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By REINNIER PADILLA, B.Sc.

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AUTHOR: Reinnier S. Padilla
Hon.B.Sc (University of Windsor)

SUPERVISOR: Gianni Parise, Ph.D.

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LAY ABSTRACT

Skeletal muscle health is partially maintained by a population of stem cells, referred to as satellite cells. The effect of resistance training on satellite cells has been widely studied, although less is known about the role of satellite cells in muscle adaptations following endurance training. The intensity in which endurance exercise is performed is suggested to play a role in both the way satellite cells respond to exercise as well as the growth of fine branching blood vessels that deliver oxygen, nutrients and removes wastes between the blood and muscle tissue (also known as capillaries). Generally, when higher exercise intensities are employed in endurance training, they are performed as short bursts of intense aerobic exercise interspersed with periods of recovery (also known as interval exercise). We sought to determine whether this rest-to-work cycle associated with interval exercise plays a role in the satellite cell response and capillary growth. We demonstrate enhanced activation of satellite cells following interval endurance training employing this rest-to-work cycle compared to work- and intensity-matched continuous endurance training.

ABSTRACT

Skeletal muscle satellite cells (SC) are essential in muscle repair and regeneration. The role of SCs in mediating hypertrophic adaptations following resistance training has been widely studied. Recent evidence from endurance training studies suggest that SCs may also play a role in mediating non-hypertrophic adaptations. Indeed, it has been shown that satellite cells respond to endurance training. Work in rodent models suggest that exercise intensity may play an important role in expanding the SC pool whereas the results of endurance training studies in humans are much less consistent. Limited evidence also suggest that exercise intensity may be important in mediating exercised-induced capillarization following endurance training in humans. In both instances, it is unknown whether the on-and-off pattern characteristic of interval training (i.e. the rest-work cycles) plays a role in the magnitude of these skeletal muscle responses to this type of exercise. Thus, we sought to determine if the rest-to-work cycle plays a role in the scope of these skeletal muscle responses by comparing the SC response and capillarization to two distinct work-matched protocols that are performed at the same intensity but at two different exercise patterns (interval versus continuous). We hypothesized that interval exercise training will elicit a greater SC response and induce greater capillary growth compared to work-matched continuous exercise training. Ten young active individuals performed 12 sessions of counterweighted single-leg cycling over 4 weeks. Each leg was randomly assigned to *Interval (INT)* (10 x 3-min intervals at 50% of single leg peak power output (PPO), with 1 min recovery) or *Continuous (CONTIN)* (30 min at 50% PPO, followed by 10 min recovery), which were performed 5 min apart on each day, in an alternating order. Resting muscle biopsies were obtained from the *vastus lateralis* pre- and post-training. Immunofluorescent microscopy of muscle cross sections was used to assess muscle fibre capillarization, SC expansion and

activation. Western blot analysis was performed on pro-angiogenic factors, specifically VEGF and VEGFR2. Mixed muscle SC activation increased in the *INT* leg compared to the pre-training time-point (3.2 ± 0.5 vs. 1.5 ± 0.2 $Pax7^+/MyoD^+$ cells/ 100 myofibers, respectively; $P < 0.05$). Mixed muscle SC activation did not increase significantly in the *CONTIN* leg compared to the pre-training time-point and there was no significant difference in activation between *CONTIN* and *INT* post-training. With regards to capillarization, no differences in type I or type II muscle fibre CC (capillary contacts), C/Fi (individual capillary-to-fibre ratio) or CFPE (capillary-to-fibre perimeter exchange index) were observed post-training or between legs. No significant expansion of the SC pool occurred in either legs post-training and no increases in fibre cross-sectional area was observed. This study presents novel evidence of mixed muscle SC activation following interval exercise training that is not observed following work- and intensity-matched continuous exercise training. This suggest that the rest-to-work cycle associated with interval exercise may dictate, to some extent, SC activation whereas it may not be a primary stimulus for training-induced changes in capillarization.

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LIST OF ABBREVIATIONS

AMPK	5' adenosine monophosphate-activated protein kinase
ANG	angiopoietin
ANOVA	analysis of variance
BMI	body mass index
BSA	bovine serum albumin
Bx	muscle biopsy
C/Fi	individual capillary-to-fibre ratio
CC	capillary contacts
CFPE	capillary-to-fibre perimeter exchange index
CONTIN	leg that performed continuous exercise training
CS	citrate synthase
CSA	cross sectional area
DAPI	4', 6-diamidino-2-phenylindole
DL	double-leg
DSHB	Developmental Studies Hybridoma Bank
ECM	extracellular matrix
eNOS	endothelial nitric oxide synthase
GS	goat serum
HGF	hepatocyte growth factor
HIIT	high-intensity interval training
HR_{max}	maximal heart rate
INT	leg that performed interval exercise training
IGF-1	insulin-like growth factor-1
IL-6	interleukin 6
MHC	myosin heavy chain
MICT	moderate-intensity continuous training
MMP	matrix metalloproteinase
MRF	myogenic regulatory factor
mRNA	messenger ribonucleic acid
OCT	optimum cutting temperature
p38 MAPK	p38 mitogen-activated protein kinase
Pax	paired box
PBS	phosphate buffered solution
PFA	paraformaldehyde
RPM	revolutions per minute
SC	satellite cell
SEM	standard error of the mean
SIT	sprint interval training
SL	single-leg
TE	time to exhaustion
TIMP-1	tissue inhibitor of matrix metalloproteinase
TSP-1	thrombospondin-1
VEGF	vascular endothelial growth factor
VEGFR2	vascular endothelial growth factor receptor 2
VO_{2max}	maximal oxygen consumption
VO_{2peak}	peak oxygen uptake
W_{peak}	peak power output

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DECLARATION OF ACADEMIC ACHIEVEMENT

R. Padilla was the principal contributor for conceptualizing the research question, research hypothesis, experimental design, collection of muscle tissue cross-section, data analysis and data interpretation. G. Parise assisted with research question, research hypothesis, experimental design, and data interpretation. M. Gibala and L. Skelly conceptualized the training protocol. L. Skelly recruited the participants, conducted the exercise performance tests and oversaw the training program. R. Padilla assisted with training the participants. M. Tarnopolsky conducted the muscle biopsies. S. Joanisse, I. Khan, M. Kamal, and N. Jacobs assisted with data analysis.

REVIEW OF THE LITERATURE

1. Introduction to Skeletal Muscle and Satellite Cells

Skeletal muscle comprises approximately 40% of the human body weight and is involved in pivotal roles in locomotion, metabolic responses and regulation of metabolic homeostasis (30). It is made up of multinucleated muscle cells called muscle fibers (myofibers) that are composed of contractile units called actin and myosin filaments. These myofibers are innervated by slow-contracting, fatigue-resistant type I motor units and fast-contracting, low fatigue-resistant type II motor units (13). The myofibers express proteins associated with their respective neurons and are accordingly classified as type I (expressing myosin heavy chain (MHC) I) and type II (expressing MHC II) myofibers. The contractile property of an individual muscle is determined by the proportion of each fiber type.

Skeletal muscle is capable of remarkable plasticity owing to muscle-specific stem cells known as satellite cells (SC) (13). SCs, which reside in the space between the sarcolemma and basal lamina (also known as the SC niche), are essential for muscle repair and regeneration (69, 107). Most SCs are quiescent (transcriptionally inactive) in healthy skeletal muscle and generally become activated in response to skeletal muscle damage (21, 113). Once activated, SCs undergo multiple rounds of cell division and enter one of two pathways: 1) return to a quiescent state or 2) proliferate, differentiate and eventually fuse to existing muscle fibers (21, 104) in a process referred to as the myogenic program (**Figure 1**). By fusing to existing muscle fibers and donating their nuclei, SCs support skeletal muscle fiber repair (107) and growth (4, 78). SC activation, nonetheless, is not restricted to the site of damage – SCs along the same fiber can be activated and migrate to the site of regeneration (125). Proliferating SCs and their progeny are referred to

as myogenic precursor cells (MPCs). The myogenic program is governed by a set of transcriptional networks collectively referred to as the myogenic regulatory factors (MRFs). The key transcription factors, together with Pax7, play a critical role in the initiation and progression of the myogenic program. These MRFs include myoblast determination factor (MyoD), myogenic factor 5 (Myf5), muscle-specific regulatory 4 (MRF4) and myogenin.

A SC in the quiescent state can be identified by the expression of Pax7, although it remains expressed during SC activation (104). For entry into the myogenic program, upregulation of Myf5 and MyoD are required for SC activation and proliferation. Myf5 has been shown to be necessary for enhanced proliferation and delayed differentiation, with the upregulation of MyoD furthering the progression of proliferating SCs into terminal differentiation (125). Downregulation of Pax7 and Myf5, concurrent with the upregulation of myogenin and MRF4, lead to terminal differentiation (125). A subpopulation of SCs down-regulate MyoD, and return to cellular quiescence to renew the SC pool (27, 126). Nevertheless, at the onset of the myogenic program, MyoD has been shown to be an important driver of myoblast proliferation and remains up-regulated at the onset of differentiation (68, 85, 104). Hence, MyoD is often used as a marker to identify activated SCs that are no longer in a quiescent state.

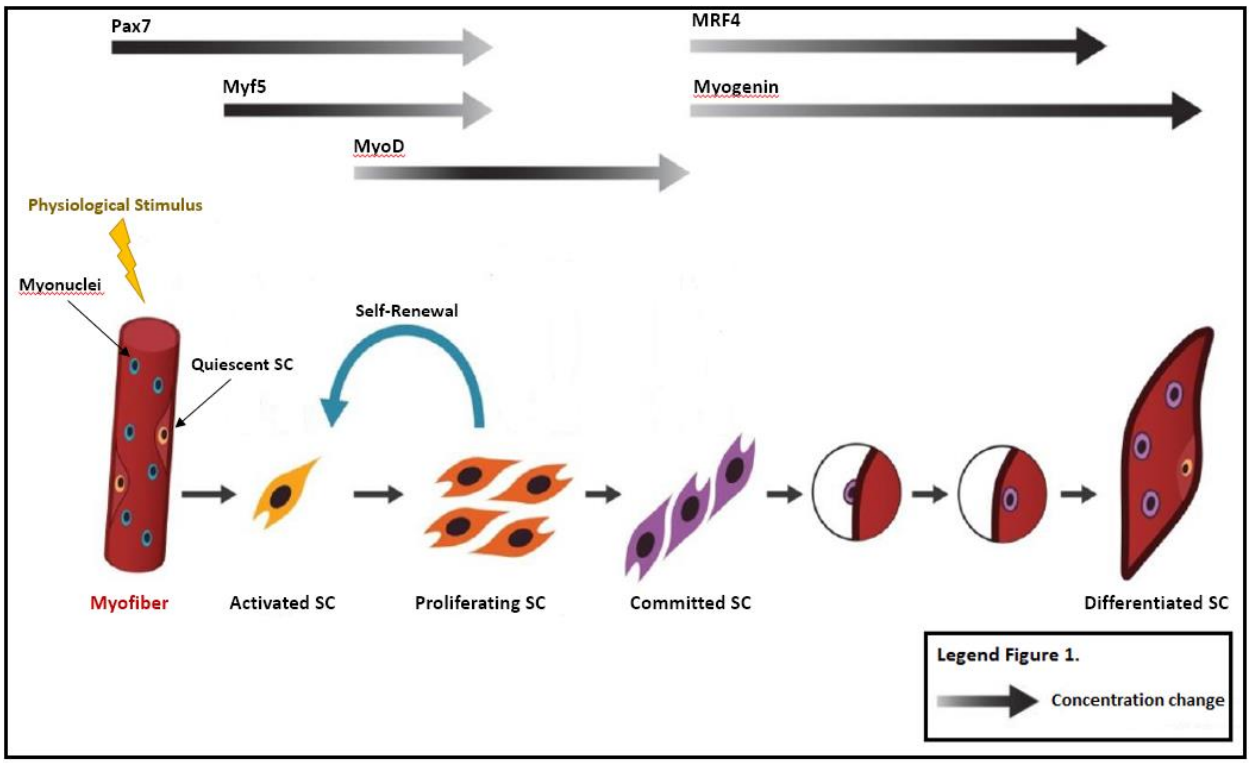

Figure 1. Schematic representation of the myogenic program in response to a physiological stimulus (adapted from (90)). Satellite cells (SC) typically reside in their quiescent state within a specialized niche located beneath the basal lamina and sarcolemma of the myofiber. Quiescent SCs are characterized by their expression of Pax7. Following stimuli, for example as a result of muscle damage from exercise, SCs enter the myogenic program, which is orchestrated by key transcription factors known as myogenic regulatory factors (MRFs). The SCs become activated and then begin to proliferate, expressing Myf5 initially and eventually upregulating MyoD to further the progression of the proliferating SCs. Following proliferation, the SCs can commit to differentiation by down-regulating Myf5 and Pax7 while concomitantly upregulating the expression of MRF4 and myogenin. Terminal differentiation occurs when the committed SCs fuse to themselves forming new myofibers, or fuse to existing myofibers to donate their nuclei. A subset of SCs during proliferation will retain Pax7 expression while down-regulating MyoD and return to their quiescent state, thus renewing the satellite cell pool.

