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ABSTRACT

We examined the influence of graded cold-water immersion (CWI) on global and regional quadriceps muscle perfusion using positron emission tomography (PET) and [^{15}O]H_2O. In thirty healthy males (33±8 yrs; 81±10 kg; 184±5 cm; percentage body fat: 13±5%; $\dot{V}O_{2peak}$: 47±8 mL·kg^{-1}·min^{-1}) quadriceps perfusion, thigh and calf cutaneous vascular conductance (CVC), intestinal, muscle and local skin temperatures, thermal comfort, mean arterial pressure and heart rate were assessed prior to and following 10-min of CWI at 8°C, 15°C or 22°C. Global quadriceps perfusion did not change beyond a clinically relevant threshold (0.75 mL·100g·min^{-1}) in any condition, and was similar between conditions [range of the differences (95% confidence interval [CI]); 0.1 mL·100g·min^{-1} (-0.9 to 1.2 mL·100g·min^{-1}) to 0.9 mL·100g·min^{-1} (-0.2 to 1.9 mL·100g·min^{-1})]. Muscle perfusion was greater in vastus intermedius (VI) compared with vastus lateralis (VL) (2.2 mL·100g·min^{-1}; 95%CI 1.5 to 3.0 mL·100g·min^{-1}) and rectus femoris (RF) (2.2 mL·100g·min^{-1}; 1.4 to 2.9 mL·100g·min^{-1}). A clinically relevant increase in VI muscle perfusion after immersion at 8°C and a decrease in RF muscle perfusion at 15°C were observed. A clinically relevant increase in perfusion was observed in the VI in 8°C compared with 22°C water (2.3 mL·100g·min^{-1}; 1.1 to 3.5 mL·100g·min^{-1}). There were no clinically relevant between-condition differences in thigh CVC. Our findings suggest that CWI (8-22°C) does not reduce global quadriceps muscle perfusion to a clinically relevant extent, however, colder-water (8°C) increases deep muscle perfusion and reduces (15°C) superficial muscle (RF) perfusion in the quadriceps muscle.

NEW & NOTEWORTHY

Using positron emission tomography, we report for the first time, muscle perfusion heterogeneity in the quadriceps femoris in response to different degrees of cold-water immersion (CWI). Noxious CWI temperatures (8°C) increases perfusion in the deep quadriceps muscle whilst superficial quadriceps muscle perfusion is reduced in cooler (15°C) water. Therefore, these data have important implications for the selection of CWI approaches used in the treatment of soft tissue injury, while also increasing our understanding of the potential mechanisms underpinning CWI.

INTRODUCTION

The application of cryotherapy (i.e., cold therapy) is widely used as a recovery modality in the treatment of soft tissue injuries (6, 18, 25). The proposed benefits of acute cryotherapy (e.g., cold-water immersion or extreme air-cooling) exposure are related to reductions in body/local temperatures, muscle microvascular blood flow, oedema, perceived soreness and possibly muscle damage (18). Therefore, understanding the change in muscle perfusion in response to cryotherapy is key in providing appropriate advice for effective intervention strategies.

The current theory that cooling causes reductions in lower limb muscle blood flow is based on studies employing techniques that only allow the inference of hemodynamic, e.g., Doppler ultrasound alongside simultaneous cutaneous blood flow measures (14, 27, 28) or volume changes within the limb (9, 12, 19, 43). Positron emission tomography (PET) alongside oxygen-15 water radiotracer [^{15}O]H_2O kinetics, provides a unique tool for the direct measurement of skeletal muscle perfusion (35). With knowledge of [^{15}O]H_2O kinetics in the arterial blood and specific tissues, it is possible to provide quantitative perfusion measurements

in the muscles of interest (20, 36). PET and [^{15}O]H_2O has been employed previously to determine muscle perfusion responses of the lower limb to local and whole body heating (16), and thereby provides an excellent model to determine muscle perfusion changes during cooling.

