TITLE

Whole-body cryotherapy (-110°C) following high-intensity intermittent exercise does not alter inflammatory, hormonal or muscle damage biomarkers in trained males

RUNNING TITLE

Cryotherapy after exercise: Biomarkers of acute recovery

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ABSTRACT

Purpose: This study examined the acute effects of a single session of Whole-body Cryotherapy (WBC) following severe intermittent running exercise on biomarkers of inflammation, muscle damage and stress.

Methods: Endurance-trained males (n=11) were tested twice using a within-participant, balanced cross-over design that consisted of 5 x 5 min of high-intensity running (HIR) followed by either 3 min of WBC at -110°C or a passive control condition (CON). Before the HIR and after 60 min of recovery a ramp-test was completed. At seven time points up to 24 hrs post exercise venous blood samples were analyzed for serum levels of interleukin 6 (IL-6), interleukin 10 (IL-10), c-reactive protein (CRP), soluble intercellular adhesion molecule-1 (sICAM-1), myoglobin, cortisol, and testosterone.

Results: HIR induced significant increases in all biomarkers except sICAM-1 in both recovery conditions, respectively. Compared to the CON condition WBC did not attenuate exercise- induced changes in IL-6, IL-10, sICAM-1, myoglobin, cortisol, testosterone or their ratio. Increased levels of cortisol following exercise were negatively correlated with subsequent running performance in both conditions (WBC: $r = -0.61$, $p = 0.04$; CON: $r = -0.64$, $p = 0.04$).

Conclusion: The results of this study suggest that the postulated physiological mechanisms by which WBC is proposed to improve recovery, i.e. reductions in inflammation and muscle damage, may not be accurate.

KEYWORDS

Cryostimulation, Biomarkers, Cytokines, Acute recovery, Athletes

HIGHLIGHTS

- WBC did not affect changes in IL-6, IL-10, or myoglobin after high-intensity exercise
- Similar data were recorded for testosterone, cortisol, and their ratio
- sICAM-1 was not altered by intermittent exercise or WBC
- ∆cortisol following exercise was negatively correlated with subsequent performance

1. INTRODUCTION

Whole-body Cryotherapy (WBC) or cryostimulation is a popular and widely used recovery modality in sport and exercise medicine following intensive training and competition. It consists of brief exposures (typically 2 to 4 minutes) to very cold air (-110°C and below) in cryogenic chambers with individuals minimally dressed [1,2]. Originally utilized in a clinical setting for treating symptoms of various rheumatic diseases, WBC is purported to reduce pain, edema, and inflammation [3]. Therefore, WBC has become very popular with both recreational and elite athletes.. To date, there is a limited body of evidence regarding its efficacy and empirical data detailing the potential mechanism(s) by which this treatment could be effective is sparse [1].

Several authors have speculated that reductions in inflammation is the primary mechanism by which WBC after strenuous exercise is believed to be effective [1,2,5]. It is well established that intense exercise, especially if the athlete is unaccustomed to such modalities and/or the exercise is eccentric [6], leads to sarcomere disruptions and cell membrane damage [7]. Following cell damage leukocytes are mobilized to the injured tissue by soluble intercellular adhesion molecule-1 (sICAM-1), producing reactive oxygen species and pro-inflammatory cytokines in the injured tissue, resulting in intramuscular degradation and an amplification of muscle damage [8]. This mechanism is defined as secondary muscle damage [8] and may also be related to the increased levels of pro- and anti-

inflammatory cytokines observed following exercise [9]. It is believed that exercise- induced inflammation, e.g. indicated by augmented Interleukin (IL)-6 impairs athletic performance [10,11]. As WBC reduces skin-, muscle- and core-temperature [12], leading to vasoconstriction and reduced blood vessel permeability to immune cells, it is plausible that fewer leukocytes are mobilized to the injured tissue, leading to a reduced pro-inflammatory response and consequently less secondary muscle damage [13]. However, the recent debate regarding the effects of WBC on modulating the expression of sICAM-1 is still inconsistent and conflicting [5,14], possibly due to the timing of the intervention post exercise. [14]To the best of our knowledge, the effect of WBC on sICAM-1 following intense exercise has not been compared to a control intervention.

