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Abstract

Carbohydrate (CHO) supplementation during prolonged exercise is widely acknowledged to blunt *in vitro* immunoendocrine responses but no study has investigated *in vivo* immunity. Purpose: To determine the effect of CHO supplementation during prolonged exercise on *in vivo* immune induction using experimental contact hypersensitivity (CHS) with the novel antigen Diphenylcyclopropenone (DPCP).

Methods: In a double-blind design, 32 subjects were randomly assigned to 120 minutes of treadmill exercise at 60% $\dot{V}O_2$max with CHO (Ex-CHO) or placebo (Ex-PLA) supplementation. Responses were also compared to 16 resting control (CON) subjects from a previous study (for additional comparison with a resting non-exercise condition). Standardised diets (24 h pre-trial) and breakfasts (3.5 h pre-trial) were provided. Subjects received a primary DPCP exposure (sensitisation) 20 min after trial completion and exactly 28 d later the strength of immune reactivity was quantified by magnitude of the cutaneous response (skin-fold thickness and erythema) to a low dose-series DPCP challenge. Stress hormones and leukocyte trafficking were also monitored.

Results: CHO supplementation blunted the cortisol and leukocyte trafficking responses but there was no difference (P > 0.05) between Ex-CHO and Ex-PLA in the *in vivo* immune responses (e.g. both ~46% lower than CON for skin-fold response).

Conclusions: CHO supplementation does not influence the decrease of *in vivo* immunity seen after prolonged exercise. The effects with more stressful (or fasted) exercise remain to be determined. However, there appears to be no benefit under the conditions of the present study, which have practical relevance to what many athletes do in training or competition.

Key words: running; immune; contact hypersensitivity; diphenylcyclopropenone; glucose; whole integrated immune response

Introduction

It is widely reported that athletes engaged in regular prolonged and/or strenuous exercise, and those in physically demanding occupations (e.g. soldiers), have a higher than normal incidence of upper respiratory tract illness (URTI) symptoms, which may be related to an exercise-induced impairment of immune function (immunodepression) [1, 2]. It has previously been suggested that the primary mechanisms contributing to such exercise-induced immunodepression include the exercise-induced stress responses (i.e. increased stress hormones, in particular cortisol; cytokines; inflammatory responses); and the redistribution of leukocytes and their subsets (e.g. [3–5]). Many nutritional interventions have been investigated as potential countermeasures to exercise-induced immunodepression and acute supplementation with carbohydrate (CHO) is often cited as the most efficacious [6–8]. Supplementation with CHO has been consistently shown to blunt many of the exercise-induced immune and endocrine perturbations (e.g. increased cortisol, cytokine, and leukocyte redistribution responses; decreased neutrophil degranulation and oxidative burst functions, lymphocyte proliferation and functions, natural killer cell function [4–12]) although it is not clear how this may influence infection risk [8, 12]. Such *in vitro* markers provide useful insight into the impact of nutritional interventions but alone they are considered to lack clinical relevance as the extent to which changes in these *in vitro* or *ex vivo* measures are related to increased infection risk is unclear [13, 14]. To date, the vast majority of the supportive evidence for CHO supplementation is based on *in vitro* measures (e.g. [4, 5, 9–11]), with little evidence on URTI [15] and no evidence on *in vivo* immunity. In one study, Nieman et al. [15] studied approximately 100 marathon runners randomly allocated to receive placebo or CHO beverages (providing ~60 g per hour) during a competitive marathon race. Carbohydrate supplementation was associated with higher post-race plasma glucose and insulin concentrations, and lower post-race cortisol and growth hormone concentrations.

However, they observed little benefit of CHO, compared to placebo, on the response of a range of salivary immune parameters (including IgA secretion rate, IgA relative to protein, and absolute IgA concentration, which actually decreased less in the placebo group). Nieman et al. [15] were also able to obtain URTI data for the 15-day post-race period from 93 of the participants, and found no difference in URTI incidence between the CHO and placebo groups. However, the validity of using self-reported URTI has been questioned [14].

The normal immune response (and hence host defence and protection) involves a complex and co-ordinated network of molecules, cells and tissues working in synergy. Hence, *in vivo* immune markers are considered particularly valuable [13, 14]. Cutaneous measures of *in vivo* immunity, such as delayed type hypersensitivity responses to intradermal injection of antigens, or contact hypersensitivity (CHS) responses to epicutaneous application of antigens, represent challenge-type measures, which have recently been suggested to be most useful in immunonutrition studies, when considering biological relevance, sensitivity and feasibility [14]. The clinical relevance of examining cutaneous *in vivo* immunity is supported by studies that have used such measures to monitor immune-competence in HIV-infected patients [16], and studies showing that the magnitude of the responses relate to URTI risk in children [17] or predict mortality in patients with surgical infections [18]. It has been previously demonstrated that the CHS response, using the novel antigen diphenylcyclopropenone (DPCP), is significantly reduced after 2 hours of treadmill running. This includes both the induction of immunity (-53%), in participants with no prior exposure, and the elicitation of immunity (-19%), in participants with well-established immune memory, to DPCP [19]. We have now demonstrated this as a controllable, reproducible, sensitive, and valid *in vivo* marker of exercise-induced immunodepression [19, 20]. Furthermore, we have recently demonstrated no effect of the same exercise (2 h of treadmill running) on the skin's response

to the irritant croton oil [20], providing further support for the suggestion that the observed decrease in *in vivo* immune induction to DPCP is an antigen-specific, T cell mediated, response.

