# Effects of repeated local heat therapy on skeletal muscle structure and function in humans

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**Running Title**: Skeletal muscle adaptations to repeated local heat stress

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## ABSTRACT

The purpose of the present study was to examine the effects of repeated exposure to local heat therapy (HT) on skeletal muscle function, myofiber morphology, capillarization and mitochondrial content in humans. Twelve young adults (23.6±4.8 years, BMI 24.9±3.0 kg/m$^2$) had one randomly selected thigh treated with HT (garment perfused with water at ~52ºC) for 8 consecutive weeks (90 min, 5 days/week) while the opposite thigh served as a control. Biopsies were obtained from the vastus lateralis muscle before and after 4 and 8 weeks of treatment. Knee extensor strength and fatigue resistance were also assessed using isokinetic dynamometry. The changes in peak isokinetic torque were higher ($p=0.007$) in the thigh exposed to HT than in the control thigh at weeks 4 (Control: 4.2±13.1 Nm vs. HT: 9.1±16.1 Nm) and 8 (Control: 1.8±9.7 Nm vs. HT: 7.8±10.2 Nm). Exposure to HT averted a temporal decline in capillarization around type 2 fibers ($p<0.05$), but had no effect on capillarization indices in type 1 fibers. The content of eNOS was ~18% and 35% higher in the thigh exposed to HT at 4 and 8 weeks, respectively ($p=0.003$). Similarly, HT increased the content of small heat shock proteins HSPB5 ($p=0.007$) and HSPB1 ($p=0.009$). There were no differences between thighs for the changes in fiber CSA and mitochondrial content. These results indicate that exposure to local HT for 8 weeks promotes a pro-angiogenic environment and enhances muscle strength but does not affect mitochondrial content in humans.



**Key words**: heat therapy, skeletal muscle

## NEW & NOTEWORTHY

We demonstrate that repeated application of heat therapy to the thigh using a garment perfused with warm water enhances the strength of knee extensors and influences muscle capillarization in parallel with increases in the content of endothelial nitric oxide synthase and small heat shock proteins. This practical method of passive heat stress may be a feasible tool to treat conditions associated with capillary rarefaction and muscle weakness.

## INTRODUCTION

Repeated exposure to whole-body passive heat therapy (HT) in the form of hot water immersion, sauna, or environmental chambers has been shown to promote a plethora of health benefits in young individuals (4, 5, 7, 19) as well as in elderly patients with chronic heart failure (30, 35) and other cardiovascular diseases (20, 34, 36). For example, a recent population-based study revealed that frequent sauna bathing is associated with a significantly lower risk of fatal cardiovascular disease events and all-cause mortality (26). The salutary effects of HT are thought to stem in part from beneficial changes in the cardiovascular system, including improved endothelial function, reduced arterial stiffness, and blood pressure (4, 5). However, it is increasingly evident that HT also elicits positive changes in skeletal muscle structure and function. Treatment with whole-body HT for 6 weeks increased skeletal muscle capillary density and endothelial cell-specific endothelial nitric oxide synthase (eNOS) content in young individuals (19). Moreover, as few as eleven days of daily exposure to heat stress in an environmental chamber improves skeletal muscle contractility, as evidenced by an increase in evoked peak twitch amplitude and maximal voluntary torque production (32).

Although whole-body HT modalities have received the greatest attention, emerging evidence indicates that local HT may also promote skeletal muscle remodeling in humans. Both superficial (e.g. hot packs, heat wraps, water-circulating garments) and deep tissue (e.g. shortwave diathermy) local HT modalities are extensively used in rehabilitation settings for the management of muscle injuries as well as other conditions associated with pain and stiffness (28, 29). Contrary to whole-body HT, heating of a small area or body segment typically induces minimal or no change in body core temperature. Goto and co-workers first reported that repeated local thigh heating increased isometric force production of the knee extensors in humans (13).

Hafen and co-workers reported that short-term heat treatment promotes mitochondrial adaptations (16) and attenuates immobilization-induced atrophy in human skeletal muscle (15). We previously demonstrated that a single session of local thigh heating enhances the mRNA expression of factors associated with vascular growth, including vascular endothelial growth factor (VEGF) (25). Together, these studies indicate that local HT may be a practical tool to enhance skeletal muscle mitochondrial content and capillarization and improve contractile function. Nonetheless, the long-term skeletal muscle adaptations to repeated local heat stress in humans remain poorly defined.

The goal of the present study was to comprehensively examine the effects of 8 weeks of exposure to local HT (5 days/week) on muscle strength, myofiber morphology, capillarization and mitochondrial content in humans. Healthy young adults had one randomly selected thigh treated with HT using a water-circulating garment perfused with water at ~52ºC for 90 min, while the opposite thigh served as a control. This heat modality and protocol were selected because: 1) a single 90-min session of local HT increases the skeletal muscle expression of heat shock proteins and angiogenic factors (25); and 2) five daily 90-min sessions of local HT hastens functional recovery following eccentric exercise-induced muscle damage (22). Based upon these previous findings, we hypothesized that daily exposure to heat stress would enhance muscle strength, promote muscle capillary growth and the expression of angiogenic mediators, and increase muscle mitochondrial content.

## METHODS

### *Participants*

105 Twelve healthy young adults (10 males, 2 females) volunteered to participate in this
106 study (mean ± SD: 23.6±4.8 y, 172.9±8.6 cm, 74.5±10.3 kg). Participants were asked to fill out a
107 health and medical history questionnaire prior to enrollment. Exclusion criteria were: pregnancy,
108 obesity (body mass index (BMI) > 30 kg/m$^2$), hypertension (resting systolic/diastolic blood
109 pressure > 140/90 mm Hg), smoking, intake of medications and vitamin supplements, and
110 history of deep vein thrombosis. Individuals that participated in any kind of supervised physical
111 activity or engaged in physical activity more than 3 days a week were also excluded. Participants
112 were informed about risks and discomforts related to the different tests and procedures of the
113 study before providing their written informed consent to participate. The experimental
114 procedures adhered to the standards in the latest revision of the Declaration of Helsinki and were
115 approved by the Institutional Review Board at Purdue University (1604017606).
116
117 *Experimental design*
118 Participants initially visited the laboratory on four separate occasions over a 2-3 week
119 period. On visits 1 and 2, participants were familiarized with muscle testing on the isokinetic
120 dynamometer. On visit 3, participants underwent the baseline assessment of muscle strength and
121 fatigability as described in detailed below. These initial testing sessions were separated by a
122 minimum of 48 hours. At least one week after visit 3, resting muscle biopsies were collected
123 from the vastus lateralis of the left and right legs of each subject (22, 25). The 8-week
124 intervention protocol commenced at least three days after the muscle biopsy procedures. Using a
125 within-subject design, the legs of participants were assigned in a counterbalanced fashion to
126 receive HT or no treatment. Participants were asked to report to the laboratory 5 days per week
127 for a total of 40 sessions. The length of the intervention (8 weeks) was based on the reports by

Brunt and co-workers that 8 weeks of whole-body HT improves conduit vessel and cutaneous microvascular function (4, 5). Muscle strength and fatigability were reassessed after 4 and 8 weeks of treatment. These experimental sessions took place approximately 24 hours after the previous HT session. At least 48 hours after the completion of muscle testing, muscle biopsies were taken from each thigh.

