Angus Lindsay, Sam Carr, Mohd Izani Othman, Edward Marks, Sian Davies, Carl Petersen, Nick Draper and Steven P. Gieseg*

The physiological and mononuclear cell activation response to cryotherapy following a mixed martial arts contest: a pilot study

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Abstract: Cold water immersion is thought to reduce the inflammatory response to injury. Using cultured mononuclear cells and human subjects in a mixed martial arts (MMA) contest, we examined the effect of cryotherapy on 7,8-dihydroneopterin and neopterin generation. Urine was collected from 10 elite male mixed martial artists before, immediately post and 1, 2, 24 and 48 h following a contest. Myoglobin was analysed by reverse-phase high performance liquid chromatography, and urinary neopterin and total neopterin (neopterin+7,8-dihydroneopterin) were measured by strong cation exchange high-performance liquid chromatography. Cold water immersion and passive recovery were compared using changes in these markers, while cryotherapy tested total neopterin production in γ-interferon and phorbol myristate acetate (PMA)-stimulated blood-derived mononuclear cells (monocytes/T cells). Myoglobin significantly increased (p<0.05) at 1 h post-contest, neopterin significantly increased at 1 and 24 h (p<0.05), total neopterin significantly increased (p<0.05) at 1 h post for the passive group only, and significant individual variation was observed for all markers (p<0.01). Cold water immersion attenuated total neopterin production (p<0.05), while cryotherapy significantly reduced total neopterin production in PMA-stimulated mononuclear cells (p<0.01). Cryotherapy attenuates the post-exercise inflammatory response following an MMA contest. The evidence also suggests that the mechanisms responsible for this may be related to direct immune cell suppression.

Keywords: cold water immersion; inflammation; mixed martial arts; mononuclear cells; muscle damage.

Introduction

Recovery from impact-induced physiological trauma is critical for sustained training and improved performance without risking the development of non-functional over-reaching or over-training syndrome. Cold water immersion (CWI) has been shown to be an effective post-exercise intervention following impact trauma [1] and resistance training-induced muscle damage [2] that is more readily available and similarly efficient at cooling in comparison to more modern whole body cryotherapy (WBC) strategies. Commonly used for its analgesic properties and shown to reduce muscle temperature by ~6–9°C [2, 3], CWI is capable of alleviating symptoms of delayed onset muscle soreness, improving muscle power recovery, reducing post-exercise increases in creatine kinase [4] and enhancing the adaptation response through mitochondrial biogenesis [3].

Cold water immersion is thought to impart its benefits through reduced peripheral blood flow, vasoconstriction [5] and hydrostatic pressure [6]. However, the direct effect of cryotherapy on immune cell activation in vitro is, to the best of our knowledge, currently unknown and a potential alternative explanation for these observed effects. Icing has been shown to retard macrophage abundance by ~1–2 days [7] following muscle damage; however, the
potential to modulate the cellular inflammatory response by cold exposure remains untested. Although there is equivocal evidence surrounding the positive effect of CWI on systemic markers of inflammation and muscle damage [8, 9], which in itself suggests further investigation is required, the majority of studies have measured performance-based parameters [10]. These investigations are currently in abundance, while physiological responses are typically assessed in an invasive manner to obtain information pertaining to the efficacy of the interventions and are quite often assessed in a controlled laboratory setting.

Neopterin, total neopterin (neopterin+7,8-dihydroneopterin) and myoglobin are key physiological markers of oxidative stress, total immune system activation and muscle damage, respectively. They have been quantified in urine to assess exercise-induced physiological stress [11] and their measureable changes present credible and reliable information pertaining to immune system activation and loss of muscle membrane integrity. Innate inflammation is characterised by T-cell stimulation of macrophages through the release of γ-interferon, which causes monocytes and macrophages to generate 7,8-dihydroneopterin, a potent antioxidant in its own right [12], which may protect the macrophages from their own oxidants during inflammation. The combined assessment of these three non-invasive and specific biological markers may provide critical and new physiological information relating to the effectiveness of cold water immersion following exercise-induced trauma.