Challenging the skin using novel antigens permits the investigation of the influence of stressors on *in vivo* immune induction and allows rigorous control of both the dose and timing of sensitisation [19]. We are not aware of any previous study that has utilised such an *in vivo* marker to determine the effects of nutritional interventions such as CHO supplementation on exercise-induced immunodepression. To date, the most supportive evidence for a nutritional strategy to limit the exercise-induced immune perturbations is for CHO supplementation, but no study has investigated *in vivo* immunity. Therefore, the aim of the present study was to determine the effects of acute CHO ingestion, before, during and after prolonged exercise, on induction of the CHS response to DPCP. We hypothesised that CHO supplementation would blunt the prolonged exercise-induced decrease of CHS induction.

Experimental Methods

This study was conducted according to the guidelines laid down in the Declaration of Helsinki (2008, including 2013 amendments) and all procedures were approved by the local University Research Ethics Committee. Written informed consent was obtained from all subjects. Subjects also completed a pre-exercise screening questionnaire (Physical Activity Readiness Questionnaire: PAR-Q) before participating in each exercise test.

Subjects

Thirty two men were included in this study (age 28.8 ± 8.1 years, body mass 76.2 ± 10.1 kg, maximal oxygen uptake, $\dot{V}O_2$max, 58.3 ± 6.4 mL·min^{-1}·kg^{-1}; means $\pm$ standard deviation for all 32 subjects; values for individual groups are summarised in Table 1). All participants were healthy, non-smoking, and recreationally active (familiar with running) with no previous history of exposure to DPCP. Subjects were excluded if they were taking any medication or dietary supplements, had a history of atopy or any other immune-related or inflammatory dermatological or relevant medical conditions. Other exclusion criteria included current (or within two weeks of recovery from) infection prior to the study, having any vaccinations in the 2 months preceding the study, and donating blood in the four weeks before the study. Participants were asked to abstain from strenuous exercise in the 24 hours before and 48 hours following each trial (exercise/sensitisation and elicitations). Participants were asked to abstain from the consumption of alcohol or caffeinated drinks 48 hours ahead of each trial.

Study design

In the current study, 32 subjects were randomly assigned to either 120 minutes of treadmill exercise at 60% $\dot{V}O_2$max with CHO supplementation (Ex-CHO, n = 16), or 120 minutes of treadmill exercise at 60% $\dot{V}O_2$max with placebo (Ex-PLA, n = 16). As a point of reference for the normal response in rested individuals, we also statistically compared the Ex-CHO and Ex-PLA groups' *in vivo* immune induction responses to the control group (CON, n = 16, who completed 120 minutes of seated rest) from Diment et al. [20]. Subjects were allocated randomly into either the Ex-CHO or Ex-PLA groups after being matched for physical characteristics, speed at gas exchange threshold (GET) and speed at $\dot{V}O_2$max (and hence relative exercise intensity in the main trials). The PLA and CHO conditions were administered in a double-blind manner. All subjects undertook two preliminary tests ($\dot{V}O_2$max determination and familiarisation) and one main trial.

Determination of maximal oxygen uptake ($\dot{V}O_{2max}$)

$\dot{V}O_{2max}$ was determined by an incremental exercise test on a motorised treadmill (H/P/Cosmos Sport and Medical, Nussdorf-Traunstein, Germany) in accordance with the methods of Diment et al. [20]. The test began at a treadmill speed of 5 km·h^{-1}, with a 1% incline, for 3 minutes. The speed was then increased by 1 km·h^{-1} every minute until a speed of 18 km·h^{-1} was attained and then further increases were achieved by increasing the gradient by 1% every minute until volitional exhaustion. Heart rate was recorded throughout using a short range telemetry heart rate monitor (Polar 810i, Polar Electro, Kempele, Finland); rating of perceived exertion (RPE) was recorded at the end of each minute [21]; breath-by-breath gas exchange was recorded using an online system (Cortex Metalyser 3B, Cortex Biophysik, GmbH, Leipzig, Germany) throughout the duration of the test. The gas analysis system was calibrated prior to use according to the manufacturer's guidelines using a calibration gas of known composition and a three-litre syringe (Hans Rudolf Inc, Kansas, USA). $\dot{V}O_{2max}$ was recorded as the highest average $\dot{V}O_2$ over any 30 second period of the test. The speed required to elicit 60% $\dot{V}O_{2max}$ was estimated from the linear part of the $\dot{V}O_2$ - speed relationship, and later verified in the familiarisation trial. The intensity at 60% $\dot{V}O_{2max}$ was also expressed as a percentage of the difference between GET and $\dot{V}O_2$max (%Δ) in accordance with the methods of Lansley et al. [22], to verify that the relative intensity (and hence relative physiological stress) were equal between exercise groups.

Familiarisation

Subjects completed a familiarisation session one week prior to the main trial. Participants completed a warm-up for 5 minutes at 5 km·h^{-1} and 1% gradient on the same treadmill used for the $\dot{V}O_{2max}$ test. This was immediately followed by a 60 minute run at a speed equivalent

to 60% $\dot{V}O_{2max}$ to familiarise subjects to the main trial exercise procedures and confirm accuracy of the calculated speed (to elicit 60% $\dot{V}O_{2max}$). Minor speed adjustments were made during the first 15 min if necessary to attain the target 60% $\dot{V}O_{2max}$. Subjects were provided with 5 ml·kg^{-1} body mass water bolus 20 min before exercise commencement and on trial completion and 2 ml·kg^{-1} was provided every 15 min during the trial to familiarise subjects with the fluid provision schedule to be followed in the main trials. Heart rate and RPE were recorded every 5 min.

