Post-exercise cold water immersion does not alter high intensity interval training-induced exercise performance and Hsp72 responses, but enhances mitochondrial markers

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Abstract This study aims to evaluate the effect of regular post-exercise cold water immersion (CWI) on intramuscular markers of cellular stress response and signaling molecules related to mitochondria biogenesis and exercise performance after 4 weeks of high intensity interval training (HIIT). Seventeen healthy subjects were allocated into two groups: control (CON, $n = 9$) or CWI ($n = 8$). Each HIIT session consisted of 8–12 cycling exercise stimuli (90–110 % of peak power) for 60 s followed by 75 s of active recovery three times per week, for 4 weeks (12 HIIT sessions). After each HIIT session, the CWI had their lower limbs immersed in cold water (10 °C) for 15 min and the CON recovered at room temperature. Exercise performance was evaluated before and after HIIT by a 15-km cycling time trial. *Vastus lateralis* biopsies were obtained pre and 72 h post training. Samples were analyzed for heat shock protein 72 kDa (Hsp72), adenosine monophosphate-activated protein kinase (AMPK), and phosphorylated p38 mitogen-activated protein kinase (p-p38 MAPK) assessed by western blot. In addition, the mRNA expression of heat shock factor-1 (HSF-1), peroxisome proliferator-activated receptor gamma coactivator-1 α (PGC-1 α), nuclear respiratory factor 1 and 2 (NRF1 and 2),

mitochondrial transcription factor A (Tfam), calcium calmodulin-dependent protein kinase 2 (CaMK2) and enzymes citrate synthase (CS), carnitine palmitoyltransferase I (CPT1), and pyruvate dehydrogenase kinase (PDK4) were assessed by real-time PCR. Time to complete the 15-km cycling time trial was reduced with training ($p < 0.001$), but was not different between groups ($p = 0.33$). The Hsp72 ($p = 0.01$), p38 MAPK, and AMPK ($p = 0.04$) contents increased with training, but were not different between groups ($p > 0.05$). No differences were observed with training or condition for mRNA expression of PGC-1 α ($p = 0.31$), CPT1 ($p = 0.14$), CS ($p = 0.44$), and NRF-2 ($p = 0.82$). However, HSF-1 ($p = 0.007$), PDK4 ($p = 0.03$), and Tfam ($p = 0.03$) mRNA were higher in CWI. NRF-1 decrease in both groups after training ($p = 0.006$). CaMK2 decreased with HIIT ($p = 0.003$) but it was not affected by CWI ($p = 0.99$). Cold water immersion does not alter HIIT-induced Hsp72, AMPK, p38 MAPK, and exercise performance but was able to increase some markers of cellular stress response and signaling molecules related to mitochondria biogenesis.

Keywords Post-exercise recovery · Cold water immersion · Heat shock protein · High intensity interval training · Mitochondria biogenesis

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Introduction

Exercise alters body temperature regulation, metabolism and hydration state, as well as local and central fatigue (Stanley et al. 2014). In some cases (i.e., unaccustomed or high intensity exercise), the ensuing muscle fatigue reduces the maximal

voluntary contraction capacity for several days after exercise. Cold water immersion (CWI) is a common strategy used by athletes and non-athletes to facilitate post-exercise recovery (Vaile et al. 2008; Peiffer et al. 2009; Stanley et al. 2014). The theoretical main purpose of CWI use is based on the effects of hydrostatic pressure and heat exchange on muscle blood flow reducing exercise-induced increase in body temperature, muscle inflammation, metabolism, and edema (reviewed by Versey et al. 2013). Additionally, CWI improves ratings of well-being, thereby facilitating subjective recovery (Haddad et al. 2012). Several studies have tested the effects of acute post-exercise CWI on performance recovery (Roberts et al. 2014), demonstrating faster restoration, allowing individuals to improve performance in subsequent exercise sessions (Crampton et al. 2013; Rowsell et al. 2014).

Only few studies have evaluated the effect of CWI during endurance training (Yamane et al. 2006; Halson et al. 2014; Ihsan et al. 2015) and the results are not consistent. Yamane et al. (2006) reported that one leg post-exercise CWI over 4 to 6 weeks of training attenuated cycling performance improvements when compared to the control leg (no CWI). Conversely, Halson et al. (2014) found that post-exercise CWI four times per week over 3 weeks of intensified training, followed by taper, did not impair adaptation to training in competitive cyclists. Instead, the CWI group demonstrated a marginal (non-significant) increase in performance when compared with the control group. It is notable that these two previous studies did not evaluate intramuscular signaling induced by CWI during the period of exercise training. Recently, Ihsan et al. (2014) showed that acute post-exercise CWI can interfere with intramuscular muscle oxidative capacity-related signaling, evaluated by real-time PCR, which could facilitate improvements in endurance performance. In fact, the same group of investigators has recently shown that regular post-exercise CWI enhances intramuscular mitochondrial biogenesis-related signaling such as total protein content of adenosine monophosphate-activated protein kinase (AMPK), p38 mitogen-activated protein kinase (p38 MAPK), phosphorylated-acetyl-CoA carboxylase (p-ACC), and mitochondrial subunits representative of respiratory complexes (C-I, C-III, and C-IV) (Ihsan et al. 2015). The authors concluded that CWI is an effective strategy to improve changes in molecular signaling that lead to mitochondrial biogenesis and a possible enhancement of exercise performance. However, the CWI protocol used in that study does not allow for distinguishing between the performance improvements for CWI and the control groups, as one of the subjects' leg was immersed while the other served as control. Of note, the performance test performed before and after the training period was done with both legs. Additionally, the authors did not observe significant changes on transcriptional coactivator peroxisome proliferator-activated receptor gamma coactivator-1 α (PGC-1 α). Therefore, it is clear from those results that the

effect of CWI on exercise training-induced functional and molecular adaptations needs further investigation.

One important family of intracellular molecules that could be modulated by CWI during exercise training is the heat shock proteins (HSPs). Increased HSPs are induced by various stimuli observed during exercise, such as hyperthermia, hypoxia, energy substrate depletion, acidosis, and reactive oxygen species (as reviewed by Kregel 2002). It has also been suggested that HSP induction by exercise is important for cell homeostasis, to repair damaged proteins as well as participation in signaling pathways (Locke et al. 1994; Tupling et al. 2004). Also, HSPs are involved in activation of signaling pathways in skeletal muscle related to exercise adaptation in experimental models (Takahashi et al. 1998; Reid 2005). For example, the overexpression of heat shock protein 72 kDa (Hsp72) in mice increases mitochondrial transcription factor A (Tfam) expression, an important gene for mitochondrial biogenesis stimulation that increases skeletal muscle oxidative capacity (Henstridge et al. 2014). It has been demonstrated that Hsp72 overexpression in skeletal muscle of mice can protect this tissue against the deleterious effect of exhaustive exercise by increasing the enzyme superoxide dismutase (SOD), decreasing serum lactate, muscular matrix metalloproteinase or myeloperoxidase activity, and muscle damage (Liu et al. 2013).

