Mawhinney, C, Heinonen, I, Low, DA, Han, C, Jones, H, Kalliokoski, KK, Kirjavainen, A, Kemppainen, J, Di Salvo, V, Lolli, L, Cable, NTIM and Gregson, W

Cool-Water Immersion Reduces Postexercise Quadriceps Femoris Muscle Perfusion More Than Cold-Water Immersion

Cool-water immersion reduces post-exercise quadriceps femoris muscle perfusion more than cold-water immersion

Chris Mawhinney[1,2], Ilkka Heinonen[3,4,5] David A. Low[1], Chunlei Han[3], Helen Jones[1], Kari K. Kalliokoski[3], Anna Kirjavainen[3], Jukka Kemppainen[3], Valter Di Salvo[6,7], Lorenzo Lolli[1,7], N. Tim Cable[8] and Warren Gregson[1,7]

[1]Research Institute for Sport and Exercise Sciences, Liverpool John Moores University, Liverpool, U.K.
[2]College of Sports Science and Technology, Mahidol University, Salaya, Thailand
[3]Turku PET Centre, University of Turku and Turku University Hospital, Turku, Finland
[4]Department of Clinical Physiology and Nuclear Medicine, University of Turku, Turku, Finland
[5]Rydberg Laboratory of Applied Sciences, University of Halmstad, Halmstad, Sweden.
[6]Department of Movement, Human and Health Sciences, University of Rome "Foro Italico", Rome, Italy
[7]Football Performance & Science Department, Aspire Academy, Doha, Qatar
[8]Institute of Sport, Manchester Metropolitan University, Manchester, UK

Running Head: Muscle perfusion after cold-water immersion

Subject area: Human/Environmental Exercise Physiology

Address for correspondence:
Professor Warren Gregson, PhD,
Aspire Academy,
PO Box 22287,
Doha,
Qatar
Email: warren.gregson@aspire.qa
Tel: (+974) 44136127
Fax: (+974) 44136060

ABSTRACT

Purpose: The muscle perfusion response to post-exercise cold water immersion (CWI) is not well understood. We examined the effects of graded post-exercise CWI upon global and regional quadriceps femoris muscle perfusion using positron emission tomography (PET) and [^{15}O]H_2O.

Methods: Using a matched-group design, 30 healthy men performed cycle ergometer exercise at 70% $\dot{V}O_{2peak}$ to a core body temperature of 38°C, followed by either 10 min of CWI at 8°C, 22°C or seated rest (control). Quadriceps muscle perfusion, thigh and calf cutaneous vascular conductance (CVC), intestinal, muscle, and local skin temperatures, thermal comfort, mean arterial pressure, and heart rate were assessed at pre-, post-exercise and following CWI.

Results: Global quadriceps perfusion was reduced beyond the pre-defined minimal clinically relevant threshold (0.75 mL·100 g·min^{-1}) in 22°C water versus control (difference [95% confidence interval (CI)]: -2.5 mL·100 g·min^{-1} [-3.9 to -1.1]). Clinically relevant decreases in muscle perfusion were observed in the rectus femoris (-2.0 mL·100 g·min^{-}1 [-3.0 to -1.0]) and vastus lateralis (VL; -3.5 mL·100 g·min^{-}1 [-4.9 to -2.0]) in 8°C water, and in the vastus lateralis (-3.3 mL·100 g·min^{-}1 [-4.8 to -1.9]) in 22°C water versus control. The mean effects for vastus intermedius and vastus medialis perfusion were not clinically relevant. Clinically relevant decreases in thigh and calf CVC were observed in both cooling conditions.

Conclusion: The present findings revealed that less noxious CWI (22°C) promoted clinically relevant post-exercise decreases in global quadriceps muscle perfusion whereas noxious cooling (8°C) elicited no effect.

Key words: Cooling; Recovery; Blood flow; Exercise

INTRODUCTION

Cold-water immersion (cryotherapy) is widely applied after strenuous exercise to facilitate recovery from exercise-induced muscle damage (1). It has been suggested that the physiological effects associated with cryotherapy are partly underpinned by reductions in microvascular blood flow to the exercised/injured muscle (2), which then subsequently reduce edema and induction of inflammatory events (3). Given the potential importance of changes in muscle perfusion in mediating the effects of post-exercise cold-water immersion on recovery, further investigation is warranted to enhance the efficacy of such intervention strategies.

We and others have conducted a number of studies using continuous Doppler ultrasound assessments of the femoral artery alongside simultaneous measures of cutaneous blood flow, and demonstrated that limb blood flow at rest and following exercise can be markedly reduced by cold-water immersion (4, 5, 6). These findings are consistent with other studies, which employed venous occlusion plethysmography (7) and near infrared spectroscopy (NIRS; 4, 8, 9). However, the above-mentioned techniques are limited by their inability to provide a direct assessment of perfusion changes within the muscle, and therefore permit only qualitative and indicative interpretations of the efficacy of cold-water immersion.

Recently, under resting conditions, we used positron emission tomography (PET) with an oxygen-15-labelled water radiotracer ($[^{15}O]H_2O$) to provide a quantitative assessment of quadriceps femoris muscle perfusion to different degrees of cold-water immersion applied over 10 minutes (10). We reported, for the first time, increased perfusion in deeper lying quadriceps muscle following noxious (8°C) cold-water immersion, whereas superficial quadriceps muscle perfusion was reduced in cooler (15°C) water. Furthermore, work from our laboratory combining Doppler artery ultrasound alongside simultaneous cutaneous blood flow measures, has indicated that the hemodynamic response to varying water immersion temperatures (8°C and 22°C for 10 min) is different under resting (11) and post-exercise conditions (5, 12).