Main trials

Standardisation and pre-exercise preparation of subjects: Dietary intake was controlled during the 24 h before the main experimental trial by providing subjects with 35 mL·kg^{-1}·d^{-1} water and food from a standard food list to meet their estimated daily energy requirement (11.4 ± 1.5 MJ·d^{-1}, which was comprised of 15%, 60%, and 25% of energy from protein, CHO and fat respectively, and was equivalent to ~1.3, 5.4 and 1.0 g·kg^{-1} body mass, respectively). They were also provided with a standard breakfast at 07:30 on the morning of the main trial to provide ~0.03 MJ·kg^{-1} (equivalent to 2.4 ± 0.3 MJ, comprised of ~0.2, 1.0 and 0.2 g·kg^{-1} of protein, CHO and fat, respectively). To further standardise dietary intake subjects were also provided with lunch after the final blood sample collection (see timings below), to provide ~0.02 MJ·kg^{-1} (equivalent to 1.6 ± 0.2 MJ, comprised of ~0.2, 1.0 and 0.2 g·kg^{-1} of protein, CHO and fat, respectively).

Experimental procedures

Subjects were provided with breakfast (as detailed above) at 07:30 before beginning the 120 min exercise (Ex-CHO and Ex-PLA) at 11:00. They were also provided with a quantity of water for the 3 h period between 07:30 and 10:30 proportional to their daily requirement but

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pro rata for this period (based on their calculated requirement for the prior 24 h, equating to approximately 6.6 mL·kg^{-1}). During this period they were required to be restful but were allowed to undertake light activities such as reading or using a computer. They were then required to sit for 10 min with minimal movement before resting blood samples were collected. Subjects received a bolus of 5 mL·kg^{-1} of their respective drink (CHO or PLA) 20 min before and immediately after exercise and 2 mL·kg^{-1} every 15 min during exercise. Exercise commenced at 11:00 and immediately after the trial, post-exercise blood samples were collected, then subjects showered and returned to the laboratory within 15 minutes of completion for patch application at exactly 20 min post-exercise (i.e. 13:20) to sensitise subjects to DPCP (details below). A final blood sample was also obtained 1 h post-exercise. During exercise, expired gas was analysed during minutes 10, 20, 40, 50, 70, 80, 100, and 110 of exercise using the Douglas bag method. Gas was analysed using a gas analyser (Servomex, Crowborough, UK) and a dry gas meter (Harvard Apparatus, Edenbridge, UK). The gas analyser was calibrated prior to use according to the manufacturer's guidelines using calibration gasses of known composition. Heart rate and RPE were recorded every 15 min during exercise. Elicitation was then performed (details below) exactly 28 days after this trial.

Drink composition: The CHO drink was prepared with a lemon flavoured CHO-based sports drink powder (Go Energy, Science in Sport, Nelson, UK) according the manufacturer's instructions (to give a CHO concentration of 10% w/v). The placebo was CHO-free and prepared from sugar-free, artificially sweetened lemon flavoured drink concentrate (Tesco, Dundee, UK) diluted 4 parts water to 1 part concentrate. The concentrations were determined so that drinks were taste matched as closely as possible. Even though this may not be necessary with the between groups design employed here, we taste matched drinks as closely as possible to avoid potential loss of blinding (i.e. subjects discussing their drinks outside of

the study/laboratory). A laboratory technician, independent of the study, was responsible for the drink preparation to maintain double-blind procedures. The drinking schedule provided approximately 60 g CHO per hour during exercise (in addition to the pre- and post-exercise boluses of ~40 g each) for an average sized subject.

Induction of contact sensitivity (sensitisation)

Subjects were sensitized to DPCP at 13:20, exactly 20 min post-exercise (to ensure cutaneous blood flow had returned to baseline) in accordance with the methods of Harper Smith et al. [19] and Diment et al. [20]. Sensitisation was achieved by the application of an occluded patch, constituting a 12 mm aluminium Finn chamber (Epitest Oy, Tuusula, Finland) on scanpor hypoallergenic tape containing an 11 mm filter paper disc. The disc was soaked in 22.8 µl of 0.125 % DPCP in acetone and allowed to dry for 5 minutes before being applied to the skin on the lower back, for exactly 48 h. This provides a dose of 30 $\mu g \cdot cm^{-2}$ DPCP on the patch.

Elicitation

The magnitude of *in vivo* immune responsiveness was quantified by measuring the responses elicited by secondary exposure to the same antigen, also in accordance with the methods of Harper Smith et al. [19] and Diment et al. [20]. That is, exactly 28 days after the initial sensitisation to DPCP, all subjects received a challenge with a low concentration dose-series of DPCP on individual patches, each comprising an 8 mm aluminium Finn chamber on scanpor hypoallergenic tape containing a 7 mm filter paper disc. Patches were applied to the inner aspect of the upper arm in the following doses of DPCP per patch: 1.24 $\mu g \cdot cm^{-2}$; 1.98 $\mu g \cdot cm^{-2}$; 3.17 $\mu g \cdot cm^{-2}$; 5.08 $\mu g \cdot cm^{-2}$; 8.12 $\mu g \cdot cm^{-2}$ and an additional acetone only control patch for background subtraction. Patches were applied in randomly allocated order at the

local site in order to minimize any anatomical variability in responses. This pattern was matched between subjects in each of the groups however (i.e. for every allocation pattern used in one group there was an equivalent identical pattern used in the other). Elicitation patches were removed after precisely 6 h and the strength of immune reactivity was assessed as cutaneous responses at 48 h post-application. This method allows the magnitude of *in vivo* immune responsiveness to be quantified (e.g. establishment of immune memory in response to the sensitisation induced during the acute post-exercise period).

