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Effect of a single and repeated dose of caffeine on antigen-stimulated human natural killer cell CD69 expression after high intensity intermittent exercise

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Abstract

Several studies investigating the effect of caffeine on immune function following exercise have used one large bolus dose of caffeine. However, this does not model typical caffeine consumption. Therefore the purpose of this study was to investigate whether small repeated doses of caffeine ingested throughout the day would elicit a similar response as one large bolus dose ingested 1 h prior to exercise on antigen-stimulated NK cell CD69 expression following strenuous intermittent exercise. In a randomized cross-over design, 15 healthy males completed six 15 min blocks of intermittent running consisting of maximal sprinting interspersed with less intense running and walking. Participants had ingested either 0 (PLA), 2 $mg \cdot kg^{-1}$ body mass (BM) caffeine on 3 separate occasions during the day (3xCAF) or 1 dose of 6 (1xCAF) $mg \cdot kg^{-1}$ BM caffeine, 1 h before exercise. At 1 h post-exercise the number of antigen-stimulated $CD3^{-}CD56^{+}$ cells expressing CD69 was lower on 1xCAF compared with PLA [$P<0.05$; PLA: 42.0 (34.0)$x10^{6}$ $cells \cdot L^{-1}$, 1xCAF: 26.2 (25.0)$x10^{6}$ $cells \cdot L^{-1}$], with values on 1xCAF at this time-point remaining close to pre-supplement. 1xCAF tended to attenuate the exercise-induced increase in geometric mean fluorescence intensity of CD69 expression on antigen-stimulated $CD3^{-}CD56^{+}$ cells 1 h post-exercise [$P=0.055$; PLA: 141 (28)%, 1xCAF: 119 (20)%]. These findings suggest that although one large bolus dose of caffeine attenuated the exercise-induced increase in antigen-stimulated NK cell CD69 expression 1 h following strenuous intermittent exercise, this attenuation at no point fell below pre-supplement values and as such caffeine does not appear to depress NK cell CD69 expression.

Keywords: methylxanthine, CD69, exercise, immune, lymphocyte

Introduction

Caffeine is a common element in most people's diets (Heatherley et al. 2006), yet it is not a typical nutrient nor is it essential for health (Graham 2001). Caffeine is legally and socially acceptable and as such is the most widely consumed behaviour-influencing substance in the world (Graham 2001). It is estimated that the mean daily intake of caffeine in the U.K is ~241 ± 173 $mg \cdot day^{-1}$, which is the equivalent to around 2-4 cups of coffee (Heatherley et al. 2006). However, daily caffeine intake can vary widely depending upon country. For example the daily caffeine intake in Sweden, Norway and Netherlands is estimated to be approximately 400 $mg \cdot day^{-1}$ (Fredholm et al. 1999).

Natural killer (NK) cells are a subset of lymphocytes that have innate immune functions, representing approximately 5–20 % of all lymphocytes in peripheral blood (Andoniou et al. 2006). NK cells are capable of recognizing and destroying certain tumour cells and virally-infected cells without prior sensitization or specific antigen recognition, and therefore are distinct from other lymphocytes. At rest (unstimulated) NK cells tend to express small amounts of functionally active CD69 (Borrego et al. 1999), however following activation this antigen is rapidly induced (Ziegler 1994). Although the exact role of CD69 has not as yet been elucidated, *in vitro* studies have demonstrated that once expressed on lymphocytes, this antigen appears to act as a co-stimulatory receptor helping to trigger subsequent cell proliferation, cytotoxicity and secretion of cytokines (such as tumour necrosis factor-α and interferon-γ) (Borrego et al. 1999). However, *in vivo* studies have demonstrated that the behaviour of CD69 is more complex, and also appears to act as an immunoregulatory molecule (reviewed in Sancho et al. 2005). Several studies have demonstrated that CD69 cross-linking appears to induce the production of the anti-inflammatory cytokine transforming

growth factor-β (TGF- β) in lymphocytes, that appears to be able to downregulate the inflammatory response as well as tumour lysis by NK cells (Esplugues et al. 2003; Sancho et al. 2003).

Acute strenuous exercise causes a temporary perturbation in a range of immune cell functions usually lasting between ~3-24 h. It is suggested that during this "open window" of altered immunity, viruses and bacteria may gain a foothold, increasing the risk of subsequent infection (Nieman 2000). Several studies have demonstrated that for several hours following both high intensity continuous and intermittent exercise NK cell number and function are suppressed to below baseline measures (Kappel et al. 1991; McFarlin et al. 2004; Neiman et al. 2007). NK cells express both adenosine receptors (A_1, A_{2A} and A_{2B}) and β_2-adrenoreceptors (Priebe et al. 1990; Shephard 2003). Caffeine is a non-selective adenosine receptor antagonist and also stimulates epinephrine release from the adrenal medulla; as such caffeine has the potential to either increase (via A_{2A} receptor antagonism) or decrease (via A_1 receptor antagonism and/or increased epinephrine stimulation) NK cell activation depending upon the principal mechanism. Strenuous exercise increases the plasma concentrations of both adenosine and epinephrine (Nieman et al. 2007; Vizi et al. 2002) and it has been proposed that these increases predominately inhibit NK cell function *in vitro* and *in vivo* (Kappel et al. 1991; Priebe et al. 1990), via A_{2A} adenosine receptor and β_2-adrenoreceptor activation of adenylate cyclase and increased intracellular cAMP (Raskovalova et al. 2006). It is likely that in response to caffeine ingestion and exercise both adenosine and epinephrine mechanisms will be involved. However, the overall affect on NK cell activation may depend upon which mechanism predominates.

Caffeine has been shown to increase sympathoadrenal medullary system (SAM) activity and as such plasma epinephrine concentration (reviewed in Graham 2001). Salivary α-amylase activity is stimulated by increased activity of the sympathetic nervous system (Rohleder et al. 2004). Therefore, it has been suggested that elevations in α-amylase concentration can be considered a good indicator of enhanced sympathetic activity (Anderson et al. 1984; Rohleder et al. 2004). As such salivary α-amylase has been chosen as a surrogate marker for plasma epinephrine concentration in the present study.

