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Passive Heat Therapy in Sedentary Humans Increases Skeletal Muscle Capillarisation and eNOS Content but Not Mitochondrial Density or GLUT4 Content

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Short Title: Passive Heating on Muscle Microvascular

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Abstract

Passive heat therapy (PHT) has been proposed as an alternative intervention to moderate intensity continous training (MICT) in individuals who are unable or unwilling to exercise. This study aimed to make the first comparison of the effect of PHT and MICT on 1) skeletal muscle capillarisation and endothelial specific eNOS content and 2) mitochondrial density, GLUT4 and IMTG content. Twenty young sedentary males (21±1years, BMI 25±1kg.m^{-2}) were allocated to either 6-weeks of PHT (n=10; 40-50min at 40°C in a heat chamber, 3x/wk) or MICT (n=10; time matched cycling at ~65% VO_{2peak}). Muscle biopsies were taken from the vastus lateralis muscle pre- and post-training. Immunofluorescence microscopy was used to assess changes in skeletal muscle mitochondrial density (mitochondrial marker COXIV), GLUT4 and IMTG content, capillarisation and endothelial specific eNOS content. VO_{2peak} and whole body insulin sensitivity were also assessed. PHT and MICT both increased capillary density (PHT 21%; MICT 12%) and capillary–fibre perimeter exchange index (PHT 15%; MICT 12%)(P<0.05), and endothelial specific eNOS content (PHT 8%; MICT 12%, P<0.05). However, unlike MICT (mitochondrial density 40%; GLUT4 14%; IMTG content 70%: P<0.05) PHT did not increase mitochondrial density (11%, P=0.443), GLUT4 (7%, P=0.217) or IMTG content (1%, P=0.957). Both intervention improved aerobic capacity (PHT 5%; MICT 7%) and whole body insulin sensitivity (PHT 15%; MICT 36%, P<0.05). 6 weeks PHT in young sedentary males increases skeletal muscle capillarisation and eNOS content to a similar extent as MICT, however, unlike MICT PHT does not affect skeletal muscle mitochondrial density, GLUT4 or IMTG content.

Keywords

Aerobic capacity, capillary density, endothelial nitric oxide synthase, insulin sensitivity, passive heating

News and Noteworthy

The effect of 6 weeks passive heat therapy (PHT) compared to moderate intensity continous training (MICT) was investigated in young sedentary males. PHT induced similar increases in skeletal muscle capillarisation and endothelial specific eNOS content to MICT. Unlike MICT, PHT did not improve skeletal muscle mitochondrial density, GLUT4 or IMTG content. These microvascular adaptations were paralleled by improvements in VO_{2peak} and insulin sensitivity, suggesting that microvascular adaptations may contribute to functional improvements following PHT.

Introduction

The prevalence of chronic inactivity-related diseases including obesity, insulin resistance, type 2 diabetes mellitus and cardiovascular disease has reached global epidemic proportions (9). Conversely, endurance training has been shown to induce a number of adaptations which delay or in many cases prevent health burdens (8). Adaptations within a number of tissues are important for the beneficial effects of endurance training, but skeletal muscle and it associated microvasculature in particular show extraordinary plasticity in response to training (27, 30, 31). As skeletal muscle comprises ~ 40% of body mass, improvements within this tissue not only improve local function, but also strongly contribute to improvements in whole-body metabolic health and performance.

Endurance training leads to a number of basic functional and structural adaptations within the skeletal muscle myocyte and its microvasculature which contribute to sustained health throughout the lifespan. Firstly, endurance training has been shown to stimulate mitochondrial biogenesis (25, 35). Importantly, mitochondrial content has been linked to the development of metabolic impairments and type 2 diabetes (29). Secondly, endurance training leads to robust increases in skeletal muscle GLUT4 (glucose transporter 4) content (20, 33). As skeletal muscle is the primary site for insulin dependent glucose uptake (36, 60) increased skeletal muscle GLUT4 content is likely to improve whole body glycemic control. Endurance training has also been shown to increase intra-muscular triglyceride (IMTG) content within skeletal muscle (50, 52). It is hypothesized that the large regularly used IMTG stores in trained individuals (52, 56) act as a sink for free fatty acids transported into the muscle, protecting trained individuals against the formation of lipid intermediates known to induce insulin resistance (12, 51). Finally, endurance training is well known to increase skeletal muscle capillary density (13, 14) and skeletal muscle microvascular eNOS content (13, 14). A growing body of literature suggests that reductions in skeletal muscle capillarization (23), and impairments in the vasodilatory responsiveness of the muscle microvasculature to physiological stimuli (insulin, and shear stress), are instrumental in the development of functional impairments and chronic disease (6, 19, 21, 59).

However, there are a number of patient groups who find traditional endurance training difficult to achieve, for example, spinal cord injured, heart failure, frail elderly and diabetic patients. Therefore, alternative therapeutic interventions that can be used in combination with or prior to effective exercise programs are needed. However, it is

essential that such methods elicit similar skeletal muscle adaptions to exercise. Passive heat therapy (PHT) using hot baths or saunas may be such an intervention, with a recent prospective cohort study showing that increased sauna use reduced all-cause mortality (41). Importantly, work in C2C12 myotubes has shown that heat stress (40ºC, 60 min/day, 5 days) induces mitochondrial biogenesis (42). Recent work has also demonstrated that PHT improves brachial artery endothelial function (5) and cutaneous microvascular function in sedentary humans (11), via improved nitric oxide-dependent dilation. However, to date no studies have investigated the effects of PHT on skeletal muscle adaptations in humans.

The mains aims of current study were two-fold. Firstly, we sought to investigate whether 6 weeks (3 sessions/wk) of PHT induces similar increases in skeletal muscle mitochondrial density, GLUT4 and IMTG content to moderate intensity continuous exercise training (MICT) in sedentary young males. Secondly, we aimed to investigate the effect of MICT and PHT on skeletal muscle capillarisation and endothelial specific eNOS content. We hypothesised that PHT would induce similar adaptations within the skeletal muscle myocyte and associated microvasculature compared to MICT.

Materials and methods

Participants and Ethical Approval

Twenty young sedentary males (defined as performing less than 150 minutes of organised exercise per week; in sports clubs, university or commercial gyms or sports classes) participated in the study (Table 1.). Participants were randomised to either

moderate intensity continuous training (MICT) or passive heat therapy (PHT) groups, in a matched fashion based on age, BMI and VO_{2peak} (n=10). All participants gave written informed consent to a protocol adhering to the Declaration of Helsinki and approved by the Institutional Ethics Committee.

Experimental Protocol

Participants first completed an incremental exercise test to exhaustion on an electromagnetically braked cycle ergometer to determine VO_{2peak} (14), using an online gas collection system (Oxycon Pro, Viasys, Wurzberg, Germany). VO_{2peak} corresponded to the highest value achieved over a 10 second period.