2. The Role of Satellite Cells in Skeletal Muscle Growth

Because myofibers are post-mitotic, SCs play an important role in skeletal muscle growth, repair and remodelling. Myofiber size dynamically respond to external stimuli (93). Changes in either the number of myonuclei or the myonuclear domain facilitate increases or decreases in myofiber size (113). The myonuclear domain is described as the defined volume of

cytoplasm within a multinucleated myofiber that is regulated by the gene products of a single myonucleus (93). Initial hypertrophy is associated with an increase in protein synthesis and the resulting growth in myonuclear domain without the addition of myonuclei (98). Once a "ceiling" is reached or the finite size for the myonuclear domain, then additional myonuclei contributed by the SCs are necessary for further and/or substantial muscle hypertrophy (67, 98). Specifically, no changes in myonuclear domain are postulated to occur when changes in muscle fiber are below 15% and it is believed that a muscle fiber will grow until the myonuclear domain hits a ceiling of approximately 2000μm^2/myonucleus (67, 98). SCs fuse with pre-existing myofibers and donate their nuclei to re-establish the ratio of DNA to cytoplasmic volume – this process ensures that muscle growth is sustainable (93). Rodent studies, however, demonstrate that SCs may not be necessary for muscle hypertrophy (58, 80). In humans, an expansion of the SC pool is tightly associated with an increase in myofiber size (111), although recent work has shown that an increase in myofiber CSA can occur without a concomitant expansion of the SC pool (33).

3. The SC Response to an Acute Bout of Resistance Exercise

The role of SCs in mediating hypertrophic adaptations following resistance training has been widely studied (4, 67, 78, 99). Specifically, eccentric contractions have typically been used to study SC function. A significant expansion of the SC pool has been observed as early as 6 hours following a single bout of eccentric exercise (81, 82), with the peak typically occurring around 72 hours post-exercise (111). In young recreationally-active men, a single bout of resistance exercise-induced SC activation occurred 24 hours post-exercise, and additional increases in SC activation was not observed 72 hours post-exercise (92). Resistance exercise can

also induce a fiber-type specific change in the SC pool, with eccentric exercise inducing an expansion of the SC pool associated with type II myofibers in young men (12). An expansion of the SC pool is commonly associated with an increase in muscle fibre CSA, often as a result of resistance exercise (67, 98, 113). Following a single bout of resistance exercise, there is a robust activation and expansion of the SC pool (4, 84, 122). However, recent evidence from exercise training studies suggest that SCs may also play a role in mediating non-hypertrophic adaptations (14, 60, 64, 120). Recent work has demonstrated that an increase in fiber cross-sectional area (CSA) is not always associated with expansion (33). While inducing a less robust SC response than resistance exercise, HIIT and MICT have been shown to be capable of inducing a SC response without a concomitant increase in muscle fiber CSA across a range of individuals, including healthy, young and older individuals (61, 79).

4. Physiological adaptations to endurance training

4.1 Interval versus continuous aerobic exercise training

Consistent with the principle of training specificity, endurance or aerobic exercise training is associated with an improved capacity for aerobic energy metabolism and enhanced fatigue resistance, whereas resistance training is known to induce muscle hypertrophy and increase strength (30, 42). Chronic involvement in aerobic exercise over a period of weeks, months or years is referred to as aerobic exercise training and the extent of adaptations are dependant on the duration, intensity and type of aerobic exercise performed. Nevertheless, physiological adaptations generally associated with aerobic exercise training are increases in mitochondrial density (mitochondrial biogenesis) (10, 36), increases in mitochondrial content

(75, 77) and capillarization (20, 26). Increased mitochondrial content following training lessens glycogen degradation and lactate production, eventually leading to increased lactate threshold; thereby, individuals are able to exercise for longer durations (30, 66). Thus, given its crucial role in exercise performance, considerable attention has been placed on elucidating the factors mediating exercise-induced mitochondrial adaptations (7, 75).

For comparative purposes, researchers have used the term moderate intensity continuous training (MICT) to describe traditional endurance exercise training. The other major form of endurance training is interval training, which can be described as short bursts of intense aerobic exercise interspersed with periods of recovery. Two basic types of interval training based on exercise intensity are generally employed in the literature: high-intensity interval training (HIIT) performed at an intensity that elicits ≥80% of maximal heart rate and sprint interval training (SIT) defined as efforts performed at intensities equal to or greater than the pace that would elicit peak oxygen uptake (VO_{2peak}), including 'all-out' and 'supramaximal' efforts (e.g. repeated Wingate tests) (75, 123). When matched-work comparisons are made within the same individual, two weeks of HIIT elicited greater increases in mitochondrial content (assessed by citrate synthase maximal activity) and mitochondrial respiration (assessed by oxidative phosphorylation (OXPHOS) capacity) compared to MICT (77). However, when matched-work comparisons are made between individuals (i.e. parallel-group designs), HIIT and MICT elicited similar increases in mitochondrial content (2, 45). It is suggested that differences in experimental design partly account for the lack of consensus among these studies (75). At the whole body-level, a meta-analysis reported greater gains in maximal oxygen consumption (VO_{2max}) in healthy adults following high-intensity interval training compared to continuous endurance training (86).

The role of exercise intensity is highlighted in studies reporting similar skeletal muscle adaptations between SIT and MICT despite the much lower training volume associated with SIT (75). Comparing low-volume SIT to MICT, similar increases in mitochondrial content were found after 2 (36), 6 (10) and 12 (38) weeks of training. Furthermore, low-volume SIT induced similar increases in VO_{2max} compared to MICT over a 12-week period (38). Acutely, higher exercise intensities appear to induce greater metabolic signaling than moderate intensities (75). For instance, an acute bout of high-intensity exercise (~36 min at 80% of peak oxygen uptake (VO_{2peak})) induced activation of signaling pathways associated with the regulation of mitochondrial biogenesis, specifically phosphorylation of AMPK (5' adenosine monophosphate-activated protein kinase) and CaMKII (Ca^{2+}/ calmodulin-dependent protein kinase II), whereas work-matched low-intensity exercise (~70 min at 39% of VO_{2peak}) did not (29). The greater activation of these kinases elicited by high- compared to low-intensity exercise was associated with greater expression of mRNA for PGC-1α (peroxisome proliferator-activated receptor-gamma coactivator 1-alpha), which is a primary regulator of mitochondrial biogenesis (29). The effect of exercise intensity on exercise-induced mitochondrial adaptations and VO_{2max} is well-established whereas it is less clear for other physiological variables, such as capillary density (77).

A fundamental question that remains unclear is whether the on-and-off pattern associated with interval training (i.e. rest-work cycles) could partially account for the skeletal muscle responses to this mode of exercise (75). It is during the initial on- and off-transient phases of interval exercise that reaction rates change rapidly and metabolic disturbances occur (i.e. a disruption of cellular homeostasis) (73). The repetition of these metabolic disturbances during the duration of the interval exercise can be referred to as metabolic fluctuations (22). Such

metabolic fluctuations alongside exercise intensity (29) is thought to explain why high-intensity, low-volume interval training may induce similar muscular adaptations to those observed after low-intensity, high-volume continuous training (10, 36). Indeed, interval exercise has been demonstrated to elicit greater metabolic fluctuations along with greater phosphorylation of AMPK and p38 MAPK (p38 mitogen-activated protein kinases) compared to work-matched continuous exercise performed at the same exercise intensity (22). Still, it is unknown whether these acute differences in signalling patterns would translate to different chronic effects (75). Although there was a lack of direct comparisons between the two exercise programmes, Cochran and colleagues demonstrated that CS maximal activity was unaltered following a continuous exercise training program of 4 min all-out bouts of cycling performed 3 days per week for 6 weeks compared to an interval exercise training program comprising of the same volume of work performed as four 30 s all-out bouts, interspersed with recovery periods, in which CS maximal activity did increase after 6 weeks (18). Thus, the intermittent nature of interval training is suggested to dictate, to some extent, the skeletal muscle responses following aerobic exercise training (18, 75).

4.2 Using single-leg cycling to examine physiological adaptations to endurance training

A cost- and time-effective method to study the differences in physiological responses between interval and continuous endurance training is the use of unilateral exercise models, such as single-leg (SL) cycling (76). The needle biopsy technique (6) is used for direct sampling of human skeletal muscle, which can provide insight into the biochemical and histological responses resulting from exercise-induced changes. However, the muscle biopsy technique is invasive, and analyses are often expensive, thereby putting restraints on the sample size. Hence, the unilateral model (a within-subject comparison in which 2 limbs of a participant are

randomized to 1 of 2 treatments) provide greater control of potential sources of variation (e.g. diet, sleep, stress), and increase the statistical power by reducing the amount of between-person variability (76). A lack of transfer to the contralateral limb of many important skeletal muscle adaptations, such as muscle capillarization (59) and mitochondrial content (74), has been shown using the unilateral model (76). However, important limitations exist using the unilateral model, including restrictions in the modes of exercise, number of simultaneous comparisons and potential outcomes variables (76). Specifically, as it relates to single-leg cycling, the reduced O_2 uptake of the inactive leg allows the active muscles to be potentially supplied with significantly more oxygenated blood, leading to the mean power output during the single-leg intervals being more than half that during the double-leg interval (1). Additionally, a lower cardiorespiratory stimulus is associated with single-leg cycling compared to double-leg cycling and no increase in single-leg VO_2 has been reported (77, 103, 106). Nevertheless, one way that the single-leg cycling model has been used to mimic double-leg cycling is with the addition of a counterweight to the contralateral pedal, which assists with the upstroke phase of the revolution, making the perceptual 'feel' of counterweighted single-leg cycling similar to double-legged cycling (11). Lastly, single-leg cycling induces greater adaptations, specifically as it relates to metabolic and oxidative physiological adaptations, than double-legged cycling due to the increased relative workloads (1).

4.3 The effect of endurance training on the SC response

While the classic physiological adaptations to aerobic exercise have been extensively studied, such as mitochondrial adaptations, less explored is the impact of aerobic exercise training on the SC response (64). Rodent studies consistently demonstrate that aerobic exercise training results in an increase in SC content (62, 70, 71, 109, 110). Additionally, aerobic exercise

training in rodents suggest that exercise intensity may play an important role in SC pool expansion (71). When considering acute aerobic exercise, exercise intensity may also mediate the SC response in humans. Following an acute bout in older men, a greater increase in SC activation was found following high-intensity exercise compared to continuous exercise, although no expansion of the SC pool or increase in CSA was observed for either exercise types (88). However, aerobic exercise training in humans show less consistent results (64) (**Table 1**). Some aerobic exercise training studies in humans demonstrate an increase in SC content concomitant with an increase in muscle fiber CSA whereas others show no increase in SC content (33, 64, 87, 112) (**Table 1**). For instance, after 14 weeks of interval aerobic training, an increase in SC content and an increase in type IIa fiber CSA was observed in older adults (14, 120). Recently, two different studies that implemented moderate-intensity continuous training (MICT) for 12 weeks in middle-aged adults both reported an expansion of the SC pool for only type I fibers despite an increase in CSA for all fiber types (33, 87). On the other hand, work by Snijders *et al.* (2011) show no change in SC content in older participants with type 2 diabetes following a more traditional lower-intensity endurance training program (112). Following 6 weeks of interval endurance training, expansion of the basal SC pool was only observed in hybrid fibers and there was no increase in muscle fibre CSA (60). Lastly, a 6-week training program consisting of MICT and 2 different sprint-interval training (SIT) protocols demonstrated an increase in SC activation without an apparent expansion of the SC pool in the absence of hypertrophy for all forms of aerobic exercise used (61). Collectively, these results suggest that SCs are responsive to aerobic exercise, although the results from human studies are much more variable than what is observed in rodent studies due to the variable populations used as well as the variety of aerobic training programs implemented (64). Considering that 16 weeks of

resistance exercise training can alter SC activation in response to an acute bout of resistance exercise (92), the SC response to an aerobic exercise training program is also likely influenced by the training status of the participant (i.e. whether or not the individual is accustomed to the exercise stimulus). Nevertheless, in addition to mediating the activation of signalling pathways linked to mitochondrial biogenesis (75), it appears exercise intensity may also play an important role in mediating SC pool expansion with respect to aerobic exercise (61). Considerably less understood is the role of exercise intensity in mediating changes in skeletal muscle capillarization (75).