Yamane et al. (2006, 2015) observed in non-athlete subjects detrimental effects of regular post-exercise CWI on exercise performance and speculated that this recovery strategy could inhibit HSPs expression, thereby reducing exercise training-induced muscular adaptations leading to attenuation performance improvements. However, it has not been investigated whether post-exercise CWI can affect the intramuscular response of Hsp72 after a training period and thus affect performance improvements.

In this context, it is important to understand the effects of CWI on intramuscular Hsp72 and markers of muscle oxidative capacity, and exercise training-induced physical performance. Therefore, the purpose of the present study was to evaluate the effect of CWI on intramuscular Hsp72 and signaling related to oxidative capacity and exercise performance after 4 weeks of high intensity interval training (HIIT).

Methods

Participants

Seventeen healthy, physically active, young male subjects (age, 23 ± 3 years old; body mass, 68.7 ± 9.2 kg; height, 171 ± 7 cm; body fat, 23.5 ± 5.2 %, mean $\pm$ SD) participated in this study. Before agreeing to participate, the subjects gave their written informed consent, which was previously approved by the institutional review board of the Universidade Federal dos Vales do

Jequitinhonha e Mucuri (protocol # 014/2011). Subsequently, participants underwent medical screening and clearance.

Experimental protocol

A medical questionnaire was completed by the subjects to exclude individuals taking medications or having recent musculoskeletal injuries. The physical activity readiness questionnaire (PAR-Q) was used to exclude individuals at risk for performing high-intensity exercise. The time line of the procedures is presented in Fig. 1.

Prior to the high intensity interval training, volunteer's anthropometric measurements (height, body mass, and fat percentage) were assessed, followed by a graded exercise test. Body fat was estimated using dual X-ray absorptiometry (DXA, GE, Lunar iDXA). Peak oxygen consumption ($\text{VO}_{2\text{peak}}$) was measured by using a customized ramp protocol, performed on a mechanically braked cycle ergometer (Ideal, Maxx, Brazil) as described elsewhere (De Matos et al. 2014). The ramp protocol consisted initially of 3 min of warm-up at 30 W. Then, the workload was increased every 60 s at an individualized rate until fatigue occurred despite verbal encouragement. Cadence was set to 50 rpm, and the test was ended when subject physically indicated test termination or could not maintain the preset cadence. The workload increments were based on the volunteer training history to induce fatigue between 8 and 12 min. Due to technical issues with the metabolic cart (K4b2, Cosmed, Italy), we used the equation described below to calculate the $\text{VO}_{2\text{peak}}$ on cycle ergometer (ACSM 2014):

$$\text{VO}_2 = 1,8(\text{work rate})/\text{body mass} + 7$$

Where VO_2 is the oxygen consumption ($\text{mL}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$), body mass in kilograms, and work rate in kilograms/meter.

A 15-km cycling time trial was used to evaluate performance pre and post 4 weeks of HIIT. The volunteers were

instructed to cover 15 km in the shortest possible time, on a mechanically braked cycle ergometer (Ideal, Maxx, Brazil), with an initial load (in kg) corresponding to 50 % of the maximum load (pedaling at 50 rpm) achieved in the $\text{VO}_{2\text{max}}$ test (adapted from Little et al. 2010). During the time trial, the volunteers were free to change the cadence and, consequently, the power output. The distance was measured using a cateye. Every 3 km, the subject was informed of the distance covered and requested to indicate his rating of perceived exertion (RPE) (Borg 1982), but no information was provided about test duration. The test was conducted in an environmental chamber controlled at 22 °C dry bulb temperature and 60 % relative humidity. Volunteers were not allowed to drink water during the test and HR was recorded, in addition to the time to cover every kilometer and 15 km. Volunteers performed a familiarization session 76 to 96 h before performance was assessed.

The subjects were allocated into two groups: control (CON, $n = 9$) and cold water immersion (CWI, $n = 8$). We matched the groups pairing every two subjects according to the time to complete the 15-km time trial. The HIIT protocol was performed using a cycle ergometer (Movement, BM 2800 PRO) during 4 weeks, 3 days per week, totaling 12 training sessions (adapted from Little et al. 2010). Each HIIT session consisted of 8–12 cycling exercise bouts with intensity between 90 and 110 % of the peak power for 60 s followed by active recovery of 75 s at 30 W. The cycling bouts (8–12) and exercise intensity (90–110 % of the peak power) were increased progressively and similarly between groups (Table 1). During every HIIT session, heart rate and rating of perceived exertion were measured by the end of each stimulus and after active recovery. After each HIIT session, the CWI group had their lower limbs immersed into a plastic container (dimensions: 90 cm height and 70 cm radius) containing water at 10 °C (± 1 °C) for 15 min. This temperature and duration were chosen based in previous studies that investigated the effect of CWI after one bout of exercise (Bailey et al. 2007;

Fig. 1 Timeline of all experimental procedures before, during, and after the 4 weeks of weeks of high intensity interval training for both experimental groups

Table 1 HIIT program for the 12 sessions

Session	Number of intervals	Interval intensity (% maximal power output)	Interval duration (seconds)	Active recovery duration (seconds)	Total session duration (minutes)
1	8	90	60	75	20.75
2	8	100	60	75	20.75
3	9	100	60	75	23
4	9	100	60	75	23
5	10	100	60	75	25.25
6	10	100	60	75	25.25
7	11	100	60	75	27.5
8	11	100	60	75	27.5
9	12	100	60	75	29.75
10	12	110	60	75	29.75
11	13	110	60	75	32
12	13	110	60	75	32

Vaile et al. 2008; Dunne et al. 2013) and after a training period (Ihsan et al. 2015). Water was stirred every 3 min through circular movements, avoiding temperature gradients. The water temperature was measured every 5 min with a digital thermometer (YSI 4600). The control group recovered on a chair without leg immersion in a temperate room. During recovery, heart rate was measured every minute. Every session was supervised by a graduate student.

All volunteers had adherence to training program superior to 90 % (completed at least 11 training sessions: CON 12 ± 0 and CWI 11.6 ± 0.3 sessions; $p = 0.1$). The training was offered in three shifts, morning (8:00 to 11:00), afternoon (14:00 to 17:00), and night (18:00 to 21:00) allowing the volunteer to attend one of these shifts. Each volunteer trained in the same period of the day. Environmental thermal conditions were recorded using an environmental thermometer (Alpha Mare, AM-208).