Another key issue not yet considered when examining the impact of cooling on limb perfusion, is that individual skeletal muscles respond to cold differently (8, 42). For example, glucose metabolism, muscle perfusion and oxygen consumption have been shown to increase, particularly in deeper centrally located cervico-upper thoracic skeletal muscles compared to superficial muscles, as a response to cold-induced shivering thermogenesis (8, 42). This deep muscle activation, which cannot be investigated by surface electromyography (EMG), has been interpreted as a physiological response to maintain core temperature as a result of cold exposure (15). However, to date, the heterogeneity in the muscle perfusion response to cooling has only been documented in the upper body muscles as part of brown fat activation studies (42). While it has been shown that perfusion is spatially and heterogeneously distributed in the quadriceps femoris muscle at rest and during exercise (24), it remains unclear how cooling may influence the directional change in global and regional muscle perfusion in the lower body. Therefore, our aim was to examine the effects of lower body cooling with 8°C, 15°C and 22°C water on global and regional quadriceps muscle perfusion, using the PET-radiowater technique. We hypothesized that colder water would elicit the greatest reductions in global quadriceps muscle perfusion but would increase muscle perfusion within the deep lying quadriceps muscles.

METHODS

Ethical Approval

All procedures performed in the study were in accordance with the latest revision of the declaration of Helsinki, and was approved by the Ethical Committee of the Hospital District of South-Western Finland and National Agency for Medicines. The purpose, potential risks and nature of the study were fully explained to each participant before their written informed consent to participate was given.

Participants

Thirty recreationally active healthy males (age: 33 ± 8 yrs; body mass: 80.9 ± 9.5 kg; height: 183.9 ± 4.7 cm; percentage body fat: 12.9 ± 5.3%; $\dot{V}O_{2peak}$: 47.4 ± 8.1 $mL \cdot kg^{-1} \cdot min^{-1}$; peak power output on cycle ergometer (PPO): 343 ± 45 W; means ± standard deviation) volunteered to participate in this study. The participants were asked to abstain from alcohol and caffeine containing beverages for at least 24 h before the commencement of the experiments and asked to avoid strenuous exercise within 48 h of commencing the experimental protocol. Participants had no history of cardiovascular or neurological disease, or skeletal muscle abnormality, and were not currently taking any pharmacological medication. Given the exploratory nature of our study, a formal sample size estimation is not presented. Our sample of 10 participants per condition was chosen to be representative of the usual between-subject experiments in this domain (48).

Study Design

Participants were randomly allocated to one of three conditions: 8°C water immersion, 15°C water immersion, or 22°C water immersion (9, 43) using covariate adaptive randomization (40), after their first visit to the hospital. A within-subject crossover design was not permitted due to ethical restrictions concerning radioactive exposure limits and invasive arterial cannulation. The groups (n = 10) were matched for potentially confounding covariates which could influence changes in muscle perfusion, namely aerobic fitness ($\dot{V}O_{2peak}$) and anthropometric indices (height, body mass, body surface area, muscle mass and thigh skinfold thickness).

Experimental Protocol

Each participant attended the hospital on two separate occasions. On the first visit, the participants were familiarised with the experimental protocol, had anthropometric measurements taken, and completed a peak oxygen uptake ($\dot{V}O_{2peak}$) test. The participant's height was measured using a stadiometer (KaWe, Asperg, Germany) and body mass was obtained using electronic scales (Seca 703, Seca, Hamburg, Germany). Limb girths (circumferences) were then measured using a tape measure (Seca 201, Seca, Hamburg, Germany) placed around the participant's right mid-thigh, forearm and calf at pre-identified landmarks (38). These measurements enabled calculation of each participant's estimated muscle mass (26). Skinfold thickness measures using calipers (HSK BI; Baty International, West Sussex, U.K.) were then taken at seven body sites (21) to permit calculation of body fat percentage (%Bfat) (37). Following anthropometric assessments, each participant completed a maximal incremental cycling protocol on a cycle ergometer (Tunturi Ergometer E85, Tunturi, Finland) while simultaneous breath by breath ($\dot{V}O_2$) measurements were recorded (Oxycon

Mobile, Jaeger, Germany). The cycling protocol commenced at 75 W and was increased 25 W every 2 min until volitional exhaustion was reached. Peak Power Output (PPO) was derived as the highest power output attained at this point. $\dot{V}O_{2peak}$ ($mL \cdot kg^{-1} \cdot min^{-1}$) was recorded as the highest 30 s average recorded before volitional exhaustion.