Despite the reported widespread and frequent use of caffeine within society (Heatherley et al. 2006), very few studies have investigated the effect of caffeine on immune cell responses to exercise in healthy individuals. Of the handful of studies that have been published, all have administered 6 $mg \cdot kg^{-1}$ body mass caffeine in one bolus dose following a 60 h abstinence period (Bishop et al. 2005, 2006; Fletcher and Bishop 2010; Walker et al. 2006, 2007, 2008). However, caffeine is typically consumed in separate doses throughout the day, following an overnight abstention (Fredholm et al. 1999). To the authors knowledge no study to date has attempted to model natural caffeine consumption in terms of dose and time of consumption when investigating caffeine's effects on immune cell function following exercise. Recent pilot work from our group has suggested that a small dose of caffeine (2 $mg \cdot kg^{-1}$) ingested prior to exercise increases antigen-stimulated NK cell CD69 expression 1 h following high intensity cycling to the same extent as that of a dose 3 times as large (6 $mg \cdot kg^{-1}$). Therefore the aim of this study was to investigate whether small repeated doses (2 $mg \cdot kg^{-1}$) of caffeine ingested throughout the day (9:00 h, 12:00 h & 15:00 h) would elicit a similar response as one large (6 $mg \cdot kg^{-1}$) bolus dose ingested 1 h prior to exercise (15:00 h) compared with placebo on antigen-stimulated NK cell CD69 expression following 90 min of high intensity intermittent exercise. It was hypothesised that both caffeine trials would increase NK cell

CD69 expression to a similar extent following high intensity intermittent exercise, and that this increase would be significantly greater when compared with placebo.

Methods

Participants

Fifteen healthy male games (football and rugby) players [mean (SD): age 22 (2) years; body mass 76 (8) kg; $\dot{V}O_{2\,max}$ 54 (3) $ml \cdot kg^{-1} \cdot min^{-1}$] volunteered to participate in this study. All participants were fully informed about the rationale for the study and the design of the exercise tests and trial protocol, along with their possible risks and discomforts, before providing written informed consent. The local Ethical Advisory Committee approved the study. Participants did not report taking any medications or experiencing any symptoms of upper respiratory tract infection in the 4 weeks prior to the study. All participants were moderate caffeine users (50 – 250 $mg \cdot day^{-1}$), with an average daily caffeine intake of 159 (61) $mg \cdot day^{-1}$.

Preliminary testing

Approximately 2 weeks before the beginning of the main trials, each participant was required to perform a progressive shuttle run test to volitional fatigue to estimate maximal oxygen consumption ($\dot{V}O_{2\,max}$) (Ramsbottom et al., 1988). From this estimate, running speeds corresponding to 55% and 95% $\dot{V}O_{2\,max}$ were calculated from the tables for predicted $\dot{V}O_{2\,max}$ values (Ramsbottom et al., 1988). Participants came back into the lab on a separate occasion to undertake a familiarization trial, which required them to perform 3x15 min blocks (45 min) of the LIST. Heart rates (Polar FS1, Polar Electro Oy, Kempele, Finland) were monitored continuously during the familiarization trial.

Experimental trial procedures

Participants were instructed to avoid alcohol consumption and strenuous physical activity in the 24 h preceding each experimental trial. Participants were also instructed to stop consuming caffeine from 21:00 h the night before each trial. In an attempt to standardize nutritional status, participants completed a 24 h food diary the day before and the day of the first main trial. Participants were then asked to follow their food diaries for the second and third main trials. Participants performed 3 main exercise trials, each separated by 1 week. Participants were randomly assigned to either the PLA, 1xCAF (1 single dose of 6 $mg \cdot kg^{-1}$ caffeine) or 3xCAF (3 repeated doses of 2 $mg \cdot kg^{-1}$ caffeine) trial and acted as their own controls in a repeated-measures, single-blind, cross-over design. Participants arrived at the laboratory at 08:50 h following an overnight fast of 12 h. After 10 min of resting quietly an initial (pre-supplement) blood sample was obtained from an antecubital forearm vein by venepuncture. Following the blood sample, an initial (09:00 h, pre-supplement) saliva sample was obtained. Participants then ingested 0 (PLA) or 2 (3xCAF) $mg \cdot kg^{-1}$ body mass of caffeine powder (BDH Laboratory Supplies, Poole, UK) taken in the form of cellulose capsules (G & G Food Supplies Ltd, West Sussex, UK) with 300 ml plain water. For the 0 $mg \cdot kg^{-1}$ dose, participants ingested 2 $mg \cdot kg^{-1}$ body mass of dextrose powder (BDH Laboratory Supplies, Poole, UK). The specific timings of PLA or CAF ingestion along with blood and saliva samples are illustrated in Fig. 1. Participants then rested quietly in the laboratory for 1 h before a further (10:00 h) saliva sample was taken, after which participants were free to leave the laboratory and to start eating. Participants were required to return to the laboratory at 12:00 h to ingest another set of either 0 (PLA) or 2 (3xCAF) $mg \cdot kg^{-1}$ caffeine capsules and to provide a further saliva sample (12:00 h). After the saliva sample participants left the laboratory until 15:00 h, at which time they provided another saliva sample (15:00 h).

Participants stopped eating from 15:00 h. Participants then ingested a final set of either 0 (PLA), 2 (3xCAF) or 6 (1xCAF) $mg \cdot kg^{-1}$ caffeine capsules immediately after the saliva sample. Participants then rested quietly in the laboratory for 1 h before a further venous blood and saliva sample (16:00 h, pre-exercise) were taken. Immediately following venous blood and saliva samples pre-exercise body mass (in shorts only) was recorded, after which, participants began six 15 min periods of intermittent shuttle running, which consisted of ~10 cycles of walking, maximal sprinting, jogging and cruising (Fig. 2). Participants were required to run between two lines separated by 20 m at various speeds that related to previous $\dot{V}O_{2\,max}$ values. Each 15 min bout of running was separated by a 3 min rest period. During this period participants were allowed to consume plain water *ad libitum* and were asked to rate their perceived exertion (RPE) on a Borg 6-20 scale. Heart rates were recorded prior to, during and immediately after each 15 min period of intermittent shuttle running. A further venous blood and saliva sample (17:45 h, post-exercise) was obtained immediately after cessation of exercise, before post-exercise body mass (in shorts only) was recorded. Participants then consumed 5 $ml \cdot kg^{-1}$ body mass of plain water and rested quietly in the laboratory for a further hour before a final venous blood and saliva sample (18:45 h, 1 h post-exercise) was obtained. During this time no additional food or fluid was administered to participants. For all venous blood samples, 20 ml of blood was obtained, with all samples taken from participants in an upright-seated position. For all saliva samples, participants were asked to swallow to empty the mouth before timed unstimulated whole mixed saliva collections were made into pre-weighed sterile vials (7 ml capacity bijou tubes with screw-top). All collections were made over a 2 min period, unless insufficient volume had been produced, in which case the collection period was increased to 3 min, as necessary. All collections were made with participants seated; leaning forwards and with their heads tilted downwards. Participants were instructed to allow the saliva to dribble into the tube with

minimal orofacial movement. Laboratory conditions throughout the study were 20.5 (0.9) °C and 35.7 (5.4) % relative humidity.