Moreover, blunting of the vascular response to sympathetic stimulation during exercise and whole-body heat stress (13, 14, 15) may persist following exercise (13) and modify the muscle perfusion response to cooling. Therefore, quantitatively determining the muscle perfusion response to post-exercise cooling is necessary.

We aimed to determine the effects of post-exercise lower body cooling with 8°C and 22°C water on global and regional quadriceps muscle perfusion, using [^{15}O]H_2O and PET imaging. We hypothesised that 8°C and 22°C water would elicit a similar reduction in muscle perfusion in deep-lying and superficial quadriceps muscles following exercise.

METHODS

Ethical Approval

The Ethical Committee of the Hospital District of South-Western Finland approved this study, with all study procedures performed in accordance with the standards set by the latest revision of the declaration of Helsinki. All test procedures and potential risks were fully explained prior to attaining each participant's written informed consent to participate.

Participants

Thirty recreationally active healthy males (means ± SD: 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) volunteered to participate. The participants were requested to abstain from alcohol and caffeine containing beverages for at least 24 h before the commencement of the experiments, and to avoid strenuous exercise within 48 h of commencing the experimental protocol. Participants were screened for history of cardiovascular disease, neurological disease, and skeletal muscle abnormality, and were excluded if currently prescribed pharmacological medication.

Study Design

The present investigation formed part of a larger research project, which also examined muscle perfusion under resting conditions using the same participant cohort (10). The design adopted a principled approach to planning (16), with sample size decisions established on cost-efficiency information and procedures relevant to subject condition allocation from existing parallel-arm experiments in this area of research (17). After undertaking preliminary assessments on their initial visit to the hospital, the participants were randomly allocated to one of the three conditions: 8°C water immersion, 22°C water immersion, or a control (rest in a semi reclined position), using covariate adaptive randomization (18). The nature of performing repeated PET/CT measures has ethical considerations in regards to radioactive exposure limits and invasive arterial cannulation. Therefore, a between subject design was employed to meet the necessary ethical requirements, with the groups (n = 10) matched for confounding covariates ($\dot{V}O_{2peak}$, height, body mass, body surface area, muscle mass and thigh skinfold thickness), which could potentially influence changes in muscle perfusion (Table 1).

Experimental Protocol

The participants attended the hospital on two separate occasions: the first visit was a preliminary test day to familiarize the participants with the experimental protocol, enable anthropometric measurements to be taken, and to assess peak oxygen uptake ($\dot{V}O_{2peak}$). The anthropometric assessments included taking measurements of the participants' height (KaWe stadiometer, Asperg, Germany), body mass (Seca 703 electronic scales, Seca, Hamburg, Germany), and limb circumferences at the right mid-thigh, forearm, and calf (Seca 201 tape measure, Seca, Hamburg, Germany) (19). These measurements were subsequently used to provide an estimation of each participant's muscle mass (20). In addition, skinfold measures (HSK BI calipers; Baty International, West Sussex, U.K.) were taken across 7-sites (21) to

permit the calculation of each participant's body fat percentage (%Bfat) (22). Next, and as previously described (10), a maximal incremental cycling protocol (Tunturi Ergometer E85, Tunturi, Finland) was completed until volitional exhaustion was attained to enable the assessment of each participant's Peak Power Output (PPO) and $\dot{V}O_{2peak}$ ($mL \cdot kg^{-1} \cdot min^{-1}$).

On the second visit to the hospital, the participants were asked to undertake a number of preparatory steps before conducting the main experimental test procedures. The participants were asked to fast overnight, ingest a disposable temperature sensor pill (CorTemp, Human Technologies Inc., Florida, USA) immediately prior to sleeping, and consume 5 mL·kg bodyweight of water within two hours prior to arrival at the hospital (arrival: 0700-0800) to help maintain hydration status (23). After changing into a pair of shorts, the participants were asked to lay semi-reclined on a hospital bed to enable the attachment of equipment: heart rate telemetry belt (Polar M400, Kempele, Finland), laser Doppler probes, and skin temperature thermistors. An anaesthesiologist then cannulated the radial artery under local anaesthesia to permit blood sampling during PET measurements. After providing ≥ 20 min to ensure physiological status was stabilised, baseline thermometry measures were taken. The skin thermistors were then unattached and the participant was taken by wheelchair to another room (temperature ~ 21.6°C) to undergo simultaneous PET/CT and laser Doppler measures.

In the same room (next to the PET/CT scanner), each participant was then asked to undertake a submaximal exercise protocol on a cycle ergometer (Tunturi Ergometer E85, Tunturi, Finland) at 70% $\dot{V}O_{2peak}$ until a core temperature of 38°C was obtained. This core temperature was selected to examine whether a relatively small thermal load could override an increase in deep muscle perfusion (speculated due to shivering) observed in cold-water (8°C) under resting conditions (10). Upon completion, the participants were moved to the adjacent PET/CT scanner to undertake post-exercise muscle perfusion measurements. Next, each participant was then taken by wheelchair to undergo the assigned experimental treatment. The

skin thermistors were then re-attached and the participants were either immersed in a semi-reclined position up to the navel in an inflatable water bath (iSprint, iCool, Queensland, Australia) for 10 min, or rested in a semi-reclined position for the same duration (control). Dependent on the participant's group allocation, the water temperature was pre-set to one of the two temperatures (8.8±0.7°C, 21.8±0.7°C), using a heating/chiller water system (Boyu CW Series, Guangdong, China); and validated using a skin thermistor (MHF-18050-A, Ellab, Rodovre, Denmark). Upon removal from the immersion bath, the participant's legs were carefully dried as not to stimulate blood flow, and taken by wheelchair to undergo PET and laser Doppler measures (commenced 10 min post-immersion). Post-immersion thermometry measures were subsequently recorded. Heart rate was continuously measured, and ratings of perceived exertion (RPE) (24) was recorded during the exercise protocol.