On the second visit, each participant arrived at the hospital (0700-0800 h) in a fasted state and after having consumed 5 mL·kg bodyweight of water two hours prior to their arrival to help maintain hydration status (2). Each participant ingested a disposable temperature sensor pill (CorTemp, Human Technologies Inc., Florida, USA) on the evening (before sleeping) prior to arrival for experimental testing. The participant changed into a pair of shorts, and was fitted with a chest heart rate telemetry belt (Polar M400, Kempele, Finland) before resting in a semi-reclined position while laser Doppler probes and skin temperature thermistors were attached to the body. An anaesthesiologist then cannulated the radial artery under local anaesthesia to permit tracer administration and blood sampling during PET measurements. After resting semi-reclined for $\geq$ 20 min, to ensure physiological status was stabilised, baseline thermometry measures were taken. The skin thermistors were then unattached (laser Doppler probes remained affixed to skin), and the participant was taken by wheelchair to another room to undergo simultaneous PET/CT and laser Doppler measures. The participant was then immersed in a semi-reclined position up to the navel into an inflatable water bath (iSprint, iCool, Queensland, Australia) for a period of 10 min. The water temperature was pre-set to one of the three temperatures (8.7±0.3°C, 15.1±0.3°C, 22.0±0.46°C) using a heating/chiller water system (Boyu CW Series, Guangdong, China) dependent on the participant's group allocation. The water temperature was continuously monitored using a skin thermistor (MHF-18050-A, Ellab, Rodovre, Denmark) to validate the water temperature. Upon completion of the immersion protocol, the participant's legs were dabbed dry (as not to stimulate blood flow) to enable the skin thermistors to be re-attached before being returned to the PET/CT room (via wheelchair)

to undergo PET and laser Doppler measures (commenced 10 min post-immersion). Our previous work has shown that CWI-induced (8°C & 22°C) decreases in deep muscle temperature, limb and cutaneous blood flows are further exacerbated over a 30 min recovery period following immersion under normal ambient temperatures (14). The 10 min period following CWI and the final PET and laser Doppler measures would therefore not have minimised the impact of CWI on these hemodynamic measures.

Heart rate, intestinal, skin and muscle temperatures were measured at baseline and after the post immersion PET/CT scan. Thigh and calf cutaneous blood flow and mean arterial pressure were measured during each PET/CT scan. Perceived thermal comfort, rated using a 9-point Likert scale (0 = unbearably cold to 9 = very hot; (49), was recorded at baseline and during immersion.

Thermometry

Upon arrival at the hospital, the ingestible core temperature sensor pill was immediately checked for location in the gastrointestinal tract by sipping 100 ml of cold water. If the temperature varied by <0.1°C, it was deemed that the ingestible senor pill was sufficiently sited down the gastrointestinal tract to enable commencement of the experimental protocol (5). The sensor pill was remotely connected to a data logger worn around the waist of each participant during resting PET/CT measures and held next to the participant (umbilical level) during immersion. Local skin temperature was measured at four sites using skin thermistors (MHF-18050-A, Ellab, Rodovre, Denmark) affixed to the chest, forearm, thigh and calf using tape (Medipore, 3M). Mean skin temperature was subsequently calculated as a weighted average of these four measurement sites (34). Thigh muscle temperature was measured via insertion of a temperature thermistor (13050; Ellab, Rodovre, Denmark). The area of insertion was marked

over the muscle belly of the vastus lateralis by measuring half the length between the head of the femur and the lateral condyle. The depth of probe insertion was then determined by measuring skinfold thickness with calipers (HSK BI; Baty International, West Sussex, U.K.) and dividing by two to determine the subcutaneous fat layer. The probe was inserted to a depth of 3 cm, plus one-half of the skinfold measurement, for the determination of deep (3 cm) muscle temperature (11). The thermistor was then withdrawn at 1 cm decrements for the determination of muscle temperature at 2 cm and 1 cm below the subcutaneous layer. Muscle and skin temperature were recorded using an electronic measuring system (CTF-9004, Ellab, Rodovre, Denmark).

Blood flow measurements and analysis

Radiowater positron emitting tracer [^{15}O]H_2O was produced using a Cyclone 3 cyclotron (IBA Molecular, Belgium) and a PET/CT scanner (STE General Electric Medical systems, Milwaukee, USA) was used in three dimensional (3D) mode for image acquisition to measure muscle perfusion with [^{15}O]H_2O. A dynamic scan (6 min) was performed 20 seconds following an intravenous injection of ~455 MBq of [^{15}O]H_2O with dynamic scanning images performed in the following frames: 6x5 seconds, 12x10 seconds, 7x30 seconds and 12x10 seconds.