Several studies have investigated the physical, psychological, and physiological effects of WBC following exercise (for a review see [1,2,15]) Many [13,16–19], but not all [20,21] have reported that WBC might facilitate the recovery process after exercise. Actually, for most biomarkers (e.g. pro- and anti-inflammatory cytokines, creatinkinase (CK)) contradictory findings have been reported in the literature. These conflicting results may be due to large differences in methodology, such as exercise duration and intensity, numbers of exercise bouts and WBC sessions and time points of biomarker assessment. Thus, practical applications and recommendations for athletes and their coaches are often difficult to conclude. As daily high-quality performance and multiple competitions per week are required in many sports, athletes often require interventions to enhance recovery within hours

or a few days. To date, only a few studies [13,16,19–23] investigated the effects of a single WBC-treatment on acute recovery after high-intensity exercise. In nine well-trained runners, performing a simulated trail run peak torques of the knee extensors along with perceived sensation of pain and tiredness was not significantly different in the WBC recovery condition compared to passive rest [13]. With regard to biomarkers, no strong conclusions can be made as alterations in some biomarkers (C-reactive protein (CRP), Interleukin (IL)-1 β, IL-1 ra) indicated reduced inflammation, while others (IL-6, IL-10, tumor necrosis factor (TNF)-α) remained unchanged compared to passive recovery [19]. WBC applied after repeated sprint exercise in professional soccer players induced a greater salivary testosterone response compared to a control condition, but the changes did not result in improvements in jump performance, blood lactate, CK concentrations or perceived recovery [20]. [16][16]Other studies demonstrated no beneficial effects of WBC on muscle force recovery [21] or mixed results regarding perceived pain sensation and maximal physical performance after hamstring damaging exercise [23]. As the majority of previous studies focused on performance parameters it remains unclear whether one session in combination with high-intensity exercise alters biomarkers of hormonal status, inflammation and muscle damage.

Accordingly, the aim of the present study was to investigate the acute effects of a single WBC-session during intermittent exercise on biomarkers associated with exercise-induced inflammation, muscle damage and stress. We hypothesized that a single exposure of WBC would reduce markers of

inflammation and muscle damage, and alter the cortisol-testosterone ratio following high-intensity intermittent exercise

2. METHODS

2.1 Participants

A convenient sample size of 11 healthy, endurance-trained, male athletes participated in the study (mean ± SD age: 25.9 ± 2.1 yrs; height: 183.4 ± 3.4 cm; mass: 76.3 ± 6.6 kg; body mass index: 22.7 ± 1.7 $kg \cdot m^{-2}$; body fat: 10.7 ± 1.9 %; lean body mass: 68.1 ± 5.9 kg; peak oxygen uptake: 59.3 ± 5.3 $mL \cdot kg^{-1} \cdot min^{-1}$; performance level 3 and 4, according to De Pauw et al. [24]).. Inclusion criteria involved a history in endurance training (running) of at least 8 years with 3 or more training sessions per week as well as being familiar with high-intensity interval training, i.e. short intervals of 2-8 min at 90-95% of maximal heart rate, separated by equally short periods of recovery [25]. Athletes were excluded if they had any contraindications to WBC, such as claustrophobia, cold hypersensitivity or abrasion injuries during medical checkup as described elsewhere [2]. Participants were instructed to refrain from consuming alcohol and caffeine 24 hrs prior to the tests and to maintain their normal diet during the testing period. Intensive exercise was not permitted for up to 48 hrs prior to the two test sessions and the individual training was identical in the preceding week, respectively. Additional recovery methods, including the use of non-steroidal anti-inflammatory drugs, were not permitted. After demonstration and briefing about the

potential risks, all participants provided their written informed consent. The study was conducted in accordance with the Declaration of Helsinki and approved by the ethical committee of the German Sports University Cologne.

2.2 Experimental design

This study was part of a larger project investigating effects of WBC on performance variables, and the experimental design has been described in detail elsewhere [16]. Briefly, a within-participant, balanced cross-over design (with 7-days washout) was employed. Before participation, the athletes underwent a medical checkup and familiarization with WBC. Furthermore they carried out an incremental step test (starting velocity: 2.4 $m \cdot s^{-1}$; increase 0.4 $m \cdot s^{-1}$ every 5 min; treadmill gradient 1%) on a treadmill (Woodway ELG 90/200 Sport, Lörrach, Germany) until individual exhaustion to determine VO_2max (MetaLyzer 3b, Cortex, Leipzig, Germany) and the individual intensities for High-intensity-running (HIR) during the two main tests. The experimental protocol of these tests is presented in Figure 1.