Mixed martial arts (MMA) is a relatively new sport that incorporates several forms of martial arts from Jiu-Jitsu to kickboxing. In a sport that revolves around grappling, striking and wrestling, the level of associated muscle damage and inflammation is relatively unexplored, despite studies having identified MMA fights having injury rates ranging from 23.6% to 40.3% to at least one of the competitors [13, 14]. Busse [15] identified 14.1% of all MMA contests end because of musculoskeletal stress, while Jiu-Jitsu, Taekwondo and Greco-Roman wrestling training/competition cause significant elevations in haematological stress parameters [16, 17]. It is therefore plausible that significant elevations in oxidative stress, inflammation and muscle damage may transpire.

The increasing popularity and participation in MMA warrant further research to understand the stresses MMA athletes endure and how different recovery protocols and measureable parameters may be used to improve recovery and performance. The purpose of this research was to quantify the level of inflammation [total neopterin (neopterin+7,8-dihydroneopterin), oxidative stress (neopterin) and muscle damage (myoglobin)] following a simulated MMA contest and to determine whether CWI is more effective at attenuating these physiological responses in comparison to passive recovery. It also hopes to provide an in vitro understanding of the effect of cryotherapy on monocyte/T-cell activation and determine whether this can offer an alternative explanation to vasoconstriction in the hypothesized reduction in inflammation following CWI.

Materials and methods

All solutions and reagents were prepared with water purified using the NANOpure ultrapure water system from Barnstead/Thermolyne (Dubuque, IA, USA). Chemicals and reagents were supplied from Sigma Chemical Company (St Louis, MO, USA), Abcam (Melbourne, Australia), BDH Chemicals New Zealand Ltd. (Auckland, New Zealand) for HPLC solvents and 7,8-dihydroneopterin and neopterin were supplied by Schircks Laboratories (Jona, Switzerland).

Participants

Ten elite amateur and professional MMA athletes volunteered for the study (age: 27.3±3.3 years; mass: 79.5±0.5 kg; height: 1.77±0.04 m), with a combined MMA record of 3.9±3.4 wins and 2.1±1.7 losses. The experimental protocol was approved by the University of Canterbury Human Ethics Committee (Christchurch, New Zealand), and all participants were informed of the risks involved in the study before their written consent was obtained.

Experimental protocol and design

An MMA contest can last anywhere from a few seconds to 25 min. As a result of this uncontrollable circumstance, each participant was weight matched with an opponent in a full-contact contest lasting three rounds of 5 min each. To gain a complete understanding of potential structural stress accompanying a longer length contest regardless of how many times a participant was “beaten”, the entire contest duration was completed. If a participant received a lesion or any form of concussion that was deemed too dangerous to continue with (diagnosed by a clinical physician onsite), the contest was halted immediately.

As semi-professional athletes, all participants adhered to a strict diet and pre-fight preparations provided by their manager. These included a 24-h rest period before and 48 h after the contest day and abstinence from any form of exercise other than the contests themselves. All participants consumed approximately 1 L of H2O, a carbohydrate-protein beverage, and slept for at least 8 h following the contest. They each completed their usual post-contest recovery protocol in a randomised fashion through a simple tossing of a coin. These included either CWI immediately post and on the next two consecutive mornings (~12 and 36 h post-contest) (n=5) or passive recovery that required participants to sit down for 15 min with minimal movement (n=5). The CWI protocol used by the participants involved...
submerging themselves, except the neck and head, for 15 min in a 10°C bath, which is commonly used in CWI studies [2].

Urine sample collection and preparation

Urine samples were collected pre, immediately post, and 1, 2, 24 and 48 h post-contest. Each participant provided a specimen midstream (from the bladder) into a 70-mL collection bottle. All samples were placed on ice immediately and transported to the laboratory for analysis. For myoglobin quantification, 5 mL of urine was aliquoted at the time of collection into a sterile 15-mL tube containing 1 mL of 0.2 mol/L of NaOH, whose pH was adjusted to approx. 7–9 as described previously [11]. Samples were prepared in minimal light at all times after collection to prevent oxidative loss of 7,8-dihydronopterin from UV light as described previously [18].