Table 1. Summary of studies in humans describing the SC response to endurance training.

Study (Reference)	Age (yrs)	Subject Characteristics	Exercise Type	SC Response
Charifi et al. 2003 **(14)**	73 ± 3	Healthy active males (n = 11)	Interval training, 14 wk, 4 d•wk^{-1}, on cycle ergometer: 7 bouts of 4 min @ 65-75% VO_{2peak} followed by 1 min @ 85-95% VO_{2peak}	↑ SC/total fiber
Verney et al. 2008 **(120)**	73 ± 4	Healthy active males (n = 10)	Concurrent training, 14 wk, 3 d•wk^{-1} Interval training on cycle ergometer: 3 bouts of 12 min consisting of 2 sequences of 4 min @ 75-85% HR_{max} interspersed by 2 sequences of 1 min @ 80-95% HR_{max} followed by active recovery (10^{th} to 12^{th} min)	↑ SC/type II fiber ↑ SC/total fiber
Snijders et al. 2011 **(112)**	61 ± 6	Obese type 2 diabetic males (n = 15)	Endurance exercise, 6 mo, 3d•wk$^{-1,}$ walking, cycling, and cross-country skiing; total time of 40 min @ 75% VO_{2peak}	NC in SC/type I fiber NC in SC/type II fiber
Joanisse et al. 2013 **(60)**	27 ± 8	Overweight females (n = 15)	HIT, 6 wk, 3 d•wk^{-1}, on cycle ergometer: 10 × 60-s bouts of cycling @ 90% HR_{max} interspersed with 60 s of recovery	NC in SC/type I fiber NC in SC/type II fiber ↑ SC/hybrid fiber
Fry et al. 2014 **(33)**	47.6 ± 8	Overweight males (n = 6) & females (n = 17)	END, 12 wk, 3 d•wk^{-1} on cycle ergometer: 45 min @ 70% HR reserve	NC in SC/type II fiber ↑ SC/type I fiber ↑ SC/total fiber
	21 ± 4	Healthy active males & females (n = 9)	MICT, 6 wk, 4 d•wk^{-1} on cycle ergometer: 30 min @ 65% VO_{2peak}	NC in SC/type I fiber NC in SC/type II fiber ↑ Pax7+/MyoD+ cells/ fiber (active SC) ↑ Pax7-/MyoD+ cells/fiber (differentiating SC)
Joanisse et al. 2015 **(61)**	21 ± 2	Healthy active males & females (n = 10)	SIT, 6 wk, 4 d•wk^{-1} on cycle ergometer: 8 × 20-s intervals @ 170% of VO_{2peak} interspersed with 10 s of rest for total time of 4 min	NC in SC/type I fiber NC in SC/type II fiber ↑ Pax7+/MyoD+ cells/ fiber (active SC) ↑ Pax7-/MyoD+ cells/fiber (differentiating SC)
	29 ± 9	Overweight men (n = 7) & women (n = 7)	SIT, 6 wk, 3 d•wk^{-1} on cycle ergometer: 3 × 20-s sprint against 0.05 kg•kg^{-1} body mass interspersed by 2 min low-intensity cycling with 3-min cooldown for total time of 10 min	NC in SC/type I fiber NC in SC/type II fiber ↑ Pax7+/MyoD+ cells/ fiber (active SC) ↑ Pax7-/MyoD+ cells/fiber (differentiating SC)
Murach et al. 2016 **(87)**	56 ± 5	Inactive females (n = 7)	END, 12 wk, 3 d•wk^{-1} on cycle ergometer: 45 min @ 65% VO_{2max}	NC in SC/type II fiber ↑ SC/type I fiber ↑ SC/total fiber

END = endurance, HR_{max} = heart rate maximum, VO_{2peak} = peak oxygen consumption, NC = no change, ↑ = significant increase

5. The Effect of Endurance Exercise on Skeletal Muscle Capillarization

5.1 Effect of exercise intensity on capillary growth

Angiogenesis refers to the formation of new capillaries from pre-existing vessels (31). It is stimulated by mechanical factors, such as shear stress and passive stretch of the muscle fiber (44, 54, 102), as well as by signals originating from changes in metabolic demand and hypoxia (55). To provide the exercising muscle with oxygen, nutrients, growth factors and cytokines, while carrying away carbon dioxide and metabolic by-products, there must be adequate muscle fiber perfusion (118). A higher capillary density can increase the muscle-to-blood exchange surface, decrease oxygen diffusion distance and increase red blood cell mean transit time – all of which contribute to optimal diffusion conditions for oxygen from the blood to the muscle (8, 31). It should be noted that capillary growth is not only induced by aerobic exercise training, but can also be induced by supramaximal exercise (59) and resistance training (47). Nevertheless, endurance training is a potent stimulus of angiogenesis (31, 43, 50), although it takes weeks to months to realize endurance training-induced capillarization in skeletal muscles in humans (20, 26, 121). For instance, after 6-8 weeks of exercise training, 10-30% increase in capillarization was observed in untrained subjects whereas elite aerobic athletes with years of aerobic exercise training expressed a capillary-to-muscle fiber ratio of more than 200 % of that of untrained individuals (57).

The effect on skeletal muscle capillarization appears to be dependent on the type, duration and intensity of the aerobic exercise training protocol (59). HIIT is associated with increasing work loads, metabolic demand, blood deoxygenation and blood flow increases; hence, it would be reasonable to assume that high-intensity training would be a strong stimuli for

angiogenesis (59). However, it appears that higher exercise intensities induce less of an increase in capillarization compared to lower exercise intensities (39). For instance, no changes in capillarization was observed following 4 weeks of SIT training whereas the 4-week preconditioning period of MICT induced an increase in capillarization (52). When comparing work-matched HIIT versus MICT, increases in skeletal muscle capillary density were greatest following MICT (26). However, two separate studies has shown that capillary density increases were similar between low-volume SIT and MICT after 6 weeks of aerobic cycling (20, 108). A recent study comparing 4 weeks of MICT versus low-volume SIT also found similar increases in capillarization (19).

It has been proposed that oxidative remodeling is more likely to occur in type II fibres following interval exercise since there is greater activation of type II fibres with increasing exercise intensity (28). However, similar increases in indices of capillary growth among fibre types have been reported following 4 weeks of SIT (19), 4 weeks of HIIT (59), 6 weeks of SIT (108) and 6 weeks of HIIT (114). It is hypothesized that the duration of shear stress exposure outweighs the magnitude of shear stress for inducing capillarization (31); therefore, low-volume HIIT may not be a sufficiently potent stimulus to induce substantial vascular remodelling in type II fibres (114). On the other hand, a longer HIIT intervention may be required to observe robust vascular remodelling in type II fibres, which appears to require longer periods of training (50). Nonetheless, similar vascular remodelling among type I and type II fibres have also been reported following short-term MICT (19, 108), suggesting that short-term (4-6 weeks) endurance training does not result in preferential vascular remodelling in terms of fibre type. No known study has examined fibre-specific capillarization following a long-term (≥ 12 weeks) endurance training protocol (50, 114). Although Ingjer found that elite cross-country skiers had 7.79, 6.63

and 4.5 capillaries surrounding Type I, Type IIA and Type IIB fibres, respectively (57). In another study on ultramarathon runners, a similar number of capillaries around Type I versus type II fibres was observed (24). It is unknown, however, if these elite athletes incorporated both forms of endurance exercise (interval and continuous) in their training program. Given the limited data, it is unclear whether interval or continuous exercise training results in preferential vascular remodelling with respect to fibre type.

The limited data available suggest that MICT was more or equally as effective for increasing capillary density compared to HIIT/SIT (75). Human data is lacking that address how exercise duration or training frequency impacts skeletal muscle capillarization, and considering the important relationship between capillary density and exercise performance (56), this area of research needs to be examined further (75).

5.2 The response of angio-regulatory factors to endurance exercise

Mechanical signals can originate from shear stress forces induced by the blood flowing through the vessel or from mechanical stretch and compression of the vascular structures as the muscle contracts (43). The mechanical signals induce angiogenic processes by up-regulating angio-regulatory proteins that either promote, modulate or inhibit angiogenesis (31, 43). The most well-studied, important pro-angiogenic factors in skeletal muscle are vascular endothelial growth factor (VEGF), endothelial nitric oxide synthase (eNOS), and angiopoietin 2 (ANG-2), whereas the angiostatic (anti-angiogenic) factors include thrombospondin-1 (TSP-1) and tissue inhibitor of matrix metalloproteinase (TIMP-1) (31, 41, 43, 50).

Among the angio-regulatory factors, VEGF, a 35- to 45-kDa peptide growth factor, is recognized as the most important for basal and exercise-induced skeletal muscle capillary growth

(115). Unlike some of the angio-regulatory factors that are involved in one or the other, VEGF is reported to play an important role in both passive stretch- and shear-stress induced angiogenesis (31, 43, 115). There are several isoforms of VEGF (A-D), although the most important for skeletal muscle angiogenesis is VEGF-A (43). An essential step in the angiogenic process is the release of VEGF from the skeletal muscle cells to the extracellular fluid where it can activate the high-affinity tyrosine kinase VEGFR-2 receptor located on the endothelial cells (the cells comprising the blood vessels) (43). The activation of VEGFR-2 leads to the initiation of multiple signalling pathways related to endothelial cell stimulation, proliferation, migration and gene expression (17, 31, 43). In response to muscle contraction, VEGF stored in vesicles in skeletal muscles fibers translocate to the sarcolemma and these vesicles release their contents to the extracellular fluid (53). Several human studies have shown a 4-7-fold increase in muscle interstitial VEGF levels in response to acute low- and moderate-intensity exercise (35, 51, 53, 54). Nevertheless, the release of VEGF from skeletal muscle cells does not appear to require protein synthesis since secretion of VEGF occurs rapidly following the onset of exercise and continues throughout the activity (34, 49). Instead, the up-regulation of VEGF mRNA occurs initially after termination of exercise (34, 49). Therefore, although VEGF levels in the muscle are sufficiently high that it remains unaltered after a bout of exercise, the up-regulation of VEGF mRNA after exercise allows for a faster replenishment of the VEGF stores in the muscle fibers (43). The lack of training-induced effect on basal muscle VEGF protein levels and VEGF secretion suggests that increased VEGF levels may not be a prerequisite for exercise-induced capillary growth in healthy muscle (51).

Analogous to the response observed with capillary growth, VEGF mRNA expression following an acute bout of endurance exercise seems to be dependent on the exercise intensity.

For instance, increasing the number of all-out efforts while reducing the total training volume resulted in decreased expression of VEGF in skeletal muscle (40). In another study, similar or greater increases in the expression of angio-regulatory factors were found following a SIT session compared to MICT, although the concentration of muscle interstitial VEGF protein and the proliferation of cultured endothelial cells were lower following the SIT session (52).

