μm^{-2})	6908 ± 1463	6998 ± 1415	1.3	5896 ± 963	6364 ± 1639	7.9	0.47	0.29	0.23
Ki-67/fibre	0.07 ± 0.05	0.06 ± 0.04	-19.7	0.05 ± 0.06	0.10 ± 0.09	103.4	0.06	0.29	0.85

Values are mean ± SD. Abbreviations: CC Type I, capillary contacts of type I muscle fibres; CC Type II, capillary contacts of type II muscle fibres; CD, capillary density; C:F, capillary to fibre ratio; CSA Type I, cross sectional area of type I muscle fibres; CSA Type II, cross sectional area of type II muscle fibres and Ki-67/fibre, Ki-67 positive capillaries per fibre. All data are CON n = 7 and BFR n = 9, except Ki-67/fibre which is CON n = 6 and BFR n = 9.

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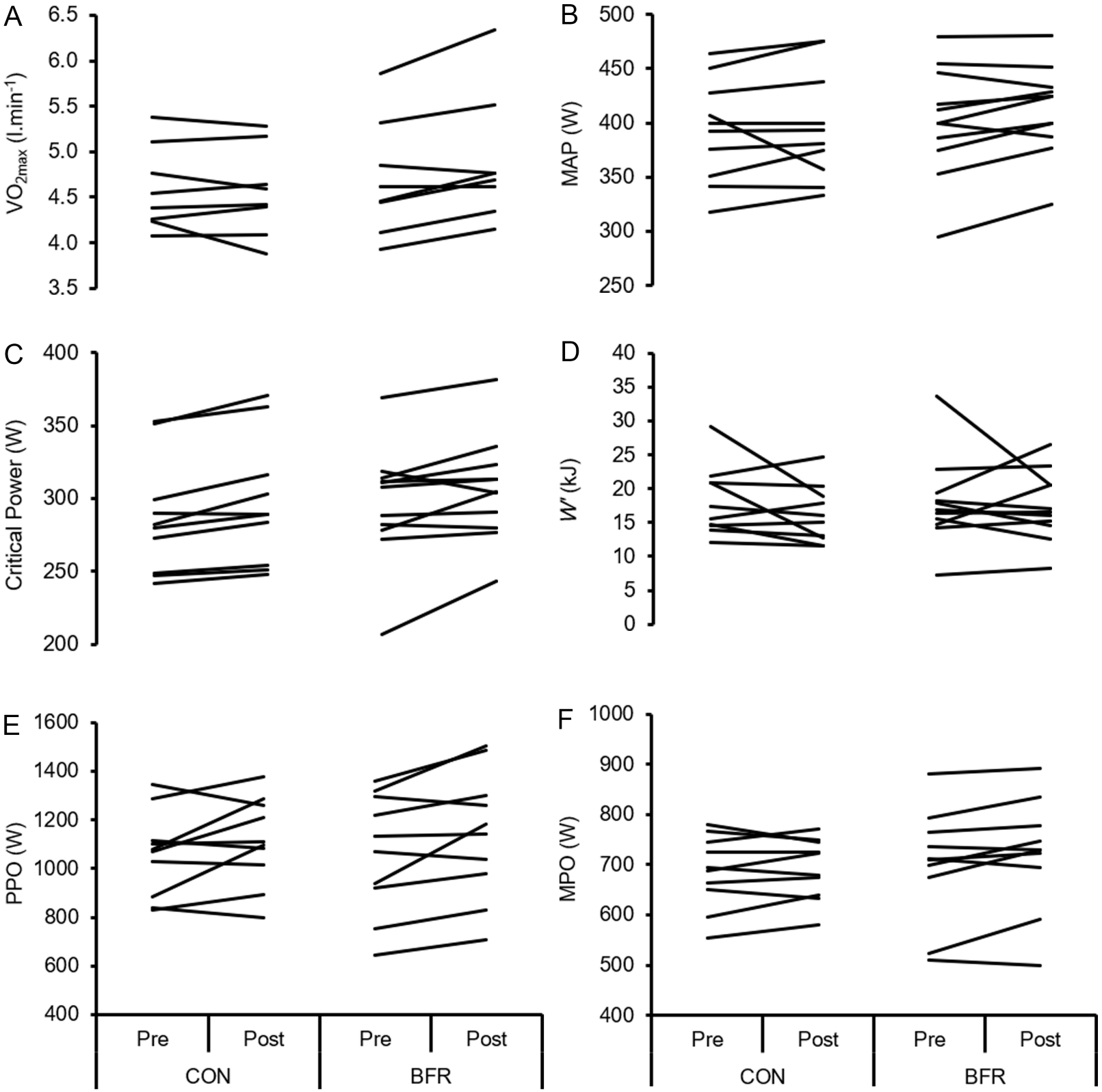