Total lymphocyte count and plasma volume

Blood samples were collected into two evacuated monovette tubes (Starstedt, Leicester, UK), one containing K_3EDTA (1.6 mg EDTA ml^{-1} blood) and one containing no additive to obtain serum, as well as a sterile 5 ml bijou tube (Sterilin, Staffordshire, UK) containing 15 µl sodium heparin (15 IU heparin ml^{-1} blood). Blood collected into the K_3EDTA monovette (7.5 ml) was analyzed for total and differential leukocyte counts, hematocrit and hemoglobin contents using a hematology analyzer ($A^C.T^{TM}$ 5diff analyzer, Beckman Coulter, UK). Plasma volume changes were estimated according to Dill and Costill (1974).

Lymphocyte culture

Five ml of whole blood transferred into the sterile bijou tube containing heparin was immediately placed on ice and mixed (Thermo Denley, Spiramix) for 20 min before setting up cultures. Due to the large time gap (6 h) between pre-supplement and pre-exercise venous blood samples, 2 lots of cultures were set up: one set was the pre-supplement sample, the other set was the pre-exercise, post-exercise and 1 h post-exercise samples. Cells were cultured in Falcon 12 x 75 mm-polystyrene tubes with caps (Becton Dickinson Biosciences, Oxford, UK) and for all samples stimulated and unstimulated cultures were set up. Tubes were stimulated by a 1:400 or 1:800 Pediacel vaccine (Sanofi Pasteur MSD Ltd, Berkshire, UK), containing diphtheria toxoid, tetanus toxoid, acellular pertussis bacteria, poliovirus and haemophilus influenza type b. Zero or 20 µl of Pediacel working vaccine (either 1:400 or 1:800) was added to 200 µl of heparinized whole blood, giving a final stimulant concentration

of 1:4000 (optimum) or 1:8000 (sub-optimum) before being incubated for 20 h at 37 °C, 5% CO_2. All cell culture work was carried out in a class II laminar flow hood (Esco GB Ltd, Salisbury, UK) by using aspectic techniques.

Assessment of lymphocyte subsets and CD69 expression

After incubation, peripheral blood cells were labelled with a cocktail of Pharmingen monoclonal antibodies (Becton Dickinson Biosciences, Oxford, UK) against human lymphocyte cell surface markers, as follows: 5 µl CD3 FITC, 20 µl CD56 PECy5 and 10 µl CD69 PE. All samples were then vortexed and placed on ice for 20 min, after which erythrocytes were lysed and leukocytes fixed using FACS Lyse (Becton Dickinson, Oxford, UK). Leukocytes were washed once in 3 ml ice cold phosphate-buffered saline (PBS) containing 0.1% bovine serum albumin (BSA) and 2 mM EDTA (PBS/BSA/EDTA) before being resuspended in 400 µl PBS/BSA/EDTA. Three-colour flow cytometric analysis was performed using a FACS Calibur flow cytometer with Cell Quest analysis software (Becton Dickinson, Oxford, UK). Standard gating procedures using side scatter versus forward scatter plots were used to gate on the lymphocyte population. An unstained unstimulated sample was used to set quadrant boundaries to allow accurate acquisition of stained samples. All samples were set to collect 30,000 lymphocyte events per analysis.

$CD3^+$ (T cell region) and $CD56^+$ (NK cell region) populations were acquired on quadrant dot plots of FL1 (CD3 FITC) and FL3 (CD56 PE-Cy5), along with quadrant dot plots of FL1 (CD3) and FL2 (CD69 PE) and quadrant dot plots of FL3 (CD56) and FL2 (CD69). This data was then displayed as histogram plots and the percentage of $CD3^-$ cells (total lymphocyte region minus the $CD3^+$ region) expressing $CD56^+$ were derived and from this the percentage of total lymphocytes that were $CD3^-CD56^+$ was determined. $CD3^-CD56^+$ cells were then

gated into a separate region and a $CD69^+$ histogram plot of the cells within this region was used to calculate the percentage expression and geometric mean fluorescence intensity (GMFI) expression of CD69 of $CD3^-CD56^+$ cells. Cell counts of $CD3^-CD56^+$ were calculated by multiplying the percentage of these cells with the absolute lymphocyte count. The number of $CD3^-CD56^+$ cells expressing CD69 were determined by multiplying the percentage of cells expressing CD69 by the total number of $CD3^-CD56^+$ cells. To facilitate intersubject comparisons, pre-exercise, post-exercise and 1 h post-exercise CD69 GMFI were expressed as a percentage of the pre-supplement value, as according to Timmons et al. (2006).

Saliva flow rate

Saliva samples were stored in their plastic containers at –80 °C prior to analysis. Saliva volume was estimated by weighing the bijou tubes to the nearest mg before and after saliva collection. Saliva density was assumed to be 1.00 $g \cdot ml^{-1}$ (Cole and Eastoe 1988) and from this, the saliva flow rate ($\mu l \cdot min^{-1}$) was determined by dividing the volume of saliva by the collection time.

Saliva α-amylase

Saliva α-amylase activity was measured using a commercially available kit (Infinity™ Amylase Liquid Stable Reagent, Thermo Scientific, UK), with proportional reduction of volumes so that the assay could be carried out in a microtitration (96-well) plate. Briefly, 20 µl saliva diluted 1:100 with 1.0 mM $CaCl_2$ was mixed with 180 µl of amylase reagent. The plate was incubated for 1 min at 25°C and then the increase in absorbance at 405 nm was recorded every min for a further 4 min period on an automated plate reader. Purified α-amylase from human saliva (A1031, Sigma) was used as a standard. The secretion rate of α-

amylase ($U \cdot min^{-1}$) was calculated by multiplying the saliva flow rate by the α-amylase activity. The intra assay coefficient of variation for α-amylase was 1.1%

Serum and saliva caffeine concentration

Serum was obtained from whole blood collected into a serum monovette (5.5 ml), which was left to clot for 1 h before being centrifuged at 1500 g for 10 min in a refrigerated centrifuge at 4 ºC. Saliva samples were transferred into eppendorfs and centrifuged at 12000 rpm for 2 min in a high speed microcentrifuge. Serum and saliva caffeine concentration was determined using a commercially available kit (Emit Caffeine Assay, Dade-Behring, Milton Keynes, UK) on an automatic photometric analyzer (COBAS Miras Plus, Roche Diagnostic Systems, Switzerland). The intra assay coefficient of variation for serum and saliva caffeine concentration was 2.6% and 1.7%, respectively.