Whereas VEGF plays an important role in both shear-stress- and stretch-induced capillary growth, NO is only a major player in shear-stress induced angiogenesis (43). In mice, it has been shown that inhibition of NOS abolished shear-stress-induced capillary growth, although stretch-induced angiogenesis still occurred (124). Although a close relationship is shown to exist between VEGF and eNOS in mice – for example, shear stress fails to cause an up-regulation of VEGF mRNA expression in eNOS-knockout mice (3) – VEGF mRNA expression is readily upregulated following acute aerobic exercise whereas eNOS gene expression remains unchanged (51, 52).

Although matrix metalloproteinases (MMPs) are not part of the family of angio-regulatory factors, they are essential in sprouting angiogenesis due to their ability to induce proteolytic degradation of the extracellular matrix which allow the endothelial sprout to protrude from the existing capillary (43). Accordingly, stretch-induced capillary growth is more dependent on MMPs than shear-stress-induced angiogenesis (31, 43). The MMP9 isoform appears to be the most responsive to aerobic exercise as its expression and activity level appear to increase after an acute bout (51, 105). Ang1 and Ang2 are both present in skeletal muscle and they compete for binding for the Tie2 receptor (43). Ang2 functions similar to MMPs in that it is involved in the degradation of the basement membrane whereas Ang1 opposes the effect of Ang2 and thereby promotes vessel stability (43). Following an acute bout of MICT, but not

HIIT, an increase in the ratio of Ang2/Ang1 mRNA expression was found and this finding corresponds with previous reports of a lower muscle interstitial VEGF level with intense endurance exercise (51, 52).

Collectively, only a few angio-regulatory compounds have been extensively examined in physiological angiogenesis in skeletal muscle (31, 43). Considering the importance of vascularization in skeletal muscle health (63), the role of angio-regulatory factors in capillarization as well as its interaction with other physiological processes occurring in the muscle requires further examination. Nevertheless, it appears that the mRNA expression of pro-angiogenic compounds, specifically VEGF, VEGFR2 and Ang2/Ang1 ratio, increase to a greater extent following MICT compared to HIIT or SIT.

6. The Importance of Vasculature in Mediating the SC Response

It is believed that the microvasculature circulation (that is muscle perfusion from the capillary bed) delivering oxygen, nutrients and growth factors to skeletal muscle is critical for the optimal maintenance of muscle mass and health (63). In addition to maintaining muscle health, revascularization is integral to the regenerative process after injury to skeletal muscle (63). Regenerating muscles have a greater metabolic demand and require sufficient vascularization to supply the muscle with nutrients and oxygen during repair (95). The importance of angiogenesis following injury is depicted by the association between delayed angiogenesis and delayed muscle regeneration as early as 14 days following injury in rodent models (5, 65). As previously discussed, SCs are essential in muscle repair and regeneration. Hence, it is not surprising that the vasculature is considered a critical aspect of many stem cell niches as stem cells are often in close proximity to capillaries (100).

Skeletal muscle tissue has been referred to as an 'endocrine organ' since it produces and releases various cytokines that act in a paracrine, autocrine or endocrine fashion (97). In accordance, the systemic environment has been reported to play a critical role in SC function (23). Regulatory signals can originate locally or they can be derived from other organs and the broader circulatory system (119) (**Figure 2)**. Thus, capillaries are suggested to play an important role in delivering these signals, such as key growth factors, to the muscle tissue (16, 63) (**Figure 2**). Various growth factors (e.g. insulin-like growth factor-1 (IGF-1), hepatocyte growth factor (HGF), interleukin 6 (IL-6), myostatin) are suggested to be regulators of SCs as they progress through the myogenic program (82-84, 94). Delivery of these systemic growth factors to the muscle SC would be partially dependent on their proximity to local microvascular flow (89) (**Figure 2**). Indeed, it has been recently established that there is a spatial relation between SCs and capillaries in humans (64) and adequate muscle fiber perfusion may be an essential mediating factor to allow optimal SC activation (63). For instance, it has been reported that there is a greater distance between SCs associated with Type II myofibers and their nearest capillary in older as compared to young men (89). Additionally, numerous studies have shown that activated SCs are found at closer proximity to capillaries than their quiescent counterparts (15, 16, 89) and there is a known "cross-talk" between SCs and endothelial cells (5, 15). Thus, it is suggested that there is a critical relationship between muscle capillarization and the ability of the SC pool to activate and expand following exercise (91). For instance, it was shown that an altered activation of the SC pool in response to an acute bout of resistance exercise was accompanied by increased capillarization following 16 weeks of resistance training (92). More recently, Nederveen *et al*. (2018) demonstrated that an enhanced capacity for muscle perfusion or a reduction in the distance of a SC to its nearest capillary was associated with an enhanced activation and

expansion of the SC pool following eccentric exercise-induced muscle damage in young healthy men (91). It appears that muscle capillarization is positively related to SC pool activation and expansion following muscle damage (89, 91). SCs closer in proximity to capillaries are thought to have enhanced exposure to circulating factors, leading to maximal SC activation (63). However, the critical interaction between SC and capillaries have been only observed following an acute bout of resistance exercise (89, 91, 92, 112). Aerobic exercise training is a potent stimulus for capillary growth, and if used in concert, could maximize the outcome of a resistance training program, especially as it relates to SC function (64). As previously discussed, aerobic exercise training can induce both SC expansion and SC activation although the mechanism in which aerobic exercise training causes these responses is unknown. Nonetheless, it is proposed that the increase in muscle capillarization, and thereby the reduction in the distance between SC and capillaries, is a potential mechanism in which aerobic exercise improves SC function (64).

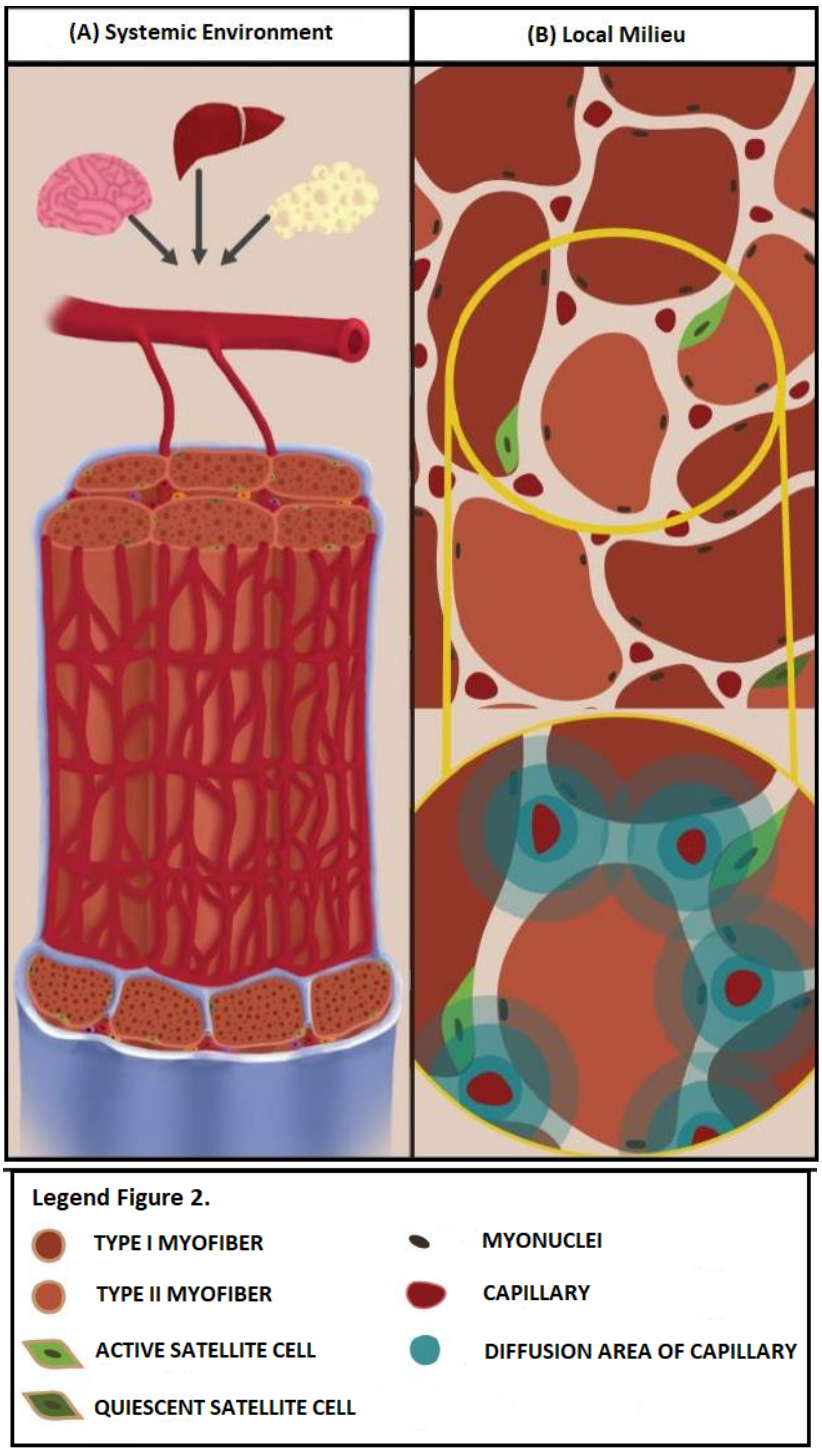

Figure 2. Schematic representation of skeletal muscle microvasculature and muscle fiber cross sections (adapted from (90)). (A) Delivery of systemic signals derived from other organs and the broader circulatory system to the skeletal muscle fiber could be a key event in SC recruitment, thereby supporting muscle repair and/or remodelling. **(B)** The delivery of these systemic signals to the muscle SC would partially depend on their spatial proximity to capillaries. The signaling molecules delivered via capillaries may be crucial for the regulation of satellite cell activation.

7. Rationale and Objectives of the Study

Collectively, studies on endurance training in humans suggest that exercise intensity may play an important role in mediating the SC response as well as capillarization. However, it has yet to be determined whether the on-and-off pattern characteristic of interval training (i.e. rest-work cycles) could partially explain the skeletal muscle responses to this type of exercise. Compared to work-matched continuous exercise, exercise using an interval pattern of contraction has been shown to induce greater activation of skeletal muscle signaling proteins (e.g., AMPK) (22). However, whether these acute differences translate to different skeletal muscle adaptations to work- and intensity-matched interval and continuous training is unknown. Recently, a great amount of attention has been directed at determining the effectiveness of different forms of aerobic exercise, such as HIIT, and how its health benefits compare to more traditional endurance exercise. HIIT is a more time-efficient alternative to traditional endurance exercise. Additionally, it has been reported to induce similar health benefits, have similar adherence during the training intervention, and be well tolerated by a range of health and diseased populations compared to traditional endurance exercise (25, 37, 117). Nevertheless, in cases where the high-intensity associated with HIIT is not tolerable for specific populations, it is worth exploring whether the intermittent nature of interval training (i.e. the interval pattern of contraction) is still worth implementing rather than continuous endurance exercise. Considering the critical role capillarization and SCs play in maintaining overall skeletal muscle health (63), examining whether the interval pattern of contraction mediates these skeletal muscle responses would further our knowledge of developing an exercise prescription for healthy and diseased populations.

Hence, the purpose of this study was to explore the potential of the work-to-rest cycle as a driver of muscle adaptation by comparing the SC response and capillarization to two different work-matched protocols performed at the same intensity but using two distinct exercise patterns (interval vs. continuous). The specific objectives were to:

1. Examine the SC response to work- and intensity-matched interval versus continuous exercise training
2. Assess whether the interval contraction pattern will affect capillarization as well as the interaction between SCs and capillaries (indicated by the SC-distance-to-nearest-capillary as previously reported (89))
3. Determine whether the interval contraction pattern will play an important role in the changes in VEGF and VEGFR2 protein levels in response to the training program

We believe that the rest-to-work cycles associated with interval exercise will be an overall greater stressor of the muscle. We hypothesized that interval exercise will elicit a greater SC response and induce greater capillary growth as well as greater increases in pro-angiogenic protein levels than continuous exercise.

METHODS

Note: This project was part of a collaborative research project that investigated skeletal muscle response to training, with performance and other measures reported elsewhere. Additionally, the number of participants recruited in this study was based on the power needed for detecting potential differences in citrate synthase between the two exercise training interventions.

