TEMPERATURE
2024, VOL. 11, NO. 2, 157–169
https://doi.org/10.1080/23328940.2024.2313954

Taylor & Francis
Taylor & Francis Group

RESEARCH PAPER

OPEN ACCESS Check for updates

Pre-exercise hot water immersion increased circulatory heat shock proteins but did not alter muscle damage markers or endurance capacity after eccentric exercise

Xiang Ren Tan [a,b], Ivan C. C. Low [b,c], Tuck Wah Soong [c], and Jason K. W. Lee [b,c,d,e]

[a]Health and Social Sciences, Singapore Institute of Technology, Singapore, Singapore; [b]Human Potential Translational Research Programme, Yong Loo Lin School of Medicine, National University of Singapore, Singapore; [c]Department of Physiology, Yong Loo Lin School of Medicine, National University of Singapore, Singapore; [d]Heat Resilience and Performance Centre, Yong Loo Lin School of Medicine, National University of Singapore, Singapore; [e]Campus for Research Excellence and Technological Enterprise (CREATE), Singapore, Singapore

ABSTRACT

Pre-exercise passive heating attenuates muscle damage caused by eccentric exercise in rats where the induction of heat shock proteins (HSPs) confers a myoprotective effect. We investigated whether pre-exercise hot water immersion (HWI) confers similar benefits in humans. Eleven recreational male athletes were immersed in 41°C water up to 60 min or until rectal temperatures reached 39.5°C. After a 6 h rest, the participants performed an eccentric downhill run for 1 h at −4% gradient to induce muscle damage. An endurance capacity test at 75% VO_{2max} was conducted 18 h later. The control trial was similar except that participants were immersed at 34°C. Blood samples were collected to assess HSPs levels, creatine kinase, and lactate dehydrogenase activities. Plasma eHSP70 was higher post-immersion in HWI trials (1.3 ± 0.4 vs 1.1 ± 0.4; $p = 0.005$). Plasma eHSP27 was higher before ($p = 0.049$) and after ($p = 0.015$) endurance test in HWI. Leukocytic p-HSP27 was increased 18 h after HWI (0.97 ± 0.14 vs 0.67 ± 0.11; $p = 0.04$). Creatine kinase and lactate dehydrogenase activities were increased by 3-fold and 1.5-fold, respectively, after endurance test in HWI but did not differ across trials ($p > 0.05$). Mean heart rates were higher during eccentric run and endurance test in HWI as compared to control ($p < 0.05$). Endurance capacity was similar between trials (57.3 ± 11.5 min vs 55.0 ± 13.5 min; $p = 0.564$). Pre-exercise heating increased the expression of plasma eHSPs and leukocytic p-HSP27 but did not reduce muscle damage nor enhance endurance capacity.

ARTICLE HISTORY
Received 30 November 2023
Revised 29 January 2024
Accepted 31 January 2024

KEYWORDS
Passive heating; muscle damage; heat shock response; hyperthermia; pre-conditioning

Introduction

Prolonged or high-intensity exercises can lead to muscle damage which results in muscle stiffness, soreness, and loss of strength [1]. Endurance capacity was impaired after the induction of exercise-induced muscle damage (EIMD) where participants rated a higher perception of exertion than the control group [2]. Therefore, interventions that attenuate EIMD may be helpful to sustain long-term training regime or physical performance during competition seasons. Several pre- and post-exercise interventions such as stretches, cryotherapy, compression, and water immersion have been used to relieve muscle damage [3]. However, the use of hot water immersion as a pre-exercise intervention to reduce EIMD and preserve exercise capacity is not well established.

The rationale behind the use of pre-exercise passive heating as a potential muscle protective intervention is derived from research findings in animal models. It has been shown that acute heat stress induced in rats 48 h prior to exercise helped to attenuate the muscle damage caused by downhill running [4]. Heat shock proteins (HSPs) were expressed due to heat exposure where they can bind and interact with various damaged proteins to preserve or restore their functions during stressful conditions. Furthermore, transgenic mice with

CONTACT Xiang Ren Tan XiangRen.Tan@singaporetech.edu.sg

*These authors contributed equally to the work.

This article has been corrected with minor changes. These changes do not impact the academic content of the article.

Supplemental data for this article can be accessed online at https://doi.org/10.1080/23328940.2024.2313954.

HSP72 overexpression have displayed greater endurance capacity as well as greater tolerance against EIMD [5]. On the contrary, the ablation of inducible HSP70 in mice delayed the inflammatory response toward EIMD and severely impaired muscle regeneration [6]. Smaller heat shock proteins such as HSP27 have also been implicated in protective stress responses, where they facilitate the proper refolding of damaged proteins [7]. When phosphorylated, HSP27 reorganizes into smaller oligomers which can bind to myofilaments to stabilize and maintain ultrastructural integrity in muscles [8,9]. Importantly, there have been studies in humans [9,10] that suggest the potential of HSPs in the protection against EIMD, as well as facilitating the repair and restoration of damaged myofibrils.

Passive heating as a pre-exercise intervention has also been examined in several human studies. For instance, Nosaka and colleagues [11] demonstrated that the induction of arm muscle hyperthermia via microwave diathermy one day prior to eccentric exercise conferred protective effects against muscle damage. Another study [12] showed that leg immersion in hot water before stretch-shortening exercises reduced the levels of serum creatine kinase (CK) and alleviated muscle soreness. Furthermore, localized heat preconditioning has been shown to preserve maximal voluntary contractions and the range of motion in the upper limb after eccentric exercises [13]. Therefore, these findings supported the use of acute heat exposure to preserve or restore skeletal muscle integrity. However, there are limited studies on the use of whole-body immersion as a systemic pre-heating strategy [14–19] and it is unclear if this protective effect of passive heating can aid in preserving human endurance capacity.

Therefore, this study sought to investigate whether pre-exercise passive heating could protect against EIMD from prior exercise bout through systemic heat shock response, thereby maintaining the endurance capacity. We evaluated the potential protective effect of passive heating by comparing hot water immersion at 41°C to a control immersion at 34°C where changes in circulatory biomarkers of muscle damage and heat shock response were assessed. We hypothesized that passive hyperthermia induced through hot water immersion could promote the release of HSPs to confer protective effects against EIMD and thus, improve endurance capacity relative to the control condition.

Methods

Participants

Eleven healthy recreational male athletes between 21 and 27 years of age were recruited. The participant characteristics were as follows (mean ± SD): age, 25 ± 2 years; body mass, 64.1 ± 6.4 kg; height, 1.73 ± 0.06 m; body fat percentage, 11 ± 4%; maximal oxygen consumption (VO_{2max}), 55 ± 7 ml/kg/min. All protocols were approved by the institutional review board (IRB) of the National University of Singapore under the NUS-IRB reference code of B-16-096 and approval number of NUS 3023. Details of the trial procedures, risks involved, and precautions taken were made clear to the participants before written informed consent. All participants completed a health declaration form and were certified fit-for-trials by a physician prior to commencement.

Study design

The trials were randomized, counterbalanced, and conducted under a controlled laboratory setting. The study included four sessions, the first session comprised a maximal oxygen uptake (VO_{2max}) test and anthropometry measurements; the second session was a familiarization trial (FAM); the third and fourth sessions were experimental trials: hot water immersion (HWI) and control (CT) trials. The two experimental trials (HWI and CT) were randomly assigned to participants, with each trial being at least 7 days apart to allow recovery. In this crossover design, each participant underwent both trials where they served as their own controls. All the trials were conducted with dry bulb temperature and relative humidity maintained at 22.9 ± 0.5°C and 50 ± 3%, respectively. All experimental trials were conducted at the same time of day to avoid circadian influences.