Input function was obtained from arterial blood, which was continuously withdrawn using a pump during scanning (5 ml·min^{-1}). Radioactivity concentration in blood was measured using a two-channel online detector system (Scanditronix, Uppsala, Sweden), cross-calibrated with an automatic gamma counter (Wizard 1480 3", Wallac, Turku, Finland) and the PET scanner. Arterial function was pre-processed with a delay correction. Muscle perfusion was subsequently measured using the 1-tissue compartment model. Data analysis were performed

using in-house developed programs (Carimas software, http://www.turkupetcentre.fi/carimas). Muscle perfusion was determined in a blinded fashion by the same individual for the specific regions of the right quadriceps muscle group, namely the rectus femoris (RF), vastus lateralis (VL), vastus intermedius (VI) and vastus medialis (VM; Figure 1). Blood pressure and MAP were recorded using a blood pressure monitor (Apteq AE701f, APTEQ, Finland) during the final 1 min of each PET scan.

Red blood cell flux was used as an index of skin blood flow using laser Doppler flowmetry (Periflux System 5001; Perimed Instruments, Jarfalla, Sweden). An integrated laser Doppler probe (Probe 455; Perimed, Suffolk, U.K) was positioned on the right anterior thigh halfway between the inguinal line and the patella, and on the calf in the region of the largest circumference. The probes remained in situ on the skin throughout the testing period. Cutaneous vascular conductance (CVC) was calculated as the ratio of laser Doppler flux to MAP. The data were transformed with natural logarithm using %CVC baseline and post-immersion data and expressed as percentage change from baseline values.

Statistical Analysis

We employed an ANCOVA model with the change score (post immersion minus baseline) as the dependent variable and baseline value as the covariate to control for any between-group imbalances (44). The least significant difference (LSD) test was used for post-hoc pairwise comparisons of the fixed effects. This ANCOVA model was used to examine the fixed effect of CWI Condition (8°C, 15°C, 22°C) under resting conditions on global muscle perfusion and skin blood flow (i.e., our primary outcomes measures), MAP, heart rate, intestinal temperature, mean and thigh skin temperature, muscle temperature, and thermal comfort (secondary outcomes measures). Following this, we employed an ANCOVA model,

again with the change score as the dependent variable and baseline as a covariate, and examined the fixed effect of CWI Condition (8°C, 15°C, 22°C) on muscle perfusion in each individual quadriceps muscle group (Muscle: rectus femoris (RF), vastus lateralis (VL), vastus intermedius (VI), vastus medialis (VM)). This model also assessed Condition*Muscle group interactions. The same ANCOVA model assessed the fixed effect of Depth (3 cm, 2 cm, 1 cm) and Condition*Depth interactions on muscle temperature. The LSD test was used for all post-hoc pairwise comparisons of the fixed effects and interactions.

For muscle perfusion, the fixed effects of CWI Condition, Muscle, and CWI Condition*Muscle interactions, were assessed for clinical relevance against a minimal clinically important difference (MCID) of 0.75 $mL \cdot 100g \cdot min^{-1}$. This value was based on the comparable reduction of resting muscle perfusion with nitric oxide synthase inhibition (17). Changes in skin blood flow were assessed against an MCID of a 19% CVC reduction. This value was based on our previous work (27, 28, 29), with a ~6°C decrease in skin temperature after 22°C lower body cooling causing a reduction in thigh %CVC by ~19%. For our primary outcome measures (muscle perfusion and skin blood flow), statistical inference was then based on the disposition of the lower limit of the 95% confidence interval (95% CI) for the ANCOVA adjusted mean differences to our MCID's, with differences deemed clinically relevant when the lower confidence interval was equal to or exceeded the MCID. Differences not reaching this threshold were declared not clinically relevant. *P* values are also presented but not interpreted, as the *p*-value does not measure the size of an effect nor the practical importance of a result (13, 45). Interpretation of our cardiovascular and thermoregulatory responses (secondary outcome measures) were based on non-overlapping of 95% CI's for the ANCOVA adjusted change scores, with non-overlap of the CI's constituting a clear difference. Here, we purposefully placed less inferential emphasis on our secondary outcomes as these data were provided to describe the differential cardiovascular and thermoregulatory response of the lower

body cooling. Jamovi statistical software, version 0.9.2.8 (https://www.jamovi.org) was used for all statistical analysis. Data in the text are presented as means and 95% CI.