Three to 7 days following the incremental exercise test participants attended pre-intervention testing. Following an overnight fast, and having refrained from exercise and alcohol consumption the day before testing, a resting muscle biopsy was taken, followed by a 2h oral glucose tolerance test (OGTT).

Participants then underwent 6 weeks of either MICT or PHT. Post-intervention VO_{2peak} was assessed the day before the final MICT/ PHT session. A minimum of 48 hours after the final MICT or PHT session post-intervention testing was conducted using methods and timings identical in all respects to pre-intervention testing.

Muscle Biopsy

A resting muscle biopsy was taken from the lateral portion of the *m. vastus lateralis* using the conchotome biopsy technique under local anaesthesia (0.5% Marcaine). Samples were embedded in Tissue-Tek OCT Compound (Sakura Finetek Europe,

Zoeterwoude, Netherlands) and immediately frozen in liquid nitrogen-cooled isopentane (Sigma-Aldrich, Dorset, UK). Samples were then stored at –80°C until analysis.

Oral Glucose Tolerance Test

Following insertion of a cannula into the antecubital vein a resting 15ml blood sample was taken. Participants then completed a 2 hour OGTT (14). Briefly, samples (5ml) were collected 30, 60, 90 and 120 minutes after the consumption of an oral glucose load (75g glucose made up to 300ml in water). Plasma was separated by centrifugation (10 min at 1800g at 4°C) and stored at −80°C until analysis. Plasma insulin concentrations were determined by enzyme linked immuno-sorbent assay (ELISA), using a commercially available kit (Invitrogen, UK). Plasma glucose concentrations were analysed using an automated analyser (Randox Laboratories, Antrim, UK). Area under the curve (AUC) for insulin and glucose during the OGTT and the insulin sensitivity index (ISI) Matsuda were calculated as described by Matsuda and DeFronzo (44).

Intervention

MICT/ PHT programmes were initiated approx. 48 hours after pre-intervention testing. Participants in the MICT group completed 40-50 minutes of cycling on an electromagnetically braked cycle ergometer at an intensity eliciting approx. 65% VO_{2peak}, 3 times per week. Therefore, by the final weeks of the intervention participants completed 150 minutes of MICT, in line with World Health Organisation guidelines for physical activity (61). Participants in the PHT group rested in a heat chamber at 40°C and approx. 40% humidity. PHT was time matched to the MICT intervention, with

participants completing 3 sessions per week of 40-50 minutes. All MICT and PHT sessions were supervised.

Core and skin temperature were monitored on the first and last training sessions of the 6 week intervention. Mean skin temperature (T_{sk}) was obtained from the area weighted average of 4 regional temperatures measured using thermocouples (Squirrel Data Logger, Grant Instruments, Cambridge, UK) taped to the calf, lateral thigh, upper arm and chest (48). Core body temperature (T_{core}) was measured using a sterile rectal thermistor probe (Grant Instruments, Cambridge, U.K), inserted approx. 10cm past the anal sphincter. Mean body temperature (T_{Body}) was calculated as follows (17):

$$T_{Body} = 0.8 \times T_{core} + 0.2 \times T_{sk}$$

Immunoflurescence microscopy

Details of the specific quantification techniques can be found below and all techniques have been described in detail previously, including antibody specificity experiments (10, 14, 15, 51, 52). All techniques used frozen muscle biopsy samples, orientated to provide cross sections, cryosectioned to a thickness of 5μm onto uncoated glass microscope slides. For eNOS content, eNOS ser[1177] phosphorylation, GLUT4 content and capillarisation measures sections were fixed in acetone and ethanol (3:1) for 5 minutes. For mitochondrial density and IMTG analysis sections were fixed in 3.7% formaldehyde for 1 hour, rinsed briefly (3 x 30s) in deionized water, and permeabilized in 0.5% Triton-X 100 for 5 minutes. Subsequently, slides underwent incubation with appropriate primary antibodies against OXPhos Complex IV (Invitrogen, Paisley, UK), eNOS (Transduction Laboratories, Lexington, KY, USA), eNOS ser[1177] (Cell Signalling Technology, Beverly, MA, USA) or GLUT4 (Abcam, Cambridge, UK). Muscle fibre type (used during analysis of mitochondrial density, IMTG content and capillarisation) was

determined using an anti-myosin antibody for slow twitch fibres (A4.840 was deposited to the developmental studies hybridoma bank (DSHB) by Blau, H.M. (DSHB Hybridoma Product A4.840)). Following primary antibody incubation sections were incubated in appropriate secondary antibodies and UEA-I-FITC (Sigma-Aldrich, UK) (eNOS content and phosphorylation and capillarisation) and/ or wheat germ agglutinin-350 (WGA-350; Invitrogen) (mitochondrial density, GLUT4 content, IMTG content and capillarisation) as markers of the endothelium and plasma membrane, respectively. Finally, for IMTG visualisation sections were incubated with Bodipy (Sigma-Aldrich).

Images for mitochondrial density and capillarisation were acquired using a Lecia DM6000FS widefield microscope and 40x 0.6NA objective. Images for eNOS, eNOS ser^{1177} phosphorylation, GLUT4 and IMTG content were acquired using an inverted confocal microscope (Zeiss LSM-710, Carl Zeiss, Germany) with a 40x 1.3NA oil immersion objective. Identical settings were used for all image capture within each participant. All image analysis was performed using ImagePro 5.1 Software (Media Cybernetics Inc, Bethesda, MD, US).

Image Analysis

Mitochondrial density and GLUT4 content

Mitochondrial density (measured through COXIV protein expression, a mitochondrial marker which has previously been shown to correlate with mitochondrial fractional area (40)) and GLUT4 content were assessed using the methods described by Shepherd et al. (52) and Bradley et al. (10), respectively. Briefly, fluorescence intensity was quantified by measuring the signal intensity within the intracellular regions of a mask created by the WGA-350 stain in a fibre type specific manner. 106±7

fibres (43±3 type 1 fibres; 63±5 type 2 fibres) and 89±5 fibres were assessed per participant for mitochondria and GLUT4, respectively.

IMTG Analysis

Fibre type specific IMTG analysis was assessed using the method described in Shepherd et al (51). Briefly, an intensity threshold was uniformly selected to represent a positive signal for IMTG. IMTG content was expressed as the positively stained area fraction relative to the total area of each muscle fibre. The area of individual lipid droplets (IMTG objects) was used as a measure of size. Lipid droplet density was calculated as the number of IMTG objects relative to area. 104±6 fibres (49±4 type 1 fibres; 54±5 type 2 fibres) were assessed per participant.

eNOS content and ser^{1177} phosphorylation

Quantitative Immunoflurescence microscopy was used to assess eNOS content and ser^{1177} phosphorylation as described by Cocks et al (15). Briefly, the endothelial (UEA-I-FITC) outline was overlaid onto corresponding eNOS images. Fluorescence intensity of the eNOS signal was then quantified within the endothelial specific area. The CV for eNOS and eNOS ser^{1177} assays has previously been shown to be 7% (15). 87±4 vessels were assessed per participant.