Cell culture and sample preparation

Mononuclear cells (monocytes and lymphocytes) were prepared from whole human blood supplied by the New Zealand blood service under human ethics committee approval by centrifugation with lymphoprep as previously described [19]. Cells were plated in RPMI1640 supplemented with 10% human serum, at 10 × 10^6/mL with 2 mL of cells per well, and incubated at 37°C in 5% CO₂. After 24 h, the cells were treated with either 5 μmol/L of PMA (dissolved in dimethyl sulfoxide) or 500 U of γ-interferon, while the addition of interferon stimulates the monocytes directly. After 1 h of incubation at 37°C, selected plates were placed in a fridge at 4°C for 1 h before returning to the 37°C incubator. At indicated time points during the treatments, 100 μL of media was removed for total neopterin analysis.

HPLC analysis of neopterin

Urine samples were diluted 1:40 with phosphate buffer [20 mmol/L of ammonium phosphate (pH 2.5)] for neopterin analysis. For total neopterin analysis, 100 μL of diluted sample was treated with 20 μL of acidic iodide solution (5.4% I₂/10.8% KI in 1 mol/L of HCl) and incubated for 15 min at room temperature in the dark. Ten microlitres of a sample was injected onto a Luna 5 μm RP 300 Å, 150×4.6-mm column supplied by Phenomenex NZ Ltd., Auckland, New Zealand. Ten microlitres of the sample was injected onto a Jupiter C5 (20 mmol/L, pH 2.5) pumped at 1 mL/min as described previously [18]. The eluting neopterin was detected using the native fluorescence at wavelength (emission 438 nm, excitation 353 nm) as previously described [18].

HPLC measurement of urinary myoglobin

HPLC measurement of myoglobin was performed using the same Sil-20A HPLC autosampler and RF-20Axl Fluorescence detector and an SPD-20A photo diode array detector (Shimadzu) used for the neopterin analysis. The pH-stabilised urine samples were diluted 1:10 with 10 mmol/L of ammonium acetate (pH 7) before reverse-phase HPLC (RP-HPLC). Ten microliters of the sample was injected onto a Jupiter C5 5 μm RP 300 Å, 150×4.6-mm column supplied by Phenomenex NZ Ltd., Auckland, New Zealand. The mobile phase was generated by mixing 0.1% of trifluoroacetic acid (v/v; pH 2.5) in water (A) with 100% acetonitrile (B) by pre-pump mixing to give 10% A/90% B at 1 mL/min. The myoglobin was eluted with a linear gradient from 10% to 70% B over 15 min and followed by 70% B for a further 5 min. The column was returned to the starting condition by 70% to 10% B gradient over 2 min and holding at 10% B for 10 min, making a total run time of 32 min. Myoglobin was detected by absorbance at 400 nm. All analytes were quantified by peak area using the Shimadzu Class VP software and conducted in duplicate.

Specific gravity

Specific gravity (SG) was calculated using a hand-held refractometer (N-20, Atago, Japan). Fifty microlitres of each sample was added to the refractometer and calculated using distilled water as a zero standard. SG was calculated using the following formula based on the normal population SG<sub>1.020</sub> [21]:

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\text{SG-corrected concentration} = \left( \frac{1.020-1}{\text{SG}_{\text{sample}}^{-1}} \right) \times \text{[neopterin] (nmol/L)}
\]

Video analysis

Notational analysis was performed on the video recordings from the referee head-mounted camera (GoPro®, San Mateo, CA, USA). This quantified takedowns, standing punches (including elbows), kicks (including knees) attempted and respective percentage of each landed [22].

Statistical analysis

The effect of contest variables (number and type of impacts, contest outcome and recovery protocol) on the change in biomarker concentrations was tested in a linear mixed effects model fitted with restricted maximum likelihood, conducted in the lme4 package [23] in R version 3.1.1. The linear mixed effects model contains both fixed and random effects, and is used to assess repeated measures variables. p-Values and r² for coefficients of fixed effects (effect sizes) were calculated using Satterthwaite’s method for denominator synthesis, conducted in the lmerTest package [24] for R. Each biomarker was analysed as the response variable in a separate model. The fixed predictors in each model were the number and type of impacts sustained by the participant during the contest and recovery protocol. Participant identity was included
as crossed random effects to account for the non-independence of marker measures from each participant. A two-way analysis of variance was similarly used to compare the differences in total neopterin production from a mixed cell culture incubated at different temperatures with Tukey’s test post hoc analysis. Data were presented as the mean ± standard error of the mean unless otherwise stated.