Anthropometric measurements

Body mass was measured to the nearest 0.01 kg with an electronic precision balance scale (Mettler-

Toledo GmBH, Giessen, Germany), while the height was measured to the nearest 1 mm with a stadiometer (NAGATA BW-110 H, Yong Kang, Tainan). Skinfold (4-site) at the biceps, triceps, subscapular, and suprailiac were measured using skinfold calipers (Model HSK-BI-3; Harpenden, Baty International, United Kingdom) and mean value was used to calculate total skinfolds. The total body fat percentage was estimated with the equation of Siri (1961).

Incremental exercise test

Peak aerobic capacity (VO_{2max}) of each participant was measured using incremental exercise protocol (Bruce, 1972) during the first visit. Participants ran at four different speeds on a treadmill (h/p/cosmos Mercury, Germany), starting at a speed 1 km/h slower than their expected pace for a 10 km race, with increments of 1 km/h every 3 min, for a total of 12 min. Heart rate (HR) and ratings of perceived exertion (RPE) were recorded during the last 10 s of each 3 min stage. Oxygen uptake (VO_2) was assessed over the final minutes of each stage through a mouthpiece connected to a metabolic cart (Parvomedics, USA). Following 5–10 min rest, the participant performed another run with a fixed speed at moderate exertion (to be determined by the researcher based on the preceding 12 min run) with an initial gradient of 1%, and increased by 1% every min until volitional exhaustion is reached. VO_{2max} was recorded as the mean oxygen uptake over the last minute before volitional exhaustion. The relationship between VO_2 and speed for each participant was used to determine their individual running speeds at 70% and 75% VO_{2max} in subsequent experimental trials.

Experimental trials

Participants were reminded to have a standardized diet, remain hydrated by consuming sufficient water, have at least 8 h of sleep, refrain from alcoholic/caffeinated beverages, and refrain from strenuous physical activity for 24 h before each trial. For the experimental trials, participants completed a 24 h dietary and physical activity questionnaire to facilitate their compliance with the study requirements.

There are three phases in each trial: (a) immersion phase, (b) eccentric downhill run (ER) and (c) endurance capacity test (ET) over the course of 2 days (Figure 1). In FAM or HWI trial, participants were passively heated in a water bath (seated and immersed up to shoulder level) maintained at 41°C for a maximum of 60 min or until a rectal temperature (T_{re}) of 39.5°C is reached. The mean immersion duration was 41 ± 12 min across all

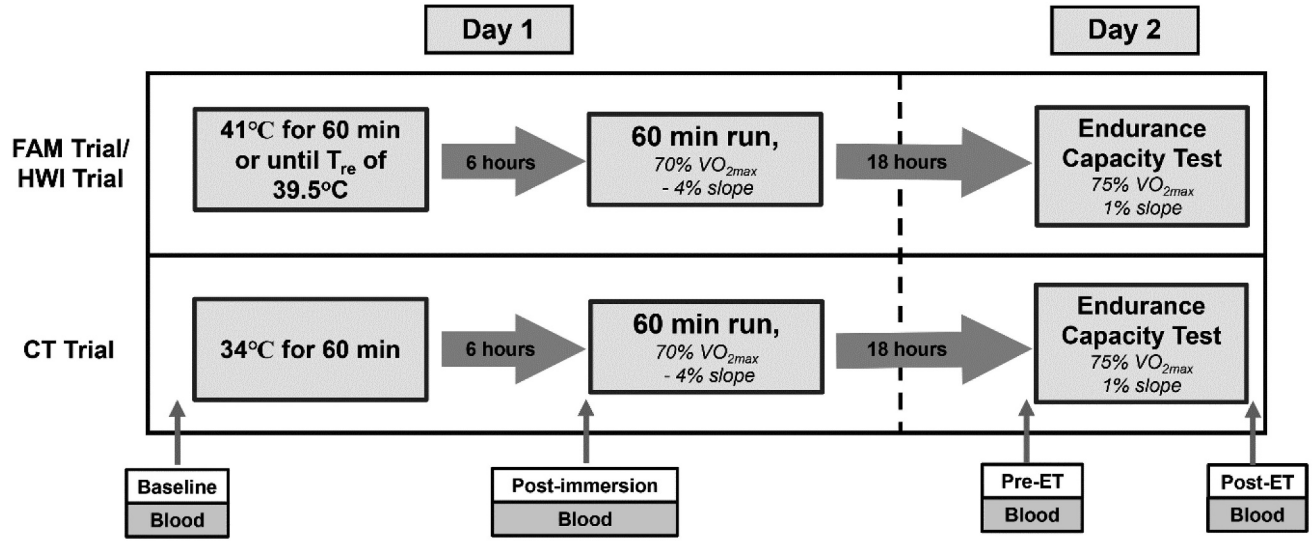

Figure 1. Experimental design. For familiarization and hot water immersion trials, the participants were immersed in 41°C for 60 min or until rectal temperature reached 39.5°C. In control trials, the participants were immersed in 34°C for the same duration. 6 h later, they performed a downhill eccentric run at –4% gradient and 70% VO_{2max}. on the following day (18 hours later), an endurance capacity test was conducted at 1% gradient and 75% VO_{2max}. FAM – familiarization, CT – control, HWI – hot water immersion.

participants, with only one participant exposed to the maximal predetermined duration of 60 min. After immersion, they rested for a period of 6 h for recovery of body temperature before performing an ER at 70% VO_{2max} on a treadmill at −4% gradient for 1 h. The next day (18 h later), an ET was conducted at 75% VO_{2max} with 1% treadmill incline where the participants ran till volitional exhaustion. For both runs, ambient temperature water (1.5 g/kg) was given every 15 min for hydration. For the CT trial, the protocol was similar to HWI trial except that the participants were immersed in 34°C water to maintain resting body temperature. Blood sampling was performed at four timepoints: (1) baseline, (2) post-immersion, (3) pre-ET and (4) post-ET (Figure 1).

HR and T_{re} of each participant were, respectively, monitored using a cardiotachometer (Polar A800, Polar Electro Oy, Kempele, Finland) and a disposable rectal thermistor probe inserted 10 cm into the rectum (Measurement Specialties Inc., OH, USA). Readings were recorded every 5 min during the run. VO_2, RPE (Borg scale [20]), and ratings of thermal sensation (RTS, Young et al., 1987 [21]) were recorded at 15-min interval during the run. Perceived muscle soreness was measured at baseline (before immersion), before ET, and 24 h after ET using a visual analog scale, where participants indicated their perceived thigh muscle soreness along a 10 cm line while maintaining a half-squat position (knee flexed at 90°) for 2 s.

Blood sampling and processing

Approximately 20 ml of venous blood was withdrawn from the median cubital vein of subjects into three EDTA-containing tubes (BD Vacutainer K2-EDTA, Becton Dickinson, New Jersey, USA) and a serum tube (BD Vacutainer serum tubes, Becton Dickinson, New Jersey, USA). The serum tube was used for the analysis of serum osmolality via Osmomat freezing point depression (Osmomat 030-D, Gonotec, Germany). Peripheral blood mononuclear cells (PBMCs) were isolated from EDTA-treated blood following the Sigma-Aldrich PBMC isolation protocol (Sigma-Aldrich, Missouri, USA). The plasma top layer was used for the CK and lactate dehydrogenase (LDH) activity assays and ELISA to detect extracellular HSPs (eHSPs). The isolated PBMC layer was used for the western blot analysis of leukocytic HSP levels.

CK/LDH assays and ELISA for plasma eHsps

Enzyme activities of CK and LDH (muscle damage biomarkers) were quantified in the extracted plasma with commercial assay kits. CK activity was quantified with the Sigma-Aldrich CK Activity Assay Kit (MAK116, Sigma-Aldrich, Missouri, USA; detection range: 30–1800 units/L CK activity), and LDH activity was quantified with the Sigma-Aldrich LDH Activity Assay Kit (MAK066, Sigma-Aldrich, Missouri, USA; detection range: 0 to 250 units/L LDH activity) in accordance with the manufacturers' protocol. Intra-assay analytic correlation variation (%) assessed upon three test subjects' samples, standards, and positive control were 5.3% and 2.5%, respectively, for CK and LDH assays. Plasma levels of eHSP27 and eHSP70 were quantified using ELISA kits (Human HSP27 and HSP70 DuoSet ELISA, R&D Systems, Inc., Minnesota, United States) following the manufacturer's protocol. CK/LDH activity or plasma HSPs levels were normalized to CT baseline values and expressed as fold change (i.e. the value of 1 indicates no difference from the control baseline level).