Assessment of cutaneous responses

Skin oedema (inflammatory swelling) is considered the key measure of CHS elicitation responses [19]. This was assessed as mean skin-fold thickness from triplicate measurements at each elicitation site using modified spring-loaded skin callipers (Harpenden Skin-fold Calliper, British Indicators, England), to the nearest 0.1 mm (measuring skin-fold only: i.e. no subcutaneous fat) as described previously [19, 20]. Skin erythema was determined from triplicate measurement at each patch site using an erythema meter (ColorMeter DSM11, Cortex Technology, Hadsund, Denmark) as previously described [19, 20]. Mean background values were determined from triplicate measurements at the acetone only control patch site for thickness and erythema. In order to determine the increase in thickness and erythema in response to DPCP, the value from the acetone only control site was subtracted from each patch site value. The values for increase in skin-fold thickness, and erythema over all the doses were summed to give a representative measure of the overall reactivity of each subject to DPCP [19, 20]. The dose-response curve was also used to conduct sensitivity analyses (i.e. identify the minimal dose required to elicit a positive response, for the skin-fold measure) in accordance with the methods of Harper Smith et al. [19].

Blood samples

Blood samples were obtained by venepuncture, with minimal stasis, from an antecubital vein and collected into vacutainer (Becton Dickinson, Oxford, UK) tubes (containing lithium heparin or K_3EDTA). All blood samples were obtained while subjects were in the seated position. Subjects were asked to sit without changes of posture and minimal movement for 10 min before all blood samples were drawn, except the Post-Ex sample, which was drawn as soon as possible after completing the exercise (within 5 min). A small aliquot was taken from the K_3EDTA tube for haematological analysis and then all samples were centrifuged immediately at $1500 \times g$ for 10 minutes at 4°C. Aliquots of plasma were stored at -80°C for later analysis.

Analytical methods

Haematological analysis was performed on the K_3EDTA treated blood aliquot using automated haematology analysers (Gen-S, Beckman Coulter, High Wycombe, UK; ABX Pentra 60C+, Horiba Medical, France).

*** Please insert table 1 near here ***

Glucose concentration was determined in heparinised plasma using an automated analyser (YSI 2300 Stat Plus, Yellow Springs, OH, USA). Plasma cortisol concentration in heparinised plasma was determined using a commercially available enzyme linked immunosorbent assay (ELISA) kit (DRG, Germany; Biomercia, Ca, USA). Plasma epinephrine and norepinephrine concentrations in K_3EDTA plasma were determined using a commercially available CatCombi ELISA (IBL International, Hamburg, Germany). All plasma samples were thawed only once prior to analysis.

Data analysis

Statistical analyses were carried out using SPSS (IBM SPSS Statistics for Windows, Version 21.0, Armonk, NY: IBM Corp.). Values were compared between groups using 1-way independent ANOVA or t-tests, or for variables with repeated measures, 2-way mixed ANOVA. If data were not normally distributed they were first normalised with log transformation using the natural log (training hours, %Δ @ 60% $\dot{V}O_{2max}$, neutrophil count, neutrophil-to-lymphocyte ratio, adrenaline, noradrenaline, summed skin-fold responses), or $1/x^2$ (total leukocyte count) transformations. Post hoc Holm-Bonferroni corrected paired (for within groups comparisons) or independent (for between groups) t-tests were applied where appropriate. All results are presented as mean ± standard deviation unless otherwise stated.

Results

Participants were well matched for physical characteristics with no significant differences for any of the physical parameters except $\dot{V}O_{2max}$, (Table 1). However, exercise responses were similar between groups (Table 2) and, importantly, there was no difference in the trial relative exercise intensity (this holds true whether expressing relative intensity as % $\dot{V}O_{2max}$ during the trial or %Δ, whereby exercise intensity is expressed relative to the individual GET and $\dot{V}O_{2max}$).

*** *Please insert Table 2 near here* ***

CHS responses to DPCP

Oedema responses: For the skin-fold responses, 1-way independent ANOVA (including comparison to the CON group from [20]) showed a main effect of group ($F_{2,45} = 3.370$, P =

0.043). There was no difference between the Ex-CHO or Ex-PLA groups (post hoc analysis: between Ex-PLA and Ex-CHO, P = 0.460; mean difference 1.04 mm, 95% confidence intervals 0.45 to 2.41 mm) but there was a difference between the CON and Ex-PLA (P = 0.026, mean difference 2.29 mm, 95% confidence intervals 1.11 to 4.71 mm) and between the CON and Ex-CHO (P = 0.036, mean difference 2.38 mm, 95% confidence intervals 1.13 to 5.02 mm, see Figure 1). The exercise trials were both ~46% lower (P < 0.05) than the CON responses reported by Diment et al. [20]. Sensitivity analysis revealed the average threshold dose for a response to DPCP was 0.45 $\mu g \cdot cm^2$ for the CON group but a dose ~3-fold higher was required (2.81-fold for PLA and 2.88-fold for CHO) in order to elicit a positive response in the exercise groups.

*** *Please insert Figure 1 near here* ***

Erythema responses: One-way independent ANOVA (including comparison to the CON group from [20]) showed a main effect of group ($F_{2,45}$ = 3.219, P = 0.049). There was no difference between the Ex-CHO or Ex-PLA groups (post hoc analysis: between Ex-PLA and Ex-CHO, P = 0.290, mean difference 4.22 AU, 95% confidence intervals -11.16 to 19.60 AU), no difference between CON and Ex-PLA (P = 0.070, mean difference 14.29 AU, 95% confidence intervals -1.25 to 29.83 AU) and a significant difference between CON and Ex-CHO (P = 0.036, mean difference 18.51 AU, 95% confidence intervals 2.6 to 34.41 AU, see Figure 2).