**Results**

Notational analysis identified an average (±SD) of 53.5 (30.5) punches, 15 (9.7) kicks and 3.3 (2.95) takedowns sustained per contest.

Urinary myoglobin increased significantly (p = 0.005, \( n_{\text{partial}} = 0.39 \)) above pre-contest concentrations at 1 h post (Figure 1A) and returned to undetectable levels by 24 h. It was detected in five of the ten participants, with concentrations ranging from 0.95 to 13.24 μg/mL. Concentrations were not correlated to the number or type of impacts a participant experienced or to the outcome of the contest.

Urinary neopterin significantly increased (p = 0.02, \( n_{\text{partial}} = 0.35 \)) after 1 and 24 h post-contest (p = 0.04, \( n_{\text{partial}} = 0.48 \)). Concentrations remained marginally elevated (p = 0.0894, \( n_{\text{partial}} = 0.33 \)) above pre-contest levels after 48 h (Figure 1B) with significant (p < 0.01)

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**Figure 1:** Urinary myoglobin (A), urinary neopterin (B), urinary neopterin of selected individuals with a varied response (C), urinary total neopterin (D) and urinary total neopterin of selected individuals with a varied response (E). Data are presented as the mean ± standard error of the mean (SEM). *p < 0.05, **p < 0.05.
inter-individual variation (Figure 1C). There was also a moderate correlation ($r=0.51$) between the percentage change in neopterin within the first 2 h post-fight and the concentration of myoglobin.

Total neopterin analysis provided insufficient evidence that the MMA contest caused any meaningful change (Figure 1D); however, there was a significant increase in the passive recovery group ($p<0.01$, $n_{\text{partial}}^2=0.50$) and a significant ($p=0.01$) inter-individual variation (Figure 1E). The number and type of impacts and the outcome of the contest had no effect on neopterin and total neopterin concentrations, while no one fight seemed to cause more or less change ($p>0.05$).

Recovery intervention analysis indicated that urinary neopterin concentration was marginally increased ($p=0.07$, $n_{\text{partial}}^2=0.55$) in the CWI group at 1 h post (Figure 2A) in comparison to the passive recovery group. However, urinary total neopterin was significantly lower ($p=0.04$, $n_{\text{partial}}^2=0.63$) at 1 h post-contest for the participants completing a CWI immediately following the contest in comparison to passive recovery (Figure 2B). At 24 and 48 h post-contest, no difference was observed between the groups.

The incubation of the blood-derived mononuclear cells at $4^\circ \text{C}$ did not visibly alter them morphologically (data not shown). There was no evidence to suggest ($p=0.19$, $n_{\text{partial}}^2=0.01$) that incubating at $4^\circ \text{C}$ had any effect on total neopterin production following $\gamma$-interferon incubation over 24 h compared to $37^\circ \text{C}$ (Figure 3A). There is, however, sufficient evidence ($p<0.01$, $n_{\text{partial}}^2=0.70$) that total neopterin production was reduced following incubation with PMA at $4^\circ \text{C}$ compared to $37^\circ \text{C}$ (Figure 3A). When this change was observed as a time course (Figure 3B), there was an immediate reduction in total neopterin production

Figure 2: The effect of CWI and passive recovery on changes in urinary neopterin (A) and total neopterin (B). Data are presented as the mean±SEM. *$p<0.05$.

Figure 3: Effect of cold treatment on total neopterin production by mononuclear cells. Blood-derived mononuclear cells consisting of a mixture of T cells and monocytes were stimulated with either 500 U of $\gamma$-interferon or 5 $\mu \text{mol/L}$ of PMA. (A) After 1 h of incubation, selected plates (containing $10^6 \times 10^3$/well) as indicated were transferred to a $4^\circ \text{C}$ fridge for 1 h before being returned to the $37^\circ \text{C}$ incubator. The remaining plates were left at $37^\circ \text{C}$ for the entire 24 h before the media was sampled for total neopterin release. (B) Release of total neopterin into the media was measured at the indicated time points following stimulation with 5 $\mu \text{mol/L}$ of PMA using the cold treatment specified for (A). Each data point is the mean of three individual wells for each time point consisting of $5 \times 10^6$ cells/well. Data were log transformed and presented as the mean±SEM. *$p<0.05$. 