Western blotting for leukocytic HSPs

PBMC (includes all leukocytes except granulocytes) was isolated from the blood samples and used as a surrogate for HSP expression in the muscle cells. Leukocytic heat shock response was shown to be an appropriate surrogate for the heat shock response in skeletal muscles [22]. Circulating leukocytes in the blood were well exposed to both the systemic signals and to the signals released by the perfused tissues, so they likely exhibit similar cellular response as peripheral tissues [23].

PBMC pellets were first lysed in a Triton X-100-based lysis solution (25 mM HEPES, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100). Fresh protease and phosphatase inhibitors (PhosSTOP, Roche, Basel, Switzerland) were added, followed

by a 1 h agitation and a 15 min centrifugation (13,000 rpm) at 4°C. The supernatant was collected in a fresh tube and incubated on ice. Protein quantification was conducted via a spectrophotometer using the Bradford Assay kit (Quick StartTM Bradford Protein Assay, Bio-Rad, California, USA). Fifty micrograms per lane of protein was separated on a 10%/15% gradient resolving gel, transferred to a PVDF membrane, and the membranes were subsequently blocked with blocking buffer (TBST with 5% milk). Primary antibodies used were HSP70/72 monoclonal mouse antibody (C92F3A-5, Enzo), HSP27 monoclonal mouse antibody (G3.1, Enzo), phospho-HSP27 monoclonal rabbit antibody (Ser82, Enzo), and β-actin mouse monoclonal antibody (A5441, Sigma-Aldrich).

Primary antibody incubation was performed overnight on a shaker, at 4°C. Secondary antibody incubation was performed with mouse/rabbit horseradish peroxidase-labeled antibodies (diluted 1:1000). Blots were developed using the chemiluminescent detection reagents ECL and Femto, and films were exposed with an X-ray machine. The blot was stripped and reblotted with HSP27 antibody due to the cross-reactivity between HSP27 and p-HSP27.

Statistical analysis

Power analysis was computed *a priori* to detect a power of 0.8 at an alpha level of 0.05 using two-tailed paired T-test (matched pairs). Sample size calculation was based upon a previous study which evaluated time-trial performance in healthy participants after an exercise-heat acclimation program [24]. Using the calculated effect size (1.1) from the mean time-trial performance between the intervention and control arms, a minimum sample size of $n = 9$ was determined (G*Power 3.1.9.4, gpower.hhu.de).

Statistical Package for Social Sciences version 22.0 (SPSS Inc., Chicago, IL) was used to compute all data. All data were checked for normality using a Shapiro–Wilk test. For all physiological data, two-tailed paired Student's t-tests were performed to test for statistical differences between conditions. For time-by-trial interaction effects, two-way repeated-measures ANOVA was performed to test for significance between trial conditions. Where significant interaction effects were established, pairwise differences were identified using the Bonferroni post-hoc analysis procedure adjusted for multiple comparisons. The significance level was set at $p < 0.05$. All the values were expressed as mean ± SD. Effect size values (Cohen's d) were displayed for the key outcome measures compared between trials including perceived muscle soreness, endurance capacity, and all blood measures.

Abbreviation list

EIMD = exercise-induced muscle damage; HSPs = heat shock proteins; eHSPs = extracellular HSPs; CK = creatine kinase; LDH = lactate dehydrogenase; FAM = Familiarization; HWI = hot water immersion; CT = control; ER = eccentric downhill run; ET = endurance capacity test; T_{re} = rectal temperature; HR = heart rate; PBMC = Peripheral blood mononuclear cells; RPE = ratings of perceived exertion; RTS = ratings of thermal sensation.

Results

Hydration status

On the first day of the trial involving immersion and ER, all participants started the trials euhydrated (292 ± 4 mOsmol/kg). The mean serum osmolality remained similar post-immersion (293 ± 4 mOsmol/kg; $F_{1,\ 9} = 2.96$, $p = 0.120$) and did not differ between trials ($F_{1,\ 9} = 0.01$, $p = 0.907$). On the second day involving ET, all participants started the trials euhydrated (294 ± 4 mOsmol/kg). The mean serum osmolality increased after ET (299 ± 5 mOsmol/kg; $F_{1,9} = 16.64$, $p = 0.003$) but did not differ between trials ($F_{1,\ 9} = 0.13$, $p = 0.728$).

Physiological measures

During immersion, the target hyperthermic T_{re} and resting T_{re} were achieved, with a mean end T_{re} of 39.4 ± 0.2°C for HWI and 36.6 ± 0.3°C for CT, respectively (Figure 2(a)). Prior to the start of the eccentric run, their T_{re} recovered to baseline, and participants started with similar T_{re} in both trials (HWI: 37.3 ± 0.4°C, CT: 37.2 ± 0.3°C; $p = 0.611$). During both ER

and ET phases, T_{re} rose steadily with fixed exercise intensity and were similar in both trials ($p = 0.914$, $p = 0.121$ respectively; Figure 2(b,c)). Mean end HR during the immersion phase (Figure 2(d)) was 63 ± 12 bpm for CT trials and 110 ± 13 bpm for HWI trials. The mean HR (excluding resting HR) was observed to be higher in HWI as compared to CT trials during ER (145 bpm ±7 bpm vs 141 bpm ±7 bpm) and ET (167 bpm ±6 bpm vs 164 bpm ±7 bpm) phases. There was a main effect of time on the HR changes during both the ER (5–60 min; $F_{11,\ 110} = 43.26$, $p < 0.001$) and ET (5–30 min; $F_{5,\ 40} = 72.92$, $p < 0.001$) phases. There was a main effect of trial condition on the HR changes during both the ER ($F_{1,\ 10} = 5.25$, $p = 0.045$) and ET ($F_{1,\ 8} = 9.89$, $p = 0.014$) phases (Figure 2(e,f)). There were no time-by-trial interaction effects on the HR changes during both ER ($F_{11,\ 110} = 0.63$, $p = 0.796$) and ET ($F_{5,\ 40} = 1.01$, $p = 0.427$) phases.

Subjective ratings

During the immersion phase, mean end RPE and TS were higher in the HWI trials compared to CT trials (RPE: 14 ± 4 units vs 6 ± 1 units, TS: 6 ± 1 units vs 3 ± 1 units; $p < 0.001$). Mean end RPE and TS were comparable between both trials during ER (RPE: 13 ± 2 units vs 12 ± 2 units, TS: 5 ± 1 units vs 5 ± 1 units; $p > 0.05$) and ET (RPE: 17 ± 2 units vs 17 ± 3 units, TS: 6 ± 1 units vs 6 ± 1 units; $p > 0.05$) phases. Perceived muscle soreness scores (normalized by subtracting baseline values) assessed before ET (HWI: 1.3 ± 0.5, CT: 1.4 ± 0.5; $d = 0.20$, $p = 0.585$) and 24 h after ET (HWI: 2.0 ± 0.5, CT: 2.6 ± 0.6; $d = 1.09$, $p = 0.310$) were comparable between trials.

Endurance capacity

Endurance capacity (Figure 3(a)) was similar between trials (CT: 57.3 ± 11.5 min, HWI: 55.0 ± 13.5 min; $d = 0.18$, $p = 0.564$). Data of two participants were excluded due to poor compliance to trial procedures or injury ($N = 9$ included; data exclusion did not influence results). No trial order effect was observed for the endurance capacity test as the running times to exhaustion were similar irrespective whether participants underwent hot water immersion or control immersion in the first trial (1st Trial: 55.4 ± 11.7 min, 2nd Trial: 56.9 ± 13.4 min; $d = 0.12$, $p = 0.707$).