*** *Please insert Figure 2 near here* ***

Blood leukocyte counts

It was not possible to obtain a blood sample at every time point for some individuals (for example, if a post-exercise sample could not be obtained within 5 min it was abandoned to avoid delays in patch application, as the CHS responses were of primary importance). For this reason the sample sizes for these measures were n = 12 for the Ex-PLA group and n = 11 for the Ex-CHO group. Significant main effects for ANOVA comparisons (group, time and group × time) were evident for most leukocyte parameters (total leukocyte count, neutrophil count, lymphocyte count, and neutrophil-to-lymphocyte ratio) with the exception of no significant group effects for total leukocyte count, lymphocyte count, and neutrophil-to-lymphocyte ratio (detailed in Table 3). Post hoc analysis on the time effect (in each group separately, owing to the observed interactions) showed that significant leukocyte trafficking was evident in Ex-PLA at Post-Ex (P < 0.001) and 1 h Post-Ex (P < 0.001) and Ex-CHO at Post-Ex (P = 0.002) and 1 h Post-Ex (P < 0.001) but counts were significantly lower at Post-Ex (P = 0.009) and 1 h Post-Ex (P = 0.001) in the Ex-CHO compared to Ex-PLA group (see Table 3). Significant neutrophil trafficking was evident in Ex-PLA at Post-Ex (P < 0.001) and 1 h Post-Ex (P < 0.001) and Ex-CHO at Post-Ex (P < 0.001) and 1 h Post-Ex (P = 0.004) but counts were significantly lower at Post-Ex (P = 0.003) and 1 h Post-Ex (P < 0.001) in the Ex-CHO compared to Ex-PLA group (see Table 3). A significant time effect was evident for lymphocyte count (trafficking) and a significant group × time interaction indicates that this pattern differs between groups. Post hoc comparison did not reveal any between group differences at any of the time points (Table 3) but there was a significant increase in Ex-PLA at Post-Ex (P = 0.006) but no change over time in the Ex-CHO group (P = 0.262). The neutrophil-to-lymphocyte ratio increased significantly in Ex-PLA at Post-Ex (P < 0.001) and 1 h Post-Ex (P < 0.001) and Ex-CHO at Post-Ex (P = 0.028) and 1 h Post-Ex (P = 0.020) but neutrophil-to-lymphocyte was significantly lower at 1 h Post-Ex (P = 0.002) in the Ex-CHO compared to Ex-PLA group (see Table 3).

Stress hormones and glucose

For stress hormones and glucose n = 12 for the Ex-PLA group and n = 11 for the Ex-CHO group. Significant main effects of group, time and group × time interaction were evident for plasma glucose, which was significantly higher at Post-Ex and 1 h Post-Ex in the Ex-CHO group compared to Ex-PLA (Table 4). Post hoc analysis on the time effect (in each group separately, owing to the observed interaction) revealed a significant decrease below Pre-Ex concentration at 1 h Post-Ex in the Ex-PLA group (P = 0.032) and a significant increase at Post-Ex (P < 0.001) and 1 h Post-Ex (P = 0.011) in the Ex-CHO group.

Significant main effects of time, and group × time interaction were evident for plasma cortisol, which was significantly lower Post-Ex and 1 h Post-Ex in the Ex-CHO compared to the Ex-PLA group (Table 4). Post hoc analysis on the time effect (in each group separately, owing to the observed interaction) revealed no differences in the Ex-PLA group (P > 0.05) and a significant decrease at Post-Ex (P < 0.001) and 1 h Post-Ex (P < 0.001) in the Ex-CHO group (in line with the normal expected diurnal pattern).

Significant main effects of time were evident for both adrenaline and noradrenaline (i.e. an exercise response) but there were no differences between Ex-CHO and Ex-PLA groups (interactions P > 0.05, Table 4). Post hoc analysis on the time effect (both groups considered together, owing to the lack of interaction) revealed a significant increase in plasma adrenaline concentration (P = 0.013) and noradrenaline concentration (P < 0.001) at Post-Ex.

Discussion

The aim of the present study was to determine the effects of acute CHO ingestion, before, during, and after prolonged exercise, on the *in vivo* immune response (CHS) to the novel contact antigen DPCP. The main findings were that despite CHO ingestion blunting the cortisol and leukocyte trafficking responses to prolonged treadmill running, there was no effect on *in vivo* immune response to DPCP. When comparing to the resting CON group from Diment et al. [20] *in vivo* immune induction (oedema assessed by skin-fold thickness) in the present study was significantly lower (-46%). Sensitivity analysis also revealed that the threshold dose for a response to DPCP was ~3-fold higher (2.81-fold for PLA and 2.88-fold for CHO) in order to elicit a positive response. Previous research has consistently demonstrated that prolonged exercise (2 h treadmill running at 60% $\dot{V}O_{2max}$) reduces *in vivo* immune induction as assessed by this method [19, 20]. The exercise groups in the present study exhibited similar responses to the exercise groups in both of these previous studies [19, 20]. This, in conjunction with direct statistical comparison with the CON group of Diment et al. [20], shows that the prolonged exercise in the present study reduced *in vivo* immune induction. However, the present study suggests that this decrease is not altered by acute CHO supplementation.