We used the Bergström and Hultman (1966) technique to perform pre/post exercise training biopsies in the *m. vastus lateralis* of the dominant leg under local anesthesia (2 % lidocaine with epinephrine). The biopsies occurred 1 week before the first and 3 days after the last training session. During the procedure, samples were harvested by suction, cleaned from blood, fat, and connective tissue, frozen in liquid nitrogen, and stored in -80°C until analysis.

Measurements

We performed western blot to evaluate intramuscular content of Hsp72, AMPK, and phosphorylated p38 MAPK. Approximately 50 mg of muscle samples were lysed by maceration in ice in 100 μL of lysis buffer (1 % Triton X100; 100 mM Tris pH 8.0, 20 % glycerol, 0.2 mM EDTA) containing protease inhibitor cocktail (Complete Protease Inhibitor

Cocktail, Roche, Germany) and phosphatase inhibitor cocktail (Phosphatase Inhibitor Cocktail PhosStop, Roche, Germany). The lysates were centrifuged at $2700 \times g$ for 20 min at 4°C , and the supernatant containing the cytosolic protein was collected. In the extracts, protein concentration was determined by the Bradford (1976) method using bovine serum albumin (BSA; 1 mg/mL) as standard. Approximately 30 μg of total protein was fractionated on polyacrylamide gel (12 %) containing sodium dodecyl sulfate (20 %) (SDS PAGE). We ran four gels for p-p38 and AMPK and other four gels for Hsp72 and GAPDH using the same amount of protein. The electrophoresis was performed between 50 and 150 V for about 4 h at room temperature (Mini Protean Tetra System, Power Pac Basic, BioRad). Proteins were transferred to previously activated (30 s in 100 % methanol) polyvinylidene fluoride membranes (Millipore, EUA), wetted in transfer buffer (25 mM Tris, 192 mM glycine, 20 % methanol). Transference was performed at 25 V and 1.5 A for 24 min in a semi dry system (Trans Blot Turbo, BioRad). Transference efficiency was verified by Ponceau S staining (0.5 %) in every membrane. The membranes were then dried and reactivated by exposing for 30 s in methanol and finally dried using filter paper for 30 min at room temperature. The membranes were incubated with primary antibody (1:1000, 1 % BSA in TBS-Tween buffer) Hsp72 (Stressgen), AMPK, p-p-38 MAPK, or GAPDH (14C10) (Cell Signaling) at room temperature, under gentle agitation for approximately 2 h. The membranes were washed in TBS Tween, three times for 5 min, and incubated for 1 h at room temperature with peroxidase-conjugated secondary antibody (1:5000, Cell Signaling). After washing three times for 5 min with TBS-Tween, the membranes were incubated in enhanced chemiluminescence solution for 15 min, protected from light. The membranes were then exposed to X-ray film (p-p38 using Hyperfilm, Amersham, USA and Hsp72, AMPK

and GAPDH using Kodak 811.6428, New York, USA) that was developed using developer and fixer (RP X-Omat, Kodak, USA) according to the manufacturer's recommendations. The film was scanned, and the bands were analyzed using Scion Image software (Scion Corporation based on NIH Image; National Institutes of Health, Scion Corporation, Frederick, MD, USA). Pre-training CTRL Hsp72/GAPDH, AMPK/GAPDH, and p-p38MAPK/GAPDH values (arbitrary units) were averaged and equaled to 100 %. Individual Hsp72/GAPDH, AMPK/GAPDH, and p-p38MAPK/GAPDH values were expressed by a percentage of pre-training CTRL Hsp72/GAPDH, AMPK/GAPDH, and p-p38MAPK/GAPDH values. GAPDH was loaded as the same internal control for p-p38, AMPK and HSP72 for each independent experiment.

We assessed mRNA expression of some mitochondrial biogenesis-related molecules by real-time PCR. One milliliter of TRIzol reagent (Invitrogen) was added to approximately 50 µg of skeletal muscle sample. Total RNA was isolated according to the manufacturer's guidelines and quantified using a spectrophotometer. The cDNA was prepared using total RNA (1 µg) and MultiScribe reverse transcriptase kit (Applied Biosystems, Grand Island, NY, USA). All primer sequences (Fig. 2) were obtained from Integrated DNA Technologies (Carpenter, CA) including Ca^{2+} /calmodulin-dependent protein kinase II (CaMK2), carnitine palmitoyltransferase (CPT1), citrate synthase (CS), heat shock factor 1 (HSF-1), nuclear respiratory factor 1 (NRF1 and NRF2), pyruvate dehydrogenase lipoamide kinase isozyme 4 (PDK4), PGC-1 α , and Tfam. The list of primers sequence is shown in Table 2. The expression of the genes was normalized by different reference genes determined for geNORM algorithm (Vandesompele et al. 2002). The list of reference genes includes β -actin, cyclophilin, RPL39, and tubulin. The real-time PCR was performed using the StepOne thermocycler (Applied Biosystems). The PCR conditions were set at 95 °C for 10 min, followed by 40 cycles at 95 °C for 10 s and 60 °C for 30 s. Real-time PCR data were analyzed using the Sequence Detector System 1.7 (Applied Biosystems). The relative amount of mRNA was calculated using 2 $^{-\Delta\text{CT}}$ method (Livak et al. 2001).

Statistical analysis

The software Statistica (v8.0, StatSoft, Inc.) was used for statistical analysis. Sample size was estimated based on the study of Yamane et al. (2006), using the endurance test outcome as the reference variable. The Shapiro–Wilk test was used to assess the normality of the data. Student's *t* test was performed to compare the characterization data between groups. We used a two-way repeated measure ANOVA to evaluate the effect of exercise on the parameters studied in the different groups, followed by Tukey's post hoc test when a significant *F* value

was observed. Statistical significance was adopted as $p \leq 0.05$. Data are presented as means $\pm$ SEM.

Results

Table 3 shows physical and physiological characteristics of the two experimental groups. As previously planned, there was no difference in physical and physiological characteristics between CWI and CON groups ($p > 0.05$), which were matched by the time to complete the 15-km time trial.

Table 4 shows the training-related data. Sessions mean heart rate (expressed as % maximal heart rate measured at pre-training incremental test) and mean heart rate during the 15-min recovery period were not different between groups. Training load, measured by session mean heart rate (bpm) times total session duration (min), increased on average 4 % (3.99 and 3.97 %, CON and CWI, respectively) per session and 52 % (51.8 and 52.0 %, CON and CWI, respectively) from session 1 to session 12, and it was not different between groups. These results show that training load was similarly increased for both groups during the HIIT protocol.