Thermometry

The temperature measures taken in this study (core, muscle, skin) are similar to that described in our recent work (10). Briefly, after initially checking that the ingestible core temperature sensor pill was located in the gastrointestinal tract, a data logger was positioned at the waist (or near to) to permit continuous temperature measures during immersion, exercise and PET/CT scans. Local skin temperature was measured at four sites (chest, forearm, thigh and calf) using skin thermistors (MHF-18050-A, Ellab, Rodovre, Denmark), thus allowing for weighted mean skin temperatures to also be calculated (25). Thigh muscle temperature was assessed by initially measuring thigh skinfold thickness with calipers (HSK BI; Baty International, West Sussex, U.K.) and dividing by 2 to take into account the subcutaneous fat overlaying the vastus lateralis muscle. A needle thermistor (13050; Ellab, Rodovre, Denmark) was then inserted into the vastus lateralis to a depth of 3 cm plus one-half of the skinfold measurement to represent deep muscle temperature (26). Upon the values stabilizing, the

temperature was recorded using an electronic measuring system (CTF-9004, Ellab, Rodovre, Denmark). The thermistor was then withdrawn at 1 cm increments and temperature was recorded at 2 cm and 1 cm depths below the subcutaneous layer. Muscle temperature was measured at baseline, pre-immersion, and post immersion.

Blood Flow Measurements

As recently described (10), positron emitting isotope [^{15}O] was produced using a Cyclone 3 cyclotron (IBA Molecular, Belgium) to produce the radiowater tracer ([^{15}O]H_2O). 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 PET scan (6 min) commenced 20 seconds after an intravenous injection of ~455 MBq of [^{15}O]H_2O, with dynamic scanning performed in the following subsequent time frames: 6x5 seconds, 12x10 seconds, 7x30 seconds and 12x10 seconds.

Input function was obtained from arterial blood, which was continuously withdrawn (5 ml·min^{-1}) using an electronically operating pump during the PET scans. 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, measured radioactivity concentration in blood. Arterial function was pre-processed with a delay correction. A 1-tissue compartment model subsequently measured muscle perfusion. Image data analysis was performed using an in-house developed program package (Carimas software, http://www.turkupetcentre.fi/carimas), with muscle perfusion determined in a blinded fashion by the same individual for the specific regions of the right quadriceps muscle group (rectus femoris, vastus lateralis, vastus intermedius and vastus medialis). Blood pressure and MAP were recorded using a blood pressure monitor (Apteq AE701f, APTEQ, Finland) during the final 1 min of each PET scan.

As previously described (10), integrated laser Doppler probes (Probe 455; Perimed, Suffolk, U.K) were attached to thigh and calf sites to permit skin blood flow (red blood cell flux) recordings via laser Doppler flowmetry (Periflux System 5001; Perimed Instruments, Jarfalla, Sweden). The probes were unattached from the Doppler flowmetry unit during exercise and immersion, however remained in situ on skin throughout the experimental testing. Thigh and calf cutaneous vascular conductance (CVC) was calculated using laser Doppler perfusion units (PU) and MAP (27) and expressed in percentage units as the difference between the natural logarithms of PU and MAP to address the potential allometric relationship between these variables.

Statistical Analysis

Summary statistics are presented as mean ± SD for post-exercise data. Using a constrained longitudinal model framework (28), within-subject linear mixed modelling with restricted maximum likelihood and an unstructured covariance structure estimated post-immersion *versus* post-exercise mean differences for primary and secondary outcome measures between conditions. Primary outcome measures were global and individual muscle perfusion and skin blood flow indices. Secondary outcome measures were MAP, heart rate, intestinal temperature, mean skin temperature, thigh skin temperature, muscle temperature, and thermal comfort. Condition, time, condition × time interaction term and the post-exercise value of the outcome were included as fixed effects, with individual specified as random effect plus a random intercept. Standard residual diagnostics were undertaken to assess model specification based on visual inspection of residual plots (29). The condition × time interaction term quantified post-immersion between-condition mean effects were interpreted against predefined minimally clinically important differences (MCID) of 0.75 $mL \cdot 100g \cdot min^{-1}$ for muscle perfusion (based upon a comparable reduction in resting muscle perfusion with nitric oxide

synthase inhibition) (30) and 19% CVC reduction in skin blood flow measures (5, 6, 12) with no multiplicity adjustment (31). Effects were declared clinically relevant based on the location of the 95% confidence interval (CI) for the between-condition mean difference to the predefined MCID (32) and presented using density strips to illustrate the degree of uncertainty surrounding the point estimates (33). Mean effects for the between-condition differences in cardiovascular and thermoregulatory outcomes were interpreted as descriptive statistics based on non-zero overlap of the 95%CI for the point estimate and presented with the respective *P* values (34). Post-immersion versus post-exercise effects for CVC measures were summarised as geometric mean differences. All analyses were performed using the MIXED procedure in SAS OnDemand for Academics (SAS Institute®) and figures were produced using R (version 3.6.3, R Foundation for Statistical Computing).