Participants. Ten healthy recreationally-active individuals (5 men and 5 women; aged 21 ± 1 yrs; body mass index 22.8 ± 0.8 kg/m^2; mean ± SEM) were recruited to participate in this study. All subjects were habitually active but not specifically endurance trained. The subjects completed a Physical Activity Readiness Questionnaire and provided their written informed consent prior to their participation. The Hamilton Integrated Research Ethics Board approved the protocol, and the study conformed with the *Declaration of Helsinki* (2013).

Pre-training procedures. To determine whole-body peak oxygen uptake ($VO_{2,\ peak}$), subjects initially performed a standard (double-legged) ramp test to exhaustion on an electronically-braked cycle ergometer (Excalibur Sport, version 2.0; Lode, Groningen, The Netherlands). The subjects began with a 2-minute warm-up at 50 W, and then workload was increased 1 W every 2 s until the subject reached volitional exhaustion or cadence decreased below 60 revolutions per minute (rpm). Expired gases were analysed using an online gas collection system (Moxus modular oxygen uptake system; AEI Technologies, Pittsburgh, PA, USA). The VO_{2peak} was determined from the highest 30 s average of $VO_{2,\ peak}$. The average $VO_{2\ peak}$ of the subjects was 43.2 ± 1.6 mL kg^{-1}min^{-1}.

At least 48 hours following the double-legged ramp test, subjects underwent familiarization with the single-leg cycling technique, which is based on previous work (1, 11,

77). A custom-machined pedal that held an 11.4 kg counterweight was fitted on one crank on an electronically-braked cycle ergometer (Velotron; RacerMate, Seattle,WA, USA). This counterweight helped with the upstroke phase of the revolution, thus eliminating the need to pull up on the pedal. Subjects pedalled using one leg, with the non-exercising leg resting on a stationary platform, to volitional exhaustion. The single-leg (SL) tests were similar to the double-legged (DL) tests with incremental increases in workload, except the rate at which the workload increased was reduced by half (i.e. 1 W every 4 s in SL instead of 1 W every 2 s in DL) (modelled after previous work (77)). The contralateral leg was tested 10 min after the current leg completed the exercise. Previous data have shown that fatigue does not transfer to the non-exercising leg (32). No significant differences were found for $VO_{2\ peak}$ (2.2 ± 0.1 vs. 2.2 ± 0.1 mL/kg/min, $P = 0.53$) and power output (144 ± 3 W vs. 146 ± 4 W, $P = 0.62$) for the left and right legs. Additionally, < 10 W difference in peak power output between legs was a criterion for each of the subject. Pre-training, single-leg $V_{O2\ peak}$ was 78.2 ± 3.4 % of the double-legged value.

Within 3 days after the final baseline testing session, resting muscle needle biopsies were taken from the vastus lateralis from a randomly chosen leg of each subject **(Figure 3**). Only one leg was chosen for the pre-training muscle biopsy due to ethical concerns and previous research have shown that capillarization is similar between legs of an individual at baseline (59). Subjects recorded a 24 h diet log before the first muscle biopsy to repeat this same diet for the post-training biopsy. Additionally, subjects refrained from exercise, alcohol and food for a minimum of 48, 24 and 10 hours, respectively. Samples were collected under local anaesthesia (1% xylocaine) using a Bergström needle modified for suction, as has been described (116). Upon excision, muscle samples were immediately mounted in optimal cutting temperature (OCT) compound, frozen in liquid nitrogen–cooled isopentane and stored at -80° C until further

analyses. Due to poor image quality or frozen artifact damage, we report *n* = 9 subjects for immunohistochemical analyses.

Training intervention. Exercise training was performed on the same cycle ergometer adapted for single-leg cycling as that used for baseline testing. Each leg was randomly assigned to different work-matched protocol that are performed at the same peak intensity and lasting the same duration, but at two different exercise patterns (interval vs. continuous). The training program for each protocol comprised of 12 sessions over four weeks (3 sessions per week). Training began 3–4 days after the muscle biopsy procedures **(Figure 4).**

Exercise prescriptions was based on the average W_{peak} obtained during the two single-leg tests. Legs in the *Interval* (INT) group performed ten 3-min bouts of cycling at 50% W_{peak}, each followed by a 1 min recovery period at 10% of average W_{peak}. Legs in the *Continuous* (CONTIN) group performed 30 min of cycling at 50% W_{peak}, followed by 10 min recovery at 10% W_{peak}, to match the total work [total work (kJ) = average power (W) × time (s)/1000] and intensity (W_{peak}) of the INT group (**Figure 4**). All training sessions were preceded by a 2 min warm-up at 25 W. Subjects were instructed to cycle at the same cadence (~80 rpm) throughout each session. The legs of each subject were trained consecutively on the same day, following a 5 min rest period, with the order alternating each day. If participants were able to tolerate the progression in workload, then training loads were increased by 2.5% W_{peak} every 4^{th} session. Otherwise, the intensities were held constant for the entire study.

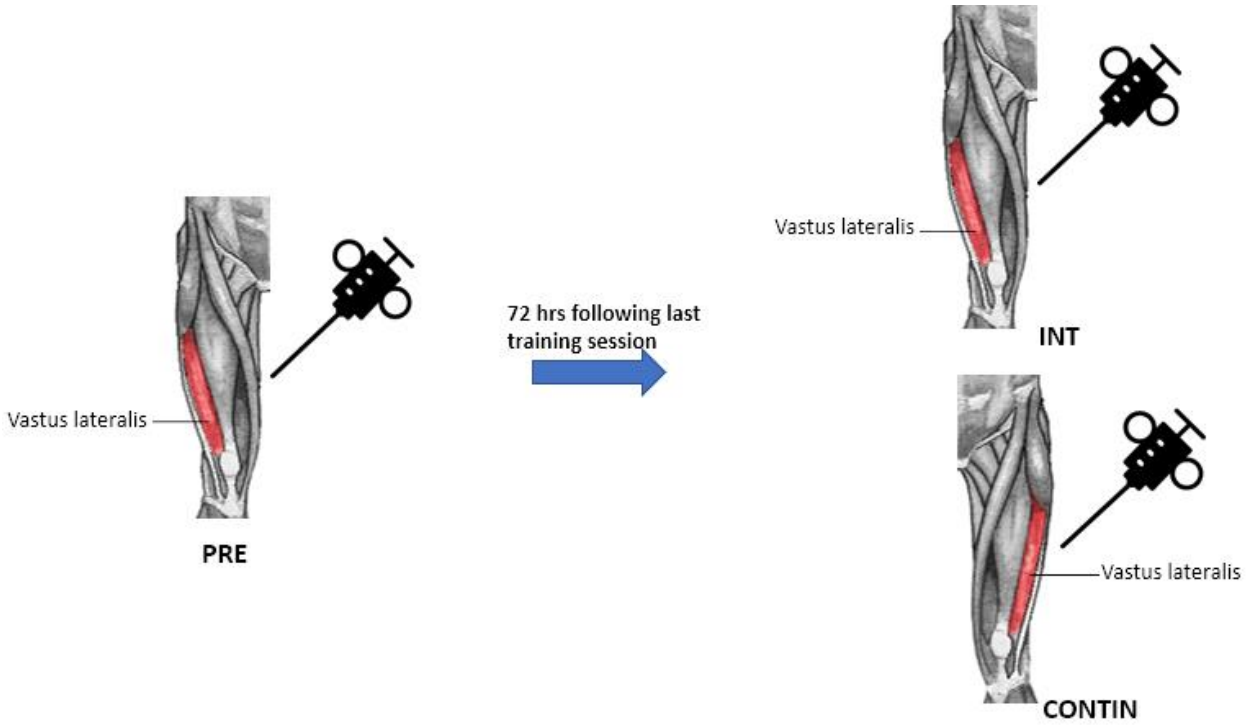

Figure 3. A schematic representation of the protocol used in taking muscle biopsies. Due to ethical concerns and after ensuring the two legs were not different with regards to peak power output, a biopsy was taken from a randomly chosen leg pre-training. Post-training, muscle biopsies were taken from both legs. PRE, pre-training leg; INT, leg that performed interval training; CONTIN, leg that performed continuous training.

Post-training procedures. A resting muscle needle biopsy was obtained from each leg 72 h after the final training session to avoid potential acute effects of exercise on mitochondrial content (72) (**Figure 3**). Additionally, previous research that examined the satellite cell response to aerobic exercise training have taken muscle biopsies 72 h following the last training session (61). The single-leg incremental tests to exhaustion were repeated 72 h later, as described above.

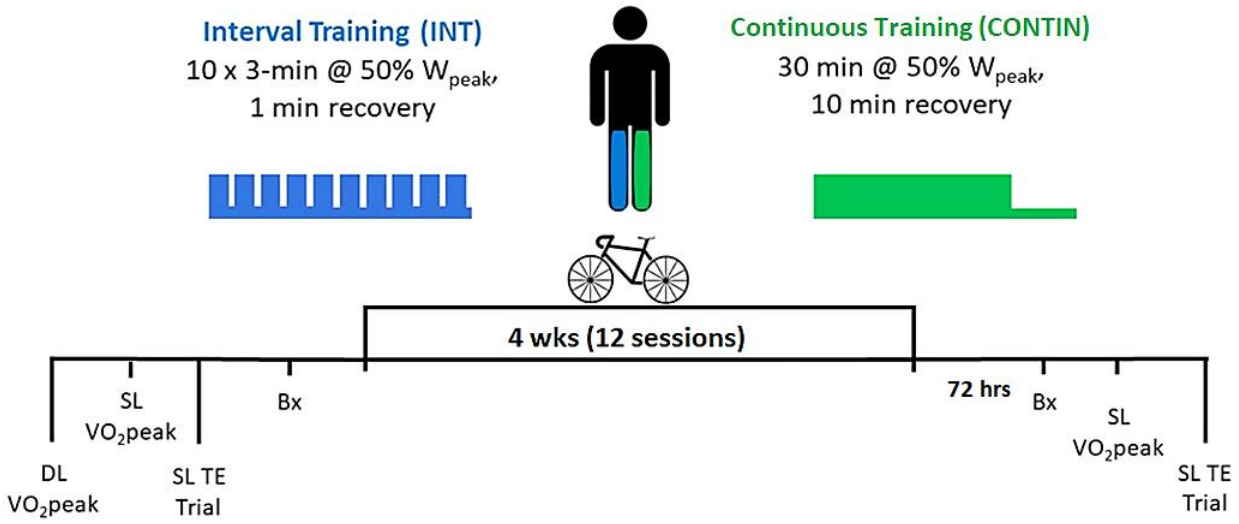

Figure 4. A schematic representation of the training program implemented in this study. DL, double-leg; SL, single-leg; TE, time to exhaustion; Bx, biopsy**.** *n* = 10 subjects.

Immunofluorescence. Muscle cross sections (5 μm) were prepared from unfixed OCT embedded samples, allowed to air dry for 30 minutes and stored at -80˚C. Muscle cross sections were fixed in 2% paraformaldehyde (PFA) for 10 mins, washed 3 × 5 min with PBST, blocked for 90 min in PBS containing 2% bovine serum albumin (BSA), 5% fetal bovine serum, 0.02% Triton X-100, 0.1% sodium azide, and 10% goat serum (GS). Afterwards, samples were stained with appropriate primary and secondary antibodies against specific antigens, found in **Table 2**. Immunofluorescence staining methods were adapted from previously published methods (89). Briefly, before cover slipping with fluorescent mounting media (DAKO, Burlington, ON, Canada), nuclei were labelled with DAPI (4',6-diamidino 2-phenylindole) (1:0000, Sigma-Aldrich, Oakville, ON, Canada). Negative and positive controls were used to verify the staining procedures and ensure appropriate specificity of staining. The Nikon Eclipse *Ti* Microscope (Nikon Instruments, Inc. USA), equipped with a high-resolution Photometrics CoolSNAP HQ2 fluorescent camera (Nikon Instruments, Melville, NY, USA) were used to view the slides. Then,

images were captured and analyzed using the Nikon NIS Elements AR 3.2 software (Nikon Instruments, Inc., USA). All images were obtained with the 20x objective. Modelled from previous data (89, 91), ≥ 200 muscle fibres/subject/time point were included in the analyses for SC content/activation status (i.e., total $Pax7^+$ cells or strictly $Pax7^+/MyoD^+$ cells), fibre cross sectional area (CSA), perimeter and fiber typing. Satellite cell analyses were divided into two parts: total $Pax7^+$ cells per 100 myofibers (indicated by the colocalization of $Pax7^+$ and DAPI) and activated satellite cells determined by the co-localization of Pax7, MyoD and DAPI (i.e., $Pax7^+/MyoD^+$). As in previous studies (60, 61, 89, 91), the anatomic location of the satellite cells within the SC niche (i.e. between the basal lamina and sarcolemma) was also another criteria used to ensure these cells were in fact satellite cells. Cell membranes were labelled with either laminin (**Table 2**) or peroxidase conjugated Wheat Germ Agglutinin (WGA) (1 ug/mL, Vector PL-1026, Burlington, ON, Canada) and realized with a substrate kit (Vector, SK-4700, Burlington, ON, Canada) as per manufacturer's instructions.