Figure 2 shows time trial-related data. Time to complete the 15-km time trial was reduced (training effect $p = 0.000$) for both groups, but there was no group ($p = 0.24$) or interaction (training $\times$ group; $p = 0.67$) effects (Fig. 2a). Time to complete each kilometer in the time trial was reduced by training ($p = 0.000$), but there was no group $\times$ training interaction ($p = 0.79$) and no kilometer $\times$ group $\times$ training interaction ($p = 0.94$) (Fig. 2b). Mean power output during the time trials (expressed as % of pre-training maximal power output) was increased from pre to post time trial ($p = 0.000$), but there was no group ($p = 0.51$) or group $\times$ training ($p = 0.67$) effects (Fig. 2c). Mean power output each kilometer was increased by training ($p = 0.000$), but there was no group ($p = 0.28$), group $\times$ time ($p = 0.98$), or kilometer $\times$ group $\times$ training ($p = 0.91$) effects (Fig. 2d). Heart rate (expressed as % of maximal heart rate) during time trials showed an increase throughout the time trials in both groups (kilometer effect, $p = 0.0000$), but no group ($p = 0.82$), training ($p = 0.73$), group $\times$ training ($p = 0.51$), or group $\times$ training $\times$ kilometer ($p = 0.97$) effects (Fig. 2e). Rating of perceived exertion scores are shown in Fig. 2f. There was a training effect ($p = 0.018$), evidencing lower scores during the post HIIT time trial. There was also an increase in the rating of perceived exertion scores during both time trials for both groups (kilometer effects, $p = 0.000$). However, there was no group ($p = 0.39$), group $\times$ training ($p = 0.43$), or group $\times$ training $\times$ kilometer ($p = 0.81$) effects.

The Hsp72 muscle values, as shown in Fig. 3a, increased with training ($p < 0.01$), but was not different between CON and CWI groups (CON 100 ± 28 to 124 ± 37 % and CWI 100 ± 31 to 131 ± 23 %, $p = 0.29$). The AMPK

Fig. 2 The 15-km time trial performance-related data before (pre) and after (post) 4 weeks of high intensity interval training for control and cold water immersion groups. Time to complete the time trial (a), time to complete each kilometer during the time trial (b), mean power output (expressed as % of pre-training maximal power output) during time trials (c), mean power output to complete each kilometer during time trial

(d), heart rate (expressed as % of maximal heart rate) during time trials (e), and rating of perceived exertion scores during time trials (f). Asterisk indicates training effect ($P < 0.05$). Data are expressed as mean $\pm$ standard error. Two-way ANOVA, CON = control, CWI = post-exercise cold water immersion, PRE = before training, and POST = after training

muscle content increased with training ($p = 0.05$), but it was not different between groups (CON 100 ± 64 to 154 ± 92 % and CWI 100 ± 45 to 155 ± 87 %, $p = 0.53$) (Fig. 3b). As shown in Fig. 3c, p-p38 MAPK muscle content also increased with training ($p = 0.048$), but it was not different between groups (CON 100 ± 12 to 128 ± 40 % and CWI 100 ± 23 to 137 ± 59 %, $p = 0.57$). These results indicate that CWI did not affect HIIT-induced muscle Hsp72, AMPK, and p-p38 MAPK increase.

Real-time PCR results are presented in Fig. 4. Neither HIIT or CWI induced changes in PGC-1 α (CON 1.0 ± 0.32 to 0.88 ± 0.10 and CWI 1.0 ± 0.23 to 0.94 ± 0.15 , $p = 0.31$), NRF-2 (CON 1.0 ± 0.26 to 0.86 ± 0.11 % and CWI 1.0 ± 0.25 to 1.19 ± 0.37 ,

$p = 0.82$), CTP1 (CON 1.0 ± 0.41 to 1.13 ± 0.24 and CWI 1.0 ± 0.37 to 1.25 ± 0.25 , $p = 0.14$), and CS (CON 1.0 ± 0.19 to 0.95 ± 0.26 and CWI 1.0 ± 0.24 to 1.20 ± 0.26 %, $p = 0.44$) mRNA. NRF-1 mRNA decreased with training ($p = 0.006$), but was not different between conditions (CON 1.0 ± 0.39 to 0.67 ± 0.18 and CWI 1.0 ± 0.34 to 0.67 ± 0.15 , $p = 0.14$). CWI increased Tfam (CON 1.0 ± 0.26 to 0.79 ± 0.29 and CWI 1.0 ± 0.22 to 1.31 ± 0.39 , $p = 0.03$), HFS-1 (CON 1.0 ± 0.38 to 0.61 ± 0.25 and CWI 1.0 ± 0.19 to 1.25 ± 0.30 , $p = 0.007$), and PDK4 mRNA (CON 1.0 ± 0.99 to 0.65 ± 0.64 and CWI 1.0 ± 0.58 to 2.26 ± 1.41 , $p = 0.03$). Also, CaMK2 mRNA decreased with HIIT (CON 1.0 ± 0.15 to 0.60 ± 0.07), but it was not affected by CWI (CWI 1.0 ± 0.22 to 1.03 ± 0.17 , $p = 0.003$).

Table 2 List of primers sequence used in the PCR analysis

	Forward	Reverse
CaMK2	5'-CACTGGCTGGACTCCTGTTT-3'	5'-AAAAGCAGTTGACAGGCACC-3'
CPT1	5'-AGGCCTATTTTGGACGTGGG-3'	5'-AACGTCACAAAGAACGCTGC-3'
CS	5'-CTACACGGTCCTGTTTGGGG-3'	5'-CTAAGGCTCGGCTCCAGATG-3'
HSF-1	5'-GAAGCAGCTGGTGCACTACA-3'	5'-AAGTAGGAGCCCTCTCCCAG-3'
NRF 1	5'-TGAGTACAGGGACAGCTCCT-3'	5'-GGTTGAGCAAGACGTGGAGA-3'
NRF 2	5'-TCGAAGTTTTCAGCATGCTACG-3'	5'-TGCCATCTCTTGTTTGTGCTGC-3'
PDK4	5'-AGAGGTGGAGCATTTCTCGC-3'	5'-ATGTTGGCGAGTCTCACAGG-3'
PGC-1 α	5'-GGAGTGACATCGAGTGTGCTG-3'	5'-ACCACTTGAGTCCACCCAGA-3'
Tfam	5'-TCAGCATGCTAAAGAGGACGA-3'	5'-TTGTGCGACGTAGAAGATCC-3'
β -actin	5'-AGACCTGTACGCCAACACAG-3'	5'-CCAGGGCAGTGATCTCCTTC-3'
Cyclophilin	5'-ACAGTGCTTGCTGGCAGTTA-3'	5'-GGCAAACCTGATTGCTGGGT-3'
Tubulin	5'-GAGGAGGTTGGTGTGGATTC-3'	5'-AAAAGCAGCACCTTTGTGAC-3'
RPL39	5'-AATCGTCCCATTTCCCAAGTG-3'	5'-CCCAGCTTGGTTCTTCTCCA-3'