RESULTS

Exercise Protocol

The exercise duration to attain a core temperature of 38°C was similar between conditions (mean ± SD: 8°C, 17.2 ± 8.8 min; 22°C, 21.9 ± 6.23 min; control, 19.8 ± 6.1 min; P =0.420).

Primary Outcome Measures

Muscle Perfusion

Post-exercise and post-immersion muscle perfusion and temperature raw data are illustrated in Table 2. The difference in global quadriceps muscle perfusion was clinically relevant for 22°C versus control conditions (-2.5 mL·100g·min^{-1}; 95% CI: -3.9 to -1.1, P = 0.001; Figure 1) in relation to the 0.75 mL·100g·min^{-1} MCID. There were no clinically relevant

differences in global quadriceps perfusion between the other cooling conditions (P = 0.026 to 0.214; Figure 1).

A clinically relevant decrease in rectus femoris (-2.0 mL·100g·min^{-1}; 95% CI: -3.0 to -1.0 mL·100g·min^{-1}; P<0.001) and vastus lateralis (-3.5 mL·100g·min^{-1}; 95% CI: -4.9 to -2.0 mL·100g·min^{-1}; P<0.001) muscle perfusion was observed in the 8°C versus control conditions (Figure 2B). A clinically relevant decrease in vastus lateralis muscle perfusion was also observed in the 22°C versus control conditions (-3.3 mL·100g·min^{-1}; 95% CI: -4.8 to -1.9 mL·100g·min^{-1}; P<0.001; Figure 2C). There were no clinically relevant differences in vastus intermedius (P = 0.014 to 0.784; Figure 2) or vastus medialis (P = 0.028 to 0.414; Figure 2) muscle perfusion irrespective of the experimental group.

Skin Blood Flow

There was a clinically relevant reduction in thigh CVC observed between the 8°C versus control (-69.3%; 95% CI: -76.1 to -60.7%; P = 0.001; Figure 3A) and 22°C versus control conditions (-52.1%; 95% CI: -62.9 to -38.1%; P<0.001 Figure 3A) when compared to the predefined -19% MCID. A clinically relevant reduction in calf CVC was also found between the 8°C versus control (-57.1%; 95% CI: -66.0 to -45.8%; P<0.001; Figure 3B) and 8°C versus 22°C conditions (-36.4%; 95% CI: -50.0 to -19.0%; P<0.001; Figure 3B), respectively.

Secondary Outcome Measures

Muscle Temperature

At 1 cm depth, the change in muscle temperature was -4.3°C (95% CI: -5.3 to -3.4°C P<0.001) for the 8°C versus control condition, and -2.1°C (95% CI: -2.9 to -1.2°C; P<0.001) for the 22°C versus control condition (Figure 4A). At 2 cm depth, the change in muscle

temperature was -3.3°C (95% CI: -3.7 to -2.8°C; $P<0.001$) for the 8°C versus control condition, and -1.2°C (95% CI: -1.6 to -0.7°C; $P<0.001$) for the 22°C versus control condition (Figure 4B). At 3 cm depth, a larger change in muscle temperature was observed for the 8°C versus control (-1.9°C; 95% CI: -2.3 to -1.5°C; $P<0.001$) compared with 22°C versus control (-0.7; 95% CI: -1.1 to -0.3°C; $P<0.001$) conditions (Figure 4C).

Intestinal and Skin Temperature

The mean change in thigh skin temperature (Figure 5) was 3.9°C; (95% CI: -4.4 to -3.4°C; $P<0.001$) for the 8°C versus control condition, and was larger than effects for the 22°C versus control condition (-2.6°C; 95% CI: -3.1 to -2.1°C; $P<0.001$). The change in mean body temperature was -0.9°C (95% CI: -1.1 to -0.6°C; $P<0.001$) for the 8°C versus control, and -0.5°C (95% CI: -0.8 to -0.2°C; $P<0.001$) for 22°C versus control conditions (Figure 5). There were no clear differences in intestinal temperature or mean skin temperature between any group comparisons (Figure 5).

Mean Arterial Pressure and Heart rate

The change in MAP for the 8°C versus control condition was 6 mmHg (95% CI: 2 to 10 mmHg; $P=0.003$), whereas effects were trivial for the 22°C versus control (-1 mmHg; 95% CI: -5 to 3 mmHg; $P=0.727$). The change in MAP for the 8°C versus 22°C condition was 7 mmHg (95% CI: 3 to 11 mmHg; $P=0.011$). There were no clinically relevant differences in heart rate responses between any group comparisons.

DISCUSSION

We demonstrated, for the first time, that non-noxious (22°C) cold-water immersion was more effective than noxious cooling (8°C) for reducing global quadriceps muscle perfusion

beyond a clinically relevant threshold after exercise. The difference in the magnitude of reduction in global perfusion between the cooling conditions was reflected in the profound effect that colder water (8°C) had on maintaining deeper vastus intermedius and vastus medialis muscle perfusion, while similar reductions in perfusion were observed in both cooling conditions across superficial muscles (rectus femoris & vastus lateralis). These findings have practical implications for practitioners who apply cold-water immersion after exercise to facilitate recovery.