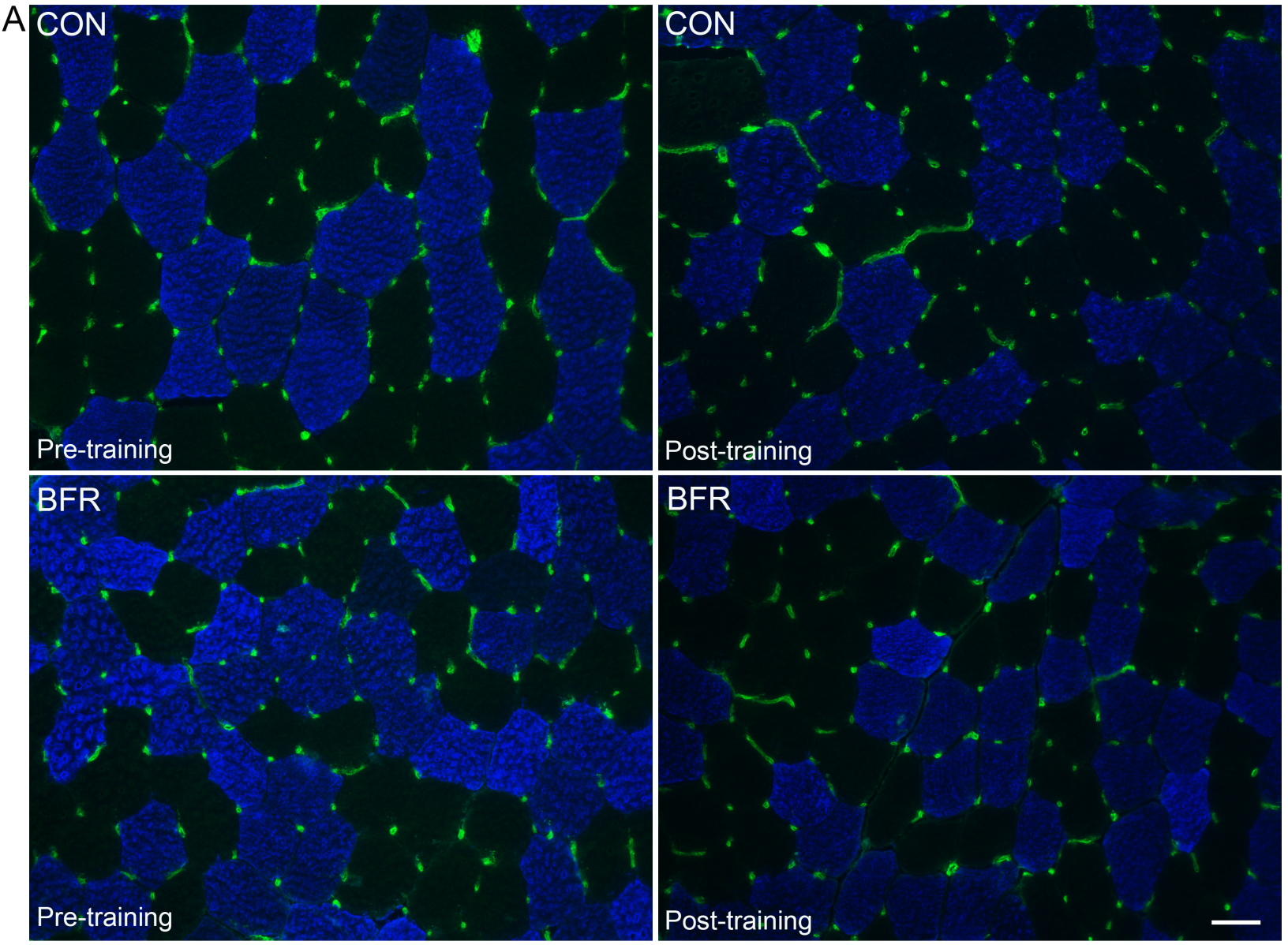

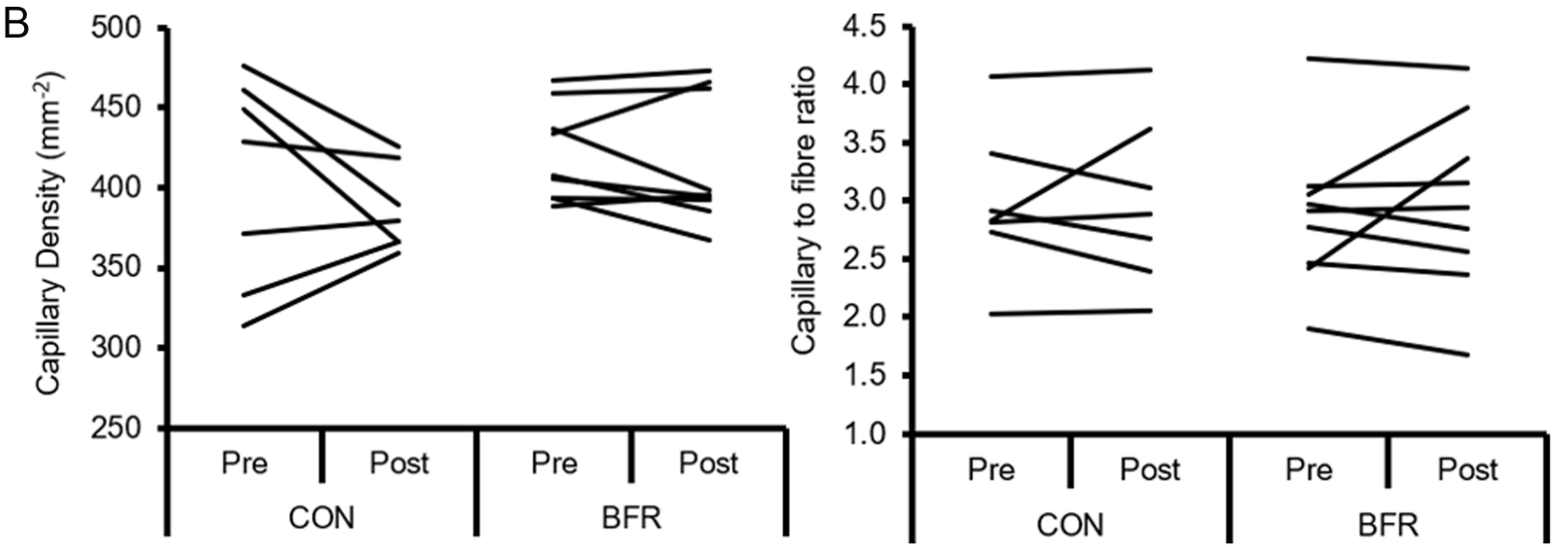

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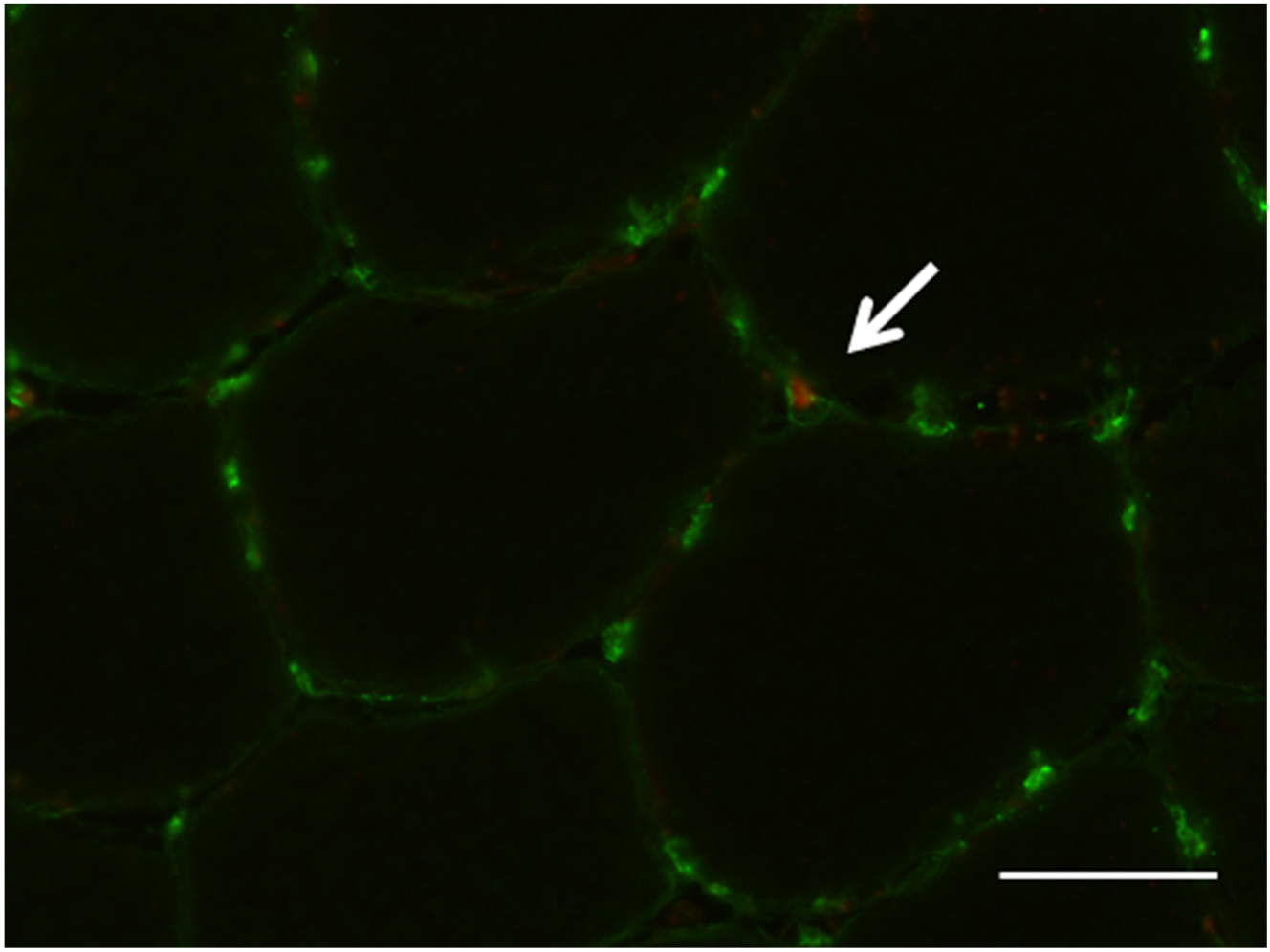

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