All visits were conducted in an environmentally controlled laboratory at a similar time of day. Participants were instructed to fast for 10-11 hours before undergoing muscle biopsies and to eat a light meal prior to the other experimental visits. Participants were instructed to abstain from vigorous physical activity in the 24 hours preceding each test and to avoid caffeine consumption on the day of testing. Participants were asked to maintain their normal dietary and exercise behavior throughout the study. At the end of each week, participants were asked to self-report the frequency, duration and intensity of physical activity performed in the preceding 5 days.

*Heat treatment*

Participants were asked to report at the same time of day for the treatment sessions. Upon arrival at the laboratory, thermocouples (MLT422; ADInstruments, Colorado Springs, CO) were taped to both thighs for measurement of skin temperature. Participants were asked to put on water-circulating trousers on top of shorts or underwear (Med-Eng, Ottawa, Canada). This garment was customized with an extensive network of medical-grade polyvinyl chloride tubing that covered the thighs and buttocks (22, 25). In the thigh assigned to receive HT, water at ~52ºC was perfused through the garment for 90 min with a goal to increase leg skin temperature to ~39.5–40°C (22, 25). Previous studies that employed a similar approach revealed that this

regimen causes muscle temperature to increase from a baseline of ~33-34ºC to approximately 37ºC (8, 17).

*Assessment of muscle strength and fatigability*

Knee extensor strength and fatigue resistance were assessed using an isokinetic dynamometer (Humac NORM, Computer Sports Medicine, Inc., Stoughton, MA, USA) as described previously (22). Participants were familiarized with the testing procedures twice before the baseline assessment. Participants were seated with hands across the chest, restraining straps over the trunk, pelvis, and thigh, and the input axis of the dynamometer aligned with the axis of rotation of the knee. The familiarization protocol included a set of 5-10 concentric knee extension contractions at 60-70% of the estimated maximal effort at an angular velocity of 180º/s, a set of three maximal contractions at an angular velocity of 180º/s, and a set of 40 consecutive maximal contractions at 180º/s.

On each experimental session, participants were allowed to warm-up for 5 min on a cycle ergometer and were then positioned on the chair of the isokinetic dynamometer with the identical apparatus setting predetermined at the first familiarization visit. Testing was performed on both legs with the order of the testing counterbalanced between participants. Participants were asked to complete 3 maximal consecutive contractions at 180º/s, with a resting period of 3 min between limbs. The maximal measured torque (Nm) was used in all analyses. Once both limbs had been tested for maximal strength, participants were allowed to rest for approximately 3 min and were then asked to perform a bout consisting of 40 consecutive maximal contractions at 180º/s. A resting period of 10 min was allowed between limbs. The total work (J) performed during the bout was computed and used as a measure of fatigue resistance of the knee extensors. The

investigator that conducted the assessment of muscle function was not blinded to the treatment assignment.

*Muscle sampling*

Muscle biopsies were obtained from the vastus lateralis under local anaesthesia (Lidocaine hydrochloride, Hospira, Lake Forest, IL) using a 5-mm Bergstrom biopsy needle (Pelomi Medical, Albruslund, Denmark). The biopsy specimens were promptly weighed, cleared from visible fat and connective tissue, and divided into three sections. Approximately 40 mg sections were mounted in transverse orientation in a disposable base mold using an embedding medium compound (Tissue-tek, O.C.T. compound, Sakura Finetek USA, Torrance, CA) and then frozen in liquid nitrogen cooled isopentane for cryosectioning. The other sections were immediately frozen in liquid nitrogen and stored at -80ºC until citrate synthase and Western blot analysis.

*Immunohistochemistry*

Transverse serial sections (10μm) of muscle were cut using a Leica CM1850 cryostat (Leica, Wetzlar, Germany) at -23°C, mounted on frosted microscope slides (Thermo Scientific, NH, USA), air-dried for 0.5-1 hours at room temperature, and stored at -80°C for subsequent analyses. Frozen sections were briefly exposed to room air and fixed with 4% paraformaldehyde for 5 min. Following 2 x 3 min washes with 1x PBS, the slides were incubated with blocking buffer (5% goat serum, 2% bovine serum albumin, 0.1% Triton X-100, and 0.1% sodium azide in PBS) for 1 h at room temperature.

196 Muscle fiber type distribution was probed using primary antibodies against the basal
197 lamina and myosin heavy chain (MHC) isoform proteins. Sections were incubated for 3 hours at
198 room temperature with the following primary antibodies: polyclonal rabbit anti-laminin IgG
199 (ab11575, 1:500; Abcam), monoclonal mouse anti-MHC I IgG2b (BA-D5, 1:100), monoclonal
200 mouse anti-MHC IIa IgG1 (A4.74, 1:100), and monoclonal mouse anti-MHC IIx IgM (6H1,
201 1:100). All MHC primary antibodies were purchased from Developmental Studies Hybridoma
202 Bank (University of Iowa, IA). After incubation, tissue sections underwent a series of 1× PBS
203 washes and incubation with fluorescently labeled secondary antibodies for 1 hour at room
204 temperature: Alexa Fluor 488 goat anti-rabbit IgG (A11008, 1:1000), Alexa Fluor 488 goat anti-
205 mouse IgG2b (A21141, 1:1000), Alexa Fluor 568 goat anti-mouse IgG1 (A21124, 1:1000), and
206 Alexa Fluor 350 goat anti-mouse IgM (A31552, 1:1000). All secondary antibodies were obtained
207 from Thermo Fisher Scientific. Following 4 x 5 min washes, slides were briefly dried and
208 mounted using fluorescent mounting medium (Dako, CA, USA) and the edges were sealed with
209 nail polish (Sally Hansen Hard as Nails, NY, USA).
210 Identification of fiber type-specific capillaries was performed in neighboring sections
211 using antibodies against mouse anti-CD31 IgG1 (550300, 1:100, BD Biosciences), rabbit anti-
212 dystrophin IgG1 (ab15277, 1:100, Abcam) and mouse anti-MHC I (BA-D5, 1:100, DSHB).
213 After 2 x 5 min washes with 1x PBS, sections were stained with appropriate secondary
214 antibodies (Alexa 350 goat anti-rabbit IgG, A11609, 1:500; Alexa 488 goat anti-rabbit IgG,
215 A11008, 1:1000; Alexa 488 goat anti-mouse IgG 2b, A21141, 1:1000; and Alexa 568 goat anti-
216 mouse IgG1, A21124, 1:1000, Thermo Fisher Scientific), diluted in 1x PBS for 1 h at room
217 temperature. Negative controls for the primary antibodies against CD31 were used to ensure
218 specificity of staining.