RESULTS

Muscle Perfusion

Baseline and post-immersion muscle perfusion and temperature data (absolute values) are included in Table 1. The change in global quadriceps muscle perfusion was not clinically relevant in any CWI condition when compared to the 0.75 mL·100g·min^{-1} MCID (p =0.233; Figure 2). The differences in global quadriceps muscle perfusion between cooling conditions also failed to reach clinical relevance (p = 0.174 to 0.791; Figure 2).

The change in muscle perfusion in VI compared to VL and RF was clinically relevant (Figure 3A). The CWI Condition*Muscle interactions also revealed a clinically relevant increase in VI muscle perfusion after immersion at 8°C (2.15 mL·100g·min^{-1}; 1.28 to 3.02 mL·100g·min^{-1}) and a decrease in RF muscle perfusion at 15°C (-1.61 mL·100g·min^{-1}; -2.47 to -0.75 mL·100g·min^{-1}, Figure 3B), respectively. In the 8°C group, clinically relevant differences in muscle perfusion were found between the VI and RF (3.1 mL·100g·min^{-1}; 1.9 to 4.4 mL·100g·min^{-1}, p<0.001) and VI and VL (3.5 mL·100g·min^{-1}; 2.3 to 4.7 mL·100g·min^{-1}, p<0.001). Similarly, after 15°C CWI, clinically relevant differences in muscle perfusion were found between the VI and RF (2.4 mL·100g·min^{-1}; 1.1 to 3.6 mL·100g·min^{-1}, p<0.001) and VI and VL (2.2 mL·100g·min^{-1}; 1.0 to 3.5 mL·100g·min^{-1}, p<0.001; Figure 3B). The change in muscle perfusion in the VI was greater after 8°C CWI when compared to 22°C (2.3 mL·100g·min^{-1}; 1.1 to 3.5 mL·100g·min^{-1}, p<0.001). All other differences in muscle perfusion between individual muscles effects did not reach clinical relevance, with the differences

293 ranging from 0.1 mL·100g·min^{-1} (95% CI, -1.2 to 1.1 mL·100g·min^{-1}, p=0.937) to 1.8
294 mL·100g·min^{-1} (0.7 to 3.0 mL·100g·min^{-1}, p=0.003).

295

296 **Skin Blood Flow**

297 There was a clinically relevant reduction in CVC at the thigh (Figure 4A) and calf
298 (Figure 4B) in each cooling condition. However, there were no clinically relevant between-
299 condition differences in CVC at either site (Figure 4C & 4D).

300

301 **Thermoregulatory and Cardiovascular Responses**

302 *Muscle Temperature*

303 There were clear differences in the changes in muscle temperature for the fixed effect
304 of Depth, with greater muscle temperature decreases at 1 cm and 2 cm depths compared with
305 3 cm (Figure 5A). At a depth of 1 cm, a clear difference in the change in muscle temperature
306 was observed in the 8°C and 15°C conditions compared with 22°C (Figure 5B). However, there
307 were no clear differences in the change in muscle temperature between conditions at depths of
308 2 cm or 3 cm (Figure 5C & 5D).

309

310 *Intestinal and Skin Temperature*

311 There were no clear differences in intestinal temperature between conditions (Figure
312 6A). A clear difference in mean skin temperature was observed in the 8°C condition compared
313 with 22°C (Figure 6A). A clear difference in local thigh skin temperature was also found in the
314 8°C and 15°C conditions compared with 22°C (Figure 6A).

Thermal Comfort

A clear difference was observed in thermal comfort ratings between the 8°C and 22°C conditions (Figure 6B).

Mean Arterial Pressure and Heart Rate

There were no clear differences observed for either MAP or heart rate responses between conditions (Figure 6C).

DISCUSSION

We show for the first time that CWI temperatures between 8°C and 22°C did not reduce global quadriceps muscle perfusion beyond a clinically relevant threshold. However, the change in muscle perfusion was not uniform across the individual muscles of the quadriceps. A clinically relevant increase in muscle perfusion was observed in the deeper vastus intermedius (VI) in the 8°C group, while muscle perfusion decreased in the more superficial rectus femoris (RF) muscle after 15°C. Taken together, our findings provide new insights regarding the influence of CWI on quadriceps femoris muscle perfusion.