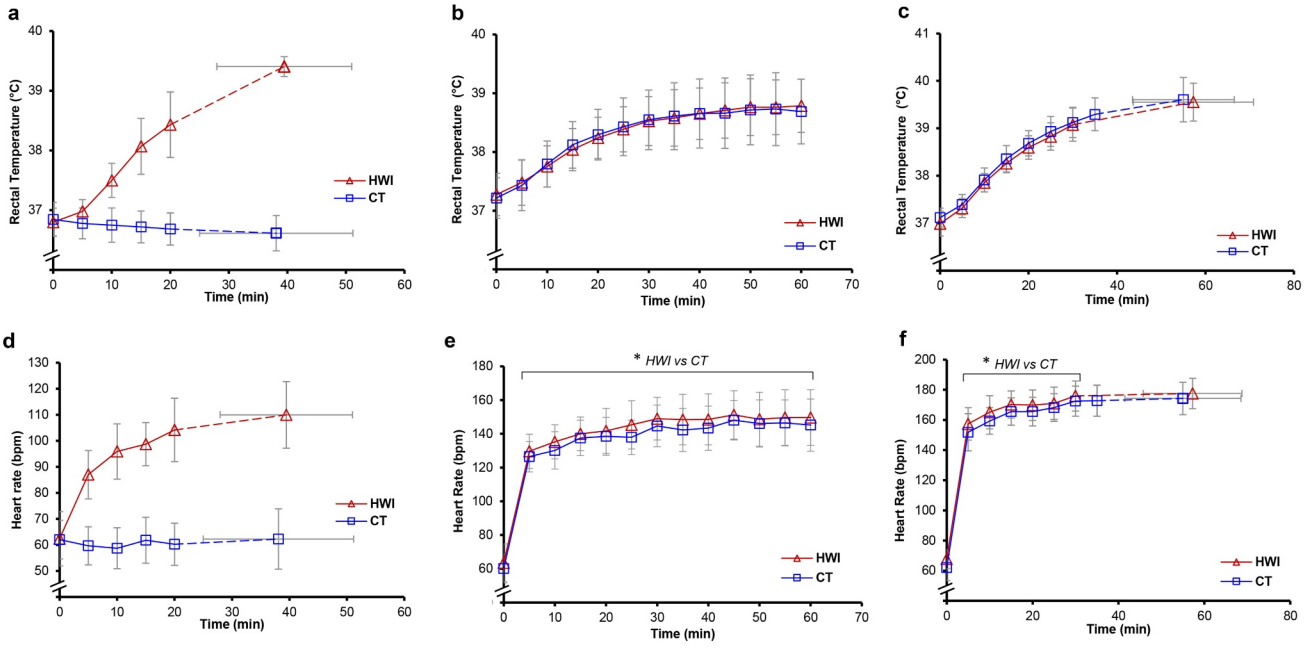

Figure 2. Mean rectal temperature profiles during (a) immersion, (b) eccentric run, and (c) endurance capacity test phases and mean heart rate profiles during (d) immersion, (e) eccentric run, and (f) endurance capacity test phases. CT – control, HWI – hot water immersion. * denotes significant difference between trials $p < 0.05$. Solid line indicates the continuation of the phase by all participants while dotted line indicates that one or more participants did not continue.

Note that the figure has been replaced - a horizontal error bar had a different colour for a subfigure in the previous figure file.

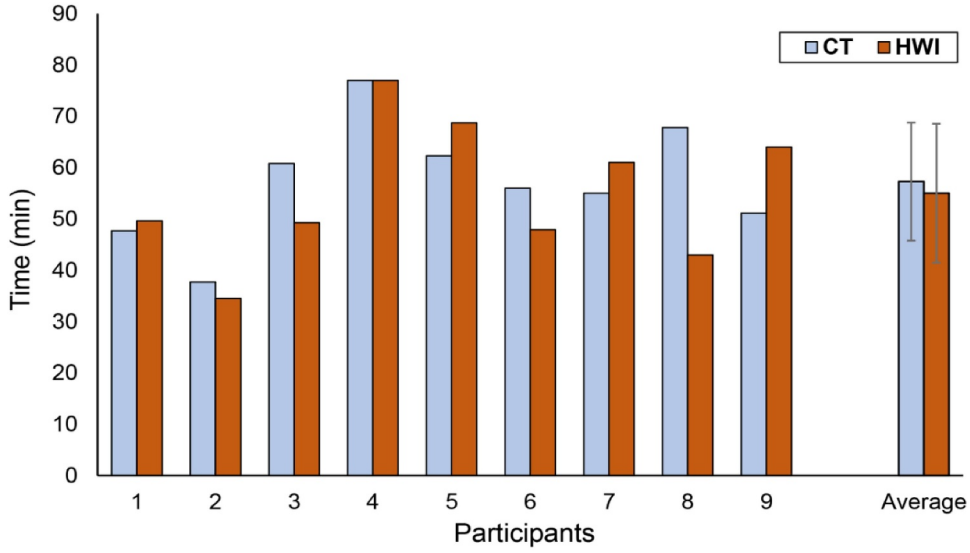

Figure 3. Endurance capacity of individual participant and averaged across participants ($n = 9$) for control and hot water immersion trials. CT – control, HWI – hot water immersion.

CK/LDH enzyme activity

Mean CK enzyme activities in HWI trial were (1) 89 ± 20 units/L at baseline, (2) 74 ± 19 units/L post-immersion, (3) 208 ± 38 units/L pre-ET, and (4) 260 ± 46 units/L post-ET. For CT trial, the mean CK activities were (1) 103 ± 21 units/L at baseline, (2) 96 ± 20 units/L before ER, (3) 235 ± 69 units/L pre-ET, and (4) 316 ± 76 units/L post-ET. After normalizing to CT baseline, the fold change in CK enzyme activity (Figure 4(a)) was higher in post-ET as compared to pre-ET in both CT (3.6 ± 1.0 vs 2.6 ± 1.0; $d = 1.00$, $p = 0.002$) and HWI (3.0 ± 0.5 vs 2.4 ± 0.4; $d = 1.33$, $p < 0.001$) trials. In HWI trials, CK activity was higher in pre-ET (1.0 ± 0.3 vs 2.4 ± 0.4; $d = 3.96$, $p = 0.004$) as compared to post immersion. No differences were observed across trials ($p > 0.05$). For trial-order effect, there were lower CK activities in the second trial for post-immersion ($d = 3.36$, $p = 0.011$), pre-ET ($d = 2.15$, $p = 0.045$) and post-ET ($d = 2.29$, $p = 0.029$).