Capillarisation

Capillaries were quantified in a fibre type specific manner manually, using the UEA-I, WGA-350 and myosin heavy chain images. Capillary density (CD) was measured as a global measure of capillarity proportional to the volume of each muscle fiber. The following fibre type specific indices of capillarity were also measured 1) capillary

contacts (CC), 2) sharing factor, 3) capillary-to-fibre ratio on an individual-fibre basis (C:FI), and 4) capillary–fibre perimeter exchange (CFPE) index. For a detailed description of the indices measured readers are encouraged to see the work of Hepple (28). Briefly, capillary contacts (CC) refers to the number of capillaries around a muscle fiber. Sharing factor calculates the number of muscle fibers a capillary is in contact with. Capillary-to-fibre ratio on an individual-fibre basis (C/FI) is the sum of the fractional contribution of each capillary around a fiber, based on the number of fibers sharing each capillary (i.e., the sharing factor) (see Fig. 1 for description). Capillary–fibre perimeter exchange (CFPE) index was then calculated as the quotient of C/FI and fiber perimeter for each fiber, allowing capillarity to be measured proportional to the 3-dimensional surface area of each muscle fiber. 35±2 fibres (16±1 type 1 fibres; 19±1 type 2 fibres) were assessed per participant.

Statistics

Mitochondrial density, IMTG content, capillary contacts, capillary-to-fibre ratio on an individual-fibre basis, capillary–fibre perimeter exchange, fibre cross-sectional area and perimeter were analysed using a three-way mixed ANOVA, with the between-group factor being 'group' (MICT versus PHT) and within-group factors 'intervention status' (pre-versus post-intervention) and 'fibre type' (type I versus type II). T_{Body}, T_{core} and T_{sk} were analysed using a three-way mixed ANOVA, with the between-group factor being 'group' (MICT versus PHT) and within-group factors 'intervention status' (pre-versus post-intervention) and 'time' (pre-versus post acute MICT/ PHT session). All other variables were analysed using a two-way mixed ANOVA, with the between-group factor being 'group' (MICT versus PHT) and repeated factor being 'intervention status' (pre- versus post-intervention). In the case of a significant interaction a

Bonferroni post hoc test was applied to locate the differences. All analyses were performed using statistical analysis software (SPSS for windows version 16.0 (SPSS,Chicago,IL,USA)). Significance was set at $P < 0.05$. Data are presented as means ± SEM. The primary aim of the study was to compare the effects of PHT and MICT on skeletal muscle capillarisation and microvascular eNOS content. The study was powered to detect between-group (PHT versus MICT) differences in these variables in response to training. G∗Power 3.1 software (G∗Power Software Inc., Kiel, Germany) was used to calculate the required sample size. The study was designed to detect a between-group effect of f=0.30, representative of a medium-sized effect (16), adopting an alpha of 0.05 and power of 0.80. An f of 0.30 was deemed to be a physiologically relevant difference, as the authors have previously observed an effect of this size following 6 weeks of sprint interval training and MICT in sedentary males (14).

Results

Temperature changes during training

There was no difference in mean skin temperature from the first to the last intervention sessions (main effect of intervention P=0.266). Mean skin temperature was significantly higher during PHT than MICT (between group difference P<0.001). Mean skin temperature increased during the sessions (main effect of time (pre-versus post acute MICT/ PHT session) P<0.001), however, a significant group x time interaction was also observed (P <0.001; Fig. 2A). When this interaction was explored participants in the PHT group had significantly higher mean skin temperatures than those doing

MICT, both pre- (P=0.01) and post-session (P<0.001). However, mean skin temperature increased from pre- to post-session in both MICT (P=0.001) and PHT (P<0.001) groups.

The interventions had no effect on core body temperature (main effect of intervention P=0.286). Core body temperature was higher during MICT than PHT (between group difference P=0.021). Core body temperature increased during the sessions (main effect of time (pre-versus post acute MICT/ PHT session) P<0.001), however, a significant group x time interaction was also observed (P<0.001; Fig. 2B). When this was investigated core body temperature was not different between MICT and PHT pre-session (P=0.410), but was significantly higher post-session in participants completing MICT compared to PHT (P<0.001). During the sessions core body temperature was increased by MICT (P<0.001), but not by PHT (P=0.299).

The interventions had no effect on mean body temperature (main effect of intervention P=0.217). There was a significant main effect of time (pre-versus post acute MICT/ PHT session) on mean body temperature (P<0.001; Fig. 2C), with mean body temperature increasing during the MICT/ PHT sessions. There was no difference between groups in mean body temperature.

Intervention effect

No differences were observed between groups at baseline for any of the variables. The interventions increased both absolute (MICT 7%, PHT 6%) and relative (MICT 7%, PHT 5%) VO_{2peak} with a main effect of intervention (absolute P=0.01, relative P=0.01; Table 1.), and no difference between groups. The Matsuda insulin sensitivity

index was also improved following the interventions (MICT 36%, PHT 15%; main effect of intervention P=0.005; Table 1.), with no difference between groups or group x intervention interaction (P=0.161). Glucose area under the curve (AUC) was reduced by the interventions (MICT 18%, PHT 7%; main effect of interventions P=0.001; Table 1.), with no difference between groups. Neither training mode reduced insulin AUC (P=0.833; Table 1.).

Skeletal Muscle Microvascular Adaptations

Both MICT and PHT increased eNOS content (MICT 12%, PHT 8%) with a main effect of intervention (P<0.001; Fig. 3C), and no differences between the interventions. In accordance, eNOS ser^{1177} phosphorylation increased following the interventions (MICT 7%, PHT 4%; main effect of intervention P=0.05; Fig. 3D), with no difference between groups. However, when eNOS phosphorylation was expressed relative to eNOS content there was a significant decrease in eNOS ser^{1177} phosphorylation (MICT -7%, PHT -3%, main effect of intervention P=0.045; Fig. 3E), with no difference between groups.

Type II fibres had a significantly larger fibre area and perimeter than type I fibres (main effect of fibre type P=0.014 and P=0.001, respectively), but neither fibre area nor perimeter were altered by the interventions (fibre area P=0.506; perimeter P=0.308). Capillary density was increased 11% following MICT and 21% following PHT (main effect of intervention P=0.015; Fig. 3B), with no difference between groups. Capillary-to-fibre ratio and capillary-fibre perimeter exchange index were both higher in type I fibres than type II fibres (main effect of fibre type P=0.048 and P<0.001, respectively), irrespective of training status. There was no significant difference between fibre types

for capillary contacts, however, there was a trend for increased capillary contacts in type I fibres compared to type II fibres (main effect of fibre type *P*=0.071). Capillary–fibre perimeter exchange index was increased by 12% following MICT and 15% following PHT with a main effect of intervention (*P*=0.012), and no differences between groups. Capillary contacts and capillary-to-fibre ratio were also increased following the interventions (Capillary contacts, MICT 9%, PHT 12%, *P* =0.049; capillary-to-fibre ratio, MICT 10%, PHT 12% *P*=0.025), with no difference between groups. Data are presented in Table 2.