Statistical analysis

Data in the text, tables and figures are presented as mean values and their standard deviation (± SD). The data were examined using a two-factor (trial x time) analysis of variance (ANOVA) with repeated measures design. If the data were not normally distributed, statistical analysis was carried out on the logarithmic transformation of the data. Assumptions of sphericity in the data were checked, and adjustments in the degrees of freedom for the ANOVA were made using the Huynh-Feldt method of correction where appropriate. Any significant data were assessed using Student's paired *t*-tests with Holm-Bonferroni adjustments for multiple comparisons. Statistical significance for this study was accepted at $P<0.05$. The observed powers of the reported main and interaction effects are all >0.8.

Results

Physiological measurements

There were no differences in ratings of perceived exertion between trials during the test [RPE of 13 (2) for all trials]. Likewise, heart rates were similar between trials during exercise [PLA: 171 (12) beat·min^{-1}, 1xCAF: 175 (13) beat·min^{-1}, 3xCAF: 177 (12) beat·min^{-1}; mean of all recordings].

Changes in body mass and plasma volume

After exercise, changes in body mass (corrected for fluid intake) were similar for all trials [PLA: -1.8 (0.3) kg, 1xCAF: -1.8 (0.3) kg, 3xCAF: -1.9 (0.4) kg]. There was no significant time x trial interaction effect for changes in plasma volume relative to the initial blood sample. After exercise plasma volume decreased by 3.3 (4.4) %, 3.4 (6.0) % and 4.8 (6.6) % on the PLA, 1xCAF and 3xCAF trials, respectively.

Serum caffeine concentration

A significant time x trial interaction was found for serum caffeine concentration ($P<0.01$), with higher concentrations at pre (16:00 h), post (17:45 h) and 1 h post-exercise (18:45 h) on both 1xCAF and 3xCAF compared with PLA ($P<0.01$; Fig. 3). Serum caffeine concentration was also significantly higher on 3xCAF at pre-exercise compared with 1xCAF ($P<0.05$). However at post-exercise and 1 h post-exercise serum caffeine concentrations on 1xCAF were significantly higher than 3xCAF (both $P<0.01$; Fig 3).

Saliva caffeine concentration

A significant time x trial interaction was found for saliva caffeine concentration ($P<0.01$), with higher concentrations at 10:00 h, 12:00 h, 15:00 h and pre (16:00 h), post (17:45 h) and 1 h post-exercise (18:45 h) on 3xCAF compared with PLA ($P<0.01$; Table 1) and at post (17:45 h) and 1 h post-exercise (18:45 h) on 1xCAF compared with PLA ($P<0.01$; Table 1). Saliva caffeine concentrations were also higher on 3xCAF at 10:00 h, 12:00 h, 15:00 h and pre-exercise compared with 1xCAF ($P<0.01$; Table 1), but lower than 1xCAF at post and 1 h post-exercise ($P<0.01$; Table 1).

Saliva flow rate

Saliva flow rate appeared to increase significantly above pre-supplement (9:00 h) values at 10:00 h, 12:00 h, 15:00 h, pre-exercise, post-exercise and 1 h post-exercise (main effect for time: all time points $P<0.01$, except post-exercise $P<0.05$; Table 1). However, when compared against pre-exercise values, saliva flow rate fell significantly at post-exercise (main effect for time: $P<0.01$; Table 1). There were no significant effects of trial/treatment.

Saliva α-amylase

A significant time x trial interaction was found for saliva α-amylase activity, which was significantly higher at pre-exercise and 1 h post-exercise on both 1xCAF and 3xCAF compared with PLA ($P<0.05$; Table 1). A significant time x trial interaction was also found for saliva α-amylase secretion rate, which was higher at 1 h post-exercise on 1xCAF compared with PLA ($P<0.05$; Table 1). There were no differences between 1xCAF and 3xCAF.

Circulating lymphocyte counts and numbers of $CD3^{-}CD56^{+}$ cells

There were no time x trial interactions for the number of circulating lymphocytes, but there was a main effect for time ($P<0.01$) with the number of circulating lymphocytes increasing above pre-supplement values at immediately post-exercise ($P<0.01$; Table 2). A significant time x trial interaction was found for the number of $CD3^{-}CD56^{+}$ cells within the circulating lymphocyte population ($P<0.01$), with a higher number of $CD3^{-}CD56^{+}$ cells found at post-exercise on both 1xCAF and 3xCAF compared with PLA ($P<0.05$; Table 2). There were no differences between 1xCAF and 3xCAF.

Number of unstimulated and antigen-stimulated $CD3^{-}CD56^{+}$ cells expressing CD69

There were no interaction effects for the number of unstimulated $CD3^{-}CD56^{+}$ cells expressing CD69. However there was a main effect for time ($P<0.01$) with the number of $CD3^{-}CD56^{+}$ cells expressing CD69 increasing significantly above pre-supplement values at pre and post-exercise, only to fall below pre-supplement values at 1 h post-exercise (all $P<0.01$; Fig. 4a). When cells were stimulated there was a significant time x trial interaction effect, with the number of antigen-stimulated (both doses) $CD3^{-}CD56^{+}$ cells expressing CD69 lower at 1 h post-exercise on 1xCAF compared with PLA ($P<0.05$); values on 1xCAF at this time-point remained close to pre-supplement (Fig. 4b & c). There were no differences between 3xCAF and PLA or 1xCAF and 3xCAF.

Geometric Mean Fluorescence Intensity of CD69 expression on unstimulated and antigen-stimulated $CD3^{-}CD56^{+}$ cells

There were no significant interaction effects for the GMFI of CD69 expression on unstimulated $CD3^{-}CD56^{+}$ cells. However there was a main effect for time ($P<0.01$), with the GMFI of CD69 expression on unstimulated $CD3^{-}CD56^{+}$ cells increasing significantly above

pre-supplement values at 1 h post-exercise ($P<0.01$; Fig. 5a). When cells were stimulated there was a significant time x trial interaction effect, with the GMFI of CD69 expression on antigen-stimulated (1:8000) $CD3^{-}CD56^{+}$ cells increasing on all trials above pre-supplement values at 1 h post-exercise ($P<0.01$; Fig. 5c). However, values tended to be lower on 1xCAF compared with PLA at this time ($P=0.055$; Fig. 5c). The GMFI of CD69 expression on antigen-stimulated (1:8000) $CD3^{-}CD56^{+}$ cells was also significantly lower at immediately post-exercise on 1xCAF compared with both PLA and 3xCAF ($P<0.05$), with values on 1xCAF at this time-point remaining close to pre-supplement values (Fig. 5c).