Mean LDH enzyme activities in HWI trial were (1) 147 ± 31 units/L at baseline, (2) 156 ± 35 units/L post-immersion, (3) 151 ± 32 units/L pre-ET,

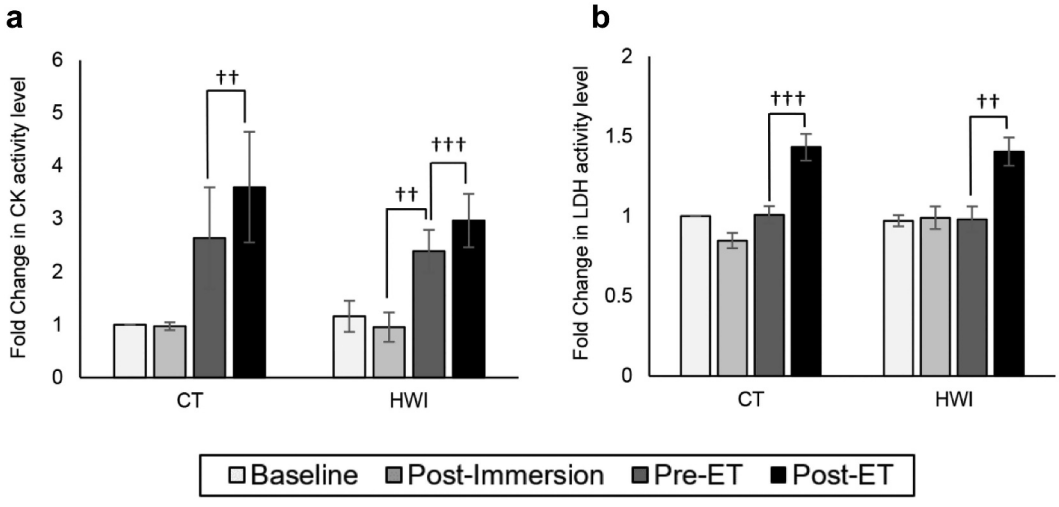

Figure 4. CK and LDH activity assay. Fold change in (a) CK and (b) LDH enzyme activity at baseline, post-immersion, pre-endurance test (pre-ET), post-endurance test (post-ET) across trials. CT – control, HWI – hot water immersion. †† and ††† denote significant differences across timepoints $p < 0.01$ and $p < 0.001$, respectively.

and (4) 199 ± 38 units/L post-ET. For CT trial, the mean LDH activities were (1) 151 ± 30 units/L at baseline, (2) 128 ± 26 units/L before ER, (3) 149 ± 29 units/L pre-ET, and (4) 206 ± 40 units/L post-ET. After normalizing to CT baseline, the fold change in LDH activity (Figure 4(b)) was higher in post-ET as compared to pre-ET in both CT (1.0 ± 0.1 vs 1.4 ± 0.1; $d = 4.00$, $p < 0.001$) and HWI (1.0 ± 0.1 vs 1.4 ± 0.1; $d = 4.00$, $p = 0.009$) trials. No differences were observed across trials ($p > 0.05$). No trial order effect was observed for LDH activity changes.

Plasma eHsps

The expression of plasma eHSP27 (Figure 5(a)) was higher in HWI as compared to CT trials for pre-ET (2.6 ± 2.9 vs 1.0 ± 0.6; $d = 0.76$, $p = 0.049$) and post-ET (3.9 ± 5.6 vs 2.4 ± 4.0; $d = 0.31$, $p = 0.015$) phases. eHSP70 (Figure 5(b)) was higher in HWI as compared to CT trials for the post-immersion (1.3 ± 0.4 vs 1.1 ± 0.4; $d = 0.50$, $p = 0.005$) and post-ET (1.7 ± 0.9 vs 1.3 ± 0.8; $d = 0.47$, $p = 0.020$) phases. No trial order effect was observed for eHSPs changes.

Leukocytic HSPs

For the densitometry results (refer to Figure 6(a) for individual western blot results), the leukocytic HSP27 and HSP70 expression levels normalized to β-actin were comparable across all phases and trials (Figure 6(b,c)). In HWI trials, the p-HSP27 (normalized to total HSP27) level was elevated in pre-ET phase as compared to the baseline (0.97 ± 0.14 vs 0.67 ± 0.11; $d = 2.38$, $p = 0.04$; Figure 6(d)). No trial order effect was observed for leukocytic HSPs changes.

Discussion

In this study, participants underwent whole-body hot water immersion prior to exercise to induce a systemic heat shock response. It was hypothesized that the released heat shock proteins reduce the extent of EIMD caused by the eccentric run, thereby preserving the endurance capacity on subsequent exercise bout. Here, our findings demonstrated that hot water immersion at 41°C induced a systemic heat shock response in the participants, as assessed from the increased HSP levels in the blood. Plasma eHSP70 and eHSP27 levels were elevated during post-immersion and pre-ET in HWI trials, respectively. Leukocytic HSP27 and HSP70 levels remained unchanged across phases in both trials. However, there was increased phosphorylation of HSP27 during the pre-ET phase after HWI.

Given that circulating leukocytes, akin to other tissues, are similarly exposed to heat stress and cell signals such as cytokines, they represent an accessible site surrogate to skeletal muscle tissues for determining the cellular heat shock response

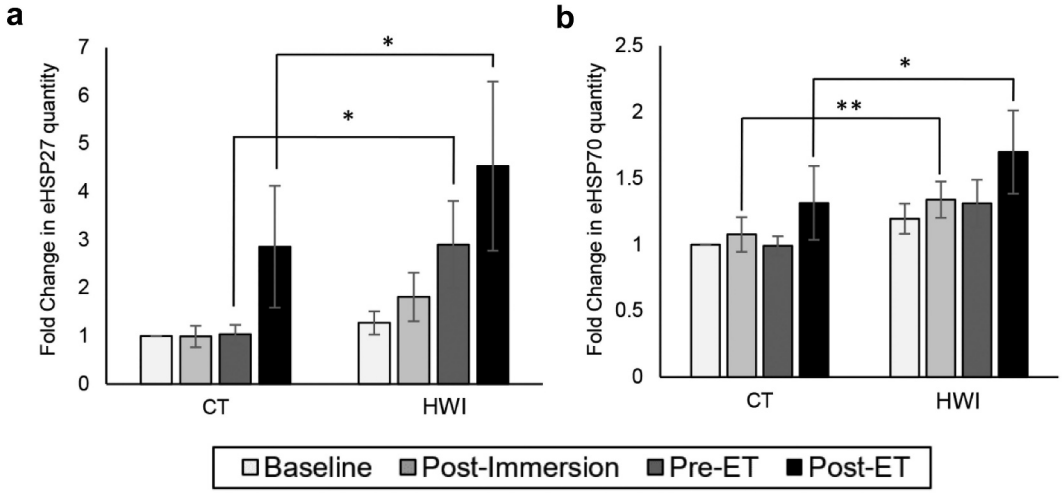

Figure 5. Extracellular HSP27 and HSP70. Fold change in (a) eHSP27 and (b) eHSP70 at baseline, post-immersion, pre-endurance test (pre-ET), post-endurance test (post-ET) across trials. CT – control, HWI – hot water immersion. * and ** denote significant difference between trials $p < 0.05$ and $p < 0.01$, respectively.