The present study is the first to directly and quantitatively determine the perfusion response to post-exercise cooling. In contrast to 8°C immersion, the application of cool water (22°C) reduced global quadriceps muscle perfusion beyond a clinically relevant threshold (> 0.75 $mL \cdot 100g \cdot min^{-1}$; Figure 1). The observed difference in global quadriceps perfusion between cooling conditions post-exercise contrasts with previous work from our laboratory (5, 6, 12) and with others (4) that employed simultaneous Doppler ultrasound alongside cutaneous blood flow measurements [4, 5, 6, 12) and NIRS (4) to provide indirect estimates of muscle perfusion. While we previously reported similar reductions in limb blood flow/volume between the different cooling conditions (range: 8-22°C), Doppler ultrasound assessment of the femoral artery only provides an indirect estimate of muscle flow in the lower limbs. This includes supply to tissue capillaries (nutritive capillary blood flow) and flow into veins via shunts that bypass the capillary bed (non-nutritive blood flow); for example, to muscle connective tissue, fat tissue and skin circulation (35, 36). In contrast, the PET $[^{15}O]H_2O$ radiotracer technique excludes the non-nutritive fraction of blood flow; suggesting that downstream changes in limb blood flow, or muscle blood volume, are not representative of the changes in the muscle microcirculation (37). Our data suggest that the measured blood flow response to cooling depends on measurement site, e.g., actually within the skeletal muscle itself (capillary level) or in conduit vessel proximal to the muscle bed (arterial level). These observations therefore

support the application of the PET [^{15}O]H_2O radiotracer method to obtain a true reflection of perfusion changes within muscle vasculature itself.

In the present study, the decrease in thigh skin blood flow exceeded the threshold of clinical relevance (Δ<19%) in both cooling conditions (Figure 3A). However, the skin blood flow response was not consistent across the leg, with calf skin blood flow only decreased beyond a clinical threshold in 8°C water (Figure 3B). This finding contrasts with previous work, which has reported similar reductions in lower limb skin blood flow after different degrees (range: 8-22°C) of lower-body cold-water immersion (4, 5, 12). Adopting a similar exercise model and cooling stimuli (12), we previously speculated that the similar skin blood flow response to different degrees of cooling was related to reduced vasoconstrictor responsiveness in the skin. While the magnitude of sympathetic nervous activity may be greater at colder water temperatures, any potential increase in vasoconstriction in the cutaneous vessels is blunted in the presence of whole body heat stress (15). Therefore, the inconsistency between our current findings and past observations make our data difficult to interpret. Furthermore, the difference between our findings are also likely related to employing a MCID for our primary outcomes in this study. In support of this, the magnitude and precision (95% CI) of the percentage change in skin blood flow between the different conditions encompassed values observed in our previous work.

We observed different perfusion mechanisms between individual quadriceps muscles with 8°C and 22°C cooling. Clinically relevant perfusion reductions in the superficial rectus femoris and vastus lateralis muscles were generally observed under both water temperatures versus control (Figure 2B & C), with only the decline in rectus femoris perfusion in 22°C water close to, but not exceeding, the clinical threshold (Figure 2C). A similar directional response to cooler water temperatures (8°C and 15°C) has been documented under resting conditions, though declines in superficial muscle perfusion were only clinically relevant in the rectus

femoris muscle (10). This greater scope to decrease perfusion towards maximal vasoconstriction after exercise may simply reflect the greater absolute capacity to reduce muscle perfusion (i.e., higher perfusion values after exercise compared with baseline at rest) (38).

In contrast to the uniform reduction in superficial muscle perfusion in both cooling conditions, a different perfusion response was observed in the deeper lying quadriceps muscles. While the degree of decline in perfusion in the deeper-lying vastus intermedius and vastus medialis muscles in 22°C water (Figure 2C) would not exclude the presence of a potential effect yet not exceeding our pre-defined MCID value (39), perfusion remained unchanged after 8°C cooling (Figure 2B). The perfusion response in deeper muscle supports our previous work under resting conditions (10), where increases in vastus intermedius muscle perfusion were speculated to reflect the occurence of low-intensity shivering in the deep-lying type 1 muscle fibers (40, 41). This putative mechanism stimulates metabolism and oxygen consumption and increases blood supply to meet the higher metabolic demand (42, 43). Taken together, the decline in superficial muscle perfusion in both cooling conditions, and the inconclusive effects observed for perfusion in deeper located muscles in 8°C water, collectively underpin the greater magnitude of global perfusion reduction with less noxious water (22°C). This suggests that non-noxious cooling (15-22°C) may have greater efficacy following exercise compared with more noxious water temperatures (<8°C). This is due to causing reductions in superfical muscle perfusion while simultaneously minimising any increases in deeper muscle perfusion that are observed at colder water temperatures. Indeed, when considered in line with previous observations at rest (10), the present data suggest that non-noxious cooling is likely to be more effective from a muscle perfusion perspective when applied either at rest or following exercise. It should be noted that the changes in perfusion must be interpreted in the context of when PET/CT measures were taken, i.e., 10 min post immersion. Since deep (3 cm) muscle

temperature continues to decrease for at least 30 min post-immersion under similar conditions (12), these perfusion responses are, however, likely to extend beyond the current 10 min period studied. Interestingly, our current findings correspond with recent work using phase change material (44), which demonstrates prolonged (>3 hours) mild cooling (15°C) ameliorates the loss in functional strength and improves subjective recovery after muscle damaging exercise. Thus, our findings may be extrapolated to other forms of cryotherapy, such as ice application, whole body cryotherapy or phase change material, which also attempt to manipulate muscle temperature and perfusion to enhance recovery.