Discussion

The main findings of this study suggest that exercise induced an increase in the number of antigen-stimulated NK cells expressing CD69 as well as in the GMFI of CD69 expression on antigen-stimulated NK cells 1 h following 90 min shuttle running that was attenuated after consuming one large bolus dose (6 $mg \cdot kg^{-1}$) of caffeine 1 h before exercise (15:00 h; 1xCAF). In contrast, ingesting small doses (2 $mg \cdot kg^{-1}$) of caffeine throughout the day to model a more typical pattern of caffeine consumption (09:00 h, 12:00 h & 15:00h; 3xCAF) had little effect on antigen-stimulated NK cell CD69 expression.

It has been suggested that caffeine's main mechanism of action is predominately via A_{2A} adenosine receptor antagonism (Fredholm et al. 1999). As such it was speculated that in the present study both caffeine trials would have increased antigen-stimulated NK cell CD69 expression in response to exercise compared with placebo as has previously been found (Fletcher and Bishop 2010). However, this was not the case in this study and as such appears to indicate that in the present study A_{2A} adenosine receptor antagonism was not the

predominant mechanism involved. NK cells also possess A_1 adenosine receptors (Priebe et al. 1990). As a non-selective adenosine receptor antagonist, caffeine could have antagonized NK cell A_1 receptors (Fredholm et al. 1999), which may have restricted the increase in antigen-stimulated NK cell CD69 expression on both caffeine trials in response to high intensity intermittent exercise compared with placebo.

It could be suggested that the large bolus dose of caffeine attenuated the exercise-induced increase in NK cell CD69 expression following high intensity shuttle running via stimulating the release of epinephrine. Epinephrine stimulates β_2-adrenoreceptors found on NK cells, leading to an increase in intracellular cAMP levels. An increase in the concentration of cAMP in the cell may have interfered with signals that are necessary for adhesion strengthening (Benschop et al. 1997) and as such could have preferentially mobilized a less active subset of NK cells into the circulation following exercise. Epinephrine has also been shown to inhibit CD69 expression on mitogen-stimulated NK cells (Shimamiya et al. 2003) and as such may have been responsible for the lower number of antigen-stimulated NK cells expressing CD69 and GMFI expression of CD69 on antigen-stimulated NK cells following the large single dose caffeine trial compared with placebo 1 h following high intensity shuttle running.

Although plasma epinephrine concentrations were not directly determined in this study, saliva α-amylase activity and secretion rate were measured as a surrogate marker, as increases in α-amylase concentration are considered a good indicator of enhanced sympathetic activity (Anderson et al. 1984; Rohleder et al. 2004). Saliva α-amylase activity was significantly higher at 1 h post-exercise on both caffeine trials compared with placebo. However, only the

large single dose caffeine trial (1xCAF) showed a significantly higher α-amylase secretion rate at 1 h post-exercise compared with placebo. As such this may explain why in the present study this was the only caffeine trial to attenuate the exercise-induced increase in both the number of antigen-stimulated NK cells expressing CD69 and their intensity of CD69 expression following high intensity shuttle running. Large (6 and 9 $mg \cdot kg^{-1}$), but not small (3 $mg \cdot kg^{-1}$), doses of caffeine have been shown to increase plasma epinephrine concentration in response to high intensity exercise (Graham and Spriet 1995; Walker et al. 2006). Therefore, it could perhaps be suggested that there may be an epinephrine threshold, above which epinephrine produces inhibitory effects upon NK cell CD69 expression. However, as plasma epinephrine concentrations were not directly determined in this study, any explanation as to how the large bolus dose of caffeine attenuated the exercise-induced increase in antigen-stimulated NK cell CD69 expression following exercise is only speculation and as such is a limitation of this study.

The increase in number of antigen-stimulated NK cells expressing CD69 following exercise on PLA could be interpreted in a number of ways due to the multiple complex actions of CD69. One could suggest that this increase may augment subsequent proliferation, cytotoxicity and pro-inflammatory cytokine release that would appear to favour a pro-inflammatory response via the actions of CD69 as a co-stimulatory receptor (Borrego et al. 1999). However, CD69 has also been shown to act as a negative regulator, downregulating the ability of NK cells to lyse target cells due to the production of TGF-β (Esplugues et al. 2003). As such the increase in the number of NK cells expressing CD69 in response to antigen may act as a regulatory response. However, given that TGF- β release by $CD69^{+}$ NK cells was not determined it is difficult to ascertain the exact role of CD69 in this study. Future *in vitro* work to determine the nature of the response (e.g. assessment of TGF-β

445 release) would provide greater insight regarding to the physiological nature and implications
446 of these findings.
447
448 Alternatively, it could be argued that as participants were habitual caffeine users, the effects
449 of caffeine on antigen-stimulated NK cell CD69 expression in response to prolonged high
450 intensity exercise in this study may have resulted from participants' tolerance to the effects of
451 caffeine. It has been demonstrated that tolerance to some of the effects of caffeine can
452 develop quite quickly in habitual caffeine users (reviewed in Fredholm et al. 1999). Studies
453 both in rats and humans suggest that following chronic caffeine intake (~14 days) A_1 and A_{2A}
454 adenosine receptors are upregulated leading to an increased sensitivity to adenosine
455 (Johansson et al. 1997; Varani et al. 2005). Therefore, as participants were only required to
456 abstain from caffeine overnight, caffeine habituation may have contributed towards the
457 findings that one large bolus dose of caffeine attenuated the exercise-induced increase in
458 antigen-stimulated NK cell CD69 expression 1 h following high intensity shuttle running yet
459 small repeated doses of caffeine had little effect at all. It should be noted that the mechanisms
460 discussed here are unlikely to occur exclusively; therefore these findings are likely to be the
461 net result of several positive and negative influences.
462
463 The issue of tolerance could also help explain why the findings of the present study contrast
464 with our previous results when caffeine ingestion (2 and 6 $mg \cdot kg^{-1}$) increased antigen
465 stimulated NK cell CD69 expression 1 h following 90 min high intensity exercise compared
466 with placebo (Fletcher and Bishop in press). Tolerance to caffeine dissipates after its
467 discontinuation, with resensitization to caffeine usually occurring within 2-3 days of
468 abstention (Benowitz et al. 1995; Varani et al. 2005). Our previous work employed a 60 h
469 caffeine abstention period and as such likely controlled for any influence of caffeine

tolerance. The mode of exercise in the present study was also different to that used in our previous work (intermittent running and continuous cycling, respectively) and may have influenced caffeine metabolism and as such caffeine's effects. However, this seems unlikely as Graham and Spriet (1991) have reported no difference in caffeine metabolism or plasma epinephrine concentration between running and cycling to exhaustion. In addition, serum caffeine concentrations immediately following 90 min continuous cycling (Fletcher and Bishop 2010) and intermittent running (present study) with prior ingestion of 6 $mg \cdot kg^{-1}$ body mass caffeine showed comparable values (continuous cycling, 44 μM; intermittent running, 43 μM).