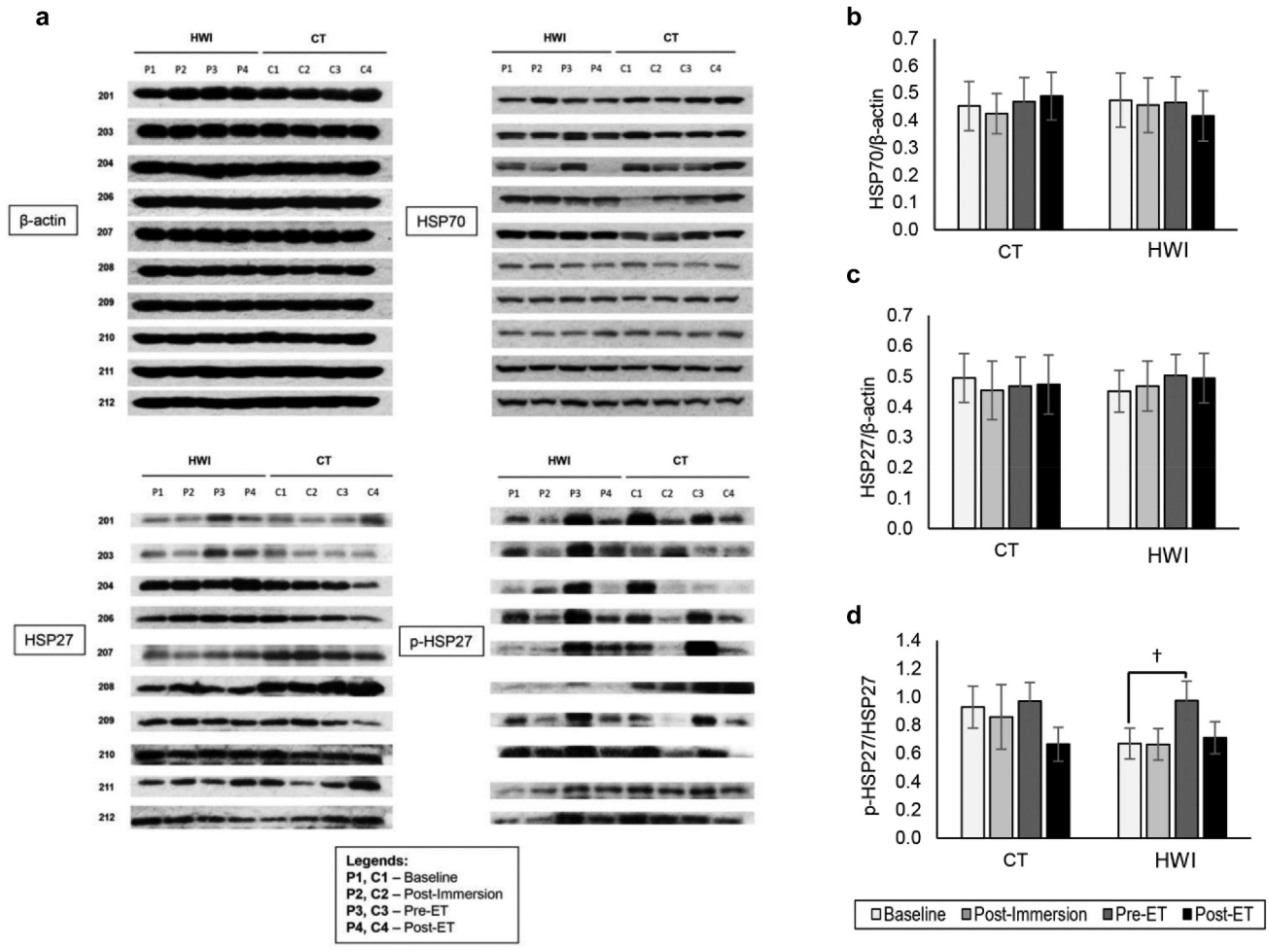

Figure 6. Western blot results depicting (a) β-actin, HSP70, HSP27, and p-HSP27 bands for individual participants, and the fold change in intracellular (b) HSP70 (normalized to β-actin), (c) HSP27 (normalized to β-actin), (d) p-HSP27 (normalized to total HSP27) at baseline, post-immersion, pre-endurance test (pre-ET), post-endurance test (post-ET) across trials. CT – control, HWI – hot water immersion. † denotes significant difference across timepoints $p < 0.05$.

[22,25]. Phosphorylated HSP27 has been proposed to play a role in protection against muscle damage. Phosphorylation of HSP27 signals for its translocation to the Z-disks of sarcomeres with severe muscle damage [9,26] where it helps to stabilize the actin filaments and maintain the integrity of cytoskeletal structure [27]. It could also promote the protection of myosin sub-fragment from heat-induced aggregation [28]. Therefore, the higher levels of p-HSP27 during the pre-ET phase could suggest a role for protection against muscle damage. However, it was noted that HSP27 phosphorylation was not immediately increased after immersion. The delayed effect could be due to the heat-induced activation of upstream kinases such as p38 and MAPKAP2/3 prior to the initiation of the HSP27 phosphorylation cascade [29]. Upon the induction of EIMD and the release of cellular damage signals after the eccentric run, this could have triggered a parallel response in skeletal muscles (matched to leukocytic response) where there is rapid phosphorylation of intracellular HSP27 for its protective effects on myofibrillar structures.

For plasma HSPs, their physiological roles are less understood, but there has been an increasing number of studies in recent years [30–32]. Circulating HSP70 has been widely implicated in modulation of immune and inflammatory responses [33–35] where it orchestrates the process of muscle repair post-exercise. Similarly, HSP27 is shown to be immunomodulatory, where it upregulates the release of cytokines via NF-κB activation [36] and modifies vascular inflammation [31]. Our study shows that plasma

HSP27 expression was higher during the pre-ET and post-ET period in the HWI as compared to CT trials, while plasma HSP70 expression was higher during post-immersion and post-ET periods in the HWI trial. The elevation in HSP27 and HSP70 expression during the post-ET period in the CT trial suggests that there could be passive release of HSPs as a result of muscle damage and cell necrosis [37]. Alternatively, these eHSPs were actively released in response to oxidative stress experienced in prolonged running and served as potential systemic signaling molecules. It has been shown that PBMCs released exosomes containing HSP70 in response to heat stress [38]. This corresponds well to the post-immersion increase in HSP70 in the HWI trials. Importantly, this circulating HSPs release could be augmented by prior heat exposure and may be important for mediating the recovery from EIMD. The exact roles of plasma HSPs in exercise recovery are largely elusive and should be explored in future studies.

To assess the impact of HSP expression on muscle damage, we measured the changes in muscle damage biomarkers (CK and LDH) and perceived muscle soreness in this study. Plasma CK levels were elevated by 2–4 folds during pre-ET (albeit not significant for CT) and post-ET phases in both trials, while LDH levels only increased by 1.5-fold during the post-ET period. This could be due to the smaller molecular size of CK molecules, which permits a more rapid efflux when membrane integrity of muscle cells is compromised [39], or the faster rate of clearance of LDH post-exercise [40]. Despite a prominent elevation in plasma HSPs and p-HSP27 levels, the increase in CK and LDH levels or perceived muscle soreness was comparable between trials. The absence of the effect could be attributed to the lack of increase in the total pool of intracellular HSP27 after HWI, or an inadequate translocation of HSPs to exercising muscles (not examined in this study), which restricts the overall protective effect. Alternatively, this may be due to the small-to-moderate level of muscle damage (2–4 folds) induced by the eccentric run, which limits the possible benefits brought about by HSP expression. Other studies have elicited greater muscle damage (4–6 folds increase in CK) with similar eccentric downhill run conducted at steeper gradients [41–44].

Concurring with the lack of effect on muscle damage indicators, pre-exercise hot water immersion did not provide clear benefits to endurance capacity. Notably, there was residual cardiovascular strain after the hot water immersion, where the mean HR was observed to be higher during ER and ET phases, as compared to control immersion. The persistence of cardiovascular strain post-heating has been reported in another study [45] where higher heart rates were observed 45 min after participants completed hot water immersion at 39°C, when compared to thermoneutral immersion. Our findings suggest that the residual strain could extend well beyond 12 h and may offset the ergogenic benefits, if any, from the heat shock response induced by passive heating. In this study, we further observed that those with poorer performance (albeit non-significant) after hot water immersion ($n = 4$) were exhibiting residual cardiovascular strain post-heating as compared to others (Appendix Figure A1). Variations in heat acclimation status may contribute to differences in baseline intracellular HSP72 levels and its expression when exposed to heat stress [46,47]. Given that HSP70 has been found to confer cardioprotective benefits such as protection against ischemia/reperfusion injury and cardiovascular stress [48–50], this could have modulated the level of cardiovascular strain incurred from heating. Therefore, individual differences in heat acclimatization status across participants may account for their differential thermotolerance and cardiovascular responses to whole-body heating [51]. Nonetheless, the relationship between post-heating residual cardiovascular strain and exercise performance remains to be further investigated.

Several limitations of this study should be noted. Due to ethical, comfort, and performance-related concerns, we relied on circulating leukocytes as a surrogate assessment site and did not obtain muscle biopsy samples to determine the cellular heat shock response directly within the skeletal muscles. Secondly, the rest period of 6 h prior to the downhill run in our study may not induce a peak HSP protective response as other studies have utilized different interval durations

(e.g. no delay, 30 min, 24 h) between heat application and eccentric exercise [11–13,16]. Future work should explore the ideal interval duration for full mounting of heat shock response with translocation of HSPs to cytoskeletal structures post-heating. Lastly, only male participants were recruited in this study and thus it is pertinent to consider that there are known sex differences in thermal sensitivity and thermoregulatory responses [52,53] which may modulate the outcome of the pre-exercise heating strategy.