Slides were viewed at ×20 magnification using an Olympus BX53 fluorescence microscope equipped with an Olympus DP72 digital camera and cellSens Dimension software. The entire specimen cross section was initially selected using the stage navigator. The multi-channel image was then acquired and two images from each channel were merged using Image J software (National Institutes of Health, USA). Histological analysis was not performed in 1 out of 72 samples due to insufficient muscle yield.

*Analysis of immunofluorescence images*

Analyses of immunofluorescence images were carried out using Adobe Photoshop CC 2015. Fiber type distributions were determined from counts of an average of 612 ± 70 muscle fibers (range 221–1260 fibers). For the quantification of muscle capillarization, all internal fibers (not bordering on a fascicle) in a cross section were initially counted (an average of 130 ± 23 fibers for type I and 153 ± 26 fibers for type II muscle fibers). A total of 25 type I and 25 type II muscle fibers were then randomly selected for analysis. Individual fibers were traced to obtain the area and perimeter of the fiber. Capillaries were quantified using the following indices: (1) the number of capillaries around a fiber (capillary contacts, CC), (2) the capillary-to-fiber ratio on an individual fiber basis (C:Fi) and (3) the number of fibers sharing each capillary (sharing factor, SF), and (4) the capillary to fiber perimeter exchange index (CFPE index), defined as the C/Fi ratio divided by the fiber perimeter of a given fiber (18). All immunofluorescent images were blinded for both treatment and time point prior to analysis.

*Protein extraction*

241 Frozen muscle samples (~30 mg) were homogenized in ice-cold homogenization buffer
242 containing 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.25% deoxycholic acid, 1% NP-40, 1 mM
243 EDTA (RIPA Lysis Buffer, EMD Milipore) with freshly added protease inhibitor cocktail
244 (P8340, Sigma-Aldrich) and phosphatase inhibitors (50 mM NaF and 0.2mM $Na_3VO_4$) at a 1:15
245 dilution of wet muscle weight using a bead mill homogenizer (BEAD RUPTOR12, Omni
246 International). The resulting homogenate was clarified by centrifugation (13,500 g) for 20 min at
247 4°C. The supernatant was collected and the protein concentration of each sample (~5 µg/µL) was
248 determined with a BCA protein assay kit (Thermo Scientific, IL, USA). All samples were
249 subsequently diluted with homogenization buffer (1.5 µg/µL) and subsequently mixed with
250 either reducing sample buffer (4x Laemmli sample buffer with 10% 2-Mercaptoethanol) or non-
251 reducing sample buffer (4x Laemmli sample buffer). Afterwards, samples were heated to 95ºC
252 for 5 min (except for mitochondrial OXPHOS protein blots), divided into small aliquots, and
253 stored at -80ºC.
254
255 *Western blot analysis*
256 For the analysis of HSP90A, HSP90B, VEGF, ANGPT1, p-eNOSser$^{1177}$, eNOS, and
257 OXPHOS, 20 µg of protein were separated by SDS-PAGE on precast Stain Free 4-15 % gels
258 (Bio-Rad, CA, USA) and transferred to polyvinylidene fluoride (PVDF) membranes using the
259 Trans-Blot® Turbo transfer system (Bio-Rad, CA, USA). Membranes were subsequently
260 blocked with 5 % non-fat milk in 1x TBST (1% tween 20) solution for 1 h at room temperature
261 (~23°C) and incubated for 3-4 hours at room temperature with primary antibodies diluted in
262 blocking buffer. The membranes were washed with 1x TBST at room temperature for 3 x 10
263 min, incubated with horseradish peroxidase-conjugated secondary antibodies diluted in 1x TBST

for 1 h at room temperature and were then washed with 1x TBST at least 3 x 10 min before being exposed to an enhanced chemiluminescent solution (Clarity Western ECL, Bio-Rad, USA) for 5 min. Membranes were visualized using a densitometer (ChemiDoc Touch Imaging System, Bio-Rad, USA), and band densities were determined using image-analysis software (Image Lab V6.0.1, Bio-Rad, USA). PageRuler Prestained Protein Ladder (Thermo Fisher, USA) was used as a molecular weight marker. Control for equal loading was performed using the stain-free technology and total protein normalization was used to calculate changes in the expression of each target protein relative to the baseline sample. The analysis of HSPB5, HSPB1, HSPA1A was performed as described previously (11). Details of the primary antibodies are provided in Supplemental Table S1 (https://figshare.com/articles/Supplemental_Table_S1_docx/11385921) https://doi.org/10.6084/m9.figshare.11385921. Recombinant proteins were used to confirm antibody specificity.

*Citrate synthase activity*

The maximal enzyme activity of citrate synthase (CS) was determined using the lysate prepared for Western blot analyses and analyzed on a spectrophotometer (Bio-Rad). Samples were analyzed in triplicate and each well (final reaction volume 210 µL, pathlength 0.57 cm) contained 10 µL of ~2 mg/ml lysate, 0.3 mM acetyl-CoA, 0.15 mM 5,5'-Dithiobis 2-nitrobenzoic acid (DTNB), 0.25% w/v Triton-X, and 1 mM oxaloacetate made to volume with 100 mM Tris buffer, pH 8.3. Oxaloacetate was added to commence the reaction, which was measured by change in absorbance (DTNB $\varepsilon$ = 14150 $M^{-1}.cm^{-1}$ at 412 nm) every 15 s over a 3-min period at 25ºC then enzyme activity was expressed as nanomoles per minute per milligram of protein.

*Statistical analysis*

All statistical analyses were conducted using SAS (Version 9.4; SAS Institute, Cary, NC) with results expressed as means ± SD. The Kolmogorov-Smirnov test was used to assess the distribution of the data. Data exhibiting skewed distribution (HSPB5, HSPB1, p-eNOS, ANGPT1, HSP90A, HSP90B) were log-transformed before statistical analysis. Descriptive results for each variable are expressed as means ± SD, or geometric mean ×/÷ geometric standard error if the variable value was log-transformed. A two-way repeated measures ANOVA was employed to compare the changes from baseline in all variables between the leg exposed to HT and the control leg. A Tukey post-hoc analysis was performed when appropriate. For all analyses, $P < 0.05$ was considered statistically significant.

## RESULTS

*Thigh skin temperature*

Figure 1 displays the temporal profile of thigh skin temperature during exposure to 90 min of HT or the control regimen. The average temperature in the thigh assigned to receive HT was 39.8±0.3ºC, while in the control leg the average temperature was 32.4±0.3ºC (main treatment effect, $p<0.001$).

*Muscle strength and fatigability*

In the thigh that received HT, maximal isokinetic peak torque of the knee extensors at 180º/s improved by 6% at week 4 and by 5% on week 8 (baseline: 140±40 Nm, 4 weeks: 149±50 Nm, 8 weeks:148±46 Nm) (Figure 2). Conversely, in the control thigh, peak torque increased by 2% and 1% at weeks 4 and 8, respectively (baseline: 142±43 N, 4 weeks: 147±44 N, 8 weeks:

144±45 N). Comparison of the changes from baseline in peak torque revealed a significant main effect of treatment ($p=0.007$), but no time effect ($p=0.333$) or treatment × time interaction ($p=0.778$). Fatigability, as assessed by the total work completed during 40 consecutive maximal contractions at 180º/s, was not altered after exposure to either HT (baseline: 4434±1232 J, 4 weeks: 4404±1310 J, 8 weeks: 4449±1281 J) or in the control thigh (baseline: 4309±1122 J, 4 weeks: 4400±1286 J, 8 weeks: 4321±1153 J) (Figure 2).