The potential benefits of reducing muscle perfusion using cold-water immersion, are often cited to be related to minimizing the underlying infilitration and accumulation of pro-inflammatory cells (45, 46); likely mediated via reductions in tissue temperature (47). In comparison with the control, the marked reductions in superficial muscle (1 cm, Figure 4A) and skin temperature (Figure 5B & C) across both cooling conditions, appeared to have been of sufficient magnitude to reduce superficial muscle perfusion to a clinically relevant degree. In the absence of any objective measurement of shivering, the difference in deeper muscle temperature (2 and 3 cm; Figure 4B & C) between the 8°C and 22°C conditions supports the occurrence of shivering, and the explanation for preservation of vastus intermedius and vastus medialis perfusion in the colder water. Indeed, work from our laboratory (6) has demonstrated that a difference in deep muscle temperature, similar to that observed in this study (~1°C), can modify femoral artery blood flow (i.e., total flow to the leg musculature). Nevertheless, without taking muscle temperature measurements across deeper lying individual quadriceps muscles, potential temperature-dependent perfusion changes cannot be directly confirmed.

It currently remains unclear whether higher exercise-induced elevations in core and deep muscle temperatures would be of sufficient magnitude to completely overide any potential shivering (and therefore perfusion) response after 8°C water exposure. For example, in

comparison with our recent observations under resting conditions (10), where we reported a clinically relevant increase in vastus intermedius muscle perfusion after 8°C cooling, there was some evidence that the relatively small heat load placed upon the body (i.e, core temperature ~38°C) negated this mechanism (i.e., vastus intermedius perfusion increase did not attain clinical relevance). Therefore, future work is required to investigate the influence of graded thermal loads upon the body (i.e., higher core and muscle temperatures) prior to being exposed to different degrees of cold-water immersion, and examine muscle perfusion responses. This will be beneficial in helping to provide individualized cold-water immersion prescriptions after different types of exercise of varying durations and intensity (i.e., different thermal loads); likely more representative of athletic training and competition.

We have previously discussed the limitations of our applied experimental approach and the potential confounding effects of muscle activation on perfusion measures (10). In particular, a key limitation was the inability to assess the shivering response in deep muscle to provide an objective interpretation of our perfusion findings. Therefore, future studies may attempt to relate changes in deep muscle perfusion in the quadriceps femoris muscle after post-exercise cold-water immersion (or cryotherapy) using suitable radiotracers (i.e.,^{18}FDG) to examine the shivering response (48). Likewise, the nature of the measurements we undertook in our investigation prevented use of a within-subject crossover design from an ethical standpoint, thereby contributing to render the width of the uncertainty around the estimated mean differences in perfusion prone to sampling error. Nevertheless, the degree of uncertainty in the effects we presented can inform sample size planning based on criteria of precision (49) for future investigations with similar, or alternative (50), experimental designs to our study. We also selected an all-male participant cohort to make it somewhat easier to conduct covariate adaptive randomization. Thus, extrapolating our perfusion data to females who typically possess different anthropometrical characteristics represents another study limitation. Finally,

it is recommended that future studies utilize more strenuous exercise protocols in order to better understand perfusion changes promoted by different degrees of cold-water immersion under conditions which more closely simulate those experienced by athletes.

CONCLUSION

We used [^{15}O]H_2O PET/CT to quantitatively measure quadriceps muscle perfusion after different degrees of post-exercise cold-water immersion. In contrast to noxious water (8°C), we observed non-noxious water (22°C) to decrease global quadriceps perfusion beyond a clinically relevant threshold. Despite both cooling temperatures reducing superficial muscle perfusion, the degree of decline in perfusion for the deeper located vastus intermedius and vastus medialis muscle with colder water would not exclude the presence of a potential effect yet not exceeding our pre-defined MCID value. Our findings therefore suggests that the selection of non-noxious water temperatures (22°C) may be more suitable for post-exercise recovery after performing exercise, which places a relatively small thermal load (< 38°C core temperature) upon the body.

ACKNOWLEDGMENTS

We sincerely thank the participants who took part in this study and all the personnel at Turku PET centre, without whose expertise this study would not have been possible.

Author Contributions

I.H., D.A.L., H.J., A.K., J.K., V.D.S., T.C., and W.G., conceived and designed the study; C.M., C.H., and L.L., analyzed the data; C.M., I.H., D.A.L., H.J., K.K.K., and W.G., interpreted the results of the experiments; L.L., prepared figures; C.M, I.H., and W.G., drafted the manuscript; C.M., I.H., D.A.L., H.J., A.K., K.K.K., J.K., L.L., and W.G. edited and revised the manuscript;

C.M., I.H., D.A.L., C.H., H.J., K.K.K., A.K., J.K., V.D.S., L.L., N.T.C., and W.G., approved the final version of the manuscript.

Funding: The data reported here are part of a research program funded by an internal Aspire Zone Foundation Research Grant.

Conflict of interest: The authors declare no conflicts of interest, financial or otherwise.

The results of the present study do not constitute endorsement by the ACSM.

We declare that the results of the study are presented clearly, honestly, and without fabrication, falsification, or inappropriate data manipulation.

REFERENCES

1. Leeder J, Gissane C, van Someren K, Gregson W, Howatson G. Cold water immersion and recovery from strenuous exercise: a meta-analysis. *Br J Sports Med*. 2012;46(4):233-40.

2. Lee H, Natsui H, Akimoto T, Yanagi K, Ohshima N, Kono I. Effects of cryotherapy after contusion using real-time intravital microscopy. *Med Sci Sports Exerc*. 2005;37(7):1093-8.

3. Merrick MA, McBrier NM. Progression of secondary injury after musculoskeletal trauma-a window of opportunity? *J Sport Rehabil*. 2010;19(4):380-8.

4. Choo HC, Nosaka K, Peiffer JJ, Ihsan M, Yeo CC, Abbiss CR. Peripheral blood flow changes in response to postexercise cold water immersion. *Clin Physiol Funct Imaging*. 2018;38(1):46-55.