Discussion

The aim of the present study was to evaluate the effect of post-exercise lower limbs CWI on physical performance, measured by 15 km time trial, and changes in intramuscular Hsp72, AMPK, p-p38 MAPK contents and markers of muscle oxidative capacity, after 4 weeks of HIIT. The present results demonstrated that HIIT-induced adaptations were not affected by CWI, measured by an increase in Hsp72, AMPK, and p-p38 MAPK muscle content and similar improvements in exercise performance in 15 km time trial testing. However, CWI up-regulated Tfam, CaMK2, and PDK4 mRNA expression, suggesting that this intervention might potentially enhance oxidative capacity. Also, HSF-1 mRNA was higher after HIIT in CWI compared with CON. These results indicate that regular post-exercise CWI induce enhancements in some markers of muscle oxidative capacity and cellular stress at the mRNA

level, but without changes in HIIT-induced endurance adaptations.

The present results demonstrate that HIIT was effective in increasing performance and improvements were similar between groups, as shown in Fig. 1. The average improvement in the time to complete the 15-km time trial was approximately 10 % for CON and 11 % for CWI, equivalent to studies using a similar training protocol (Burgomaster et al. 2006; Little et al. 2010) and confirming the effectiveness of HIIT training in improving performance during a 15-km time trial. We observed improvements in time to complete each kilometer, mean power and power during each kilometer, showing that subjects exercised at a higher intensity after the training period. There was no difference in heart rate responses between groups or between pre and post HIIT time trials, suggesting that subjects were able to maintain higher exercise intensity throughout the time trials with less cardiovascular strain. Interestingly, rating of perceived exertion scores were reduced in the post HIIT time trial, suggesting that subjects perceived the exercise as less difficult, although power output was increased and heart rate was not different in comparison with the pre HIIT time trial.

Halsen et al. (2014) observed, in professional cyclists, after an intensified training period (21 days) a marginal superior increase in performance (9.3 ± 3.0 % for CWI versus 6.5 ± 5.2 % for CON) and peak power (2.5 ± 3.5 % for CWI versus -1.8 ± 2.8 % for CON) for the CWI versus control, with no statistical differences between groups. The authors claim that although they did not observe clear changes in performance between groups, the CWI had no negative effect on the variables of exercise performance. In the present study, we also observed no negative (nor positive) effects of CWI in performance. Conversely, Yamane et al. (2006) found that post-exercise CWI decreased VO_2max ($+8.2$ % for CON and -2.2 % for CWI, $p < 0.05$) and inhibited the increase in

Table 3 Physical and physiological characteristics of the volunteers

	CON	CWI	<i>P</i> value
Age (years)	22.1 $\pm$ 2.3	25.0 $\pm$ 3.4	0.06
Body mass (kg)	70.6 $\pm$ 11.4	66.7 $\pm$ 6.1	0.39
Height (cm)	172 $\pm$ 7	170 $\pm$ 8	0.32
Body fat (%)	23.3 $\pm$ 5.0	23.7 $\pm$ 5.9	0.85
VO_2max ($\text{mL} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$)	50.1 $\pm$ 6.7	45.7 $\pm$ 4.8	0.28
Maximum power output (W)	276. $\pm$ 22.5	235.9 $\pm$ 4.7	0.08
Maximal HR (bpm)	194 $\pm$ 1.4	188 $\pm$ 4.7	0.25
Time trial (min)	42.9 $\pm$ 1.8	42.4 $\pm$ 2.4	0.62
Time trial mean power output (W)	276.7 $\pm$ 22.5	235.7 $\pm$ 14.7	0.16
Time trial mean speed (km/h)	21.45 $\pm$ 0.38	21.58 $\pm$ 0.38	0.43
Time trial mean HR (% max)	89.6 $\pm$ 1.6	88.8 $\pm$ 2.2	0.31
Time trial mean RPE (units)	15.5 $\pm$ 1.1	15.0 $\pm$ 1.2	0.55

HR heart rate, bpm beats per minute, RPE rating of perceived exertion

Table 4 HIIT-related data

	Session 1		Session 2		Session 4		Session 6		Session 8		Session 10		Session 12		CWI		P value (time)	P value (group)	P value (interaction)
	CON	CWI	CON	CWI	CON	CWI	CON	CWI	CON	CWI	CON	CWI	CON	CWI	CON	CWI			
HIIT HR(% max)	88 ± 1.5	84 ± 2.3	90 ± 1.6	88 ± 2.1	88 ± 1.6	88 ± 2.5	87 ± 1.0	86 ± 2.8	89 ± 1.3	85 ± 2.7	89 ± 1.1	84 ± 1.8	86 ± 1.4	83 ± 1.6	83 ± 1.6	83 ± 1.6	0.00	0.47	0.18
15 min Recovery HR(% max)	58 ± 3.1	57 ± 2.0	58 ± 1.6	58 ± 2.0	57 ± 1.2	5 ± 8 2.0	57 ± 0.8	56 ± 2.2	57 ± 1.2	55 ± 2.3	57 ± 0.5	55 ± 1.4	58 ± 0.9	54 ± 1.7	54 ± 1.7	54 ± 1.7	0.70	0.52	0.47
Training load (HR x duration)	3533 ± 74.9	3371 ± 67.6	3632 ± 76.8	3539 ± 65.6	3922 ± 72.8	3914 ± 92.7	4293 ± 61.6	4199 ± 110.8	4751 ± 83.4	4524 ± 129.3	5125 ± 62.4	4868 ± 93.3	5362 ± 104.6	5125 ± 174	5125 ± 174	5125 ± 174	0.00	0.23	0.09

Data are shown as mean ± SEM

HIIT high intensity interval training, HR heart rate, W Watts

Fig. 3 Representative blot and mean expression for Hsp72 (a), AMPK (b), and p-p-38 MAPK proteins (c) before (pre) and after (post) 4 weeks of high intensity interval training for control and cold water immersion groups. Data are expressed as mean ± standard error. Asterisk indicates $P \leq 0.05$ for training effects. CON = control, CWI = post-exercise cold water immersion, PRE = before training, and POST = after training. The blottings are representative of each independent experiment

femoral artery diameter after a training program of three to four times a week for 4–6 weeks, in non-athlete subjects. The authors justify the results indicating that intramuscular hyperthermia induced by exercise is considered a factor that influences endurance training adaptation, and lowering muscle temperature by CWI interfered with recovery process