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Discussion

This study used non-invasive biochemical markers of oxidative stress, inflammation and muscle damage to gauge the physiological effect of CWI following a high-intensity impact sport. The CWI protocol used in this study attenuated the initial elevations in total immune system activation (total neopterin) accompanying an MMA contest. The significant elevation in total neopterin following the contest seemed to be attenuated at least in the hours immediately following a contest, while the consumption of a protein and carbohydrate drink, water and at least 8 h of sleep did not affect the immediate inflammatory response, but did seem to be substantial enough to induce recovery 24–48 h post-exercise as previously noted [25]. This is in contrast to the lack of difference in certain haematological parameters measured following CWI and other high-intensity exercise [26], suggesting that further research into a standardized approach is still required. Moreover, the ability to measure total immune system activation with urinary total neopterin in a non-invasive manner may offer future testing options for real-world settings where venipuncture is not allowed.

The significant reduction in urinary total neopterin and the likelihood of a positive analgesic and vasoconstrictive effect of this particular type of protocol [27] suggest a reduction in immune cell transportation to sites of tissue damage. Costello et al. [28] solidified this theory through an observed restriction in red blood cell concentration and microcirculation following CWI. Our data offer an alternative mechanism that may parallel these previously identified properties of CWI. The cooling of a mixed cell culture of monocytes and T cells (blood-derived mononuclear cells) incubated with PMA and γ-interferon suggests that CWI does not have any meaningful effect on γ-interferon-stimulated monocyte activation, but rather suppresses γ-interferon production by T cells. This is clearly indicative of a latent effect that suggests cryotherapy may concurrently suppress the activation of the resident immune cells both immediately (corroborates the in vivo suppression following the MMA contest) and for a period of up to 24 h. So, while cryotherapy acts to attenuate the transportation of immune cells, it may simultaneously diminish their ability to generate a potential excessive inflammatory response. While this provides the first in vitro evidence pertaining to the cooling effect on immune cells responsible for tissue regeneration, future research into cryotherapy techniques needs to consider the impact this may have. It has to be noted, however, that macrophage activation is imperative for the muscle regenerative process (satellite cell activation, proliferation and differentiation) [29], which opens up debate on the validity of cryotherapy treatments in the role of adaptation and their effectiveness following exercise-induced muscle damage. This CWI protocol, however, did not seem to retard the adaptation and regenerative process at 24 and 48 h post-exercise in the CWI group in comparison to passive recovery.

Cold water immersion may also cause an immediate change in oxidative status, which is in contrast to the positive effects on the oxidant/antioxidant balance of WBC following exercise [30]. This contrast may be explained by an increase in reactive oxygen species through elevated mitochondrial biogenesis (increased expression of mRNA peroxisome proliferator-activated receptor γcoactivator-1α) following CWI [3] and by the adaptive response of mitochondrial thermogenesis to cold exposure [31]. Its use 12 and 36 h following exercise, however, does not seem to have any further effect on an inflammatory response, which clarifies how imperative immediate post-exercise recovery intervention is for reducing swelling and inflammation, if that be the desired effect.

This study is also the first to report changes in urinary neopterin, total neopterin and myoglobin following an MMA contest. The contests caused significant muscle damage, inflammation and oxidative stress in both group and individual contexts, which is similar to that previously noted in other physical impact sports [32] and traditional martial arts [17]. More importantly, pronounced individual variation was identified following the MMA contest, which we have previously reported in amateur and professional rugby players [11, 32] where a similar pattern of disparity warrants the need to treat every individual athlete as his/her own entity and solidifies the use of biochemical analysis for quantification of exercise-induced stress. This is particularly important for management and for providing the best opportunity for each fighter to respond and prepare optimally for a competition rather than to rely solely on group data that often mask the individual physiological response.