*Fiber type distribution and morphology*

Muscle fiber cross-sectional area (CSA), perimeter, SF as well as fiber type distribution are shown on Table 2. There were no treatment, time or treatment x time effects for the changes in fiber cross-sectional area in both fiber types. Fiber type distribution was also not significantly influenced by HT.

*Capillarization*

The number of capillary contacts in type 1 fibers declined throughout the study in both the control thigh (baseline: 5.0±1.1, 4 weeks: 4.7±0.8, 8 weeks: 4.8±0.8) and in the thigh treated with HT (baseline: 5.1±1.0, 4 weeks: 4.7±0.8, 8 weeks: 4.7±0.8) (Figure 3). Exposure to HT also had no significant effect on other capillarization indices in type 1 fibers (Figure 3). Conversely, while the number of capillary contacts around type 2 fibers declined by nearly 10% in the control thigh (baseline: 4.6±0.6, 4 weeks: 4.2±0.7, 8 weeks: 4.2±0.5), exposure to HT prevented a temporal reduction in this variable (baseline: 4.2±0.6, 4 weeks: 4.2±0.6, 8 weeks: 4.3±0.8). A significant treatment effect was observed for the changes in capillary contacts ($p=0.016$), the

capillary-to-fiber ratio on an individual fiber basis (p=0.007), and the capillary-to-fiber perimeter exchange index (p<0.001) in type 2 fibers (Figure 3).

*Mitochondrial content*

The changes in maximal citrate synthase activity and the content of OXPHOS protein complexes are shown on Table 2. There were no treatment, time, or treatment x time interaction for levels of the mitochondrial OXPHOS proteins measured.

*Expression of angiogenic factors and heat shock proteins*

A main effect of treatment was observed for the changes in skeletal muscle eNOS content (p=0.003), while eNOS $^{ser1177}$ phosphorylation (p=0.389) and eNOS phosphorylation normalized to eNOS content (p=0.201) were not altered by the intervention (Figure 6). The protein content of members of the small heat shock protein (HSP20) family, alpha B-crystallin (HSPB5) (main effect of treatment, p=0.007) and heat shock protein family B member 1 (HSPB1) (main effect of treatment, p=0.009), were also significantly higher in the thigh treated with HT (Figure 6). No treatment effect was observed for the changes in VEGF, ANGPT1, HSPA1A and the HSP90 family members (Figure 6).

## DISCUSSION

The primary findings of this study were that repeated local thigh heating for 8 weeks elicited an increase in eNOS content and averted a temporal decline in skeletal muscle capillarization indices. Conversely, HT had no effect on skeletal muscle mitochondrial content. Confirming earlier observations that exposure to local and whole-body heat stress improves

skeletal muscle contractile function (13, 32), we also report that 8 weeks of local HT enhanced the strength of the knee extensors. Combined, these findings indicate that a simple and well-tolerated HT modality significantly influences skeletal muscle morphology and function and sheds new light on the potential therapeutic use of local heat stress to treat conditions associated with skeletal muscle abnormalities.

*Experimental considerations*

We chose to apply local HT for 90 min in each session in the present study because we previously showed that this regimen elicits increased expression of heat shock proteins and angiogenic mediators in human skeletal muscle (25). Thus, participants were required to spend 90 min daily (5 days/week) sitting in the laboratory to receive HT and control treatments. One unintended consequence of this demanding protocol was that some participants reported being unable to maintain their habitual exercise routines throughout the study due to time constraints. Although we did not directly measure physical activity patterns, analysis of weekly reports by the participants revealed that seven individuals had marked decrements in exercise time throughout the study, while three others reported modest changes. The reduction in structured physical activity coupled with increased sedentary time might be partially responsible for the observed small, albeit consistent, decline in fiber cross-sectional area (Table 2) and capillarization (Figure 3), particularly in the thigh assigned to the control regimen. Several studies have shown that short periods of reduced physical activity (e.g. step reduction) impairs glucose metabolism, including insulin sensitivity (24) and lowers myofibrillar protein synthesis rates (33) in healthy young adults. More severe forms of muscle disuse, such as 2 weeks of single leg limb immobilization, lead to reduced leg lean mass and muscle capillarization in old

and young men (39). Of note, exposure to HT has been shown to attenuate the manifestations of skeletal muscle disuse in animals (37, 58) as well as in humans (15). Our findings that daily local HT prevented the decline and/or enhanced indices of capillarization (Figure 3) relative to the control intervention add to this growing body of literature that indicates that HT mitigates the detrimental consequences of physical inactivity in skeletal muscle.

*Effect of HT on muscle capillarization*

The ability of heat stress to promote a pro-angiogenic milieu in skeletal muscle and a consequent increase in capillarization was first documented by Akasaki and co-workers in a model of peripheral arterial insufficiency (1). These authors showed that mice treated with far-infrared dry sauna daily for 5 weeks had greater capillary density and eNOS expression in the ischemic muscle. Of note, chronic treatment with NOS inhibitor N(ω)-nitro-L-arginine methyl ester (L-NAME) abolished the changes in capillarization as well as the recovery in blood flow (1). Similarly, the angiogenic response to heat stress was absent in mice lacking eNOS (1). Recently, these earlier observations in ischemic mouse skeletal muscle were extended to humans. Hesketh and co-workers reported that 6 weeks of whole-body passive HT increased capillary density by 21% and endothelial-specific eNOS content by 8% in the vastus lateralis muscle of sedentary young individuals (19). The increase in eNOS content and the consequent angiogenic response to whole-body HT appears to be mediated in part by circulating factors. Brunt and co-workers showed that exposing cultured endothelial cells to serum collected from participants who had undergone whole-body HT for 8 weeks increased the abundance of eNOS and endothelial tubule formation (6). Combined, these studies provide compelling evidence implicating nitric oxide (NO) as a critical mediator of heat-induced skeletal muscle angiogenesis.

Based upon these earlier reports, we examined the effects of local HT on the content of eNOS and muscle capillarization. In accordance with the previous findings from whole body heating (19), we report that eNOS content was 18% and 35% higher in the thigh exposed to HT as compared to the control thigh at 4 and 8 weeks, respectively (Figure 6). Changes in eNOS were accompanied by significant differences in capillarization between HT and control in type 2, but not type 1 fibers (Figure 3). The mechanistic basis underlying the fiber type specific effect of HT on capillarization is unclear. Increased wall shear stress in the capillary network has been proposed to be a critical signal for promoting HT-induced skeletal muscle angiogenesis (1, 19). Studies in animals (2) as well in humans (17) have documented a modest increase in muscle blood flow during exposure to local heat stress. It is possible to speculate that type 2 fibers experienced a greater relative increase in blood flow (and wall-shear stress) during HT compared to type 1 fibers. Alternatively, it is possible that the effects of HT were mostly evident in type 2 fibers because capillarization around these fibers was more severely impacted by reduced physical activity levels (Figure 3). Of note, Hesketh and co-workers did not observe differences between fiber types in the magnitude of the increase in capillarization following 6 weeks of whole-body HT (19).