Fig. 4 Mean fold change for PGC-1 α (a), NRF-1 (b), NRF-2 (c), Tfam (d), CaMK2 (e), CPT1 (f), CS (g), HSF-1 (h), and PDK4 (i) mRNA before (pre) and after (post) 4 weeks of high intensity interval training for control and cold water immersion groups. Data are expressed as mean

$\pm$ standard error. Asterisk indicates $P \leq 0.05$ for differences training effects. Number sign indicates $P \leq 0.05$ for differences in between conditions. CON = control, CWI = post-exercise cold water immersion, PRE = before training, and POST = after training

reducing improvements in performance. Also, the authors speculated that lower intramuscular temperature induced by cooling could affect the expression of HSPs, considered an important molecule for exercise induces muscle adaptation, although they did not assess HSP content.

In the present study, we evaluated the effects of regular post-exercise CWI after 4 weeks of HIIT in HSF-1 mRNA and Hsp72 intramuscular content. We reported that HSF-1 mRNA was higher in the CWI treatment compared with CON. However, Hsp72 muscle content increased in both groups with HIIT, but was not different between them. As HSF-1 is a transcription factor for other HSPs, we speculated that higher HSF-1 mRNA induced by CWI may have resulted in changes in expression of other proteins member (such as Hsp60). As reviewed by Sonna et al. (2002), under a cold shock response, the HSFs induce an increase in different HSPs. In addition, a mechanism of response to cold stress is the changes in gene expression that involves inhibition of RNA degradation. This mechanism could explain our result as the CWI maintained HSF-1 mRNA increased even without differences in Hsp72 muscle content between groups.

Yamane et al. (2006) speculated that CWI could inhibit HSP muscle content increase by a faster reduction in muscle temperature post-exercise. In the present study, we did not measure intramuscular temperature, but a previous study has shown over 4 °C muscle temperature difference between CWI and control condition (Mawhinney et al. 2013; Broatch et al. 2014). In the present study, it seems that the different thermal stress with the two experimental conditions was not sufficient to induce a different Hsp72 response in the muscle. Several non-thermal cellular stress sources such as energy substrate depletion (Sciandra and Subjeck 1983), hypoxia (Guttman et al. 1980), and acidosis (Liu et al. 1999), which occur during exercise (Vogt et al. 2001), may stimulate the synthesis of Hsp72. We do not know if the temperature difference (cooling induced by CWI) between the experimental conditions is sufficient to alter the Hsp72 response. In fact, the present study is the first to evaluate the effects of CWI on HIIT-induced performance adaptation in two groups of physically active subjects and to evaluate intramuscular Hsp72 content.

Our results show that HIIT was efficient to increase the intramuscular AMPK and p-p38 content without differences

between groups. This result indicates that decreasing muscle temperature and blood flow using post-exercise CWI has no influence on two important signaling molecules involved in mitochondrial biogenesis. P38 MAPK (Kramer and Goodyear 2007) and AMPK (Reznick and Shulman 2006) signaling have been implicated in the regulation of PGC-1 α , considered an important regulator of mitochondrial biogenesis in skeletal muscle. PGC-1 α mRNA evaluated 72 h after the last training session was not affected by HIIT or CWI. It is possible that the induction of PGC-1 α mRNA was decreased along the 4 weeks of training. As demonstrated by Perry et al. (2010), the peak of PGC-1 α mRNA occurs ~4 h after an exercise bout and returns to pre exercise level after 24 h. The authors also showed that PGC-1 α mRNA evaluated after HIIT (~90 % VO_{2peak}) sessions 1, 3, 5, and 7 was progressively decreased. We also evaluated transcription factors downstream of PGC-1 α , such as NRF1, NRF2, and Tfam. We observed no change in NRF-2 and a decrease in NRF-1 mRNA with HIIT, but no effect of CWI. Tfam mRNA, considered an important transcriptional activator of the mitochondrial genome, was higher in CWI compared with CON after HIIT. It is likely that this increase is mediated, in part, by PGC-1 α , but we could not affirm that because we did not measure PGC-1 α content. Additionally, HIIT reduced CaMK2 mRNA, but CWI was not able to change it. Bruton et al. (2010) studied skeletal muscle function in mice exposed to a cold environment (4 °C for 4–5 weeks) with special focus on the interaction between intracellular Ca²⁺ handling, mitochondrial biogenesis, and fatigue resistance. They reported that the muscles of cold-acclimated mice showed increased expression of PGC-1 α , Tfam mRNA, and citrate synthase activity. Therefore, it is possible that CWI is enhancing positively some, but not all, indices of mitochondrial biogenesis.

The gene expression of mitochondrial enzymes CS and CPT1 was not affected by HIIT or CWI. Although there are different post-transcriptional mechanisms that determine mRNA translation into protein, we speculated that CS and CPT1 enzymes increased with HIIT and the transcription stimulus reduced along the training sessions. The mitochondrial enzyme PDK4 mRNA was up-regulated only in CWI after the HIIT training. PDK4 is considered an important element involved in fuel selection by reducing the flow of pyruvate dehydrogenate into the tricarboxylic acid cycle. Buck et al. (2002) demonstrated that PDK4 mRNA levels increase five-fold in skeletal muscle and a metabolic shift from the oxidation of carbohydrates to the use of stored fatty acids as the primary source of energy in ground squirrel during hibernation. Additionally, Wende et al. (2005) observed an increase in PDK4 mRNA in muscle following 6 h of cold exposure facilitating mitochondrial fatty acid oxidation. The fast reduction in muscle temperature with CWI reduce muscle metabolism (Ihsan et al. 2013) and may facilitate fatty acid oxidation. In this sense, we observed that CWI increased PDK4

mRNA in skeletal muscle and it may facilitate the selection of a thermogenic fuel in a diverse physiological condition.