Conclusion

In conclusion, our findings suggested that despite the increased expression of plasma eHSPs and leukocytic p-HSP27, pre-exercise passive heating using 41°C hot water immersion did not provide clear benefits to endurance capacity. This could be likely due to the residual cardiovascular strain incurred after immersion, which could be contributed by the inter-individual differences in thermotolerance. Our current work also showed that the pre-heating strategy did not successfully attenuate EIMD in humans, which contrasts with the findings from mice studies. Future studies may explore its applicability toward more intensive and potentially damaging exercises such as those involved in competitive and ultra-endurance events.

Acknowledgments

The authors would like to thank Isabella Wong, Sarah Loke, Beverly Tan, and Lyndi Loy for their assistance with data collection, and the participants for their efforts and commitment to the study.

Disclosure statement

No potential conflict of interest was reported by the author(s).

Funding

This research was supported by Defence Innovative Research Program (DIRP) Grant, PA No. [9015102335] from Defence Research & Technology Office, Ministry of Defence, Singapore. J.K.W.L. is supported by the National Research Foundation. Prime Minister's Office, Singapore, under its Campus for Research Excellence and Technological Enterprise (CREATE) programme.

Author contributions

I.C.C.L., S.T.W., and J.K.W.L. were involved in experimental conception and design. X.R.T. and I.C.C.L. conducted the study. X.R.T. and I.C.C.L. analyzed and interpreted the data. X.R.T. drafted the manuscript. I.C.C.L., S.T.W., and J.K.W.L. critically revised the manuscript. All authors have read and approved the final version of the manuscript.

Data availability statement

Data are available from the corresponding author upon request.

ORCID

Xiang Ren Tan http://orcid.org/0000-0001-9972-0080
Ivan C. C. Low http://orcid.org/0000-0003-2735-395X
Tuck Wah Soong http://orcid.org/0000-0002-0876-0325
Jason K. W. Lee http://orcid.org/0000-0003-4042-795X

References

[1] Cleak MJ, Eston RG. Muscle soreness, swelling, stiffness and strength loss after intense eccentric exercise. Br J Sports Med. 1992;26(4):267. doi:10.1136/bjsm.26.4.267
[2] Marcora SM, Bosio A. Effect of exercise-induced muscle damage on endurance running performance in humans. Scand J Med Sci Sports. 2007;17(6):662–71. doi:10.1111/j.1600-0838.2006.00627.x
[3] Connolly DA, Sayers SP, McHugh MP. Treatment and prevention of delayed onset muscle soreness. J Strength Cond Res. 2003;17(1):197–208. doi:10.1519/00124278-200302000-00030
[4] Touchberry CD, Gupte AA, Bomhoff GL, et al. Acute heat stress prior to downhill running may enhance skeletal muscle remodeling. Cell Stress Chaperones. 2012;17(6):693–705. doi:10.1007/s12192-012-0343-5
[5] Liu CC, Lin CH, Lin CY, et al. Transgenic overexpression of heat shock protein 72 in mouse muscle protects against exhaustive exercise-induced skeletal muscle damage. J Formos Med Assoc. 2013;112(1):24–30. doi:10.1016/j.jfma.2012.02.007
[6] Senf SM, Howard TM, Ahn B, et al. Loss of the inducible Hsp70 delays the inflammatory response to skeletal muscle injury and severely impairs muscle regeneration. PLoS One. 2013;8(4):e62687. doi:10.1371/journal.pone.0062687
[7] Rogalla T, Ehrnsperger M, Preville X, et al. Regulation of Hsp27 oligomerization, chaperone function, and protective activity against oxidative stress/tumor necrosis factor alpha by phosphorylation. J Biol Chem. 1999;274(27):18947–18956. doi:10.1074/jbc.274.27.18947
[8] Vidyasagar A, Wilson NA, Djamali A. Heat shock protein 27 (HSP27): biomarker of disease and

therapeutic target. Fibrogenesis Tissue Repair. 2012;5 (1):7. doi:10.1186/1755-1536-5-7

[9] Paulsen G, Lauritzen F, Bayer ML, et al. Subcellular movement and expression of HSP27, alphaB-crystallin, and HSP70 after two bouts of eccentric exercise in humans. J Appl Physiol. 2009;107(2):570–582. doi:10.1152/japplphysiol.00209.2009

[10] Paulsen G, Vissing K, Kalhovde JM, et al. Maximal eccentric exercise induces a rapid accumulation of small heat shock proteins on myofibrils and a delayed HSP70 response in humans. Am J Physiol Regul. 2007;293(2): R844–R853. doi:10.1152/ajpregu.00677.2006

[11] Nosaka K, Muthalib M, Lavender A, et al. Attenuation of muscle damage by preconditioning with muscle hyperthermia 1-day prior to eccentric exercise. Eur J Appl Physiol. 2007;99(2):183–192. doi:10.1007/s00421-006-0331-5

[12] Skurvydas A, Kamandulis S, Stanislovaitis A, et al. Leg immersion in warm water, stretch-shortening exercise, and exercise-induced muscle damage. J Athl Train. 2008;43(6):592–9. doi:10.4085/1062-6050-43.6.592

[13] Saga N, Katamoto S, Naito H. Effect of heat preconditioning by microwave hyperthermia on human skeletal muscle after eccentric exercise. J Sci Med Sport. 2008;7 (1):176–183.

[14] Gregson W, Drust B, Batterham A, et al. The effects of pre-warming on the metabolic and thermoregulatory responses to prolonged submaximal exercise in moderate ambient temperatures. Eur J Appl Physiol. 2002;86(6):526–33. doi:10.1007/s00421-002-0580-x

[15] Gregson WA, Batterham A, Drust B, et al. The influence of pre-warming on the physiological responses to prolonged intermittent exercise. J Sports Sci. 2005;23 (5):455–64. doi:10.1080/02640410410001730214

[16] Iguchi M, Shields RK. Prior heat stress effects fatigue recovery of the elbow flexor muscles. Muscle Nerve. 2011;44(1):115–25. DOI:10.1002/mus.22029

[17] Khamwong P, Paungmali A, Pirunsan U, et al. Prophylactic effects of sauna on delayed-onset muscle soreness of the wrist extensors. Asian J Sports Med. 2015;6(2):e25549. doi:10.5812/asjsm.6(2)2015.25549

[18] Sabapathy M, Tan F, Al Hussein S, et al. Effect of heat pre-conditioning on recovery following exercise-induced muscle damage. Curr Res Physiol. 2021;4:155–162. doi:10.1016/j.crphys.2021.06.002

[19] Philp CP, Pitchford NW, Fell JW, et al. Hot water immersion; potential to improve intermittent running performance and perception of in-game running ability in semi-professional Australian rules footballers? PLoS One. 2022;17(2):e0263752. doi:10.1371/journal.pone.0263752

[20] Borg GA. Psychophysical bases of perceived exertion. Med Sci Sports Exerc. 1982;14(5):377–381. doi:10.1249/00005768-198205000-00012

[21] Young AJ, Sawka MN, Epstein Y, et al. Cooling different body surfaces during upper and lower body exercise. J Appl Physiol. 1987;63(3):1218–1223. doi:10.1152/jappl.1987.63.3.1218

[22] Tuttle JA, Chrismas BCR, Gibson OR, et al. The Hsp72 and Hsp90α mRNA responses to hot downhill running are reduced following a prior bout of hot downhill running, and occur concurrently within leukocytes and the vastus lateralis. Front Physiol. 2017;8:473. doi:10.3389/fphys.2017.00473