5. Mawhinney C, Jones H, Low DA, Green DJ, Howatson G, Gregson W. Influence of cold-water immersion on limb blood flow after resistance exercise. *Eur J Sport Sci*. 2017;17(5):519-29.

6. Mawhinney C, Low DA, Jones H, Green DJ, Costello JT, Gregson W. Cold Water Mediates Greater Reductions in Limb Blood Flow than Whole Body Cryotherapy. *Med Sci Sports Exerc*. 2017;49(6):1252-60.

7. Vaile J, O'Hagan C, Stefanovic B, Walker M, Gill N, Askew CD. Effect of cold water immersion on repeated cycling performance and limb blood flow. *Br J Sports Med*. 2011;45(10):825-9.

8. Hohenauer E, Costello JT, Stoop R et al. Cold-water or partial-body cryotherapy? Comparison of physiological responses and recovery following muscle damage. *Scand J Med Sci Sports*. 2018;28(3):1252-62.

9. Ihsan M, Watson G, Lipski M, Abbiss CR. Influence of postexercise cooling on muscle oxygenation and blood volume changes. *Med Sci Sports Exerc*. 2013;45(5):876-82.

10. Mawhinney C, Heinonen I, Low DA et al. Changes in quadriceps femoris muscle perfusion following different degrees of cold-water immersion. *J Appl Physiol (1985)*. 2020;128(5):1392-401.

11. Gregson W, Black MA, Jones H et al. Influence of cold water immersion on limb and cutaneous blood flow at rest. *Am J Sports Med*. 2011;39(6):1316-23.

12. Mawhinney C, Jones H, Joo CH, Low DA, Green DJ, Gregson W. Influence of cold-water immersion on limb and cutaneous blood flow after exercise. *Med Sci Sports Exerc*. 2013;45(12):2277-85.

13. Moynes J, Bentley RF, Bravo M, Kellawan JM, Tschakovsky ME. Persistence of functional sympatholysis post-exercise in human skeletal muscle. *Front Physiol*. 2013;4:131.

14. Remensnyder JP, Mitchell JH, Sarnoff SJ. Functional sympatholysis during muscular activity. Observations on influence of carotid sinus on oxygen uptake. *Circ Res*. 1962;11:370-80.

15. Wilson TE, Cui J, Crandall CG. Effect of whole-body and local heating on cutaneous vasoconstrictor responses in humans. *Auton Neurosci*. 2002;97(2):122-8.

16. Bacchetti P. Current sample size conventions: flaws, harms, and alternatives. *BMC Med*. 2010;8:17.

17. Yeung SS, Ting KH, Hon M et al. Effects of Cold Water Immersion on Muscle Oxygenation During Repeated Bouts of Fatiguing Exercise: A Randomized Controlled Study. *Medicine (Baltimore)*. 2016;95(1):e2455.

18. Taves DR. Minimization: a new method of assigning patients to treatment and control groups. *Clin Pharmacol Ther*. 1974;15(5):443-53.

19. Stewart A, Marfell-Jones M, Olds T, De Ridder H. *International Standards for Anthropometric Assessment.* Potchefstroom, South Africa: The International Society for the Advancement of Kinanthropometry; 2011.

20. Martin AD, Spenst LF, Drinkwater DT, Clarys JP. Anthropometric estimation of muscle mass in men. *Med Sci Sports Exerc*. 1990;22(5):729-33.

21. Jackson AS, Pollock ML, Gettman LR. Intertester reliability of selected skinfold and circumference measurements and percent fat estimates. *Res Q*. 1978;49(4):546-51.

22. Siri WE. The gross composition of the body. *Adv Biol Med Phys*. 1956;4:239-80.

23. American College of Sports Medicine, Sawka MN, Burke LM et al. American College of Sports Medicine position stand. Exercise and fluid replacement. *Med Sci Sports Exerc*. 2007;39(2):377-90.

24. Borg G. Borg's perceived exertion and pain scales. Champaign, IL, Human Kinetics; 1998.

25. Ramanathan NL. A New Weighting System for Mean Surface Temperature of the Human Body. *J Appl Physiol*. 1964;19:531-3.

26. Enwemeka CS, Allen C, Avila P, Bina J, Konrade J, Munns S. Soft tissue thermodynamics before, during, and after cold pack therapy. *Med Sci Sports Exerc*. 2002;34(1):45-50.

27. Bailey TG, Cable NT, Aziz N et al. Exercise training reduces the acute physiological severity of post-menopausal hot flushes. *J Physiol*. 2016;594(3):657-67.

28. Hooper R, Forbes A, Hemming K, Takeda A, Beresford L. Analysis of cluster randomised trials with an assessment of outcome at baseline. *BMJ*. 2018;360:k1121.

29. Schabenberger O. Mixed model influence diagnostics. In: *Proceedings of the twenty-ninth annual SAS Users Group International Conference*. 2004: Cary (NC). p. 189-229.

30. Heinonen I, Saltin B, Kemppainen J et al. Skeletal muscle blood flow and oxygen uptake at rest and during exercise in humans: a pet study with nitric oxide and cyclooxygenase inhibition. *Am J Physiol Heart Circ Physiol*. 2011;300(4):H1510-7.

31. Rothman KJ. No adjustments are needed for multiple comparisons. Epidemiology. 1990;1(1):43-6.

32. Cook JA JS, Sones W, Hampson LV et al. DELTA(2) guidance on choosing the target difference and undertaking and reporting the sample size calculation for a randomised controlled trial. *Trials*. 2018;19:606.