Recently, Ihsan et al. (2015) observed that regular CWI enhances molecules involved in mitochondrial biogenesis in the *vastus lateralis*. In that study, physically active subjects were trained using a treadmill for 4 weeks (three times per week) and had one of their legs cold water immersed while the other served as control. Different from the present study, the authors reported increase mitochondrial biogenesis pathway signaling molecules protein content such as AMPK and p38 in the leg that was cold water immersed. They also observed higher oxidative enzymes protein content such as β -HAD, and some respiratory chain subunits in cooled compared with control leg. However, the exercise testing protocol used in that study did not allow for a comparison of the performance-related adaptations between the legs, as tests were conducted using both legs simultaneously. It should be kept in mind that although some markers of mitochondrial biogenesis were up-regulated in the cold water-immersed leg, suggesting higher oxidative capacity in the muscle in response to CWI, oxidative enzyme (CS, CPT-1, and succinate dehydrogenase) maximal activities were not altered by CWI. Furthermore, as stated by the authors, cold-induced mitochondrial biogenesis may not be translated into increased exercise performance, as cold-induced mitochondrial biogenesis can be associated with increase in uncoupled mitochondrial respiration. For instance, cold exposure results in increased expression of uncoupled proteins (Puigserver et al. 1998; Wu et al. 1999), which facilitates the leak of protons across the inner mitochondrial membrane, bypassing ATP synthase. This adaptation would counteract the increased mitochondrial content, resulting in decreased mitochondrial efficiency and decreased ATP production per mitochondria volume. Therefore, we believe that the assessment of exercise performance in studies that used post-exercise CWI is more important than the analyses of mitochondrial biogenesis-related proteins.

A limitation of the present study is to present mRNA instead of total protein measurement as an outcome of exercise training which may limit interpretation about cell adaptation, as proposed by Miller et al. (2016). We attempted to measure other components of the mitochondria biogenesis signaling pathways, such as PGC-1 α and CaMK2 protein, but were unable to get reliable data. It is possible that some of the changes in mRNA do not agree with protein translation. Another limitation of the present study was not measured total p-38 and presented a ratio between total and phosphorylated proteins. Therefore, caution should be used interpreting the p-p-38 result.

Conclusion

We conclude that cold water immersion does not alter HIIT-induced intramuscular Hsp72, AMPK, and p38 adaptations

and exercise performance, but it was able to increase some markers of cellular stress response and oxidative capacity related to mitochondrial biogenesis.

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References

- ACSM (2014) In: Linda S Pescatello, Ross Arena, Deborah Riebe, Paul D Thompson (eds). ACSM's Guidelines for Exercise Testing and Prescription. 9th Ed. Philadelphia: Wolters Kluwer/Lippincott Williams & Wilkins
- Bailey DM, Erith SJ, Griffin PJ, Dowson A, Brewer DS, Gant N, Williams C (2007) Influence of cold-water immersion on indices of muscle damage following prolonged intermittent shuttle running. *J Sports Sci* 25:1163–1170
- Bergström J, Hultman E (1966) Muscle glycogen synthesis after exercise: an enhancing factor localized to the muscle cells in man. *Nature* 210: 309–310
- Borg GA (1982) Psychophysical bases of perceived exertion. *Med Sci Sports Exerc* 14:377–381
- Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 72:248–254
- Broatch JR, Petersen A, Bishop DJ (2014) Postexercise cold-water immersion benefits are not greater than the placebo effect. *Med Sci Sports Exerc* 46:2139–2147
- Bruton JD, Aydin J, Yamada T, Shabalina IG, Ivarsson N, Zhang SJ, Wada M, Tavi P, Nedergaard J, Katz A, Westerblad H (2010) Increased fatigue resistance linked to Ca²⁺-stimulated mitochondrial biogenesis in muscle fibres of cold-acclimated mice. *J Physiol* 588(Pt 21):4275–4288. doi:10.1113/jphysiol.2010.198598
- Buck MJ, Squire TL, Andrews MT (2002) Coordinate expression of the PDK4 gene: a means of regulating fuel selection in a hibernating mammal. *Physiol Genomics* 8(1):5–13
- Burgomaster KA, Heigenhauser GJ, Gibala MJ (2006) Effect of short-term sprint interval training on human skeletal muscle carbohydrate metabolism during exercise and time-trial performance. *J Appl Physiol* 100:2041–2047
- Crampton D, Donne B, Warmington SA, Egaña M (2013) Cycling time to failure is better maintained by cold than contrast or thermoneutral lower-body water immersion in normothermia. *Eur J Appl Physiol* 113:3059–3067
- De Matos MA, de Oliveira Ottone V, Duarte TC, Sampaio PF, Costa KB, Fonseca CA, Neves MP, Schneider SM, Moseley P, Coimbra CC, Magalhães FC, Rocha-Vieira E, Amorim FT (2014) Exercise reduces cellular stress related to skeletal muscle insulin resistance. *Cell Stress Chaperones* 19:263–270
- Dunne A, Crampton D, Egaña M (2013) Effect of post-exercise hydrotherapy water temperature on subsequent exhaustive running performance in normothermic conditions. *J Sci Med Sport* 16:466–471
- Guttman SD, Glover CV, Allis CD, Gorovsky MA (1980) Heat shock, deciliation and release from anoxia induce the synthesis of the same set of polypeptides in starved *T. pyriformis*. *Cell* 22:299–307
- Haddad HA, Parouty J, Buchheit M (2012) Effect of daily cold water immersion on heart rate variability and subjective ratings of well-being in highly trained swimmers. *Int J Sports Physiol Perform* 7: 33–38
- Halsom SL, Bartram J, West N, Stephens J, Argus CK, Driller MW, Sargent C, Lastella M, Hopkins WG, Martin DT (2014) Does hydrotherapy help or hinder adaptation to training in competitive cyclists. *Med Sci Sports Exerc* 46:1631–1639
- Henstridge DC, Bruce CR, Drew BG, Tory K, Kolonics A, Estevez E, Chung J, Watson N, Gardner T, Lee-Young RS, Connor T, Watt MJ, Carpenter K, Hargreaves M, McGee SL, Hevener AL, Febbraio MA (2014) Activating HSP72 in rodent skeletal muscle increases mitochondrial number and oxidative capacity and decreases insulin resistance. *Diabetes* 63:1881–1894
- Ihsan M, Watson G, Lipski M, Abbiss CR (2013) Influence of postexercise cooling on muscle oxygenation and blood volume changes. *Med Sci Sports Exerc* 45:876–882
- Ihsan M, Watson G, Choo HC, Lewandowski P, Papazzo A, Cameron-Smith D, Abbiss CR (2014) Postexercise muscle cooling enhances gene expression of PGC-1 α . *Med Sci Sports Exerc* 46(10): 1900–1907
- Ihsan M, Markworth JF, Watson G, Choo HC, Govus A, Pham T, Hickey A, Cameron-Smith D, Abbiss CR (2015) Regular postexercise cooling enhances mitochondrial biogenesis through AMPK and p38 MAPK in human skeletal muscle. *Am J Physiol-Regul Integr Comp Physiol* 309:R286–R294
- Kramer HF, Goodyear LJ (2007) Exercise, MAPK, and NF- κ B signaling in skeletal muscle. *J Appl Physiol* 103:388–395
- Kregel KC (2002) Invited review: heat shock proteins: modifying factors in physiological stress responses and acquired thermotolerance. *J Appl Physiol* 92:2177–2186
- Little JP, Safdar A, Wilkin GP, Tarnopolsky MA, Gibala MJ (2010) A practical model of low-volume high-intensity interval training induces mitochondrial biogenesis in human skeletal muscle: potential mechanisms. *J Physiol* 588:1011–1022
- Liu Y, Mayr S, Opitz-Gress A, Zeller C, Lormes W, Baur S, Lehmann M, Steinacker JM (1999) Human skeletal muscle HSP70 response to training in highly trained rowers. *J Appl Physiol* 86:101–104
- Liu CC, Lin CH, Lee CC, Lin MT, Wen HC (2013) Transgenic overexpression of heat shock protein 72 in mouse muscle protects against exhaustive exercise-induced skeletal muscle damage. *J Form Med Assoc* 112:24e30
- Livak K. J., Schmittgen, T. D. Analysis of relative gene expression data using real time quantitative PCR and the 2-(Δ CT) method. *Methods*, v. 25, n. 4, p. 402–8, DEC, 2001.
- Locke M, Atkinson BG, Tanguay RM, Noble EG (1994) Shifts in type I fiber proportion in rat hindlimb muscle are accompanied by changes in HSP72 content. *Am J Physiol-Cell Physiol* 266:C1240–C1246
- Mawhinney C, Jones H, Joo CH, Low DA, Green DJ, Gregson W (2013) Influence of cold-water immersion on limb and cutaneous blood flow after exercise. *Med Sci Sports Exerc* 45:2277–2285
- Miller BF, Konopka AR, Hamilton KL. (2016) The rigorous study of exercise adaptations: why mRNA might not be enough. *J Appl Physiol* (1985). Mar 24:jap.00137.2016. doi: 10.1152/japplphysiol.00137
- Peiffer JJ, Abbiss CR, Watson G, Nosaka K, Laursen PB (2009) Effect of cold-water immersion duration on body temperature and muscle function. *J Sports Sci* 27:987–993
- Perry CG, Lally J, Holloway G, Heigenhauser GJ, Bonen A, Spriet LL (2010) Repeated transient mRNA bursts precede increases in transcriptional and mitochondrial proteins during training in human skeletal muscle. *J Physiol* 588(Pt 23):4795–4810. doi: 10.1113/jphysiol.2010.199448
- Puigserver P, Wu Z, Park CW, Graves R, Wright M, Spiegelman BM (1998) A cold-inducible coactivator of nuclear receptors linked to adaptive thermogenesis. *Cell* 92:829–839
- Reid MB (2005) Response of the ubiquitin-proteasome pathway to changes in muscle activity. *Am J Physiol-Regul Integr Comp Physiol* 288:R1423–R1431