Myocyte Adaptations

COXIV protein expression (fluorescence intensity), representing mitochondrial density, was greater in type I fibres than type II fibres (main effect of fibre type *P*<0.001). Mitochondrial density increased following the interventions (main effect of intervention *P*=0.003). However, a significant group x intervention interaction was also observed (*P*=0.035; Fig. 4E). When this interaction was explored mitochondrial density was increased following MICT (40%; *P*<0.001), but not PHT (11%; *P*=0.443). No significant differences in mitochondrial density existed between interventions.

GLUT4 content indicated by fluorescence intensity, was unchanged by the interventions (main effect of interventions *P*=0.256). However, a significant interaction was also observed (group x intervention interaction *P*=0.008; Fig. 4D). When within group differences were examined GLUT4 content was significantly increased following MICT (*P*=0.009), but no changes were observed following PHT (*P*=0.217). No significant differences in GLUT4 content existed between the interventions.

IMTG content (expressed as percentage area stained), lipid droplet size and density were significantly greater in type I fibres compared to type II fibres (main effect of fibre type $P<0.001$). IMTG content was unchanged by the interventions (main effect of intervention $P=0.063$). However, a significant group x intervention interaction was also observed (P=0.050; Fig. 4F), with IMTG content significantly increasing following MICT ($P=0.01$), but not following PHT ($P=0.957$). No significant differences in IMTG content were observed between interventions.

This finding was attributed to a significant increase in lipid droplet density following MICT as the interventions significantly increased lipid droplet density (main effect of intervention $P<0.05$). However, as above a significant group x intervention interaction was also observed ($P<0.05$). When this was investigated IMTG density was increased following MICT (pre-training type I 0.041±0.006µm^2 vs. post-training type I 0.069±0.004µm^2; pre-training type II 0.021±0.003µm^2 vs. post-training type II 0.032±0.004µm^2; $P<0.05$), but not PHT (pre-training type I 0.059±0.007µm^2 vs. post-training type I 0.062±0.008µm^2; pre-training type II 0.029±0.005µm^2 vs. post-training type II 0.028±0.004µm^2;$P=0.806$). No significant differences in IMTG density were observed between the interventions. IMTG size was unchanged by the interventions (main effect of intervention $P=0.765$), with no difference between interventions.

Discussion

The primary finding of this study is that 6 weeks of PHT increased skeletal muscle capillarisation and endothelial specific eNOS content to a similar extent as a time-

matched traditional MICT intervention in young sedentary males. However, unlike traditional MICT 6 weeks of PHT did not induce increases in skeletal muscle mitochondrial density, GLUT4 or IMTG content. Importantly these microvascular adaptations were paralleled by improvements in maximum aerobic capacity and whole body insulin sensitivity. These results add to the growing body of literature that supports PHT as a potential intervention to induce health benefits, although the data suggests improvements within the skeletal muscle microvasculature rather than myocyte are the primary adaptation.

Skeletal Muscle Microvascular Adaptations

This is the first study to investigate the effect of PHT on skeletal muscle microvascular adaptations in humans. The data suggests that PHT induces similar increases in capillarisation and endothelial specific eNOS content to traditional MICT, with the results indicating a similar magnitude of change to previous studies investigating these microvascular adaptations following MICT in young healthy individuals (4, 13, 14). The findings extend recent work by Kuhlenhoelter et al. (39) showing that acute exposure to lower body heating or local thigh heating (water circulating garment at 48-52°C) increase expression of key angiogenic factors associated with capillary growth, including VEGF and angiopoietin 2, in human skeletal muscle. It also confirms previous work in animals showing that PHT, through hot water immersion or sauna therapy, increases skeletal muscle capillarisation and eNOS expression (45, 46).

Recent *in vitro* studies and work utilising animal models have proposed a primary role for increased expression of HSPs mediating the increase in eNOS content and angiogenesis following PHT. Inhibition of HSP90 using 17-AAG in human umbilical

vein endothelial cells (HUVECs) has been shown to attenuate the expression of eNOS, likely through suppression of eNOS gene transcription rather than any effect on eNOS mRNA stability (54). Treatment with 17-AAG also inhibited endothelial cell migration under basal and VEGF stimulated conditions. The study also showed that the inhibitory effect of 17-AAG on angiogenesis was nitric oxide (NO) mediated, suggesting a role for the increased eNOS content in this study in the increased capillarisation observed. In a similar *in vitro* study Shiota et al. (53) showed that inhibition of HSP70, using KNK437, significantly decreased VEGF induced cell migration and tube formation in HUVECs. Finally, inhibition of HSP90 using 17-AAG has been shown to attenuate the beneficial effects of sauna therapy (Waon therapy using a far infrared dry sauna system at 61°C) on capillarisation in apolipoprotein E-deficient mice (45). Interestingly, the effect of a similar sauna therapy (far infrared dry sauna system at 41°C) on capillarisation was shown to be dependent on eNOS mediated NO production in apolipoprotein E-deficient mice (1). Importantly, Kuhlenhoelter *et al.* (39) have recently shown that PHT, through lower body heating or local thigh heating using a water circulating garment at 48-52°C, increases expression of HSPs in human skeletal muscle, including members of the HSP90 and HSP70 families. Another potential mechanism for the increased capillarisation and eNOS content observed could be an increase in skeletal muscle blood flow and shear stress during PHT. It has been shown that NO dependent increases in shear stress are a key stimulus for angiogenesis (34). A number of studies have shown that skeletal muscle blood flow increases with passive heat stress (26, 37, 47). Importantly, Pearson et al. (47) showed that whole-body heat stress, which increased skin but not rectal temperature (water perfused suit), induced significant increases in estimated leg tissue blood flow as well as skin blood flow. This suggests that a less extreme heating

protocol, as used in the present study, could induce significant increases in muscle blood flow.

The increase in capillarisation and endothelial specific eNOS content following PHT likely contribute to the increased aerobic capacity and insulin sensitivity observed following PHT. Increased skeletal muscle capillarisation is an established adaptation that likely contributes to the increased VO_{2peak} , increasing red blood cell transit time and decreasing diffusion distance to optimise delivery and extraction of oxygen to the working muscle (4, 7, 49). However, it should not be discounted that increases in plasma volume, not measured in the current study, could have also contributed to the increase in VO_{2peak} observed following PHT (18). In addition, pharmacological increases in angiogenesis using Prazosin (an α1-adrenergic receptor antagonist) has demonstrated the importance of capillarisation for insulin sensitivity in rats (2). Animals treated with Prazosin for 3 weeks showed a 20% increase in capillary density which resulted in a 30% increase in insulin-stimulated skeletal muscle glucose disposal, despite skeletal muscle insulin signalling being unchanged. In addition, the increase in endothelial specific eNOS content following PHT may potentially lead to greater NO production upon stimulation with insulin (59). As insulin mediated NO-dependent increases in skeletal muscle perfusion are essential for optimal glucose uptake (57, 58) the increase in eNOS content could contribute to the increase in insulin sensitivity observed. Indeed, Kubota et al. (38) demonstrated the importance of increasing eNOS content through administration of bera-prost sodium, a prostaglandin I2 analogue that increases eNOS mRNA expression and protein synthesis. This pharmacological intervention resulted in increased skeletal muscle capillary perfusion and glucose uptake in mice lacking IRS-2 in endothelial cells and fed a high-fat diet.