***** Figure 1 near here*****

All athletes were tested at the same time of day on each occasion and were randomly assigned to start with either the WBC or control intervention (CON) using research randomizer (version 4.0, retrieved from http://www.randomizer.org/). The participants arrived at the laboratory at least one hour prior to the test for acclimatization. At first, an incremental exercise protocol to individual exhaustion was performed (Ramp 1). The

protocol consisted of 3 submaximal 3-min steps at 3.2, 3.6 and 4.0 $m \cdot s^{-1}$ with a treadmill gradient of 1% and 30 s of rest after each step. Thereafter velocity was increased to 4.4 $m \cdot s^{-1}$ and remained constant while the treadmill gradient was increased by 0.5% every 30 s until exhaustion. After 5 min of recovery, HIR was carried out, consisting of 5 x 5 min at 90 % of maximum velocity (V_{max}) reached during the step test, with 4 min of active recovery in between the intervals (60 % of V_{max}). HIR was followed by 1 h of passive recovery, which was identically structured in both conditions except for the implementation of one 3-min session of WBC after 45 min of rest. During the recovery period the athletes remained seated in the conditioned laboratory (ambient laboratory temperature WBC: 21.7 ± 0.8°C vs. CON: 21.7 ± 1.0°C; humidity WBC: 36.4 ± 7.7% vs. CON: 35.8 ± 8.3%) and consumed 0.5 L of a standardized fluid intake (energy: 400 kcal consisting of 46.5g carbohydrates, 15g protein, 17g fat) to avoid dehydration and to replenish depleted glycogen stores [26]. WBC was performed in a temperature-controlled cryochamber with an electrical cooling system (Zimmer MedizinSysteme GmbH, Ulm, Germany). The chamber system consists of three separate rooms with constant temperatures of -10, -60 and -110°C and we employed a protocol similar to that previously described elsewhere [2,16]. The participants traversed the first two chambers with -10°C and -60°C quickly and remained slowly walking for 3 min within the room at -110°C. During the control intervention athletes walked slowly within the laboratory for 3 min (at 21.7 ± 0.8°C and 35.8 ± 8.3 % humidity). After a total of 60 min of recovery, athletes performed a second incremental exercise

(Ramp 2) with the same design as the first one. Time to exhaustion (t_{lim}) was defined as the time (sec.) from the beginning of the ramp test until the athlete´s volitional exhaustion [16].

2.3 Data measurement

Before and after Ramp 1 + HIR ($R1_{pre}$, $R1_{post}$) and Ramp 2 ($R2_{pre}$, $R2_{post}$) as well as 1-, 4- and 24-hrs after finishing the exercise protocol 8.5 mL venous blood samples was obtained (BD Vacutainer Blood Collection System, Beckton Dickson & Company, Plymouth, UK) from the antecubital vein following 10 min of seated rest (see Figure 1). After collection, the samples were stored at 7 °C for ~30-min for deactivation of coagulation factors before being centrifuged (1861 g for 10-min at 4°C, Rotixa 50; Hettich Zentrifugen, Mühlheim, Germany). The serum was then aliquoted (Eppendorf type) at -80°C until later analysis. In particular, we were interested in the inflammatory markers IL-6, IL-10, CRP and sICAM-1; the hormonal biomarkers cortisol and testosterone, and the muscle damage biomarker myoglobin. Serum levels of cortisol ($ng \cdot mL^{-1}$), testosterone ($ng \cdot mL^{-1}$), IL-6 ($pg \cdot mL^{-1}$), IL-10 ($pg \cdot mL^{-1}$), sICAM-1 ($ng \cdot mL^{-1}$), CRP ($mg \cdot L^{-1}$), and myoglobin ($ng \cdot mL^{-1}$) were determined using human enzyme-linked immunosorbent assay (ELISA) kits. Manufacturer instructions were followed for each of the kits and repeated freeze-thaw cycles of serum were avoided. Intra-assay coefficient of variations for cortisol, testosterone, c-reactive protein and Myoglobin (ELISA kits manufactured by DRG Instruments GmbH, Marburg, Germany) as well as sICAM-1 and IL-10 high sensitive (R&D Systems Inc, Minneapolis, USA)