- Reznick RM, Shulman GI (2006) The role of AMP-activated protein kinase in mitochondrial biogenesis. *J Physiol* 574:33–39
- Roberts LA, Nosaka K, Coombes JS, Peake JM (2014) Cold water immersion enhances recovery of submaximal muscle function after resistance exercise. *Am J Physiol-Regul Integr Comp Physiol* 307: R998–R1008
- Rowell GJ, Reaburn P, Toone R, Smith M, Coutts AJ (2014) Effect of run training and cold-water immersion on subsequent cycle training quality in high-performance triathletes. *J Strength Cond Res* 28: 1664–1672
- Sciandra JJ, Subjeck JR (1983) The effects of glucose on protein synthesis and thermosensitivity in Chinese hamster ovary cells. *J Biol Chem* 258:12091–12093
- Sonna LA, Fujita J, Gaffin SL, Lilly CM (2002) Effects of heat and cold stress on mammalian gene expression. *J Appl Physiol* 92:1725–1742. doi:[10.1152/jappphysiol.01143.2001](https://doi.org/10.1152/jappphysiol.01143.2001)
- Stanley J, Peake JM, Coombes JS, Buchheit M (2014) Central and peripheral adjustments during high-intensity exercise following cold water immersion. *Eur J Appl Physiol* 114:147–163
- Takahashi M, Chesley A, Freyssen D, Hood DA (1998) Contractile activity-induced adaptations in the mitochondrial protein import system. *Am J Physiol-Cell Physiol* 274:C1380–C1387
- Tupling AR, Gramolini AO, Duhamel TA, Kondo H, Asahi M, Tsuchiya SC, Borrelli MJ, Lepock JR, Otsu K, Hori M, MacLennan DH, Green HJ (2004) HSP70 binds to the fast-twitch skeletal muscle sarco (endo) plasmic reticulum Ca²⁺-ATPase (SERCA1a) and prevents thermal inactivation. *J Biol Chem* 279:52382–52389
- Vaile J, Halson S, Gill N, Dawson B (2008) Effect of hydrotherapy on the signs and symptoms of delayed onset muscle soreness. *Eur J Appl Physiol* 102:447–455
- Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, Speleman F. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol.*, v. 3, n. 7, JUN, 2002.
- Versey NG, Halson SL, Dawson BT (2013) Water immersion recovery for athletes: effect on exercise performance and practical recommendations. *Sports Med* 43:1101–1130
- Vogt M, Puntchart A, Geiser J, Zuleger C, Billeter R, Hoppeler H (2001) Molecular adaptations in human skeletal muscle to endurance training under simulated hypoxic conditions. *J Appl Physiol* 91:173–182
- Wende AR, Huss JM, Schaeffer PJ, Giguère V, Kelly DP (2005) PGC-1 α coactivates PDK4 gene expression via the orphan nuclear receptor ERR α : a mechanism for transcriptional control of muscle glucose metabolism. *Mol Cell Biol* 25(24):10684–10694
- Wu Z, Puigserver P, Andersson U, Zhang C, Adelmant G, Mootha V, Troy A, Cinti S, Lowell B, Scarpulla RC, Spiegelman BM (1999) Mechanisms controlling mitochondrial biogenesis and respiration through the thermogenic coactivator PGC-1. *Cell* 98:115–124
- Yamane M, Teruya H, Nakano M, Ogai R, Ohnishi N, Kosaka M (2006) Post-exercise leg and forearm flexor muscle cooling in humans attenuates endurance and resistance training effects on muscle performance and on circulatory adaptation. *Eur J Appl Physiol* 96:572–580
- Yamane M, Ohnishi N, Matsumoto T (2015) Does regular post-exercise cold application attenuate trained muscle adaptation? *Int J Sports Med* 36(8):647–653