Skeletal Muscle Myocyte Adaptations

Contrary to our initial hypothesis, and unlike MICT, PHT did not induce improvements in mitochondrial density, GLUT4 or IMTG content. This hypothesis was driven by work in C6C12 myotubes (42) and mice (55) showing that heat stress could increase mitochondrial biogenesis. As such, it would appear that human skeletal muscle does not respond to whole-body PHT in the same way as cell models or mice, however, as discussed below the potential of more extreme heating protocols should not be discounted. In addition, the potential effect of PHT in patient groups or elderly individuals should be investigated in future studies. Interestingly, a recent study in young sedentary men and women has shown that repeated exposure to local heat stress, inducing significant increases in muscle temperature, for 6 days (2h/ day of pulsed shortwave diathermy) improved mitochondrial function, measured through mitochondrial respiratory capacity (24). However, this study did not show an increase in citrate synthase activity, a common marker of mitochondrial content.

Importantly, core temperature was not increased by the PHT intervention used in the current study (40°C and 40% humidity). Much of the previous work investigating PHT has used more extreme heating protocols resulting in significant increases in core temperature, including hot water immersion (approx. 40°C)(5, 11) and sauna bathing (>70°C and 10-20% humidity)(41). It was the research team's aim to use a less extreme protocol than previous work, that was of a similar duration to a typical exercise training session, as the experience of our group and others suggests that hot water immersion and sauna therapy can be associated with heat intolerance (3) and dizziness upon standing (32). Therefore, the current work suggests that a more

tolerable PHT intervention can induce significant improvements in skeletal muscle capillarisation and eNOS content. However, Faulkner et al. (22) have recently demonstrated that the change in extracellular Heat Shock Protein (HSP) 70 following acute hot water immersion is positively correlated with the change in mean body temperature. As discussed above elevations in HSP's could be one of the primary mechanisms behind adaptations following PHT, therefore, it cannot be discounted that the previously employed protocols could have induced greater adaptations or induced improvements within the myocyte. As such, future studies should attempt to investigate the dose response to PHT in order to establish the most effective, but tolerable intervention.

As changes in mitochondrial function, GLUT4 and IMTG content have been associated with insulin sensitivity, it was hypothesised that increases in these factors could have contributed to the increased insulin sensitivity observed in the current study. As no changes were observed within the myocyte this study may suggest that PHT induces improvements in insulin sensitivity through microvascular adaptations alone. Interestingly, although increases in whole body insulin sensitivity were statistically similar using the small sample size employed in this study, the increase following PHT was approximately half that of MICT. To confirm this hypothesis future work should investigate the effect of PHT on insulin-mediated activation of the insulin signalling cascade and GLUT4 translocation. However, if true PHT could represent an important model to investigate the effect of changes in skeletal muscle microvascular function alone on insulin sensitivity in humans. This hypothesis is added to by the recent work of Marshal et al. (43) who demonstrated that muscle specific overexpression of HSP72 induced increases in whole body and skeletal muscle glucose uptake in high fat fed

mice. However, this improvement was observed without changes in the insulin signalling cascade or mitochondrial biogenesis, again pointing to a factor outside the myocyte as responsible for the improvements observed.

Limitations

We decided not to include a control group with repeated measurements in this study for comparison. This would have strengthened the design, but reduced the feasibility of completing the study (e.g. due to costs, time demands and recruitment difficulties). The study was powered to detect a medium effect size between groups for skeletal muscle capillarisation and microvascular eNOS content, and as a result the sample size was not high enough to detect between-group differences in other variables displaying a larger variability (e.g. increases in VO_{2peak} and whole body insulin sensitivity). As such, translational studies with a larger number of participants are required before it can be concluded that PHT and MICT are similarly effective in inducing adaptation in all the variables investigated in this study. We also acknowledge that the conclusions made in this study should be restricted to previously sedentary young adult males. The study used COXIV as a marker of mitochondrial density. Although COXIV shows a significant correlation to mitochondrial fractional area, future studies should aim to use markers with a higher correlation, for example Cardiolipin or citrate synthase activity (40).

Conclusion

This study provides novel information that 6 weeks of PHT can induce similar increases in skeletal muscle capillarisation and endothelial specific eNOS content to a time matched traditional MICT intervention in previously sedentary young males.

However, unlike MICT PHT did not induce increases in skeletal muscle mitochondrial density, GLUT4 and IMTG content. In addition, it is shown that these changes in skeletal muscle microvascular capillarisation and eNOS content were paralleled by improvements in maximal aerobic capacity and insulin sensitivity, suggesting that microvascular adaptations may contribute to functional improvements following PHT in young sedentary males. Finally, the study adds to the growing body of literature that suggests PHT may be an effective alternative intervention to traditional MICT for those who are unable or unwilling to exercise at an intensity great enough to achieve significant health benefits.

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Disclosures

The authors declare that there are no conflicting interests.

References

1. **Akasaki Y, Miyata M, Eto H, Shirasawa T, Hamada N, Ikeda Y, Biro S, Otsuji Y, and Tei C**. Repeated thermal therapy up-regulates endothelial nitric oxide synthase and augments angiogenesis in a mouse model of hindlimb ischemia. *Circulation Journal* 70: 463-470, 2006.

2. **Akerstrom T, Laub L, Vedel K, Brand CL, Pedersen BK, Lindqvist AK, Wojtaszewski JF, and Hellsten Y**. Increased skeletal muscle capillarization enhances insulin sensitivity. *American Journal of Physiology-Endocrinology and Metabolism* ajpendo. 00020.02014, 2014.

3. **Amano K, Yanagihori R, and Tei C**. Waon therapy is effective as the treatment of myalgic encephalomyelitis/chronic fatigue syndrome. *The Journal of The Japanese Society of Balneology, Climatology and Physical Medicine* 78: 285-302, 2015.

4. **Andersen P, and Henriksson J**. Capillary supply of quadriceps femoris muscle of man- Adapative response to exercise. *Journal of Physiology* 270: 677-690, 1977.

5. **Bailey TG, Cable NT, Miller G, Sprung V, Low D, and Jones H**. Repeated warm water immersion induces similar cerebrovascular adaptations to 8 weeks of moderate-intensity exercise training in females. *International journal of sports medicine* 37: 757-765, 2016.