Challenging the skin using novel antigens permits the investigation of the influence of stressors on *in vivo* immune induction and allows rigorous control of both the dose and timing of sensitisation [19]. However, we are not aware of any previous study that has utilised *in vivo* immune induction of CHS to determine the effects of nutritional interventions on exercise-induced immunodepression. Despite the large body of research showing CHO

supplementation to blunt the prolonged exercise-induced responses for a wide array of immune markers (e.g. [4, 5, 9–11]), there is a lack of evidence on the effects of CHO supplementation on *in vivo* immune function and/or clinically relevant outcomes. The findings of the present study, that CHO supplementation did not benefit *in vivo* immunity post-exercise, is broadly in line with Nieman et al. [15] who compared placebo or CHO beverage-supplemented runners during a competitive marathon race. In that study Nieman et al. [15] observed CHO supplementation (approximately 60 g per hour) to be associated with higher post-race plasma glucose and insulin concentrations, and lower post-race cortisol and growth hormone concentrations. However, there was no difference between the CHO and placebo groups in salivary IgA responses (considered high value markers in relation to URTI risk, [14]) or URTI incidence in the 15-day post-race period. However, as this was a field-based study in which subjects were racing, it is possible that performance would have differed (relative to each individual's current potential performance or fitness) or that the CHO could have ergogenic effects meaning it is difficult to confirm whether subjects were exposed to the same relative stress (both physiological and psychological) in each group. Hence, in the present study we investigated *in vivo* immune function using the CHS 'challenge-test' with otherwise standardised diet and exercise conditions. Given the outcomes previously reported for immune markers, with CHO supplementation, we hypothesised that CHO would protect against the exercise-induced decrease of *in vivo* immunity. However, contrary to our original hypothesis, and despite significant blunting of the leukocyte (and leukocyte subset) trafficking and cortisol responses, there was no difference between the Ex-CHO and Ex-PLA groups in *in vivo* immune function. Therefore, these results suggest that CHO supplementation does not protect against prolonged exercise-induced immunodepression when assessed using an *in vivo* immune marker, which we [19, 20] and others [14] propose to be of clinical relevance in an exercise immunology context.

For consistency between groups, we were careful to control diet and CHO intake before (i.e. on the day before and the morning of) the main trials and during the period of sensitisation so that the only difference between groups was CHO content of drinks consumed acutely before, during, and after the prolonged exercise. Many previous studies required subjects to exercise following an overnight fast and it is possible, therefore, that immune alterations would be more pronounced in these circumstances. We chose to provide a standard breakfast in order to replicate what many athletes often do in training and competition and, as such, our results have practical relevance to such athletes. This presents the possibility that CHO supplementation during exercise would be more beneficial if exercise was commenced in the fasted state, which may be used by some athletes in certain sessions (e.g. see reference [23] for a recent review on the practice of training with low CHO availability). However, consuming a CHO-containing beverage would be counterproductive to any athlete wishing to practice this 'train low' method (as it would negate the fundamental principle of training low). As such, our approach for determining the effect of acute CHO ingestion on the *in vivo* immune responses to prolonged exercise in the fed state seems the most valid and of practical relevance for athletes. In the current study, the fact that we still observed notable differences, between the Ex-CHO and Ex-PLA conditions, in plasma glucose, leukocytosis and a number of stress markers typically associated with exercise and/or CHO ingestion demonstrates that there was a meaningful difference between the Ex-CHO and Ex-PLA groups in terms of the stress response to exercise. We suggest, therefore, that the lack of difference between CHO and PLA conditions in the present study cannot be explained by the fact that subjects were fed a standard breakfast 3.5 h before the exercise trials. These findings are in line with previous studies showing that *in vivo* immune responses to DPCP are unrelated to stress hormone responses induced by exercise [20] or altitude [24]. Taken together, this offers a

feasible explanation for the lack of effect of CHO supplementation on DPCP responses, despite a blunting of many of the stress response markers typically proposed to be responsible for the immunodepressive effects associated with prolonged and strenous exercise. Induction of CHS is dependent upon dendritic cell activation at the skin, dendritic cell migration from the skin to the regional lymph nodes and subsequent clonal expansion of specific T cells [25], and represents an *in vivo* measure of the whole integrated immune response. As we have demonstrated no effect of CHO supplementaiton on this *in vivo* immune measure, and since the stress hormone (particularly cortisol) response does not seem to be implicated [20], it is now necessary to further explore the mechanisms responsible for the exercise-induced decrease in *in vivo* immunity and suitable nutritional countermeasures.

Limitations

The results for erythema responses were generally in line with the oedema responses but when comparing to the CON data of Diment et al. [20] the summed response was significantly lower in the Ex-CHO condition (~41%) but there was only a trend for lower responses in the Ex-PLA group (~32%). Importantly, however, there was no difference between the Ex-PLA and Ex-CHO groups (if anything the response was lower in the CHO group). Since the use of erythema may suffer from interference at sites of a strong response, where yellow vesicles can interfere with the erythema readings [19, 20] the oedema results of this study should be taken as the primary indication of CHS. Importantly, even if using the erythema data, the overall conclusion would remain the same, that acute CHO ingestion before, during and after prolonged exercise does not benefit *in vivo* immunity.

We recognise a limitation of the current study is that we did not concurrently measure any of the previously reported functional *in vitro* markers (e.g. neutrophil function, lymphocyte

proliferation, stimulated cytokine production) for comparison with *in vivo* responses. However, it is well-established and consistently reported that CHO supplemention blunts many of the prolonged-exercise-induced alterations in *in vitro* immune measure (e.g. [4, 5, 9– 11]). Our current results do not suggest that subjects' responses were any different from those typically observed in such studies: we observed the typical exercise-induced increases of stress hormones and leukocyte trafficking which were blunted by CHO ingestion. This study therefore supports the recent findings of Diment et al. [20] that the induction of immune memory is decreased by prolonged exercise but the circulating stress hormone responses do not appear to be responsible. This also agrees with Oliver et al. [24] who showed that reduced CHS responses after ascent to altidude were not related to the stress hormone response. The current study does support the previously reported effects of CHO supplementation on blunting of stress and leukocytosis responses to prolonged exercise (e.g. [4, 5, 9, 11]) but it does not support the use of CHO as an acute nutritional intervention of benefit to *in vivo* immunity. This also seems to support the notion that *in vitro* measures do not sufficiently capture the whole integrated immune response and as such, are not representative of *in vivo* host defence. As a result, it would be beneficial for future studies to include *in vivo* measures in order to test the utility of proposed nutritional countermeasures for immune impairment after prolonged exercise.