One limitation of this study was the absence of a resting trial to determine if caffeine acted on antigen-stimulated NK cell CD69 expression without the influence of exercise. Initial pilot work from our group that looked at NK cell responses to caffeine at rest between 08:30 h and 12:00 h suggests that caffeine at doses of 2 or 6 $mg \cdot kg^{-1}$ body mass ingested in one bolus dose at 08:30 h have little effect on antigen-stimulated NK cell CD69 expression up until 12:00 h.

In conclusion, the findings of the present study suggest that exercise induced an increase in the number of antigen-stimulated NK cells expressing CD69 as well as their intensity of CD69 expression 1 h following 90 min shuttle running that was attenuated by one large bolus dose (6 $mg \cdot kg^{-1}$) of caffeine ingested 1 h prior to exercise. In contrast, small doses (2 $mg \cdot kg^{-1}$) of caffeine ingested throughout the day to model a more typical pattern of caffeine consumption had little effect on antigen-stimulated NK cell CD69 expression. It should be noted that the attenuations seen with one large bolus dose of caffeine at no point fell below pre-supplement values and as such suggests that caffeine does not depress antigen-stimulated NK cell CD69 expression following 90 min of high intensity shuttle running.

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Ethical Standards

The authors declare that the experiments comply with the current laws of the U.K.

Conflict of Interest

The authors declare that they have no conflict of interest.

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595 **Figure captions**

596 **Fig. 1** Schematic representation of the trial protocol

597 **Fig. 2** Schematic representation of the Loughborough Intermittent Shuttle Running Test
598 (LIST). Adapted from Bishop et al. 2002

599 **Fig. 3** Serum caffeine concentrations during PLA, 3xCAF and 1xCAF trials. * significantly
600 higher than PLA ($P<0.01$). ** significantly higher than PLA and 3xCAF ($P<0.01$).
601 § significantly higher than PLA and 1xCAF ($P<0.01$). † significantly higher than pre-
602 supplement within trial ($P<0.01$). Values are means ± SD

603 **Fig. 4** Number of (a) unstimulated (b) 1:4000 antigen-stimulated, (c) 1:8000 antigen-
604 stimulated $CD3^{-}CD56^{+}$ NK cells expressing CD69 within the circulating lymphocyte
605 population during PLA, 3xCAF and 1xCAF trials. * significantly lower than PLA ($P<0.05$);
606 † significantly higher than pre-supplement within trial ($P<0.01$). Values are means + SD

607 **Fig. 5** Geometric Mean Fluorescence Intensity of CD69 expression on (a) unstimulated (b)
608 1:4000 antigen-stimulated, (c) 1:8000 antigen-stimulated $CD3^{-}CD56^{+}$ NK cells within the
609 circulating lymphocyte population during PLA, 3xCAF and 1xCAF trials. * significantly
610 lower than PLA and 3xCAF ($P<0.05$); † significantly higher than pre-supplement within trial
611 ($P<0.01$). Values are means + SD and are expressed as a percentage of the pre-supplement
612 value

613

614 Fig. 1
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616
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618
619