6. **Barrett EJ, Wang H, Upchurch CT, and Liu Z**. Insulin regulates its own delivery to skeletal muscle by feed-forward actions on the vasculature. *American Journal of Physiology-Endocrinology and Metabolism* 301: E252-E263, 2011.

7. **Bassett DR, and Howley ET**. Limiting factors for maximum oxygen uptake and determinants of endurance performance. *Medicine and Science in Sports and Exercise* 32: 70-84, 2000.

8. **Booth FW, Gordon SE, Carlson CJ, and Hamilton MT**. Waging war on modern chronic diseases: primary prevention through exercise biology. *Journal of applied physiology* 88: 774-787, 2000.

9. **Booth FW, and Hawley JA**. The erosion of physical activity in Western societies: an economic death march. *Diabetologia* 58: 1730-1734, 2015.

10. **Bradley H, Shaw CS, Worthington PL, Shepherd SO, Cocks M, and Wagenmakers AJ**. Quantitative immunofluorescence microscopy of subcellular GLUT4 distribution in human skeletal muscle: effects of endurance and sprint interval training. *Physiological reports* 2: e12085, 2014.

11. **Brunt VE, Eymann TM, Francisco MA, Howard MJ, and Minson CT**. Passive heat therapy improves cutaneous microvascular function in sedentary humans via improved nitric oxide-dependent dilation. *Journal of Applied Physiology* 121: 716-723, 2016.

12. **Chow LS, Mashek DG, Austin E, Eberly LE, Persson X-M, Mashek MT, Seaquist ER, and Jensen MD**. Training status diverges muscle diacylglycerol accumulation during free fatty acid elevation. *American Journal of Physiology-Endocrinology and Metabolism* 307: E124-E131, 2014.

13. **Cocks M, Shaw CS, Shepherd SO, Fisher JP, Ranasinghe A, Barker TA, and Wagenmakers AJ**. Sprint interval and moderate-intensity continuous training have equal benefits on aerobic capacity, insulin sensitivity, muscle capillarisation and endothelial eNOS/NAD (P) Hoxidase protein ratio in obese men. *The Journal of physiology* 594: 2307-2321, 2016.

14. **Cocks M, Shaw CS, Shepherd SO, Fisher JP, Ranasinghe AM, Barker TA, Tipton KD, and Wagenmakers AJM**. Sprint interval and endurance training are equally effective in increasing muscle microvascular density and eNOS content in sedentary males. *The Journal of Physiology* 591: 641-656, 2013.

15. **Cocks M, Shepherd SO, Shaw CS, Achten J, Costa ML, and Wagenmakers AJM**. Immunofluorescence Microscopy to Assess Enzymes Controlling Nitric Oxide Availability and Microvascular Blood Flow in Muscle. *Microcirculation* 19: 642-651, 2012.

16. **Cohen J**. A Power Primer. *Psychological Bulletin* 112: 155-159, 1992.

17. **Colin J, Timbal J, Houdas Y, Boutelier C, and Guieu J**. Computation of mean body temperature from rectal and skin temperatures. *Journal of Applied Physiology* 31: 484-489, 1971.

18. **Coyle E, Hopper M, and Coggan A**. Maximal oxygen uptake relative to plasma volume expansion. *International journal of sports medicine* 11: 116-119, 1990.

19. **de Jongh RT, Serne EH, Ijzerman RG, de Vries G, and Stehouwer CDA**. Impaired microvascular function in obesity - Implications for obesity-associated microangiopathy, hypertension, and insulin resistance. *Circulation* 109: 2529-2535, 2004.

20. **Dela F, Mikines KJ, Larsen JJ, and Galbo H**. Training-induced enhancement of insulin action in human skeletal muscle: the influence of aging. *The Journals of Gerontology Series A: Biological Sciences and Medical Sciences* 51: B247-B252, 1996.

21. **Doupis J, Rahangdale S, Gnardellis C, Pena SE, Malhotra A, and Veves A**. Effects of Diabetes and Obesity on Vascular Reactivity, Inflammatory Cytokines, and Growth Factors. *Obesity* 19: 729-735, 2011.

22. **Faulkner SH, Jackson S, Fatania G, and Leicht CA**. The effect of passive heating on heat shock protein 70 and interleukin-6: A possible treatment tool for metabolic diseases? *Temperature* 4: 292-304, 2017.

23. **Gavin TP, Stallings HW, Zwetsloot KA, Westerkamp LM, Ryan NA, Moore RA, Pofahl WE, and Hickner RC**. Lower capillary density but no difference in VEGF expression in obese vs. lean young skeletal muscle in humans. *Journal of Applied Physiology* 98: 315-321, 2005.

24. **Hafen PS, Preece CN, Sorensen JR, Hancock CR, and Hyldahl RDJJoAP**. Repeated exposure to heat stress induces mitochondrial adaptation in human skeletal muscle. 2018.

25. **Hawley JA**. Adaptations of skeletal muscle to prolonged, intense endurance training. *Clinical and experimental pharmacology and physiology* 29: 218-222, 2002.

26. **Heinonen I, Brothers RM, Kemppainen J, Knuuti J, Kalliokoski KK, and Crandall CG**. Local heating, but not indirect whole body heating, increases human skeletal muscle blood flow. *Journal of Applied Physiology* 111: 818-824, 2011.

27. **Hellsten Y, and Nyberg M**. Cardiovascular adaptations to exercise training. *Compr Physiol* 6: 1-32, 2015.

28. **Hepple RT**. A new measurement of tissue capillarity: The capillary-to-fibre perimeter exchange index. *Canadian Journal of Applied Physiology-Revue Canadienne De Physiologie Appliquee* 22: 11-22, 1997.

29. **Hesselink MK, Schrauwen-Hinderling V, and Schrauwen P**. Skeletal muscle mitochondria as a target to prevent or treat type 2 diabetes mellitus. *Nature Reviews Endocrinology* 12: 633-645, 2016.

30. **Hoier B, and Hellsten Y**. Exercise-Induced Capillary Growth in Human Skeletal Muscle and the Dynamics of VEGF. *Microcirculation* 21: 301-314, 2014.

31. **Hood DA, Irrcher I, Ljubicic V, and Joseph A-M**. Coordination of metabolic plasticity in skeletal muscle. *Journal of experimental biology* 209: 2265-2275, 2006.

32. **Hooper PL**. Hot-tub therapy for type 2 diabetes mellitus. *New England Journal of Medicine* 341: 924-925, 1999.

33. **Houmard JA, Egan PC, Neufer PD, Friedman JE, Wheeler WS, Israel RG, and Dohm GL**. Elevated skeletal muscle glucose transporter levels in exercise-trained middle-aged men. *American Journal of Physiology-Endocrinology And Metabolism* 261: E437-E443, 1991.

34. **Hudlicka O, Brown MD, May S, Zakrzewicz A, and Pries AR**. Changes in capillary shear stress in skeletal muscles exposed to long-term activity: Role of nitric oxide. *Microcirculation* 13: 249-259, 2006.