Contrary to our hypothesis, we did not observe changes in the content of VEGF and ANGPT1 levels after treatment with local HT. We previously reported that the expression of these pivotal angiogenic mediators is enhanced following a single session (25) as well as 5 days of repeated exposure to HT in injured muscle (22). It is plausible that the levels of these factors were temporarily increased early in the intervention period and later declined toward baseline levels. A similar scenario might explain the lack of effect of local HT on the content of several members of the heat-shock protein family, including HSP70 and HSP90. One important

exception was the marked increase in the content of small heat-shock proteins HSPB5 and HSPB1 in the thigh exposed to HT (Figure 6). This is an important observation because small heat shock proteins have been implicated in the regulation of angiogenesis and blood vessel function in multiple tissues (10, 21). Additional studies are warranted to define the role these molecular chaperones exert on heat-induced skeletal muscle angiogenesis.

*Heat stress and mitochondrial biogenesis*

The finding that heat stress induces mitochondrial biogenesis in C2C12 myotubes (27) has led to several investigations asking if repeated HT could potentially enhance mitochondrial content *in vivo*. Experiments in mice revealed that daily exposure to whole body heat stress (5 days/wk for 3 wk) increased mitochondrial enzyme activities and respiratory chain protein content in skeletal muscle (38). More recently, local heating of the vastus lateralis for 6 consecutive days (2 h daily) increased mitochondrial respiratory capacity and mitochondrial content (16). In contrast, we did not observe a significant effect of local HT on the content of respiratory chain proteins or maximal CS activity in the present study. Our findings align closely with the recent report of Hesketh and co-workers that repeated whole-body HT had no effect on skeletal muscle mitochondrial density despite marked effects on exercise capacity and capillarization (19). The inconsistent effect of HT on mitochondrial content may be partially explained by variations in the magnitude and duration of heat stress as well as the modality used for heat induction in skeletal muscle. Pulsed shortwave diathermy, which produces rapid and marked deep tissue heating (12), may be more effective at producing mitochondrial adaptations than superficial heat modalities as employed in the current report. It is worth noting that we have not measured the content of AMP-activated protein kinase (AMPK), peroxisome proliferator-

activated receptor gamma, coactivator-1 alpha (PGC1a) and other biomarkers of mitochondrial biogenesis nor assessed the effects of HT on mitochondrial respiration. Hafen and co-workers showed that despite no changes in citrate synthase activity, a common surrogate marker of mitochondrial content, local HT using diathermy increased the content of PGC1a and the phosphorylation of AMPK and resulted in improved mitochondrial respiratory capacity (16).

*HT and skeletal muscle strength*

Given that local heat treatment of the thigh for 8 h/day for 10 weeks improved maximal isometric force in young individuals (13), we questioned if 90 min of thigh heating over 8 weeks would significantly enhance knee extensor strength. In agreement with the findings of Goto and co-workers (13), maximal isokinetic torque increased to a greater extent in the thigh exposed to HT as compared to the control thigh with just 90 min of treatment (Figure 2). This improvement in force after treatment with local HT occurred despite no significant differences in fiber cross-sectional area between treatments (Table 2), indicating that adaptations other than changes in fiber size explain the observed improvements in force generating capacity. Of note, Racinais and colleagues demonstrated that as little as 11 days of whole-body heat stress increased peak twitch amplitude and torque production of the plantar flexors in humans (32). As it seems unlikely that major changes in fiber size would occur in this short period of time, these findings imply that alternative mechanisms, including increases in force per cross-bridge or possibly the kinetics of formation of cross-bridges contribute to strength gains to heat therapy (32). Of note, the study of Racinais et al did not include a sham-treated group and it is thus impossible to exclude the possibility that the improvement in muscle function derived partially from a time and/or familiarization effect (32). Further research is needed to explore the mechanistic basis of

enhanced force-generating capacity of muscles exposed to repeated heat stress. Additional studies are also warranted to define if in addition to muscle strength, HT may affect muscle power and improve performance during submaximal, prolonged events.

*Limitations*

An important limitation of the current study is that we have not directly measured intramuscular temperature during exposure to local HT. Studies that employed water-circulating garments perfused with warm water to heat the calf or the entire leg of healthy individuals reported average increases in intramuscular temperature ranging from 2.5 to 4ºC (8, 17). As we utilized a similar heating modality and treatment regimen, it is tempting to suggest that comparable changes in temperature occurred in the present study. Nonetheless, it worth highlighting that the time course and magnitude of changes in muscle temperature upon exposure to heat treatment may be modulated by a number of factors, including the treatment duration and the thickness of the subcutaneous fat layer (31).

Another limitation that is inherent to HT studies is the fact that participants cannot be blinded to the intervention. This imposes a challenge for the interpretation of experimental outcomes that are prone to the placebo effect, including voluntary force production. It is plausible that the observed increase in muscle strength after treatment with HT may be partially ascribed to a placebo effect. This seems unlikely given the accumulating evidence derived from animal studies that repeated heat stress enhances muscle strength and prevents disuse-induced muscle weakness. For instance, we recently reported that repeated immersion in a water bath at 37°C and 39°C for 3 weeks enhanced maximal absolute force of the soleus muscle in a model of ischemia-induced muscle damage (23). Similarly, Yoshihara and colleagues reported that 3 days

493 of whole-body HT using a heat chamber (40-41°C for 60 min) abrogated ventilator-induced
494 diaphragm contractile dysfunction in rats (40). These findings reveal that repeated heat stress
495 elicits adaptations that culminate in an improved force generation capacity. Nonetheless, it is
496 imperative that future studies in humans compare the effects of HT on muscle force with a
497 placebo rather than a control intervention (3).
498
499 *Clinical implications*
500 Water-circulating garments are amenable for home use, do not require supervision by a
501 therapist, and are practical for individuals with restricted locomotion who cannot participate in
502 exercise (e.g. severe peripheral artery insufficiency, chronic heart failure and chronic obstructive
503 pulmonary disease). Our findings that local HT enhances muscle strength and affects muscle
504 capillarization indicate that this method may be a feasible tool to treat these chronic conditions.
505 One caveat regarding the clinical use of tube-lined garments for HT is that this modality is
506 designed primarily to manipulate skin temperature (9). Prolonged exposure to this method is
507 necessary to attain significant increases in intramuscular temperature (8). For example, 1 hr of
508 perfusion of 50°C water through a garment covering a single leg raised the vastus lateralis
509 muscle temperature by ~2.5ºC (8). Substantially faster and greater increases in intramuscular
510 temperatures can be achieved with the use of deep tissue heating modalities, such as short-wave
511 diathermy. Garrett and co-workers showed that diathermy application for 20 min raised the
512 triceps surae muscle temperature by ~3.5ºC (12). Deep tissue heating modalities may therefore
513 possibly confer benefits that are similar or superior to the ones reported herein despite a
514 substantially lower treatment duration. It should be emphasized, nonetheless, that diathermy is

less accessible than superficial heating modalities because these devices are expensive, cumbersome and require a trained professional for proper operation.