Muscle perfusion responses to local and whole-body heating have previously been investigated (16), but this is the first study to quantitatively determine lower limb muscle perfusion responses to cooling. The observation of similar changes in global quadriceps muscle perfusion (<0.75 $mL \cdot 100g \cdot min^{-1}$), from baseline, and between CWI trials (see Figure 2) contrasts with previous work from our laboratory (14) and others that assessed forearm blood flow (4) under resting conditions. Using simultaneous Doppler ultrasound and cutaneous blood flow measurements, to provide indirect estimates of muscle perfusion, we reported that total

leg blood flow decreased after both 8 and 22°C CWI with greater blood flow reductions in the colder water. The contrast of the present study's findings with our previous work most likely relate to the methods used to index muscle perfusion. Nonetheless, our current observations are partly in agreement with other previous studies, which have qualitatively examined the limb blood volume/flow response to different CWI temperatures after exercise using various measurement techniques (9, 27, 29). In line with the current investigation, these studies reported similar reductions in limb blood flow/volume (clinical relevance not determined) between the different cooling conditions (range: 8 to 22°C).

Skin blood flow also contributes to total limb blood flow and was consistently reduced in all experimental conditions in the present study. Indeed, our novel findings demonstrate that cold-induced reductions in limb blood flow are likely mediated through reduced flow to the skin, superficial skeletal muscles and other tissues (i.e., subcutaneous fat). Under resting conditions, we have previously reported (14) a higher cutaneous blood flow response to noxious (8°C) versus non-noxious (22°C) cooling despite lower skin temperatures at 8°C. We speculated that this higher cutaneous blood flow response may have been due to the occurrence of cold-induced vasodilation, which could have potentially redistributed blood from the underlying muscle. In the present study, the graded decrease in skin blood flow between the cold (8°C-15°C) and cool (22°C) conditions provided no evidence of cold-induced vasodilation (Figure 4A & B). The discrepancy with our present findings may be related to our experimental design, with the group design (and selected measurement time points) utilised in this study potentially masking the identification of any cold-induced vasodilation due to the inter-individual nature of skin blood flow responses (33).

Despite not finding a change in global muscle perfusion after cooling, we observed a directionally different muscle perfusion response in the deep VI muscle compared with the superficial VL and RF muscles (see Figure 3A). The differences in the changes in perfusion

between these individual muscles were only evident with exposure to the colder water temperatures (8°C-15°C; see Figure 3B). The 8°C water also induced a clinically relevant increase in VI muscle perfusion compared with 22°C cooling (see Figure 3B). Our findings suggest that colder water temperatures modulate specific muscle perfusion responses across individual quadriceps muscles. Indeed, a spatially and heterogeneous distribution of quadriceps muscle perfusion has previously been reported at rest and after exercise (24). The observation of greater perfusion in the VI under these conditions were thought to be related to the higher proportion of slow oxidative fibres within this muscle. In addition, our findings also support the observation of greater muscle perfusion within deeper centrally located upper body skeletal muscles during cold exposure (8, 42). Therefore, our novel findings subsequently extend previous observations (8, 42) to support the view that in response to relatively intense cold exposure (8°C-15°C), deep muscle perfusion is also elevated in the lower body.

The deep lying VI muscle, located next to the femoral bone, has a higher proportion of type 1 fibres in comparison to the three other superficial muscles in the quadriceps (23). It may be speculated that shivering was responsible for the increase in VI muscle perfusion in the colder water, since burst shivering rates have been related to differences in muscle fiber compositions between individuals (7), with low intensity shivering in particular associated with type 1 fibers (15, 30). It has been proposed that this benign shivering response begins from deep muscles to maintain core temperature (8). Slight twitching of muscle fibers stimulates metabolism and oxygen consumption, with more blood supply in the form of blood flow needed to meet the increased metabolic demands (1, 22, 32) of the largely type I muscle fibers (10, 23). Nevertheless, it is difficult to ascertain with certainty that the increase in VI muscle perfusion in the 8°C condition was related to shivering thermogenesis since responses were not objectively measured. Surface electromyography (EMG) cannot be used to assess the shivering contribution in deeper muscles and limits interpretation of surface EMG signals in superficial

muscles which are in close proximity to each other (3). The use of EMG would, however, have provided an indication of the degree of shivering in superficial muscles and therefore the absence of EMG measures represents a study limitation. Blondin *et al's.,* (8) seminal work indicated that EMG measures of shivering are strongly associated with PET measures of fludeoxyglucose (^{18}FDG) uptake in superficial muscle. Future work may consider extrapolating this method to determine the relationship between the shivering and perfusion response in superficial and deeper muscles in response to cooling to confirm our present findings.