35. **Joseph A-M, Pilegaard H, Litvintsev A, Leick L, and Hood DA**. Control of gene expression and mitochondrial biogenesis in the muscular adaptation to endurance exercise. *Essays in Biochemistry* 42: 13-29, 2006.

36. **Katz LD, Glickman MG, Rapoport S, Ferrannini E, and DeFronzo RA**. Splanchnic and peripheral disposal of oral glucose in man. *Diabetes* 32: 675-679, 1983.

37. **Keller DM, Sander M, Stallknecht B, and Crandall CG**. α-Adrenergic vasoconstrictor responsiveness is preserved in the heated human leg. *The Journal of physiology* 588: 3799-3808, 2010.

38. **Kubota T, Kubota N, Kumagai H, Yamaguchi S, Kozono H, Takahashi T, Inoue M, Itoh S, Takamoto I, Sasako T, Kumagai K, Kawai T, Hashimoto S, Kobayashi T, Sato M, Tokuyama K, Nishimura S, Tsunoda M, Ide T, Murakami K, Yamazaki T, Ezaki O, Kawamura K, Masuda H, Moroi M, Sugi K, Oike Y, Shimokawa H, Yanagihara N, Tsutsui M, Terauchi Y, Tobe K, Nagai R, Kamata K, Inoue K, Kodama T, Ueki K, and Kadowaki T**. Impaired Insulin Signaling in Endothelial Cells Reduces Insulin-Induced Glucose Uptake by Skeletal Muscle. *Cell Metabolism* 13: 294-307, 2011.

39. **Kuhlenhoelter AM, Kim K, Neff D, Nie Y, Blaize AN, Wong BJ, Kuang S, Stout J, Song Q, and Gavin TP**. Heat therapy promotes the expression of angiogenic regulators in human skeletal muscle. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology* 311: R377-R391, 2016.

40. **Larsen S, Nielsen J, Hansen CN, Nielsen LB, Wibrand F, Stride N, Schroder HD, Boushel R, Helge JW, and Dela F**. Biomarkers of mitochondrial content in skeletal muscle of healthy young human subjects. *The Journal of physiology* 590: 3349-3360, 2012.

41. **Laukkanen T, Khan H, Zaccardi F, and Laukkanen JA**. Association between sauna bathing and fatal cardiovascular and all-cause mortality events. *JAMA internal medicine* 175: 542-548, 2015.

42. **Liu C-T, and Brooks GA**. Mild heat stress induces mitochondrial biogenesis in C2C12 myotubes. *Journal of Applied Physiology* 112: 354-361, 2012.

43. **Marshall JP, Estevez E, Kammoun HL, King EJ, Bruce CR, Drew BG, Qian H, Illiades P, Gregorevic P, and Febbraio MA**. Skeletal muscle-specific overexpression of heat shock protein 72 improves skeletal muscle insulin-stimulated

glucose uptake but does not alter whole body metabolism. *Diabetes, Obesity and Metabolism* 2018.

44. **Matsuda M, and DeFronzo RA**. Insulin sensitivity indices obtained from oral glucose tolerance testing - Comparison with the euglycemic insulin clamp. *Diabetes Care* 22: 1462-1470, 1999.

45. **Miyauchi T, Miyata M, Ikeda Y, Akasaki Y, Hamada N, Shirasawa T, Furusho Y, and Tei C**. Waon therapy upregulates Hsp90 and leads to angiogenesis through the Akt-endothelial nitric oxide synthase pathway in mouse hindlimb ischemia. *Circulation Journal* 76: 1712-1721, 2012.

46. **Morimoto Y, Kondo Y, Kataoka H, Honda Y, Kozu R, Sakamoto J, Nakano J, Origuchi T, Yoshimura T, and Okita M**. Heat Treatment Inhibits Skeletal Muscle Atrophy of Glucocorticoid-Induced Myopathy in Rats. *Physiological Research* 64: 897-905, 2015.

47. **Pearson J, Low DA, Stöhr E, Kalsi K, Ali L, Barker H, and González-Alonso J**. Hemodynamic responses to heat stress in the resting and exercising human leg: insight into the effect of temperature on skeletal muscle blood flow. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology* 300: R663-R673, 2010.

48. **Ramanathan N**. A new weighting system for mean surface temperature of the human body. *Journal of applied physiology* 19: 531-533, 1964.

49. **Saltin B**. Capacity of blood flow delivery to exercising skeletal muscle in humans. *The American journal of cardiology* 62: 30E-35E, 1988.

50. **Shepherd S, Cocks M, Meikle P, Mellett N, Ranasinghe A, Barker T, Wagenmakers A, and Shaw C**. Lipid droplet remodelling and reduced muscle

ceramides following sprint interval and moderate-intensity continuous exercise training in obese males. *International journal of obesity (2005)* 2017.

51. **Shepherd S, Strauss J, Wang Q, Dube J, Goodpaster B, Mashek D, and Chow LJTJop**. Training alters the distribution of perilipin proteins in muscle following acute free fatty acid exposure. 595: 5587-5601, 2017.

52. **Shepherd SO, Cocks M, Tipton KD, Ranasinghe AM, Barker TA, Burniston JG, Wagenmakers AJM, and Shaw CS**. Sprint interval and traditional endurance training increase net intramuscular triglyceride breakdown and expression of perilipin 2 and 5. *Journal of Physiology-London* 591: 657-675, 2013.

53. **Shiota M, Kusakabe H, Izumi Y, Hikita Y, Nakao T, Funae Y, Miura K, and Iwao H**. Heat shock cognate protein 70 is essential for Akt signaling in endothelial function. *Arteriosclerosis, thrombosis, and vascular biology* 30: 491-497, 2010.

54. **Sun J, and Liao JK**. Induction of angiogenesis by heat shock protein 90 mediated by protein kinase Akt and endothelial nitric oxide synthase. *Arteriosclerosis, thrombosis, and vascular biology* 24: 2238-2244, 2004.

55. **Tamura Y, Matsunaga Y, Masuda H, Takahashi Y, Takahashi Y, Terada S, Hoshino D, and Hatta H**. Postexercise whole body heat stress additively enhances endurance training-induced mitochondrial adaptations in mouse skeletal muscle. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology* 307: R931-R943, 2014.

56. **Tarnopolsky MA, Rennie CD, Robertshaw HA, Fedak-Tarnopolsky SN, Devries MC, and Hamadeh MJ**. Influence of endurance exercise training and sex on intramyocellular lipid and mitochondrial ultrastructure, substrate use, and mitochondrial enzyme activity. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology* 292: R1271-R1278, 2007.

57. **Vincent MA, Barrett EJ, Lindner JR, Clark MG, and Rattigan S**. Inhibiting NOS blocks microvascular recruitment and blunts muscle glucose uptake in response to insulin. *American Journal of Physiology-Endocrinology and Metabolism* 285: E123-E129, 2003.