The quantification of muscle fibre capillaries was performed on 50 muscle fibres/subject/time point, as previously described (89). Based on the model by Hepple and colleagues (46, 48), quantification was performed for capillary contacts (CC; the number of capillaries around a fibre) and the capillary-to-fibre ratio on an individual fibre basis (C/Fi). The capillary-to-fibre perimeter exchange index (CFPE) was calculated as an estimate of the capillary-to-fibre surface area (46). The SC-to-capillary distance measurements were performed on all SC that were enclosed by other muscle fibres, and has been described previously (89). All immunofluorescent analyses were completed in a blinded fashion.

Table 2. Antibody information. Detailed information on primary and secondary antibodies and dilutions used for immunofluorescent staining of the frozen muscle cross sections.

Antibody	Species	Source	Details	Primary	Secondary
Anti-Pax7	Mouse	DSHB	Pax7	1:1	Alexa 594, 488 goat-anti mouse 1:500
Anti- laminin	Rabbit	Abcam	ab11575	1:500	Alexa Fluor 488 anti-rabbit, 1:500
Anti-MHCI	Mouse	DSHB	A4.951 Slow isoform	1:1	Alexa Fluor 488, 647 goat anti-mouse, 1:500
Anti-MHII	Rabbit	Abcam	Ab91506	1:1000	Alexa 647 goat anti-rabbit, 1:500
Anti-CD31	Rabbit	Abcam	ab28364	1:30	Alexa Fluor 647 goat anti-rabbit, 1:500
Anti-MyoD	Rabbit	Abcam	ab133627	1:25	Alexa 594 goat anti-rabbit, 1:500

Whole muscle western blotting. Muscle was homogenized, protein concentration was detected using the Bradford assay (9), and samples were prepared at 2.5 $\mu g \cdot \mu L^{-1}$. All samples were run on 4–15% Criterion TGX Stain-Free protein gels (BioRad, Hercules, CA, USA) at 100 V for 2 hours. A protein ladder (Fermentas PageRuler Prestained Ladder, ThermoFisher Scientific, Waltham, MA, USA) and an internal control of pooled whole muscle homogenates were run on every gel. Proteins were then wet-transferred to nitrocellulose at 100 V for 1 hour and subsequently stained with Ponceau S to verify equal loading across samples. Membranes were incubated with 5% bovine serum albumin (BSA) in Tris-buffered saline with 1% Tween-20 (TBST) for 1 hr at room temperature. Proteins were probed at 4 °C overnight with the following antibodies: VEGF (Abcam 1/1000, ab46154) and VEGF receptor 2 (VEGFR2, 1/1000, ab39256; Abcam). Membranes were washed in TBST 3 × 5 min and then incubated with the appropriate horseradish peroxidase-linked secondary antibodies for 1 hr at room temperature. The membranes were visualized through enhanced chemiluminescence (Biorad) using a FluorChem SP Imaging System (Alpha Innotech Corporation, San Leandro, USA). Densitometry was performed using Image Lab analysis (Biorad, Mississauga, Canada). α-Tubulin was also

detected on each membrane to verify consistent/equal loading. Detection of all membranes for a given target was done at the same time.

Statistical analysis. Comparisons of fibre type-specific measurements were performed using a two-way repeated-measures ANOVA with leg (*PRE, INT, CONTIN*) and fibre type (Type I and Type II) as the two factors. For the mixed muscle satellite cell response (i.e. quantifying the $Pax7^+$ cell populations for the total muscle fibers), a one-way repeated-measures ANOVA was conducted for comparisons between the legs in the *PRE, INT* and *CONTIN* groups. Mauchly's sphericity test was used to validate data sets before interpreting ANOVA results, and the Greenhouse–Geisser correction was applied to data-sets that violated the assumption of sphericity. *Post hoc* testing (Bonferroni's multiple comparisons test) was performed when statistically significant interactions were detected with the two-way ANOVA or when the one-way repeated ANOVA was statistically significant. $P < 0.05$ was considered statistically significant. Data are presented as the mean ± SEM. Statistical analysis was performed using Prism, version 6.0 (GraphPad Software, La Jolla, CA, USA).

RESULTS

Subject characteristics

Complete subject characteristics are reported in **Table 3**.

Table 3. Subject characteristics

Variable	*n = 10*
Age (yrs)	21 ± 1
Height (m)	1.7 ± 0.02
Weight (kg)	67.3 ± 2.9
BMI (kg/m^2)	22.8 ± 0.8
DL VO_2 Peak (mL/kg/min)	43.2 ± 1.6

Data are means ± SEM. DL, double-leg.

Muscle fibre CSA and fibre-type distribution

Four weeks of training did not lead to a significant increase of muscle fiber CSA or perimeter and no differences were observed between the pre-training (PRE) leg, the *INT* leg and the *CONTIN* leg for either measure (**Table 4)**. Muscle fibre-type distribution did not change as a result of training and was not significantly different between the legs. There were significant main effects of fibre type for CSA and fibre type proportion ($P < 0.01$ and $P < 0.0001$, respectively*)* (**Table 4**).

Muscle fiber capillarization & fibre-specific capillary remodelling

The CFPE index (derived as the quotient of the capillary-to-fibre ratio (C/Fi) and the fibre perimeter) provides an overall indicator for muscle fibre perfusion capacity (46). No differences in type I or type II muscle fibre CC (capillary contacts), C/Fi (capillary-to-fibre ratio for each individual fibre) or CFPE (capillary-to-fibre perimeter exchange index) were observed post-training or between exercised legs (**Figure 5** and **Table 4)**. There were main effects of fibre type for CC, C/Fi and CFPE ($P < 0.02$, $P < 0.01$ and $P < 0.001$, respectively).

Whole muscle protein content of angio-regulatory factors

VEGF protein content was not significantly altered as a result of training ($P = 0.15$) (**Figure 6C).** To further elucidate the training-induced effect on VEGF and its signaling cascade, we next examined the protein content of its main receptor, VEGFR2. No changes were observed in the receptor content post-training ($P = 0.81$) (**Figure 6D).**

Table 4. Summary of muscle characteristics & muscle fibre capillarization

	Fibre type	PRE (n = 9)	INT (n = 9)	CONTIN (n = 9)
Fibre CSA (μm^2)				
	I	6356 ± 596	7153 ± 395	6690 ± 455
	II*	7248 ± 502	8381 ± 545	8093 ± 582
Fibre perimeter (μm)				
	I	305 ± 18	306 ± 8	301 ± 12
	II	320 ± 12	319 ± 12	320 ± 16
Fibre type proportion (%)				
	I	34.0 ± 3.1	32.3 ± 3.5	35.4 ± 3.2
	II*	65.4 ± 3.1	67.3 ± 3.6	63.8 ± 3.3
	Hybrid	0.3 ± 0.2	0.3 ± 0.1	0.8 ± 0.3
Capillary contacts (CC)				
	I*	5.25 ± 0.32	5.74 ± 0.11	5.70 ± 0.29
	II	5.01 ± 0.24	5.35 ± 0.13	5.34 ± 0.32
Individual capillary-to-fibre ratio (C/Fi)				
	I*	1.87 ± 0.14	2.08 ± 0.05	2.03 ± 0.14
	II	1.75 ± 0.10	1.89 ± 0.06	1.86 ± 0.15
Capillary-to-fibre perimeter exchange index (CFPE)				
	I*	6.14 ± 0.25	6.84 ± 0.17	6.73 ± 0.38
	II	5.41 ± 0.20	5.95 ± 0.23	5.76 ± 0.34
Satellite cell distance to nearest capillary (μm)				
	I	21.4 ± 1.4	22.0 ± 2.4	20.4 ± 1.5
	II	23.8 ± 2.1	22.3 ± 2.6	24.0 ± 1.9
Satellite cell (total Pax7$^+$) per 100 myofibers				
	I	3.2 ± 0.5	3.6 ± 0.5	4.1 ± 0.8
	II*	5.6 ± 0.6	7.7 ± 1.2	7.4 ± 0.7
Activated satellite cell (Pax7$^+$/MyoD$^+$) per 100 myofibers				
	I	0.5 ± 0.1	1.2 ± 0.2	0.93 ± 0.1
	II	0.9 ± 0.2	2.0 ± 0.5	1.8 ± 0.4

Data are means ± SEM.
CSA, cross sectional area.
*P < 0.05, main effect of fibre type.

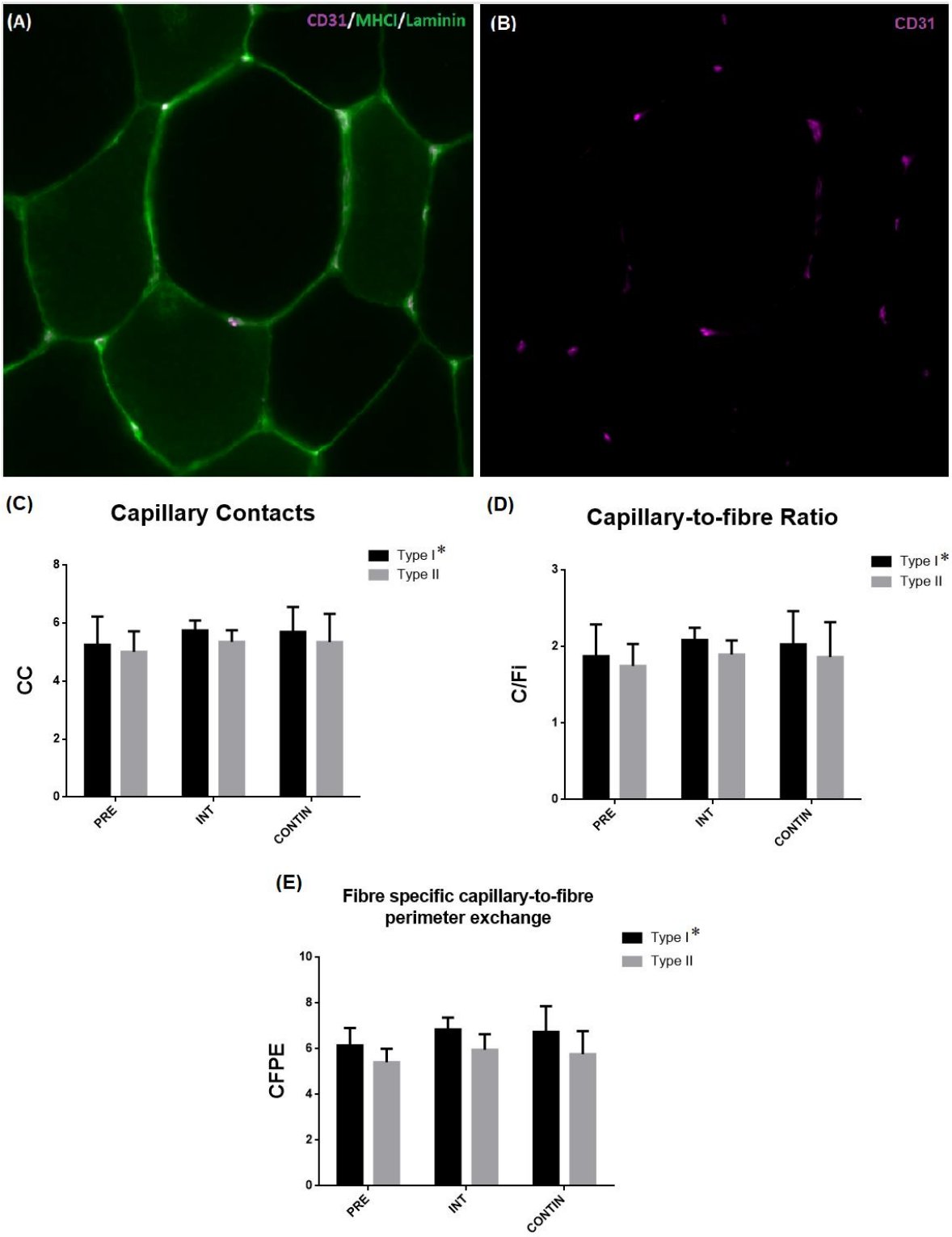

Figure 5. Fibre type-specific staining with muscle capillaries. (A) Representative image of a MHCI/laminin/CD31 stain of a muscle cross section. Single channel views of (B) CD31. (C) capillary contacts (CC). (D) Fibre-specific capillary-to-fibre ratio (C/F_i). (E) Fibre-specific capillary-to-fibre perimeter exchange index (CFPE). Values represent means ± SEM; *$P < 0.05$, main effect of fiber type.