and IL-6 high sensitive (IBL International GmbH, Hamburg, Germany) was 3.2%, 3.3%, 4.2%, 3.9%, 5.0%, 9.3%, and 4.6%, respectively. Minimum detectable serum concentrations were 2.5 $ng \cdot mL^{-1}$ for cortisol, 0.083 $ng \cdot mL^{-1}$ for testosterone, 0.03 $pg \cdot mL^{-1}$ for IL-6, 0.09 $pg \cdot mL^{-1}$ for IL-10, 0.096 $ng \cdot mL^{-1}$ for sICAM-1, 0.1 $mg \cdot L^{-1}$ for CRP, and 5.0 $ng \cdot mL^{-1}$ for myoglobin. Hematological blood analysis was performed on the day of data collection for the assessment of white blood cell count ($1 \cdot 10^{-3} \cdot \mu L^{-1}$) using Sysmex KX-21N (Sysmex Deutschland GmbH, Norderstedt, Germany).

2.4 Statistical Analyses

All statistical tests were carried out using the Statistica software package for Windows® (version 13.0, StatSoft Inc., Tulsa, OK, U.S.A). The distribution of data was assessed using descriptive methods (skewness, outliers, and distribution plots) and inferential statistics (Shapiro–Wilk test). As all data were normally distributed data are presented as means ± standard deviations (SD). A two way (treatment [WBC, Control] * time [$R1_{pre}$, $R1_{post}$, $R2_{pre}$, $R2_{post}$, 1h, 4h, 24h]) repeated-measures analysis of variance (ANOVA) was applied to compare all biomarkers. If main effects or interactions were identified, Bonferroni post-hoc analysis was applied where appropriate. Statistical significance was accepted at $P < 0.05$. Person product-moment correlations were used to detect relationships between ramp tests performance decrements ($\Delta t_{lim} = t_{lim}R2 - t_{lim}R1$) and changes in cortisol, testosterone, IL-6, IL-10, sICAM-1, CRP, myoglobin, and white blood cell count from baseline ($R1_{pre}$) to $R2_{pre}$ in both recovery conditions, respectively.

3. RESULTS

The serum concentrations of all biomarkers (mean ± SD) are detailed in table 1. At baseline ($R1_{pre}$), similar results were recorded for all outcome measures (all $p > 0.05$) and all values were within normal range for healthy individuals.

3.1 Inflammatory Markers

Significant time effects ($p < 0.01$) were observed for IL-6 (Fig 2A) and IL-10 (Fig 2B). No significant intervention or interaction effects were detected for IL-6 ($p = 0.23$ and $p = 0.51$) and IL-10 ($p = 0.53$ and $p = 0.78$), respectively. Compared to baseline (WBC: 0.85 ± 0.56; CON: 0.75 ± 0.41) IL-6 ($pg \cdot mL^{-1}$) was significantly higher at $R1_{post}$ (WBC: 2.12 ± 0.99; CON: 1.93 ± 0.51), $R2_{pre}$ (WBC: 1.19 ± 0.51; CON: 1.18 ± 0.28), $R2_{post}$ (WBC: 1.22 ± 0.56; CON: 1.10 ± 0.31) and 1h (WBC: 1.25 ± 0.47; CON: 1.19 ± 0.42) (all $p < 0.01$). IL-10 ($pg \cdot mL^{-1}$) was elevated at $R1_{post}$ (WBC: 3.62 ± 2.03; CON: 3.27 ± 0.97, $p < 0.01$) and $R2_{pre}$ (WBC: 3.03 ± 1.39; CON: 2.68 ± 0.61, $p < 0.01$) compared to baseline (WBC: 1.49 ± 0.42; CON: 1.44 ± 0.39).