Urinary myoglobin detection in five of the ten participants provides further conclusive evidence that MMA causes significant structural damage in a clinically
relevant range [33], albeit at a considerably lower level than other impact sports like rugby union [32]. Values exceeding 1 and 20 μg/mL are related to rhabdomyolysis susceptibility [33], highlighting the severity of intramuscular stress associated with MMA and the necessity of physiological stress quantification for health and recovery maintenance of elite athletes. Furthermore, the inability to identify myoglobin in all participants suggests that they are well adapted to the stress of MMA or had concentrations in an undetectable range. The former is highly conceivable due to the 6 days/week training/competing schedule they complete and perhaps explains why myoglobin may become drastically increased following unaccustomed trauma under different circumstances. It has to be noted that there were no physical characteristic or experience differences between the participants.

Meanwhile, the elevation above pre-contest concentrations in urinary total neopterin for certain participants is indicative of immune system and macrophage activation. Its sustained elevation above pre-contest levels at 24 and 48 h for a select few participants represents either a sustained inflammatory effect or a delay in macrophage activation as a result of the CWI, similar to that previously identified in rat muscle [7]. While impact numbers are significantly greater in MMA than in other impact sports [32], the duration and type of exercise may play a more significant role in macrophage activation, hence the lower values observed in this study cohort. Moreover, impacts do not seem to correlate with the extent of muscle damage and inflammation, which is in contrast to other impact-related sports [34]. Contest duration and adaptation may play a major role in this discrepancy.

The MMA contest also caused a significant change in oxidative status. This may be attributed directly to the impacts and associated inflammatory response, or simply to the metabolism increase known to generate 10.2–20.7 mmol/L of lactate following an MMA bout [22]. The sustained elevation of neopterin represents a 24- to 48-h recovery period and a potential for impaired recovery following an MMA contest owing to its ability to identify athletes in a continual state of recovery or over-training [35]. Meanwhile, the correlation with myoglobin offers an alternative hypothesis to hypochlorite oxidation of 7,8-dihydroneopterin to neopterin as previously reported [36], while also solidifying the oxidative potential of myoglobin and the suggested feedback loop intertwining muscle damage and oxidative status [36]. This relationship suggests that the greater the degree of muscle damage, the greater the likelihood of elevated oxidative stress.

Although this study does provide some interesting new innovative knowledge pertaining to structural stress accompanying an MMA contest and the potential cellular response to CWI, certain limitations do exist. The number of participants (n=10) certainly restricted the ability to definitely conclude the meaning of some results. The large inter-individual variation that exists in the biomarker response that is commonly observed in other physical impact sports [32] provides further difficulties when drawing assumptions. Considering the results of this pilot study, a sample size of 24 would be required to provide clarification to the drawn conclusions. Similarly, the real-world setting of this study and the professionalism of the participants, who are under certain dietary restrictions that cannot be controlled for, do provide certain limitations surrounding nutritional variables that may affect contest variables. However, when assessing the efficacy of a recovery protocol such as CWI, the necessity to gather real-world data should be prioritised over laboratory-controlled studies where they will be most beneficial.

**Conclusion**

CWI attenuates the immediate post-exercise inflammatory response that may be attributable to blood-derived mononuclear cell activation suppression. A mixed martial arts contest causes severe structural damage (myoglobin), inflammation (total neopterin) and change in oxidative status (neopterin), with the last one being partially dependent on the degree of muscle damage. Meanwhile, biochemical analysis of these athletes through non-invasive protocols may provide a comprehensive alternative to subjective and qualitative measures such as questionnaires about perceived soreness and fatigue and separate individualistic physiological responses.

**Perspective**

Although a significant amount of literature exists on cold water immersion, its effect on high-intensity impact sports and physiological adaptations still requires extensive research to understand its potential benefits. Where severe structural stress accompanies exercise in the form of impact sports, cold water immersion may, in fact, provide a means to alleviate the acute inflammatory response and improve recovery. More importantly, the non-invasive and simple procedure of physiological stress assessment with neopterin may offer an alternative and potentially effective means of assessing inflammatory responses following
impact exercise and recovery modalities. The observation of cryotherapy-induced mononuclear cell suppression also provides an interesting alternative mechanism for future cryotherapy research studies that normally suggest vasoconstriction as the primary means of immune system suppression.

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