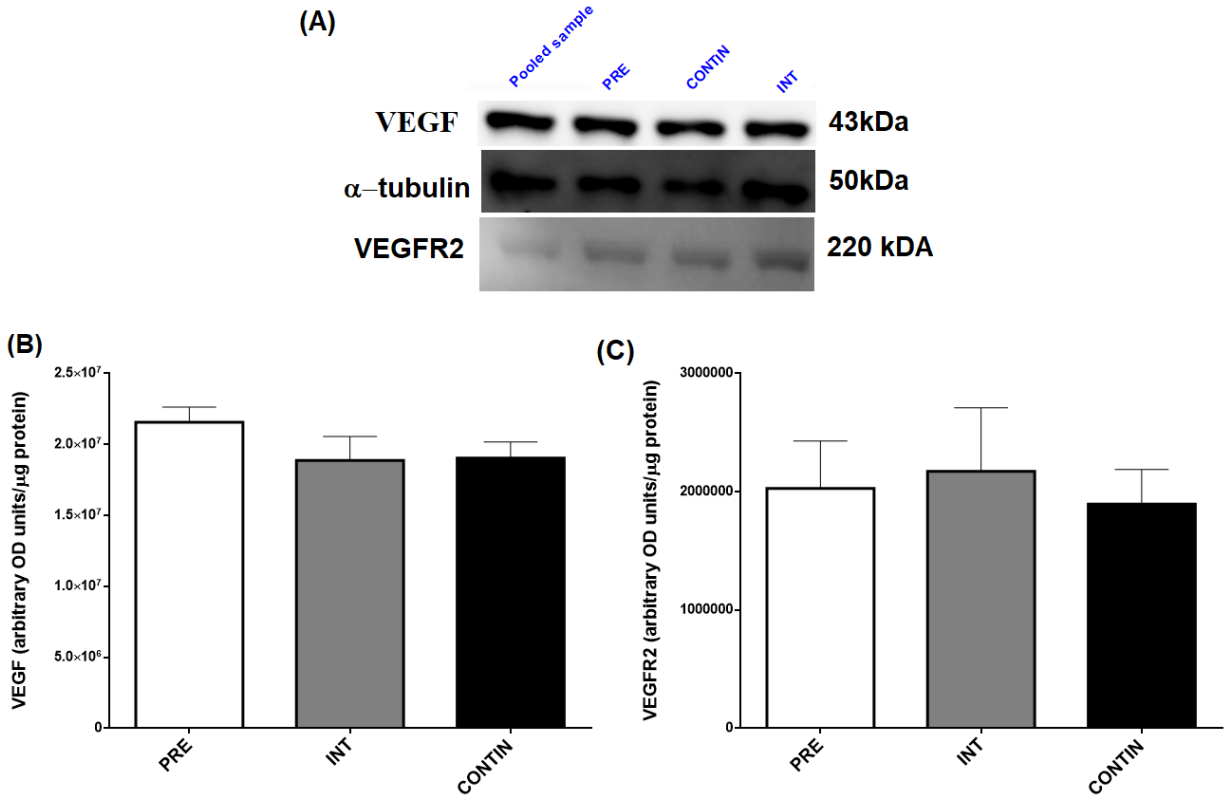

Figure 6. Western blot analysis of whole muscle. α-Tubulin is presented as loading control. (A), representative blots of VEGF and VEGFR2. B-C, density quantifications of VEGF (B) and VEGFR2 (C). Values represent means ± SEM.

Fibre-specific SC response and distance to nearest capillary

Training did not result in significant changes and no difference were observed between the two legs that underwent interval and continuous exercise training with regards to the number of $Pax7^+$ cells per 100 myofibers for either Type I or Type II fibers (**Figure 7A** and **Table 4**). There was a main effect of fibre type for the number of Pax7+ cells per 100 myofibers ($P < 0.01$, **Figure 7A, Table 4**). Following training, no significant changes in the SC distance to nearest capillary was observed (**Figure 7B** and **Table 4).** With regards to fibre type-specific SC activation (i.e. the number of $Pax7^+/MyoD^+$ cells per 100 myofibers), no significant changes were observed post-training (leg × fibre type interaction $P > 0.05$). There were significant main effects of fibre type ($P < 0.03$) and leg ($P < 0.03$) (**Figure 8B and Table 4).**

Mixed muscle SC response

No differences were observed post-training or between the two legs in terms of the number of $Pax7^+$ cells per 100 myofibers in mixed muscle (PRE: 8.8 ± 0.9, INT: 11.4 ± 1.5, CONTIN: 11.5 ± 1.1 $Pax7^+$ cells per 100 myofibers, $P = 0.2$) **(Figure 7C).** There was a small effect size for the number of Pax7+ cells per 100 myofibers in mixed muscle, indicating a weak trend for an increase (R square = 0.l9). A significant increase in activated SCs ($Pax7^+/MyoD^+$) per 100 myofibers in mixed muscle was observed in the *INT* leg compared to the pre-training time-point (before training (PRE): 1.5 ± 0.2 Pax7+/MyoD+ cells/ 100 fibers and after training (INT): 3.2 ± 0.5 Pax7+/MyoD+ cells/ 100 myofibers, $P < 0.05$; **Figure 8C**). No significant increase in the number of activated SCs in mixed muscle was observed in the *CONTIN* leg compared to the pre-training time-point (before training (PRE): 1.5 ± 0.2 Pax7+/MyoD+ cells/ 100 fibers and after training (CONTIN): 2.4 ± 0.3 Pax7+/MyoD+ cells/ 100 myofibers, $P = 0.0985$) (**Figure 8C)**. With regards to SC activation for

mixed muscle, a moderate effect size was found (R square = 0.41). No significant difference was found between the *INT* leg and *CONTIN* leg with regards to SC activation in mixed muscle (P = 0.9) (**Figure 8C**).

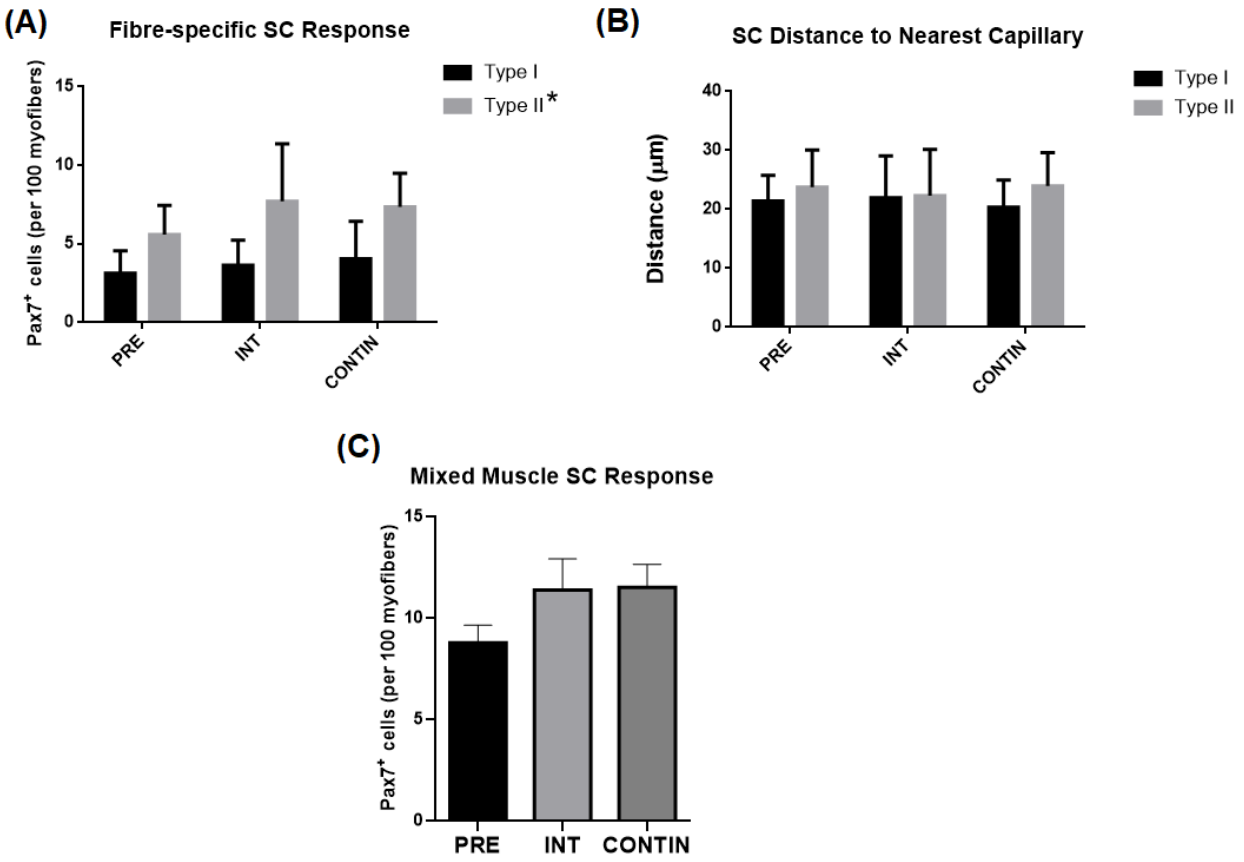

Figure 7. Satellite cell response and distance to nearest capillary. (A) The number of type I and type II satellite cells per muscle fibre. (B) The number of satellite cells per mixed muscle fibre. (C) The SC distance to the nearest capillary. Values represent means ± SEM. *P < 0.05, main effect of fibre type.