Despite an increase 24h after exercise ($p < 0.01$), CRP (Fig 2C) was not altered following WBC (intervention: $p = 0.51$; interaction: $p = 0.94$).

sICAM-1 levels following the two treatments remained similar (Fig. 2D), with no intervention ($p = 0.96$) or interaction ($p = 0.27$) effects observed. Despite

observing a significant main effect for time ($p < 0.01$), no post-hoc differences (all $p > 0.05$) using the Bonferroni correction were detected.

A significant increase over time ($p < 0.01$), but no intervention ($p = 0.73$) or interaction ($p = 0.79$) effect was observed in white blood cell count (Fig. 2E). Compared to baseline (WBC: 5.1 ± 1.4; CON: 5.4 ± 1.4) white blood cell count ($\cdot 10^9 \cdot L^{-1}$) was elevated (all $p < 0.01$) at $R1_{post}$ (WBC: 6.4 ± 1.7; CON: 6.3 ± 1.8) $R2_{pre}$ (WBC: 7.4 ± 1.9; CON: 7.0 ± 2.2), $R2_{post}$ (WBC: 10.7 ± 2.5; CON: 10.0 ± 2.3), 1h (WBC: 9.5 ± 2.4; CON: 9.3 ± 2.0) and 4h (WBC: 9.1 ± 2.1; CON: 8.7 ± 1.6).

3.2 Muscle damage

There was a significant increase in myoglobin [$ng \cdot mL^{-1}$] over time ($p < 0.01$; Fig. 2F); however no significant intervention ($p = 0.36$) or interaction ($p = 0.73$) effects were observed. Compared to baseline (WBC: 39.5 ± 7.7; CON: 39.8 ± 10.0) myoglobin was elevated at $R1_{post}$ (WBC: 81.7 ± 26.2; CON: 84.5 ± 27.0), $R2_{pre}$ (WBC: 94.1 ± 35.3; CON: 113.6 ± 52.8), $R2_{post}$ (WBC: 93-6 ± 33.4; CON: 115.9 ± 64.3), 1h (WBC: 124.9 ± 52.2; CON: 158.0 ± 108.3) and 4h (WBC: 95.9 ± 31.4; CON: 123.0 ± 95.8) (all $p < 0.01$).

***** Figure 2 near here*****

3.3 Hormonal response

A significant main effect over time was also overserved for cortisol ($p < 0.01$; Fig 3A), testosterone ($p < 0.01$; Fig 3B), and testosterone to cortisol ratio ($p < 0.01$; Fig 3C). Specifically, compared to baseline (WBC: 158.7 ± 26.7;

297 CON: 167.0 ± 29.8) cortisol (ng·mL-^{1}) was elevated (all $p < 0.01$) after the
298 first ramp test (WBC: 217.9 ± 48.6; CON: 224.0 ± 58.2), at 1h (WBC: 115.6
299 ± 29.3; CON: 130.5 ± 14.6), 4h (WBC: 48.7 ± 18.4; CON: 62.8 ± 29.1) and
300 24h (WBC: 87.1 ± 20.3; CON: 89.0 ± 26.2). Compared to baseline (WBC:
301 5.2 ± 1.4; CON: 5.4 ± 1.9) testosterone (ng·mL-^{1}) was also elevated (all $p <$
302 0.01) after the first ramp test (WBC: 6.4 ± 2.2; CON: 7.0 ± 2.1), at 1h (WBC:
303 4.0 ± 1.1; CON: 4.2 ± 1.4) and 4h (WBC: 3.2 ± 1.2; CON: 3.8 ± 1.3). Analysis
304 of the testosterone to cortisol ratio indicated that, compared to baseline
305 (WBC: 0.34 ± 0.11; CON: 0.35 ± 0.19) values increased significantly at 4h
306 (WBC: 0.80 ± 0.52; CON: 0.81 ± 0.54, $p < 0.01$) and 24h (WBC: 0.63 ± 0.21;
307 CON: 0.72 ± 0.47, $p < 0.01$). Again, no significant intervention or intervention
308 * time interaction was overserved for cortisol ($p = 0.53$ and $p = 0.93$
309 respectively), testosterone ($p = 0.67$ and $p = 0.81$ respectively), and the
310 testosterone to cortisol ratio ($p = 0.84$ and $p = 0.98$ respectively).