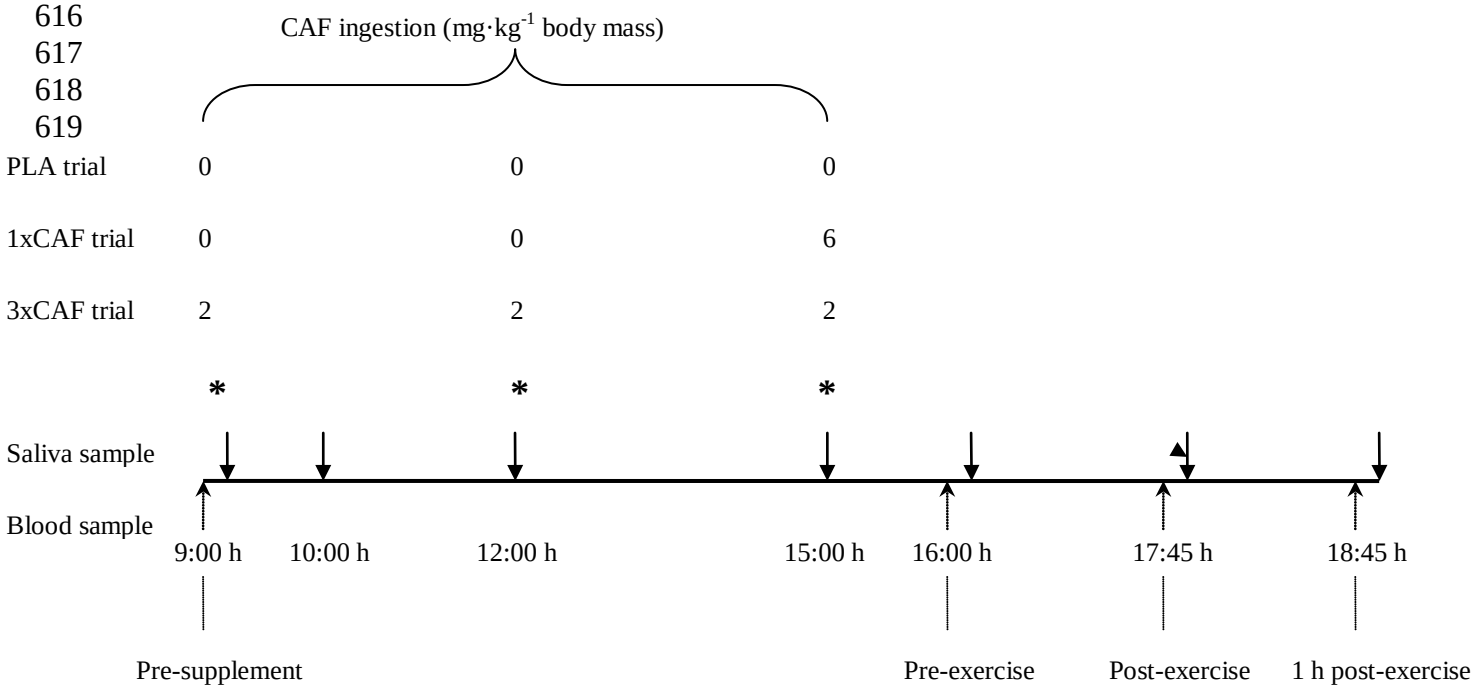

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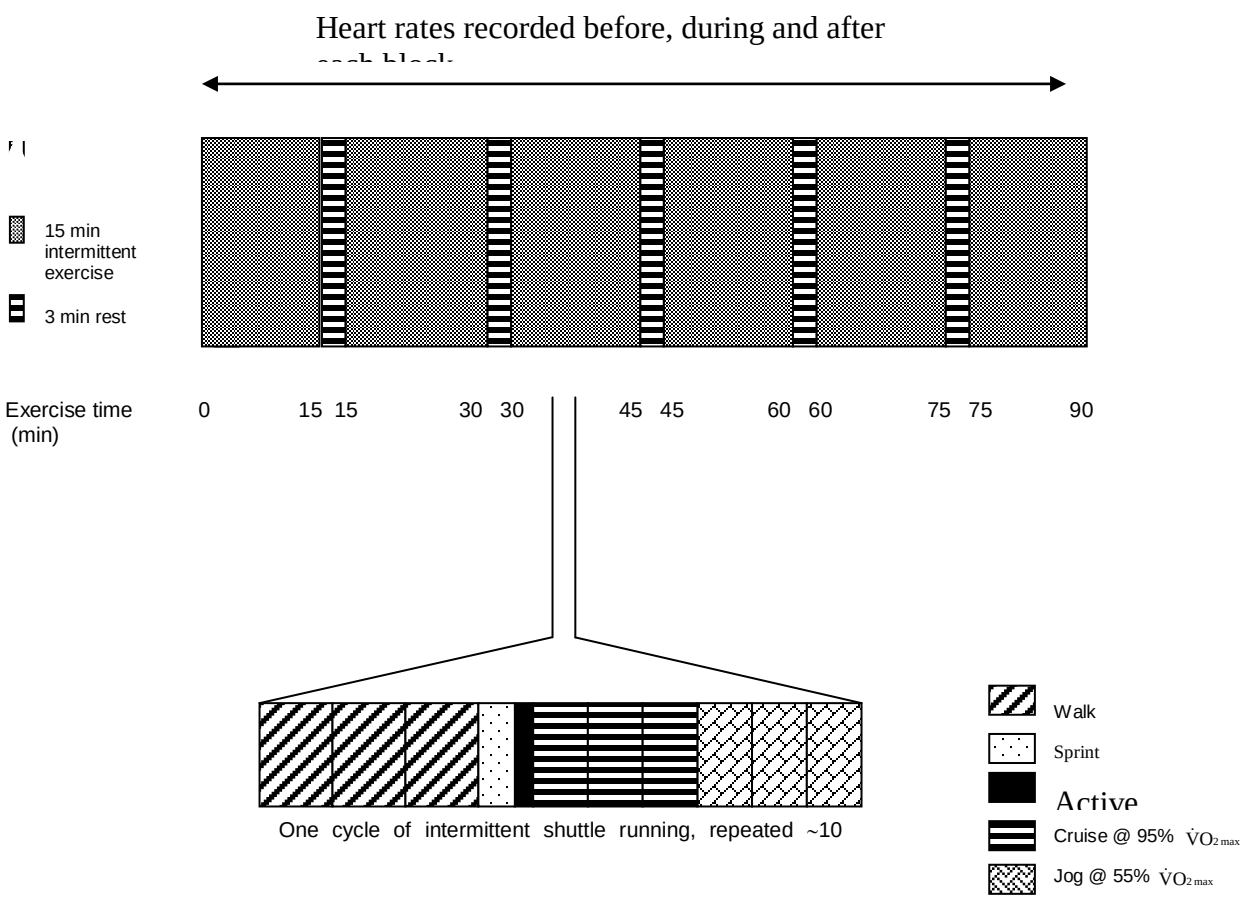
Fig. 2


Heart rates recorded before, during and after each block
15 min intermittent exercise
3 min rest
Exercise time (min)
0 15 15 30 30 45 45 60 60 75 75 90
One cycle of intermittent shuttle running, repeated ~10
Walk
Sprint
Active
Cruise @ 95% $\dot{V}O_{2\,max}$
Jog @ 55% $\dot{V}O_{2\,max}$

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659 Fig. 3
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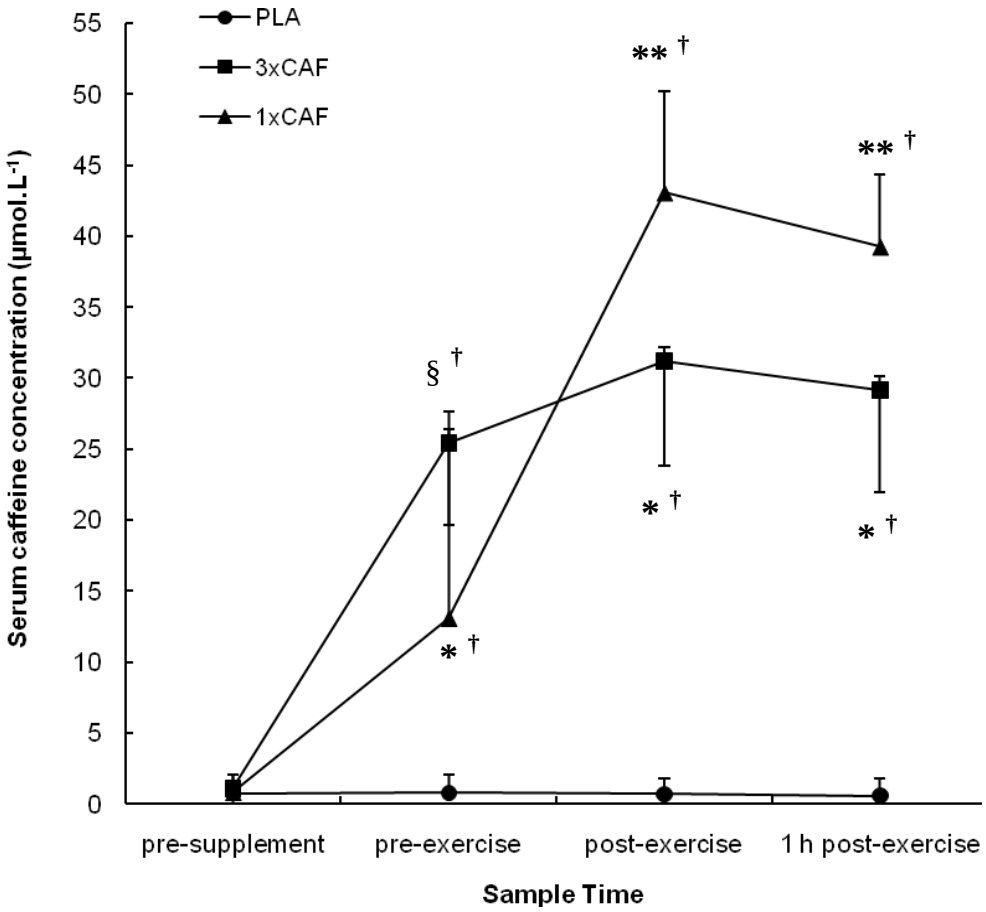