58. **Vincent MA, Clerk LH, Lindner JR, Klibanov AL, Clark MG, Rattigan S, and Barrett EJ**. Microvascular recruitment is an early insulin effect that regulates skeletal muscle glucose uptake in vivo. *Diabetes* 53: 1418-1423, 2004.

59. **Wagenmakers AJ, Strauss JA, Shepherd SO, Keske MA, and Cocks M**. Increased muscle blood supply and transendothelial nutrient and insulin transport induced by food intake and exercise: effect of obesity and ageing. *The Journal of physiology* 594: 2207-2222, 2016.

60. **Wasserman DH, Kang L, Ayala JE, Fueger PT, and Lee-Young RS**. The physiological regulation of glucose flux into muscle in vivo. *Journal of Experimental Biology* 214: 254-262, 2011.

61. **World Health Organization**. *Global recommendations on physical activity for health*. World Health Organization, 2010.

Tables

Table 1. Participant Characteristics

Variable	MICT		PHT	
	Pre-Intervention	Post-Intervention	Pre-Intervention	Post-Intervention
Age	20±1		21±1	
Height (cm)	180±1		180±2	
Weight (kg)	79±4	79±4	80±5	80±5
BMI ($kg.m^{-2}$)	24±1	24±1	25±2	25±2
VO_{2peak} ($l.min^{-1}$)*	3.6±0.2	3.9±0.2	3.6±0.2	3.8±0.2
VO_{2peak} ($ml.kg^{-1}.min^{-1}$)*	46.4±2.7	49.5±2.8	44.8±2.3	47.0±1.7
ISI Matsuda*	117±17	159±20	103±11	119±18
AUC Glucose ($mmol\ l^{-1}\ (120min)^{-1}$)*	9157±594	7338±589	10303±560	9543±558
AUC Insulin ($mmol\ l^{-1}\ (120min)^{-1}$	5501±995	4645±1715	6750±915	7302±1580

Data provided are means ±SEM (*n*=10 per group). BMI, body mass index; MICT, moderate intensity continuous training; PHT, passive heat therapy. * $P<0.05$, main effect of intervention

Table 2. Capillarisation

Variable	MICT		PHT	
	Pre-Intervention	Post-Intervention	Pre-Intervention	Post-Intervention
Overall FA (μm^2)	4035±532	3886±456	4477±448	3943±389
Type I FA (μm^2)	3844±460	3663±462	4343±437	3708±330
Type II FA (μm^2)	4242±622	4028±501	4689±554	4247±458
Overall perimeter (μm^2)	290±19	277±14	297±17	288±18
Type I perimeter (μm^2)	281±18	267±15	289±19	277±19
Type II perimeter (μm^2)	299±23	283±15	308±19	300±19
Overall CC *	3.77±0.19	4.03±0.19	3.40±0.37	3.80±0.31
Type I CC *	3.79±0.19	4.03±0.19	3.55±0.36	3.98±0.29
Type II CC *	3.80±0.20	3.73±0.27	3.29±0.44	3.76±0.34
Overall C/FI *	1.51±0.08	1.66±0.09	1.39±0.14	1.56±0.14
Type I C/FI *	1.54±0.10	1.71±0.09	1.47±0.14	1.62±0.13
Type II C/F_I *	1.50±0.08	1.53±0.13	1.33±0.17	1.54±0.16
Overall CFPE *	5.47±0.49	6.14±0.39	4.81±0.51	5.54±0.48
Type I CFPE *	5.78±0.58	6.56±0.38	5.20±0.45	6.03±0.49
Type II CFPE *	5.29±0.45	5.59±0.48	4.48±0.63	5.15±0.49

Values are means ± SEM (*n*=10 per group). CC, capillary contacts; C/FI, capillary-to-fibre ratio on an individual fibre basis; CFPE, capillary-fibre-perimeter exchange; FA, fibre cross sectional area; MICT, moderate intensity continuous training; PHT, passive heat therapy. * P<0.05, main effect of intervention

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Figures

Figure 1. Method for calculating indices of capillarisation.

The fibre indicated has a capillary contacts (CC) of six, five of these capillaries are in contact with three muscle fibres (sharing factor of three) and one capillary is in contact with two muscle fibres (sharing factor of two). Capillary-to-fibre ratio on an individual fibre basis (C/FI) is calculated by taking the sum of these two proportions: C/FI = (5 x 1/3)+(1 x 1/2) = 2.17.

Figure 2. Mean skin temperature, core body temperature and mean body temperatures during the first and last sessions of moderate intensity continuous training (MICT) and passive heat therapy (PHT).

A. Mean skin temperature during the first and last MICT and PHT sessions. *B.* Core body temperature, measured as rectal temperature, during the first and last MICT and PHT sessions. *C.* Mean body temperature during the first and last MICT and PHT sessions. * P<0.05, main effect of time (pre-versus post acute MICT/ PHT session). ¥ P<0.05 from MICT pre-session. § P<0.05 from MICT post-session. # P<0.05 from MICT pre-session. † P<0.05 from PHT pre-session.

Figure 3. Skeletal muscle microvascular adaptations to moderate intensity continuous training (MICT) and passive heat therapy (PHT).

A, representative confocal microscopy images of skeletal muscle pre- (*a, c, e*) and post-PHT (*b, d, f*). The skeletal muscle microvascular endothelium was revealed using Ulex europaeus-FITC conjugated lectin (*a, b*). Skeletal muscle eNOS content was revealed using an appropriate antibody (*c, d*). Skeletal muscle eNOS ser^{1177}

phosphorylation was revealed using an appropriate antibody (*e, f*). *B*, mean capillary density. *C*, mean fluorescent intensity of eNOS. *D*, mean fluorescent intensity of eNOS ser^{1177}. The mean level of eNOS or eNOS ser^{1177} pre-intervention was assigned a value of 1, and the relative intensity post-intervention was calculated. *E*, eNOS ser^{1177} phosphorylation normalised to eNOS content (eNOS content/eNOS ser^{1177} phosphorylation). * $P<0.05$, main effect of intervention. Bar = 25 µm.

Figure 4. Myocyte adaptions following moderate intensity continuous training (MICT) and passive heat therapy (PHT).

A, C representative Confocal microscopy images of skeletal muscle pre- (*a, e*) and post-PHT (*b, f*). *A,* GLUT4 was revealed using an appropriate antibody. *C*, Skeletal muscle intramuscular triglycerides (IMTG) were revealed using bodipy in combination with WGA-350 to mark the plasma membrane. *B* representative widefield microscopy images of skeletal muscle pre- (*c*) and post-intervention (*d*). COXIV was revealed using an appropriate antibody in combination with WGA-350 to mark the plasma membrane. *D*, mean fluorescent intensity of GLUT4. *E*, fibre type specific analysis of COXIV fluorescence intensity. *F*, fibre type specific analysis of IMTG concentration. # $P<0.05$, main effect of fibre type. † $P<0.05$ from MICT pre-intervention. Bar = 50 µm.