[23] Sonna LA, Sawka MN, Lilly CM. Exertional heat illness and human gene expression. Prog Brain Res. 2007;162:321–346. doi:10.1016/s0079-6123(06)62016-5

[24] Lorenzo S, Halliwill JR, Sawka MN, et al. Heat acclimation improves exercise performance. J Appl Physiol. 2010;109 (4):1140–1147. doi:10.1152/japplphysiol.00495.2010

[25] Niess AM, Fehrenbach E, Schlotz E, et al. Effects of RRR-alpha-tocopherol on leukocyte expression of HSP72 in response to exhaustive treadmill exercise. Int J Sports Med. 2002;23(6):445–452. doi:10.1055/s-2002-33741

[26] Koh TJ, Escobedo J. Cytoskeletal disruption and small heat shock protein translocation immediately after lengthening contractions. Am J Physiol Cell Physiol. 2004;286(3):C713–22. doi:10.1152/ajpcell.00341.2003

[27] Guay J, Lambert H, Gingras-Breton G, et al. Regulation of actin filament dynamics by p38 map kinase-mediated phosphorylation of heat shock protein 27. J Cell Sci. 1997;110(Pt 3):357–368. doi:10.1242/jcs.110.3.357

[28] Markov DI, Pivovarova AV, Chernik IS, et al. Small heat shock protein Hsp27 protects myosin S1 from heat-induced aggregation, but not from thermal denaturation and ATPase inactivation. FEBS Lett. 2008;582 (10):1407–12. doi:10.1016/j.febslet.2008.03.035

[29] Dorion S, Lambert H, Landry J. Activation of the p38 signaling pathway by heat shock involves the dissociation of glutathione S-transferase mu from Ask1. J Biol Chem. 2002;277(34):30792–7. doi:10.1074/jbc.M203642200

[30] De Maio A, Vazquez D. Extracellular heat shock proteins: a new location, a new function. Shock. 2013;40 (4):239–46. doi:10.1097/SHK.0b013e3182a185ab

[31] Batulan Z, Pulakazhi Venu VK, Li Y, et al. Extracellular Release and Signaling by Heat Shock Protein 27: Role in Modifying Vascular Inflammation. Front Immunol. 2016;7:285. doi:10.3389/fimmu.2016.00285

[32] Reddy VS, Madala SK, Trinath J, et al. Extracellular small heat shock proteins: exosomal biogenesis and function. Cell Stress Chaperones. 2018;23(3):441–54. doi:10.1007/s12192-017-0856-z

[33] Milani V, Noessner E, Ghose S, et al. Heat shock protein 70: role in antigen presentation and immune stimulation. Int J Hyperthermia. 2002;18(6):563–75. doi:10.1080/02656730210166140

[34] Somensi N, Brum PO, de Miranda Ramos V, et al. Extracellular HSP70 activates ERK1/2, NF-kB and pro-inflammatory gene transcription through binding with RAGE in A549 human lung cancer cells. Cell Physiol Biochem. 2017;42(6):2507–22. doi:10.1159/000480213

[35] Hulina A, Grdic Rajkovic M, Jaksic Despot D, et al. Extracellular Hsp70 induces inflammation and modulates LPS/LTA-stimulated inflammatory response in

THP-1 cells. Cell Stress Chaperones. 2018;23 (3):373–84. doi:10.1007/s12192-017-0847-0

[36] Salari S, Seibert T, Chen YX, et al. Extracellular HSP27 acts as a signaling molecule to activate NF-kappaB in macrophages. Cell Stress Chaperones. 2013;18 (1):53–63. doi:10.1007/s12192-012-0356-0

[37] Basu S, Binder RJ, Suto R, et al. Necrotic but not apoptotic cell death releases heat shock proteins, which deliver a partial maturation signal to dendritic cells and activate the NF-κB pathway. Int Immunol. 2000;12(11):1539–46. doi:10.1093/intimm/12.11.1539

[38] Lancaster GI, Febbraio MA. Exosome-dependent trafficking of HSP70: a novel secretory pathway for cellular stress proteins. J Biol Chem. 2005;280(24):23349–55. doi:10.1074/jbc.M502017200

[39] Wyss M, Smeitink J, Wevers RA, et al. Mitochondrial creatine kinase: a key enzyme of aerobic energy metabolism. Biochim Biophys Acta. 1992;1102 (2):119–66. doi:10.1016/0005-2728(92)90096-K

[40] Callegari GA, Novaes JS, Neto GR, et al. Creatine kinase and lactate dehydrogenase responses after different resistance and aerobic exercise protocols. J Hum Kinet. 2017;58:65–72. doi:10.1515/hukin-2017-0071

[41] Park KS, Lee MG. Effects of unaccustomed downhill running on muscle damage, oxidative stress, and leukocyte apoptosis. J Exerc Nutr Biochem. 2015;19 (2):55–63. doi:10.5717/jenb.2015.15050702

[42] Chen TC, Nosaka K, Lin MJ, et al. Changes in running economy at different intensities following downhill running. J Sports Sci. 2009;27(11):1137–44. doi:10.1080/02640410903062027

[43] Pokora I, Kempa K, Chrapusta SJ, et al. Effects of downhill and uphill exercises of equivalent submaximal intensities on selected blood cytokine levels and blood creatine kinase activity. Biol Sport. 2014;31(3):173–8. doi:10.5604/20831862.1111434

[44] Chen TC, Nosaka K, Wu CC. Effects of a 30-min running performed daily after downhill running on recovery of muscle function and running economy. J Sci Med Sport. 2008;11(3):271–9. doi:10.1016/j.jsams.2007.02.015

[45] Worley ML, Reed EL, Kueck PJ, et al. Hot head-out water immersion does not acutely alter dynamic cerebral autoregulation or cerebrovascular reactivity to hypercapnia. Temperature. 2021;8(4):381–401. doi:10.1080/23328940.2021.1894067

[46] Amorim FT, Fonseca IT, Machado-Moreira CA, et al. Insights into the role of heat shock protein 72 to whole-body heat acclimation in humans. Temperature. 2015;2(4):499–505. doi:10.1080/23328940.2015.1110655

[47] Mee JA, Gibson OR, Tuttle JA, et al. Leukocyte Hsp72 mRNA transcription does not differ between males and females during heat acclimation. Temperature. 2016;3 (4):549–56. doi:10.1080/23328940.2016.1214336

[48] Tanimoto T, Parseghian MH, Nakahara T, et al. Cardioprotective effects of HSP72 administration on ischemia-reperfusion injury. J Am Coll Cardiol. 2017;70(12):1479–92. doi:10.1016/j.jacc.2017.07.762

[49] Zhu J, Quyyumi AA, Wu H, et al. Increased serum levels of heat shock protein 70 are associated with low risk of coronary artery disease. Arterioscler Thromb Vasc Biol. 2003;23(6):1055–9. doi:10.1161/01.Atv.0000074899.60898.Fd

[50] de Jong PR, Schadenberg AWL, Jansen NJG, et al. Hsp70 and cardiac surgery: molecular chaperone and inflammatory regulator with compartmentalized effects. Cell Stress Chaperones. 2009;14(2):117–131. doi:10.1007/s12192-008-0066-9

[51] Kuennen M, Gillum T, Dokladny K, et al. Thermotolerance and heat acclimation may share a common mechanism in humans. Am J Physiol Regul Integr Comp Physiol. 2011;301(2):R524–33. doi:10.1152/ajpregu.00039.2011

[52] Kaciuba-Uscilko H, Grucza R. Gender differences in thermoregulation. Curr Opin Clin Nutr Metab Care. 2001;4(6):533–536. doi:10.1097/00075197-200111000-00012

[53] Greenfield AM, Alba BK, Giersch GEW, et al. Sex differences in thermal sensitivity and perception: implications for behavioral and autonomic thermoregulation. Physiol Behav. 2023;263:114126. doi:10.1016/j.physbeh.2023.114126