311 ***** Figure 3 near here*****

312

313 **3.4 Correlation**

314 Performance decrements (Δt_{lim}) correlated significantly with Δcortisol after
315 both the CON ($r = -0.64$, $p = 0.04$) and the WBC interventions ($r = -0.61$, $p =$
316 0.04; Fig. 4). No statistically significant correlations were detected for Δt_{lim}
317 and the change in any of the other biomarkers (all $p > 0.05$).

318 ***** Figure 4 near here*****

319 ***** Table 1 near here*****

320 ## 4. DISCUSSION

321 The current study is the most thorough investigation of the acute effects (up
322 to 24 hrs) of a single WBC-session following intermittent high-intensity
323 exercise on hormonal, inflammatory and muscle damage biomarkers to
324 date. The main findings of this investigation are as follows: (1) contrary to
325 our initial hypothesis, compared to passive recovery one session of WBC
326 did not alter the exercise-induced inflammatory, muscle damage or
327 hormonal response to high-intensity running in trained athletes, (2) the
328 exercise-induced perturbations of all inflammatory, muscle damage, and
329 hormonal biomarkers, except CRP and testosterone to cortisol ratio,
330 returned to basal levels within 24 hrs, and (3) increased levels of cortisol,
331 induced by high-intensity exercise, were negatively correlated to subsequent
332 running performance. Collectively, these data suggest that the postulated
333 physiological mechanism(s), i.e. reductions in inflammation and muscle
334 damage, by which WBC is purported to enhance recovery from EIMD
335 following high-intensity running in trained male athletes may not be accurate.

336 Reducing the inflammatory response following exercise is one of the primary
337 reasons why WBC is applied as a recovery method [2]. In the present study,
338 similar inflammatory responses were observed following WBC and the
339 control intervention, with IL-6 and IL-10 peaking immediately after exercise
340 and returning to baseline after 1-4 hrs post-exercise (see Fig 2). Pournot and
341 colleagues [19] have previously reported comparable IL-6 and IL-10

342 reactions following a simulated trail run on a treadmill in 11 well-trained
343 distance runners. While exercise duration was longer in our investigation
344 (56.3 ± 1.6 min vs 48 min), we did not include downhill running, that is known
345 to induce severe structural muscle damage and inflammation due to high
346 muscular load during eccentric contractions [13]. Selfe and colleagues [27]
347 also reported similar IL-6 values to those of the present investigation and no
348 effects of WBC on IL-6 concentration applied 10-16 hrs after a rugby training.
349 Interestingly, another study reported reduced IL-6 values after 40-min of
350 cycling in professional volleyball players following one session of WBC
351 compared to no WBC and the authors suggested that WBC might initiate
352 protective effects [28]. As WBC preceded exercise, it is likely that these
353 methodological differences caused the differences in the cytokine profile
354 compared to the present study.

355 Despite a main effect over time, sICAM-1 was not altered following the WBC
356 intervention (see Fig. 2). Thus, assuming sICAM-1 plays a key-role in the
357 inflammation response [5,14], the present data suggest that a single session
358 of WBC is insufficient to reduce the exercise-induced inflammation. Banfi
359 and colleagues [29] have previously reported reduced levels of sICAM-1
360 after five daily WBC- sessions in rugby players, yet due to the absence of a
361 control group, it is difficult to delineate if WBC in isolation was responsible
362 for these findings [29]. In contrast, Both Dugué and Leppänen [30] and
363 Buemi and colleagues [31] reported increased levels of sICAM-1 after the
364 application of cold water immersion. There is a brevity of empirical data

365 investigating the effects of WBC on the sICAM-1, especially repeated
366 exposures, thus this topic warrants further investigation [5,14].

367 In comparison to the control condition, WBC did not alter CRP (see Fig. 2).
368 Similarly, no effect of WBC on CRP response was reported in rugby players
369 after five days with continuous training and daily WBC- treatments [32].
370 Contrary, Pournot and colleagues observed reduced CRP-levels 24 hrs after
371 one session of WBC (3 min at -110°C) compared to passive recovery in
372 equally trained participants [19]. Overall higher CRP-values compared to
373 our results are most likely caused by additional eccentric contractions during
374 downhill running. Furthermore, Pournot reported decreased levels in pro-
375 inflammatory cytokine IL-1β and increased levels in anti-inflammatory
376 cytokine IL-1ra, suggesting, in contrast to our findings, that one session of
377 WBC reduces the inflammatory process [19].