In addition to its use in rehabilitation, there is evidence that HT may be an ergogenic aid to boost the adaptations to exercise training. For example, Tamura and co-workers recently showed that post-exercise whole body heat stress (40°C, 30 min/day, 5 days/wk, 3 wk) additively enhanced endurance training-induced mitochondrial adaptations in mouse skeletal muscle (38). Goto and co-workers showed that repeated heating of the elbow flexor muscles using a heating and steam-generating sheet prior to and during low-load resistance exercise resulted in greater changes in maximum isometric torque and cross-sectional area of the biceps brachii muscle as compared to resistance training alone (14). This effect of HT does not appear to occur in the lower-limb muscles. Stadnyk and colleagues recently reported local thigh heating during, and for 20 min after resistance exercise of the knee extensors in untrained individuals had no effect on training-induced hypertrophy or function (37). It remains to be determined whether HT may facilitate the adaptations to endurance and resistance training in trained individuals.

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## CONFLICTS OF INTEREST:

None

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## FIGURE LEGENDS

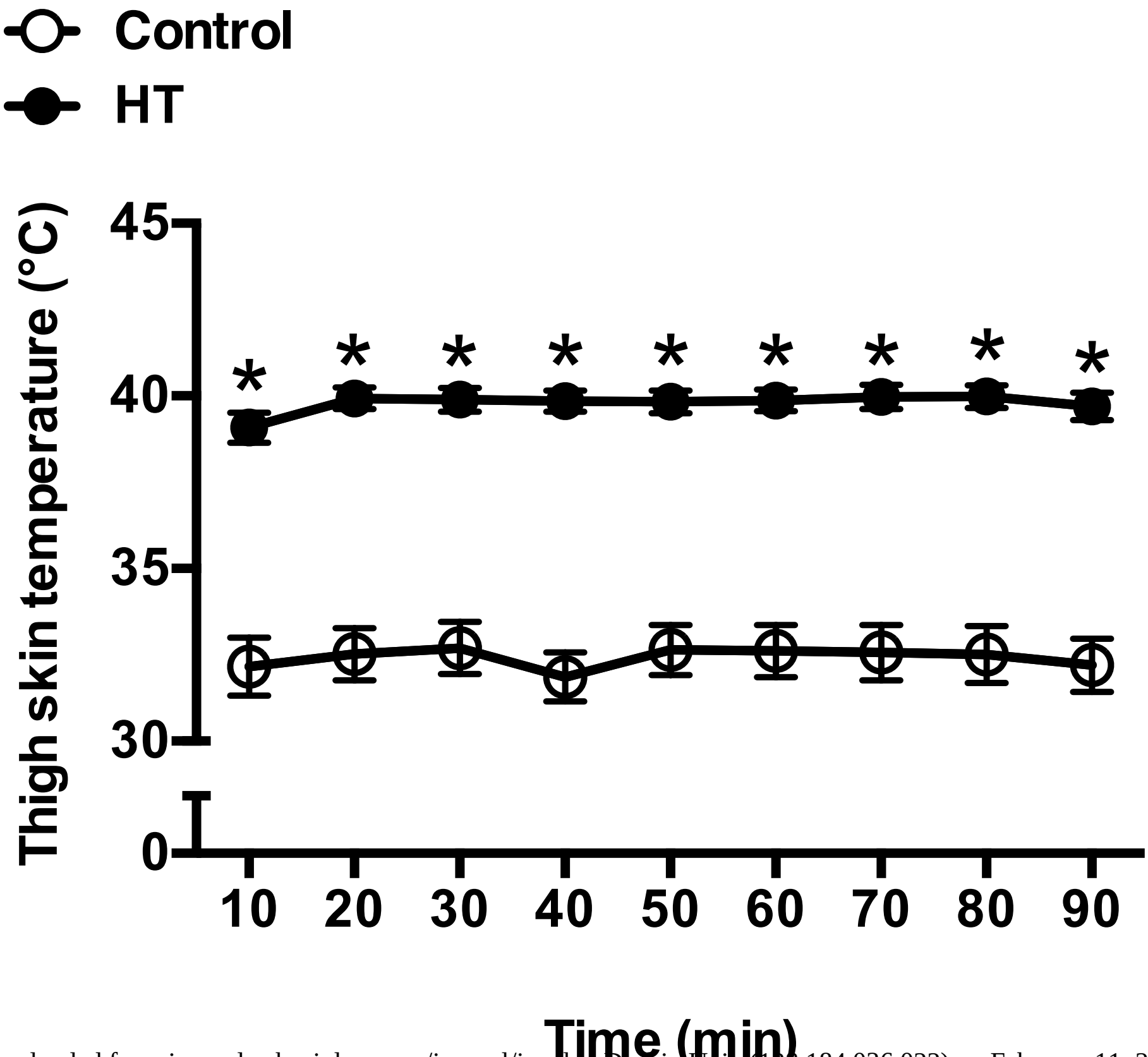
**Figure 1:** Thigh skin temperature during exposure to 90 min of heat therapy (HT, closed circles) or the control intervention (open circles). Data were analyzed with a 2-way repeated-measures ANOVA. Values are means ± SD. *$p < 0.05$ vs. Control.

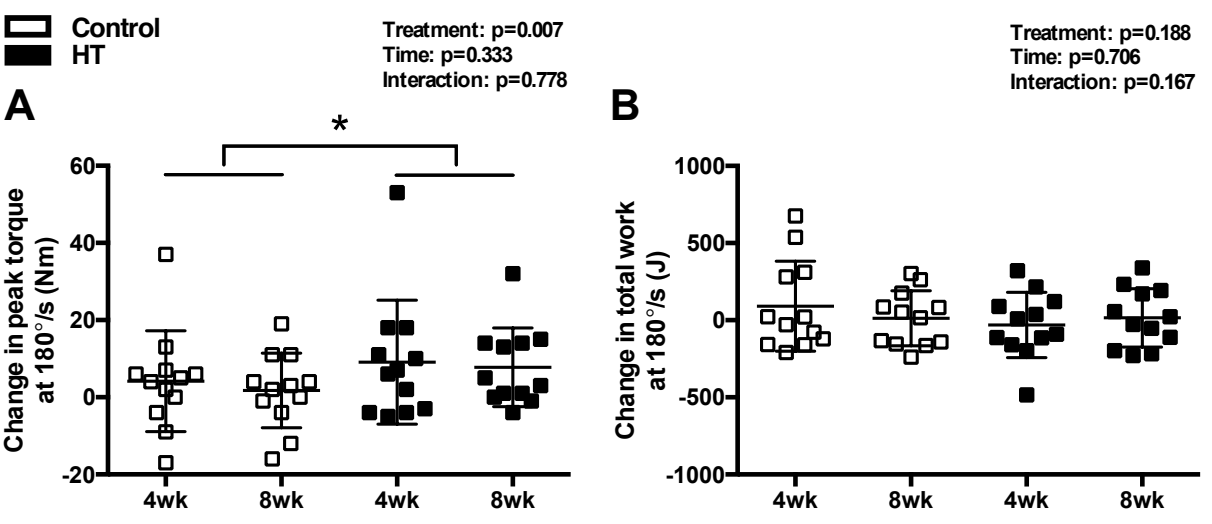
**Figure 2:** Individual and group mean changes from baseline in muscle strength (A) and fatigue resistance (B) following 4 and 8 weeks of heat therapy (HT, closed squares) or the control intervention (open squares). Data were analyzed with a 2-way repeated-measures ANOVA. *main effect of treatment ($p < 0.05$).