In the present study, the generally lower magnitude of muscle temperature reduction in the deeper tissue (3 cm depth; see Figure 5A) was associated with higher muscle perfusion in the VI compared with the RF and VL muscles across the conditions. This finding suggests that after cooling the legs with CWI (independent of water temperature), perfusion in the deeper and superficial muscle tissue does not respond in a similar manner to reductions in muscle temperature across the quadriceps musculature. Another key finding was the greater increase in VI muscle perfusion in the colder water (8°C) compared with 22°C immersion. This difference in muscle perfusion was evident despite similar changes in deep muscle temperatures (2 & 3 cm) across the conditions (Figure 5B & C). It would perhaps be expected that a difference in muscle temperature of sufficient magnitude would be required to modify the observed perfusion response between the cooling conditions (4, 29). However, it must be noted that muscle temperature was only measured at different depths within the VL muscle and therefore does not necessarily represent tissue temperature changes within other quadriceps muscles, in particular the deeper muscles (i.e., VI muscle).

Cryotherapy is widely administered in clinical and applied sport settings in the acute treatment of soft tissue injuries and exercise induced muscle damage. It is proposed that a cooling induced reduction in muscle perfusion may limit infiltration of leucocytes,

macrophages and other pro-inflammatory cells to better preserve cellular oxygen supply, which may be otherwise compromised by local swelling, oedema and capillary constriction (39, 41, 46). This may limit hypoxic cell death and damage and minimize secondary tissue damage (31, 41, 46). We demonstrate for the first time, that 10 min of lower body CWI, can lead to a clinically relevant reduction in muscle perfusion in superficial areas of the quadriceps femoris muscle. This reduction appears to be dependent on water temperature with the decline in RF muscle perfusion observed in 15°C water (Figure 3B). Nevertheless, in contrast to deep muscle(s), there was a trend for perfusion to decrease in the three superficial muscles (RF, VL and VM) across all experimental conditions. Since superficial muscles still contribute to a large part of the bulk skeletal muscle mass, our findings suggest that cold-induced reductions in superficial perfusion and skin blood flow play an important role in mediating reductions in total limb blood flow previously reported (9, 14, 27, 28, 29, 43). Taken together, our data indicates that a less noxious water temperature (15°C) may be the most viable option as a treatment for soft tissue injury by promoting a clinically relevant decrease in superficial muscle perfusion whilst minimising increases in deep (VI) muscle perfusion (Figure 3B). Moreover, the increase in deep muscle perfusion (VI) in the 8°C condition suggests that more noxious CWI cooling may potentially accentuate the inflammatory response in deeper tissues. This inference, however, warrants further investigation.

Our experimental design, using CWI as the cooling stimulus, was used to simulate real-world practice (construct validity), which required the logistics of moving participants from the bed/cold water bath to the PET scan room to undertake muscle blood perfusion measurements. We therefore used a wheelchair to move the participants from either location to try and control any muscle activation and limit any confounding of perfusion measurements. Whilst we endeavoured to limit any unnecessary muscle activation, is it important to note that participants briefly had to stand out of the wheelchair to position themselves onto the PET

scanner in a supine position. However, there was a 10 min period prior to commencing PET scans after lying supine, which is likely to have limited any potential confounding of muscle perfusion. Indeed, another limitation of the present study was that PET scan perfusion measures were only measured at one time point after cooling. We have documented (14, 27, 29) prolonged decreases in deep muscle temperatures during extended post cooling periods (30 min) due to sustained tissue heat loss via thermal conduction. In addition, the magnitude of this deep muscle temperature decrease is related to the CWI water temperature (14, 27, 29). Therefore, if tissue temperature change is of sufficient magnitude to modify muscle perfusion *per se*, it is possible that a greater change in muscle perfusion may have been observed over a longer duration post-cooling.