We also recognise that the CHS response to DPCP is benign, and the clinical significance of the response, with specific regard to URTI in athletes, has not yet been established [20]. Nevertheless, the clinical relevance of *in vivo* cutaneous immune measures has been demonstrated in other contexts e.g. relationships with the progression of immunodeficiency; progression of acute to persistent diarrhoea; and URTI risk in children [17, 26–28]. In the current study, we have used the CHS response as a marker of *in vivo* immune function but do

acknowledge that future research must confirm the relationship between this and illness (e.g. URTI) risk in athletes.

Conclusion

Based on the results of this study, acute CHO ingestion before, during and after exercise does not blunt the decrease of *in vivo* immunity observed following 2 h of moderate exercise in the fed state (exercise commencing 3.5 h after breakfast). It remains to be determined, however, whether there would be any benefit with exercise that places a greater stress on CHO availability (e.g. longer duration and/or in the fasted state), or during periods of intensified training. However, we propose that the current design is relevant to what many athletes actually do in training and competition althouh the effects of more chronic CHO supplementation (i.e. during regular training for weeks or months) require further investigation.

Ethical standards

All procedures within this study were first approved by the University local research ethics committee and were carried out in accordance with the Declaration of Helsinki (including 2013 amendments). All study participants gave written informed consent prior to their inclusion in the study.

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Table 1. Subject demographics

	PLA-Ex (n = 16)	CHO-Ex (n = 16)	t-test P value
Height (m)	1.79 (0.05)	1.78 (0.05)	0.489
Weight (kg)	76.4 (10.4)	76.0 (10.0)	0.916
BMI (kg·m^{-2})	23.8 (3.0)	24.0 (2.6)	0.827
Age (years)	29.0 (8.3)	28.5 (8.1)	0.864
Training (h)	6.8 (3.1)	6.4 (2.5)	0.721
$\dot{V}O_{2max}$ (ml·kg^{-1}·min^{-1})	55.7 (6.1)	60.9 (5.6)	0.017

Values are mean (SD)

Table 2. Exercise responses during main trial

	PLA-Ex	CHO-Ex	t-test P value
%Δ @ 60% $\dot{V}O_{2max}$	39.6 (28.7)%	38.8 (36.4)%	0.622
Mean % $\dot{V}O_{2max}$	59.6 (4.9)%	58.2 (4.7)%	0.432
HR (bpm)	140 (15)	138 (12)	0.686
RPE	11 (2)	11 (2)	0.182

Values are mean (SD)

Table 3. Leukocyte responses to exercise

		Pre-Ex	Post-Ex	1 h Post-Ex	ANOVA main effects (group; time; trial × time)
Leukocyte count ($\times 10^9 . l^{-1}$)					0.313 ; <0.001 ; <0.001
	Ex-PLA	5.1 (1.2)	10.4 (2.9)	10.2 (2.7)	
	Ex-CHO	5.6 (0.8)	7.1 (1.3) [aa]	6.2 (0.6) [aa]	
Neutrophil count ($\times 10^9 . l^{-1}$)					0.005 ; <0.001 ; <0.001
	Ex-PLA	3.2 (1.0)	7.6 (2.6)	7.9 (2.1)	
	Ex-CHO	3.2 (0.6)	4.7 (1.2) [aa]	4.1 (0.8) [aa]	
Lymphocyte count ($\times 10^9 . l^{-1}$)					0.830 ; <0.001 ; 0.037
	Ex-PLA	1.4 (0.4)	1.8 (0.7)	1.3 (0.5)	
	Ex-CHO	1.6 (0.5)	1.7 (0.4)	1.4 (0.4)	
N:L ratio					0.053 ; <0.001 ; <0.001
	Ex-PLA	2.5 (1.2)	4.9 (2.7)	6.5 (2.3)	
	Ex-CHO	2.2 (1.0)	2.9 (0.9)	3.2 (1.2) [aa]	

N:L ratio: Neutrophil-to-Lymphocyte ratio. *Values are mean (SD);*
Post hoc (between groups): Significantly different to Ex-PLA [a] (P <0.05), [aa] (P <0.01). Post hoc results for significant time effects are described in the text of results section but not shown in above table.

Table 4. Glucose and stress hormone responses to exercise

|		Pre-Ex	Post-Ex	1 h Post-Ex	ANOVA main effects (group; time; trial × time)
Plasma Glucose ($mmol.l^{-1}$)					0.002 ; 0.007 ; <0.001
	Ex-PLA	4.5 (0.8)	4.2 (0.6)	3.8 (0.9)	
	Ex-CHO	4.2 (0.8)	5.9 (0.9) [aa]	5.1 (1.1) [aa]	
Cortisol ($nmol.l^{-1}$)					0.263 ; 0.037 ; 0.001
	Ex-PLA	367 (187)	431 (218)	398 (175)	
	Ex-CHO	488 (159)	259 (98) [a]	269 (81) [a]	
Adrenaline ($nmol.l^{-1}$)					0.961 ; 0.002 ; 0.857
	Ex-PLA	0.37 (0.27)	0.96 (1.26)	0.45 (0.44)	
	Ex-CHO	0.37 (0.28)	0.62 (0.31)	0.31 (0.09)	
Noradrenaline ($nmol.l^{-1}$)					0.949 ; <0.001 ; 0.701
	Ex-PLA	3.15 (1.53)	4.71 (2.02)	3.30 (1.19)	
	Ex-CHO	2.96 (1.14)	4.98 (1.19)	3.18 (0.77)	

Values are mean (SD);
Post hoc (between groups): Significantly different to Ex-PLA [a] (P <0.05), [aa] (P <0.01). Post hoc results for significant time effects are described in the text of results section but not shown in above table.