33. Bowman AW. Graphics for uncertainty. *J R Stat Soc Ser A Stat Soc*. 2019;182:403-18.

34. Amrhein V TD, Greenland S. Inferential statistics as descriptive statistics: there is no replication crisis if we don’t expect replication. *Am Stat*. 2019;73:262-70.

35. Clark MG, Rattigan S, Clerk LH et al. Nutritive and non-nutritive blood flow: rest and exercise. *Acta Physiol Scand*. 2000;168(4):519-30.

496 36. Heinonen I, Kemppainen J, Kaskinoro K et al. Comparison of exogenous adenosine and
497 voluntary exercise on human skeletal muscle perfusion and perfusion heterogeneity. *J Appl*
498 *Physiol (1985)*. 2010;108(2):378-86.
499 37. Radegran G. Limb and skeletal muscle blood flow measurements at rest and during exercise
500 in human subjects. *Proc Nutr Soc*. 1999;58(4):887-98.
501 38. Delp MD, O'Leary DS. Integrative control of the skeletal muscle microcirculation in the
502 maintenance of arterial pressure during exercise. *J Appl Physiol (1985)*. 2004;97(3):1112-8.
503 39. Altman DG, Bland JM. Absence of evidence is not evidence of absence. *BMJ*.
504 1995;311(7003):485.
505 40. Johnson MA, Polgar J, Weightman D, Appleton D. Data on the distribution of fibre types
506 in thirty-six human muscles. An autopsy study. *J Neurol Sci*. 1973;18(1):111-29.
507 41. Meigal A. Gross and fine neuromuscular performance at cold shivering. *Int J Circumpolar*
508 *Health*. 2002;61(2):163-72.
509 42. Alexander G, Bell AW, Setchell BP. Regional distribution of cardiac output in young lambs:
510 effect of cold exposure and treatment with catecholamines. *J Physiol*. 1972;220(3):511-28.
511 43. Murrant CL, Sarelius IH. Local and remote arteriolar dilations initiated by skeletal muscle
512 contraction. *Am J Physiol Heart Circ Physiol*. 2000;279(5):H2285-94.
513 44. Kwiecien SY, McHugh MP, Howatson G. Don't Lose Your Cool With Cryotherapy: The
514 Application of Phase Change Material for Prolonged Cooling in Athletic Recovery and Beyond.
515 *Front Sports Act Living*. 2020;2:118.
516 45. Tipton MJ, Collier N, Massey H, Corbett J, Harper M. Cold water immersion: kill or cure?
517 *Exp Physiol*. 2017;102(11):1335-55.
518 46. Wilcock IM, Cronin JB, Hing WA. Physiological response to water immersion: a method
519 for sport recovery? *Sports Med*. 2006;36(9):747-65.

47. Barcroft H, Edholm OG. The effect of temperature on blood flow and deep temperature in the human forearm. *J Physiol*. 1943;102(1):5-20.

48. Blondin DP, Labbe SM, Phoenix S et al. Contributions of white and brown adipose tissues and skeletal muscles to acute cold-induced metabolic responses in healthy men. *J Physiol*. 2015;593(3):701-14.

49. Bland JM. The tyranny of power: is there a better way to calculate sample size? *BMJ*. 2009;339:b3985.

50. Senn S, Rolfe K, Julious SA. Investigating variability in patient response to treatment--a case study from a replicate cross-over study. *Stat Methods Med Res*. 2011;20(6):657-66.

Figure captions

Figure 1. The mean difference (Δ) in global quadriceps muscle perfusion between the 8°C, 22°C and control conditions (*n* = 10 per condition; mean ± 95% confidence interval (CI)). Clinical relevance was assessed against a minimal clinically important difference in muscle perfusion of ± 0.75 mL·100g·min^{-1} (dashed lines). The colour intensity of the density strip represents the degree of uncertainty around the point estimate for the mean effect.

Figure 2. The mean difference (Δ) in individual muscle perfusion between a) 8°C versus 22°C; b) 8°C versus control; and c) 22°C versus control conditions (*n* = 10 per condition; mean ± 95% confidence interval (CI)). Clinical relevance was assessed against a minimal clinically important difference in muscle perfusion of ± 0.75 mL·100g·min^{-1} (dashed lines). The colour intensity of the density strip represents the relative frequency of the data. The colour intensity of the density strip represents the uncertainty around the point estimate for the mean effect.

Figure 3. The mean percentage difference in a) thigh and b) calf cutaneous vascular conductance, between the 8°C, 22°C and control conditions (*n* = 10 per condition; mean ± 95% confidence interval (CI)). Clinical relevance was assessed against a minimal clinically important difference in muscle perfusion of ± 19% (dashed lines). The colour intensity of the density strip represents the uncertainty around the point estimate for the mean effect.

Figure 4. The mean difference (Δ) in muscle temperature at a depth of a) 1 cm, b) 2 cm, and c) 3 cm, between the 8°C, 22°C and control conditions (*n* = 10 per condition; mean ± 95% confidence interval (CI)). Non-zero overlap of the 95%CI for the mean represents clear difference between conditions. The colour intensity of the density strip represents the uncertainty around the point estimate for the mean effect.

Figure 5. The mean difference (Δ) in the secondary outcome variables of core temperature, thigh temperature, mean skin temperature and mean body temperature between the between the 8°C, 22°C and control conditions (*n* = 10 per condition; mean ± 95% confidence interval (CI)). Non-zero overlap of the 95%CI for the mean represents clear difference between conditions. The colour intensity of the density strip represents the uncertainty around the point estimate for the mean effect.