The semi-reclined immersion protocol utilized in this study is only one of several that can be chosen, for example, CWI protocols can be undertaken at a variety of depths (navel, chest, neck), positions (seated or standing), temperatures, and/or durations. In the current protocol, the hydrostatic pressure acting on the legs (whilst seated) was minimal, due to the pressure that acts on a body part being dependent on its depth in the water (46). However, changes in central hemodynamic responses and muscle perfusion associated with hydrostatic pressure will need to be accounted for when adopting greater water depths. Additionally, CWI is often used immediately after intense or muscle damaging exercise (47), when tissue temperature, and skin and muscle blood flow, are elevated. It remains to be elucidated if any potential differences in muscle perfusion would be noted when CWI is applied under these conditions. Therefore, there is greater scope for work in this area by utilizing different cooling protocols and examining perfusion responses across different muscle groups at rest and after exercise.

In summary, we used PET and [^{15}O]H_2O to quantitatively measure muscle perfusion in the quadriceps muscle after different degrees of CWI cooling. CWI (8-22°C) did not reduce

global quadriceps muscle perfusion to a clinically relevant extent, however, the muscle perfusion response to cooling was not uniform across the individual muscles composing the quadriceps. Our findings suggest that colder-water (8°C) increases deep muscle perfusion, while 15°C water reduces superficial muscle (RF) perfusion in the quadriceps muscle. Therefore, a less noxious water temperature (15°C) may be considered a viable option as a treatment for soft tissue injury.

ADDITIONAL INFORMATION

Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

WG, NTC, DAL, HJ, IH, JK and KKK conceived and designed the study. CM and IH were responsible for all data collection. JK was the responsible physician of the study and AK was responsible for the radiotracer production. MW and CM performed the statistical analysis. CM, IH, WG, DAL, HJ and MW contributed to writing the paper. IH, CH, KKK, AK and JK provided expertise for data acquisition for and from PET scans. CH, IH, KKK and CM performed PET scan analysis. All authors have approved the final version of this manuscript.

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Table 1. Baseline and post immersion absolute values of muscle perfusion and temperature variables (mean ± SD).

Figure 1. Representative cross-sectional computed tomography (CT) image of a participant's right quadriceps femoris muscle (left). The specified region of interests (ROI) are shown on the CT image (middle), which were fused with the positron emission tomography (PET) image to calculate muscle blood flow (right).

692

693 **Figure 2.** The mean Δ in global quadriceps muscle perfusion after 8°C, 15°C and 22°C cooling
694 (mean ± 95% CI). Clinical relevance was assessed against a minimally clinically important
695 difference (MCID) in muscle perfusion of ±0.75 mL·100g·min^{-1} (shaded area).

696

697 **Figure 3.** The mean difference in muscle perfusion between individual muscles independent
698 of the cooling condition (A) and the mean Δ in perfusion in each quadriceps muscle after 8°C,
699 15°C and 22°C cooling, respectively (B) (mean ± 95% CI). Clinical relevance was assessed
700 against a minimally clinically important difference (MCID) in muscle perfusion of ±0.75
701 mL·100g·min^{-1} (shaded area).

702

703 **Figure 4.** The mean Δ in thigh (A) and calf (B) cutaneous vascular conductance (CVC) from
704 baseline and the mean differences in thigh (C) and calf (D) CVC between the 8°C, 15°C and
705 22°C conditions, respectively (mean ± 95% CI). Clinical relevance was assessed against a
706 minimally clinically important difference (MCID) in CVC of ±19.0% (shaded area).

707

708 **Figure 5.** The mean Δ in muscle temperature for the fixed effect of depth (A) and at 1 cm (B),
709 2 cm (C) and 3 cm (D) depths in the 8°C, 15°C and 22°C cooling conditions (mean ± 95% CI).
710 None overlap of ±95% CI's represents clear difference between conditions.

711

712 **Figure 6.** Forest plot displaying condition main effects of secondary outcome variables:
713 temperature (A), subjective measures (B) and cardiovascular measures (C). Symbols represent

714 mean differences: 8°C (●), 15°C (■) and 22°C (▲) ± 95% CI. None overlap of ±95% CI's

715 represents clear difference between conditions.

716