378 CK is probably the most frequently analyzed biomarker to identify muscle
379 damage [33]. CK only leaks into the bloodstream when the sarcolemma is
380 damaged and is, therefore, another commonly used biomarker of muscle
381 damage [34]. However, the enzyme has a molecular mass of 84 kilodaltons
382 (kDa) and has to be transported by the lymphatic system [6]. Therefore, the
383 onset of CK-concentration in venous blood is delayed. - Depending on the
384 magnitude of muscle damage, CK peaks 24 to 96 hrs after exercise [35].
385 Therefore myoglobin, a rather small molecule (18 kDa) that is released
386 directly to the blood flow as a result of degradation of muscle proteins [34],
387 appears to be more feasible to detect the acute muscle damage effects of

388 strenuous exercise. Myoglobin typically peaks within the first hours after
389 severe exercise and is already decreasing at 24 hrs post exercise whereas
390 CK is still rising [36,37]. To the best of our knowledge, this is the first
391 investigation that has utilized myoglobin as marker for muscle damage after
392 WBC. Myoglobin peaked 1 hr after exercise and returned to baseline after
393 24 hrs with no differences between recovery modalities (see Fig 2).
394 Although this is the first study to assess myoglobin, others have reported
395 comparable results regarding CK [13,20], but also reduced levels of CK after
396 WBC [3,18,38–40] [13,20]. However, these studies have some limitations,
397 such as small sample size [38], the lack of a control group [40] or the high
398 probability that the results were influenced by the repeated bout effect
399 [18,39]. Therefore, the evidence for WBC to reduce muscle damage remains
400 very limited.

401 Testosterone, cortisol, and their ratio are often employed as biomarkers of
402 anabolic status, training responses/adaptations and motivation [41]. It is well
403 established that cortisol increases when an individual is exposed to
404 psychophysiological stress following activation of the hypothalamic-pituitary-
405 adrenal axis [42]. The physical exercise itself potentially increases cortisol
406 further, though the magnitude depends on, amongst other variables, the type
407 of exercise [43] and the environmental conditions [44] where it takes place.
408 In the present study we observed increased levels in cortisol at baseline and
409 exercise induced increments in cortisol and testosterone, respectively (see
410 Fig 3). Due to heterogeneous methodologies and conflicting results in the
411 current literature, the effects of WBC on hormone biomarkers is still unclear

412 [38,45–47]. However, to our knowledge there is only one study which has
413 investigated the effects of a single WBC-session, applied immediately after
414 exercise, on cortisol and testosterone [20]. In line with the findings of the
415 present study, Russell and colleagues [20] detected no WBC- related
416 changes in cortisol. Collectively, these findings suggest that a single WBC-
417 session does not alter the stress-related hormone cortisol. However, in
418 contrast to our findings Russell and colleagues [20] reported increased
419 testosterone levels in the WBC- group, suggesting that higher testosterone-
420 concentrations may facilitate recovery.[20]. Moreover, the authors speculate
421 that higher testosterone- values might indicate reduced inflammation as low
422 serum- testosterone concentrations are related to inflammation [20]. These
423 results and speculations cannot be confirmed by the findings of the present
424 investigation, as testosterone responded similarly after the WBC and CON-
425 interventions. The contrasting findings might be explained by the application
426 of different exercise protocols. Immediately after repeated sprint exercise,
427 Russell et al [20] detected no time effects in cortisol or testosterone, while
428 our data indicated significant elevations in both hormones, most likely due
429 to higher intensity of the exercise intervention. Therefore, it is plausible to
430 speculated that testosterone concentrations are only elevated by one
431 session of WBC if the preceding exercise itself did not induce a significant
432 hormonal response.

433 The present study also investigated the relationship between the
434 performance decrements after HIR (i.e. Δ t_{lim} R1 – R2) and the changes in
435 biomarker-levels from baseline to the end of 60 min recovery period ($R2_{pre}$

– $R1_{pre}$). No correlations were found for all biomarkers except cortisol, regardless of the recovery modality applied (see Fig. 4). These results suggest that reductions in cortisol after exercise and recovery lead to higher subsequent running-performance. Interestingly, despite no significant intervention or interaction effects in inflammatory, muscle damage and stress-related biomarkers, we observed improved running performance (time to exhaustion) immediately after a single WBC-session [16]. It can be speculated that these performance improvements are most likely attributed to i) a placebo effect, ii) perceptual reductions in pain, iii) acute changes in muscle oxygenation, iv) lower cardiovascular strain, or v) a combination of these factors.