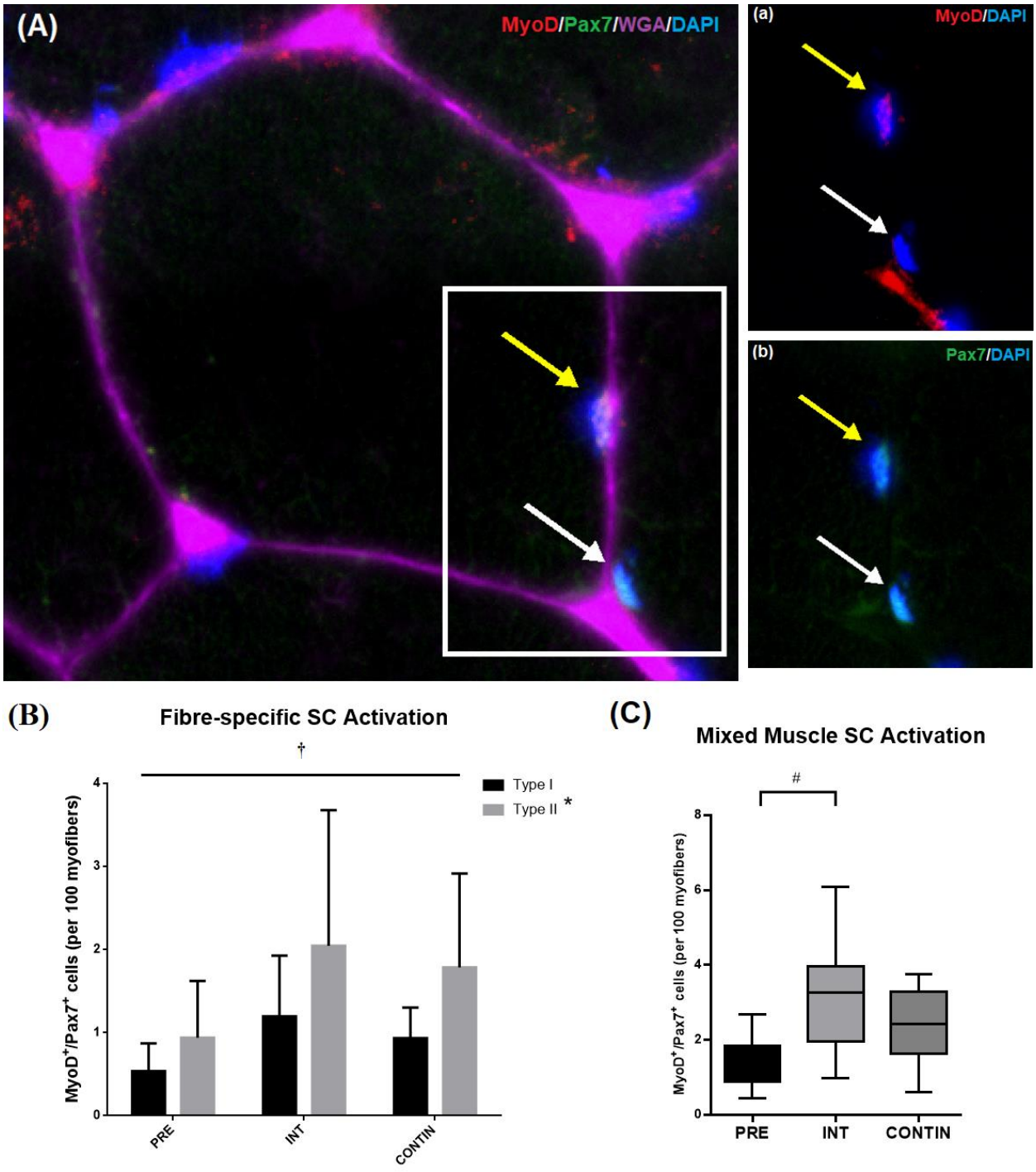

Figure 8. Muscle cross section staining of satellite cell (SC) activation. (A), representative image of a MyoD/Pax7/WGA/DAPI stain of a muscle cross section. (a)-(b), channel view of MyoD/Pax7 (a), and Pax7/DAPI (b). Characterization of the activation status of the SC pool per the two fiber types (B) and in total muscle (C). † $P < 0.05$, main effect of leg; *$P < 0.05$, main effect of fibre type; $^{\#}P < 0.05$, significantly different compared with PRE. Values represent means ± SEM. Yellow arrow indicates activated SC whereas white arrow indicates quiescent SC.

DISCUSSION

Both interval (HIIT & SIT) and continuous (MICT) exercise training have been shown to induce a SC response (33, 61, 87) and an increase in muscle fibre capillarization (20, 59, 121). The magnitude of these skeletal muscle responses is suggested to be mediated by exercise intensity. It is unknown, however, whether the on-and-off pattern characteristic of interval training (i.e. rest-work cycles) could partially explain the skeletal muscle responses to this exercise type. Following four weeks of work- and intensity-matched interval and continuous endurance training, we observed no significant changes in SC pool expansion or capillarization. However, for fibre-specific SC activation, we report a main effect of leg (i.e. the legs in the *PRE*, *INT* and *CONTIN* group) (**Figure 8B)**. We believe this main effect is driven by the difference in SC activation between the pre-training time-point (*PRE*) and the *INT* leg. When we pooled type I and Type II muscle fibres together (referred to as mixed muscle), we found an enhanced SC activation for the *INT* leg relative to the pre-training time-point whereas we observed no significant increase for the *CONTIN* leg post-training (**Figure 8C**). The rest-to-work cycles associated with an acute bout of interval exercise has been shown to induce greater metabolic fluctuations and a greater metabolic signal compared to work- and intensity-matched continuous exercise (22). We believe the exercise-induced repeated metabolic fluctuations associated with interval exercise was a more robust stimuli that cumulatively led to an enhanced SC activation following training compared to continuous exercise. However, we did not observe significant differences in mixed muscle SC activation between the two endurance-trained legs (**Figure 8C)**. A recent study by Joanisse and colleagues have shown similar increases in SC activation following 6 weeks of low-volume SIT and MICT (61). In this present study, we only observed an enhanced SC activation in the leg that underwent interval training and not in the leg that

underwent continuous training. The incongruent findings between the studies may relate to disparities in the endurance training protocols implemented. Compared to the training protocol in this present study, the training protocol implemented by Joanisse and colleagues (61) was longer by two weeks, employed double-leg cycling as opposed to single-leg cycling and was not matched for work or intensity. Hence, it is possible that we may observe small differences in SC activation between work- and intensity-matched interval versus continuous endurance training if we increase the magnitude or duration of the stimuli (and thereby increase the magnitude and/or duration of the corresponding metabolic fluctuations) by increasing the exercise intensity or the length of the training protocol.

Despite implementing a different endurance training protocol to that of Joanisse and colleagues (61), we show that endurance training is capable of inducing SC activation despite no changes in the SC pool and in the absence of an increase in fibre CSA. It has been proposed that this discernible lack of SC pool expansion can be attributed to a gradual and consistent contribution of SC-derived nuclei to muscle fibers where proliferation and differentiation are virtually matched (61). It is speculated that activated SCs may play a role in the transition in myosin heavy chain protein expression following non-hypertrophic endurance training (61, 64). However, here we report no changes in fibre type proportions following training (**Table 4**).

We did not observe changes in the SC pool post-training (**Figure 7A and 7C)** nor did we observe an increase in CSA of either type I or II fibres following training (**Table 4**). Previous research reporting a concurrent expansion of the SC pool and increases in fibre size following endurance training have recruited participants that were either overweight (33) or sedentary (87), whereas our study recruited recreationally-active, young individuals. Similar to previous research (61), our subject pool may not have been sufficiently sedentary or the exercise intensity in our

training protocol may not have been sufficiently hypertrophic in nature to induce a hypertrophic adaptation. Nonetheless, this study supports the point made by Joanisse and colleagues (61), that enumeration of the SC pool alone, as previously done in many endurance training studies (64), is insufficient to fully appreciate the role of SCs in mediating skeletal muscle adaptations to exercise training.

Exercise intensity is suggested to mediate increases in capillarization following training (39, 75). Higher exercise intensities, which are associated with HIIT and SIT, have been shown to induce less of an increase in capillarization compared to lower exercise intensities, which is associated with MICT (26, 39). Additionally, various markers of angiogenesis increase to a greater extent following lower-intensity continuous exercise compared to higher-intensity interval exercise (40, 52). However, it is unknown whether the rest-to-work cycles associated with interval exercise could partially explain this trend in capillarization following endurance training. Here, we employed a work- and intensity-matched endurance training protocol to examine the role of the rest-to-work cycles associated with interval exercise on capillarization. Post-training, we did not observe significant changes in CFPE, C/Fi or CC (**Figure 5C, 5D, 5E & Table 4**). Furthermore, in line with this observation and in accordance with the previously demonstrated temporal nature of these proteins in which VEGF protein content increases initially but returns to baseline after a few weeks of exercise training (96), we report no changes in VEGF or VEGFR2 protein content post-training (**Figure 6B & 6C)**.

We observed a main effect of fibre-type for CFPE, C/Fi and CC, indicating greater capillarization in type I fibres compared to type II fibres that did not change following training. This is consistent with previous research that examined fibre-specific capillarization at baseline in recreationally-active young individuals (59, 89, 91). Studies that observed similar

capillarization between type I and type II fibres at baseline recruited either sedentary (20) or obese individuals (114). Thus, it appears that individuals with some exposure to endurance exercise, such as the participants recruited in this present study, had fibre-specific differences in capillarization at baseline.

Oxidative remodeling is suggested to occur more robustly in type II fibres following interval exercise since there is greater activation of type II fibres with increasing exercise intensity (28). Previous research, however, has shown similar vascular remodelling in type I and type II fibres following continuous (19, 108) and interval (59, 114) endurance training. Although we did not observe an increase in capillarization, we would expect similar vascular remodelling in type I and type II fibres with prolonged training for both *INT* and *CONTIN* since we implemented an intensity-matched protocol.

In conclusion, the present study examined capillarization and SC response following single-leg work- and intensity-matched continuous and interval training. Mixed muscle SC activation increased following interval training but not after continuous training, suggesting that the rest-to-work cycles associated with interval exercise may be a more potent stimulus for SC activation than continuous exercise. Additionally, our results show that in the absence of a hypertrophic stimuli, SCs may still play a role, although likely different than that associated with resistance training. The importance of considering the activation status of the SC pool when describing their contribution to muscle adaptations following exercise is supported by our results. Lastly, our findings suggest that the rest-to-work cycles associated with interval exercise may not be a primary stimulus for training-induced changes in capillarization.

Limitations

The lack of significant increases in capillarization post-training may have been due to the length of the training program. Extensive increases in capillarization are rare in humans and most studies show that endurance training for 4-24 weeks induces an increase in capillarization between 10% and 25% (50, 51, 57, 101). The training protocol implemented here consisted of four weeks of endurance training and coupled with the fact that single-leg cycling was used as opposed to double-leg cycling, the stimulus may have been insufficient in length and potency to induce significant changes in capillarization. Additionally, it should be noted that with a sample size of 9 (considering the lost of one subject due to the poor quality of the tissue sample), it is likely that we were unable to detect changes and/or differences between legs that are biologically relevant. As mentioned earlier, this present study was part of a collaboration and the sample size was based on a power calculation designed to detect a difference in citrate synthase. Based on a post-hoc power calculation with an effect size for type I CFPE (r square = 0.2), the required sample size needed to detect a difference in capillarization between *INT* and *CONTIN* is 30.

In addition, there are also limitations to the measurements used in this study. SCs closer in proximity to capillaries are thought to have greater exposure to circulating factors, leading to maximal SC activation (89, 91). Although we did not observe changes in SC distance to nearest capillary post-training, it should be noted that this measurement was performed for overall $Pax7^+$ cells and not activated SCs (i.e. $Pax7^+/MyoD^+$ cells). Hence, it is unknown whether there would be a reduction in distance of activated SCs to their nearest capillary, as previously reported following an eccentric bout of exercise (89). Overall, even though we observed significant changes in the *INT* leg compared to the pre-training leg, we did not observe any significant differences between the *INT* and *CONTIN* legs with regards to any of the muscle responses

measured here. Hence, we cannot conclude that interval exercise (employing "rest-to-work" cycles) is better at inducing SC activation or capillarization compared to work- and intensity-matched continuous exercise.

Future directions

We propose changes to the exercise protocol to ascertain the effect of the rest-to-work cycles associated with interval exercise on the skeletal muscle responses measured here. Previous research indicates that capillarization takes weeks to months to manifest (50). We propose increasing the length of the training program to 6 weeks or more. Additionally, to tease out the true effect of the rest-to-work cycles more effectively, sedentary participants could be recruited instead of recreationally-active individuals. Lastly, as previously discussed, Combes and colleagues reported that an acute bout of interval exercise induced greater activation of skeletal muscle signaling proteins (e.g. AMPK) compared to work and intensity-matched continuous exercise (22). Here, we employed an endurance training protocol to examine the chronic skeletal muscle response to work- and intensity-matched interval versus continuous exercise. Acute measurements of AMPK and other signalling proteins, such as pro- and anti-angiogenic factors, before and after training, could shed light on whether we would observe similar findings reported by Combes and colleagues (22). The inclusion of additional acute muscle biopsies, before and after training, could have also provided further information on the progression of the SC through the myogenic program.

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