1

2

3   Figures
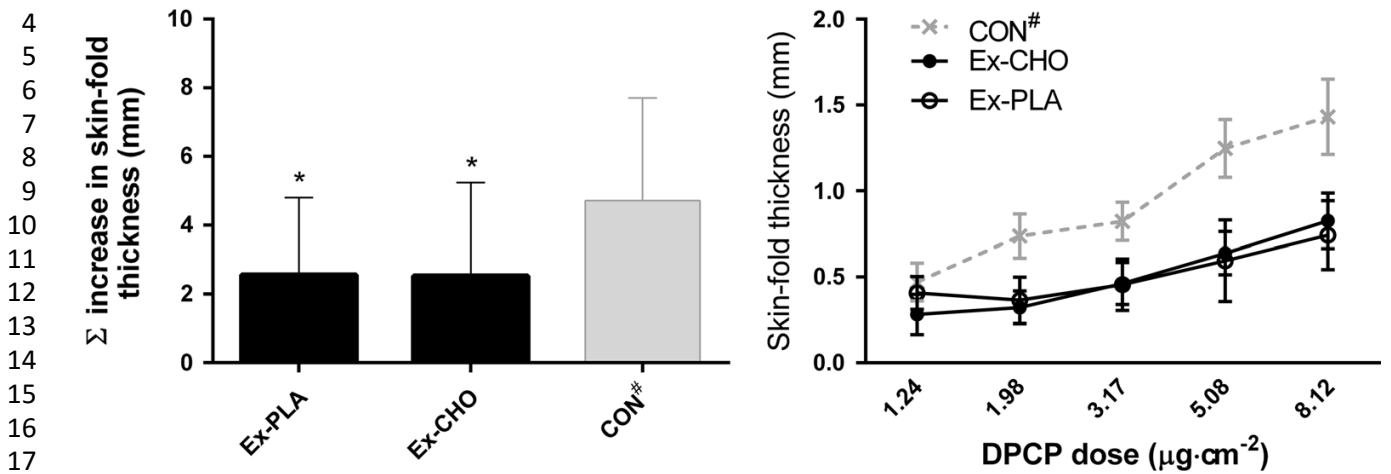
Figure 1. CHS response to DPCP elicitation 28 d after sensitisation: Summed increase in skin-fold thickness (left panel, values are mean ± SD) and full dose-series response (right panel, values are mean ± SEM), 48 h post elicitation.
#note: Responses were also compared to 16 resting control (CON) subjects from Diment et al. [20] for additional comparison with a resting non-exercise condition (n = 16, 120 minutes of seated rest).
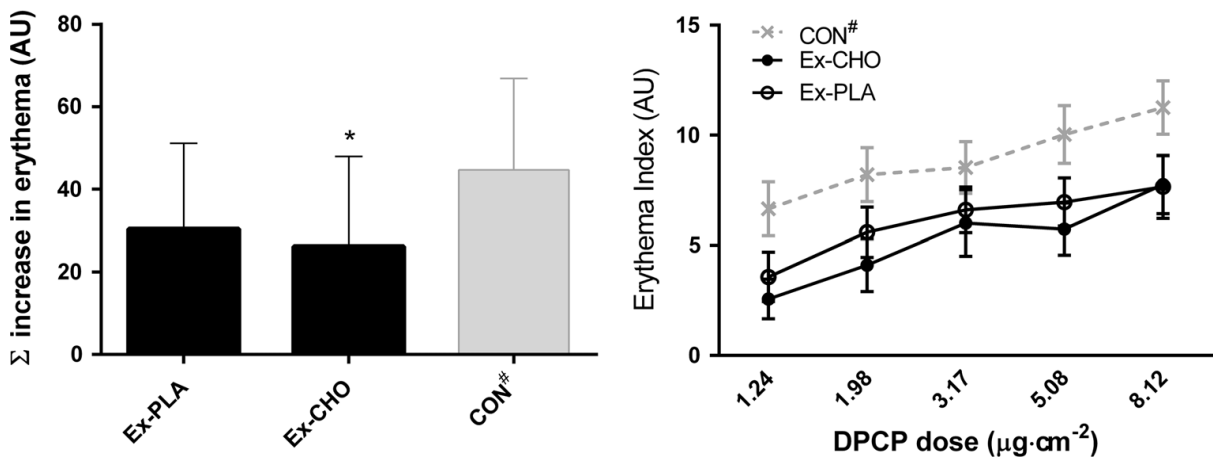** Indicates significantly different to CON (P < 0.05). There were no differences between Ex-PLA and Ex-CHO*

Figure 2. CHS response to DPCP elicitation 28 d after sensitisation: Summed increase in erythema (left panel, values are mean ± SD) and full dose-series response (right panel, values are mean ± SEM), 48 h post elicitation.
#note: Responses were also compared to 16 resting control (CON) subjects from Diment et al. [20] for additional comparison with a resting non-exercise condition (n = 16, 120 minutes of seated rest).
* Indicates significantly different to CON (P < 0.05). There were no differences between Ex-PLA and Ex-CHO