5. LIMITATIONS

Our study has limitations that warrant mention. Firstly, incorporating a range of other inflammatory cytokines (e.g. TNF-alpha, IL-8 and IL-15), and biomarkers of muscle damage (e.g. creatine kinase, lactate dehydrogenase) and stress (e.g. epinephrine, alpha amylase) would have provided further insight into the inflammatory, damage, and hormonal effects of a single WBC exposure following exercise. Secondly, despite all biomarkers except CRP and testosterone-cortisol ratio returning to basal levels a longer timeline of analysis, possibly up to 96 hrs post exercise, would have offered additional insights into the potential effects of WBC. Thirdly, although the present investigation focused on inflammatory, hormonal and muscle damage

biomarkers, functional and performance measures up to 24 hrs would have added additional practical information regarding the application of WBC. Fourthly, despite employing a cross-over study design, it is possible that the small sample-size increased the potential for type II error. Furthermore, a cross-over study design might be influenced by the repeated bout effect. However, due to the participants being very familiar with the exercise protocol and the lack of any unaccustomed exercise or downhill running, i.e. eccentric muscle damage, it is very unlikely that these results were impacted by the repeated bout effect. Future research incorporating a parallel design with a larger sample size is therefore warranted. Finally, not including an active recovery group is a limitation of this study.

6. CONCLUSION

This study is the most thorough investigation of the effects of a single session of WBC (3 min at -110°C) on biomarkers of hormonal status, inflammation, and muscle damage after acute high-intensity exercise in trained males. Despite the expected changes in IL-6, IL-10, CRP, sICAM-1, myoglobin, cortisol, testosterone, and cortisol-testosterone ratio in the 24 hrs following exercise, and contrary to our hypotheses, our results demonstrate for the first time that WBC has no acute beneficial effect compared to passive recovery in any biomarker assessed. The results of this study suggest that the postulated physiological mechanisms by which a single exposure to

WBC is speculated to improve recovery, i.e. reductions in inflammation and muscle damage, may not be accurate.

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DISCLOSURE STATEMENT

The authors confirm there are no conflicts of interest.

Figure captions

Figure 1. Schematic presentation of the experimental randomized cross over design.  denotes blood sampling at seven time points, before and after ramp test 1 ($R1_{pre}$; $R1_{post}$) and 2 ($R2_{pre}$; $R2_{post}$) and 1 (1h), 4 (4h) and 24 (24h) hrs after the intervention for whole-body cryotherapy (WBC) and control (CON) intervention, respectively.

Figure 2. Serum concentrations (mean ± SD) of interleukin 6 (IL-6; A), interleukin 10 (IL-10; B), C-reactive Protein (CRP; C), soluble intercellular adhesion molecule-1 (sICAM-1; D) and myoglobin (F) as well as white blood cell count (WBC-count; E) at seven time points ($R1_{pre}$; $R1_{post}$; $R2_{pre}$; $R2_{post}$; 1h; 4h; 24h). * $P < 0.05$ time effect compared to baseline ($R1_{pre}$), for both

interventions (whole-body cryotherapy (WBC) and control (CON)) combined.

Figure 3. Serum concentrations (mean ± SD) of cortisol (A), testosterone (B) and calculation of testosterone to cortisol ratio (C) at seven time points ($R1_{pre}$; $R1_{post}$; $R2_{pre}$; $R2_{post}$; 1h; 4h; 24h). * $P < 0.05$ time effect compared to baseline ($R1_{pre}$), for both interventions (whole-body cryotherapy [WBC] and control [CON]) combined.

Figure 4. Correlations between change (Δ) in time to exhaustion (t_{lim}) from ramp 1 to ramp 2 and change in serum cortisol from $R1_{pre}$ to $R2_{pre}$ in control (CON) and whole-body cryotherapy (WBC) intervention, respectively.

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