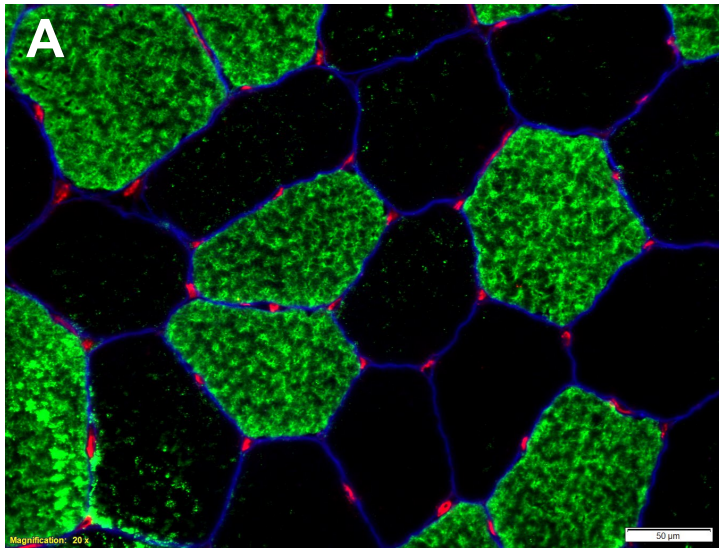
**Figure 3:** A: Representative skeletal muscle cross section displaying immunoreactivity for dystrophin (blue), CD31 (red), and myosin heavy chain type I (green). B and C: Changes from baseline in the number of capillary contacts (CC) for type I (B) and type II (C) fibers. D and E: Changes from baseline in the number of capillaries to each muscle fiber (C:Fi) for type I (D) and type II (E) fibers. F and G: Changes from baseline in the capillary-to-fiber perimeter exchange index (CFPE) for type I (F) and type II (G) fibers. Data were analyzed with a 2-way repeated-measures ANOVA. *main effect of treatment ($p < 0.05$).

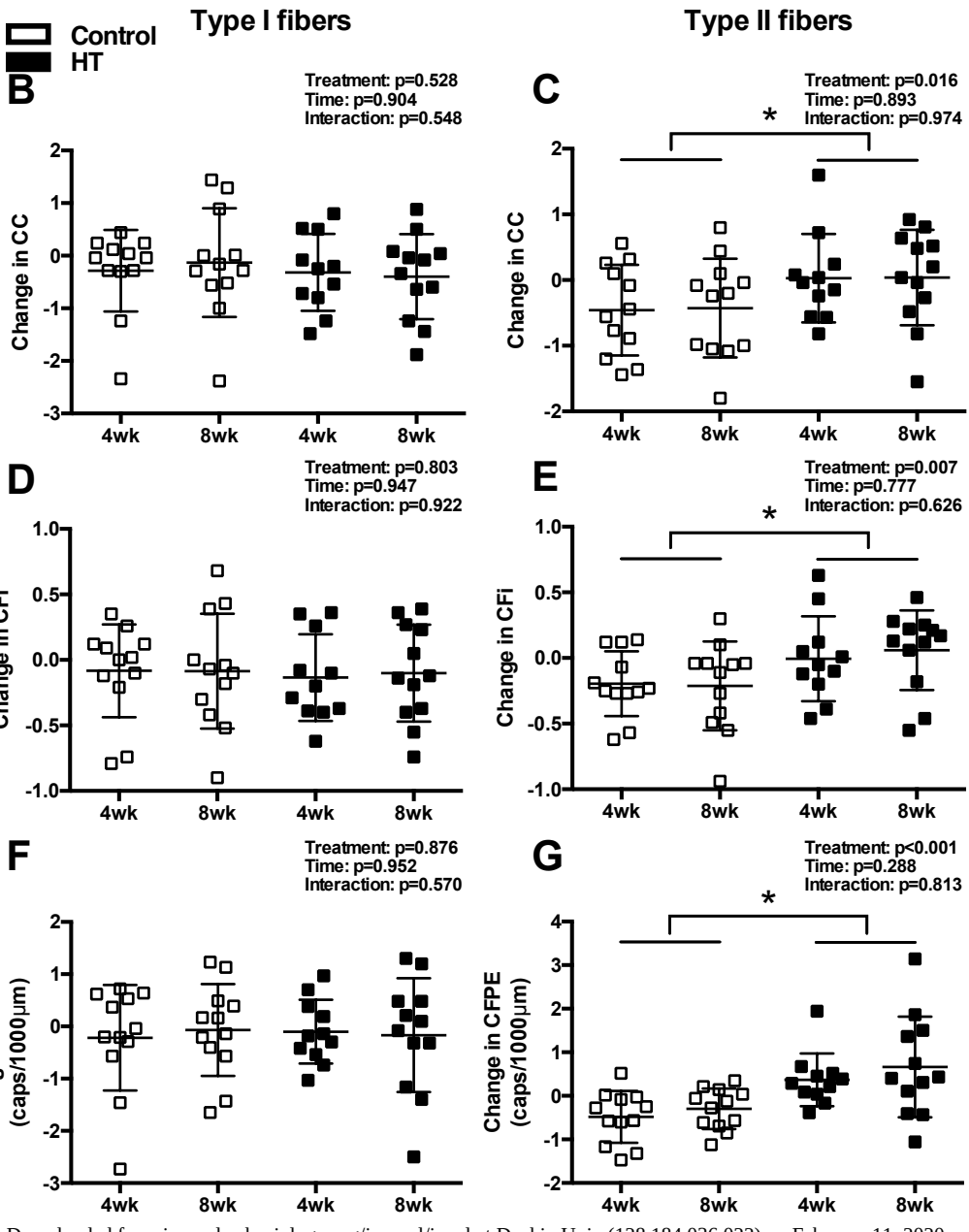
**Figure 4:** Fold changes in skeletal muscle protein expression relative to the baseline sample of select stress management and angiogenic proteins. A: Endothelial nitric oxide synthase (eNOS). B: Phosphorylated endothelial nitric oxide synthase at Ser1177 (p-eNOS $^{ser1177}$). C: The ratio of p-eNOS$^{ser1177}$ to eNOS. D: Alpha B-crystallin protein (HSPB5). E: Heat shock protein family B

member 1 (HSPB1). F: Vascular endothelial growth factor (VEGF). G: Angiopoietin 1 (ANPTT1). H: Heat shock protein 72-kDa (HSPA1A). I: Heat shock protein 90-kDa alpha class A member 1 (HSP90A). J: Heat shock protein 90-kDa alpha class B member 1 (HSP90B). The baseline sample was assigned a value of 1 and is represented by the dashed line. Data exhibiting skewed distribution (HSPB5, HSPB1, p-eNOS, ANGPT1, HSP90A, HSP90B) were log-transformed before statistical analysis. Values are means ± SD or geometric mean ×/÷ geometric standard error if the variable value was log-transformed. Data were analyzed with a 2-way repeated-measures ANOVA. *main effect of treatment ($p < 0.05$).







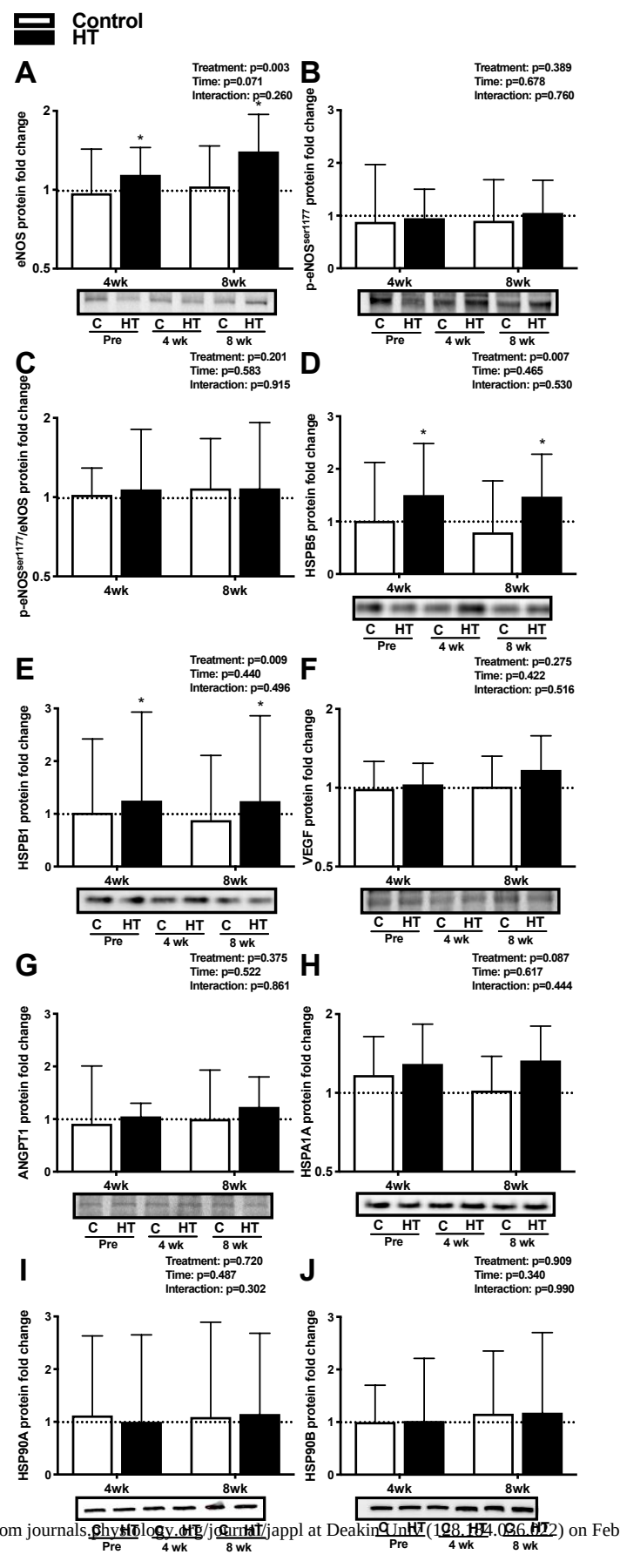


Table 1. Muscle fiber morphological measurements

| | Control | | | Heat therapy | | |
|---|---|---|---|---|---|---|
| | **Week 0** | **Week 4** | **Week 8** | **Week 0** | **Week 4** | **Week 8** |
| Type I CSA, $\mu m^2$ | 6013.3±1136.4 | -20.3±1202.2 | -533.5±1193.2 | 6394.2±1608.9 | -431.9±1717.7 | -315.5±2234.2 |
| Type II CSA, $\mu m^2$ | 7186.8±1168.2 | -443.9±1638.6 | -1192.3±1958.7 | 6960.6±1208.1 | -488.6±2141.1 | -564.3±1934.0 |
| Type I perimeter, μm | 326.2±28.1 | 7.3±34.0 | -11.7±33.5 | 339.2±42.7 | -18.4±37.5 | -6.2±67.7 |
| Type II perimeter, μm | 367.4±60.9 | -14.2±59.9 | -35.7±67.9 | 359.0±35.7 | -23.7±44.9 | -17.8±62.6 |
| Type I SF | 2.53±0.2 | -0.06±0.3 | 0.07±0.2 | 2.62±0.1 | 0.02±0.1 | -0.10±0.2 |
| Type II SF | 2.58±0.2 | -0.03±0.3 | 0.05±0.2 | 2.59±0.2 | 0.05±0.2 | -0.06±0.3 |
| Type I (%) | 38.4±6.1 | -2.01±8.9 | -1.45±5.3 | 42.5±14.0 | -3.82±9.0 | -2.73±12.2 |
| Type II (%) | 61.6±6.1 | 2.01±8.9 | 1.45±5.3 | 57.5±14.0 | 3.82±9.0 | 2.73±12.2 |

Values are means ± SD; Week 0, baseline values prior to treatments; Week 4 and week 8, changes from baseline value following 4 and 8 weeks of heat therapy or control intervention

Table 2. Changes in maximal citrate synthase activity and the content of OXPHOS protein complexes

| | Control | | Heat therapy | |
|---|---|---|---|---|
| | **Week 4** | **Week 8** | **Week 4** | **Week 8** |
| Maximal citrate synthase activity (nmol/min/mg protein) | -4.40±14.43 | 2.04±11.43 | -0.84±6.83 | -2.94±14.05 |
| Fold changes in OXPHOS protein complexes | | | | |
| Complex I | 1.03±0.35 | 1.11±0.54 | 0.98±0.26 | 1.08±0.33 |
| Complex II | 0.89±0.30 | 0.96±0.38 | 0.90±0.28 | 0.93±0.33 |
| Complex III | 1.06±0.15 | 1.06±0.21 | 1.02±0.11 | 1.03±0.13 |
| Complex IV | 1.02±0.36 | 1.10±0.46 | 0.92±0.23 | 0.97±0.44 |
| Complex V | 1.01±0.17 | 1.02±0.22 | 0.99±0.15 | 1.01±0.18 |

Values are means ± SD; Week 4 and week 8, changes from baseline value following 4 and 8 weeks of heat therapy or control intervention