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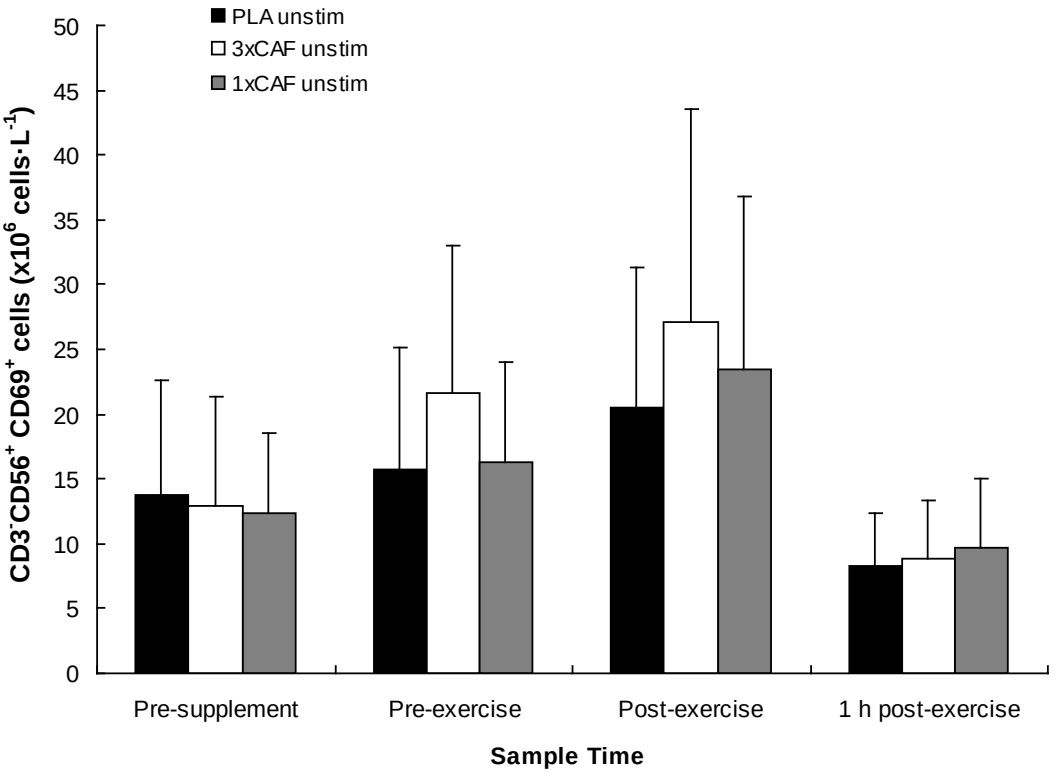
Fig. 4a



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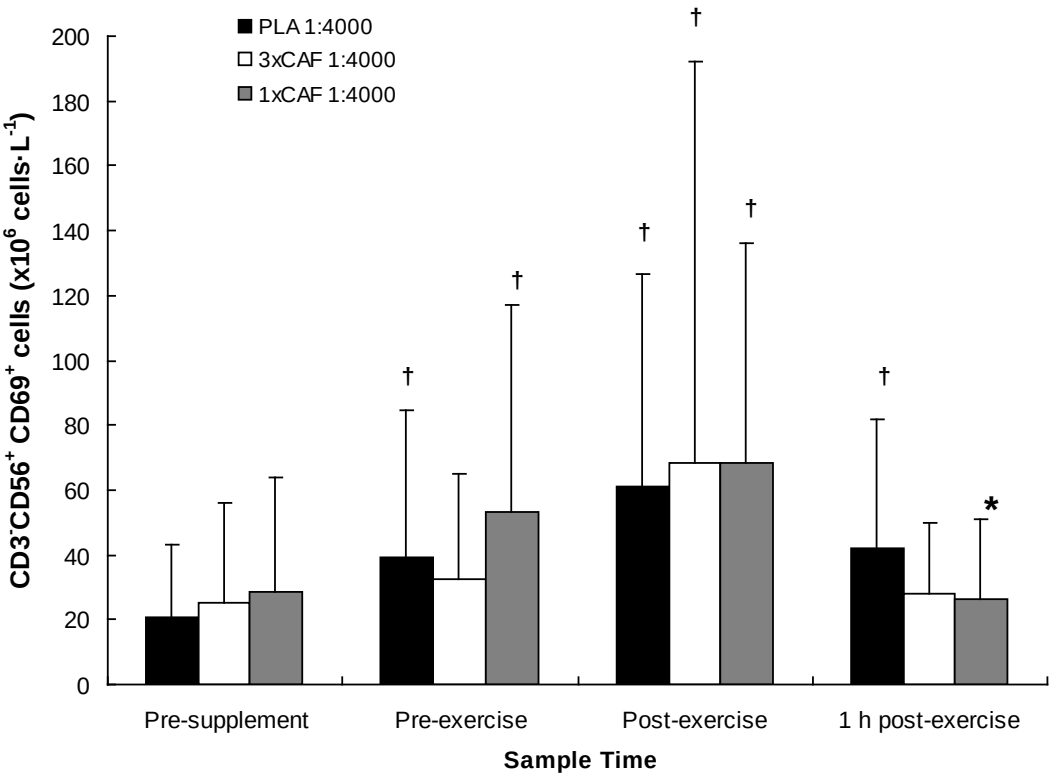
4b



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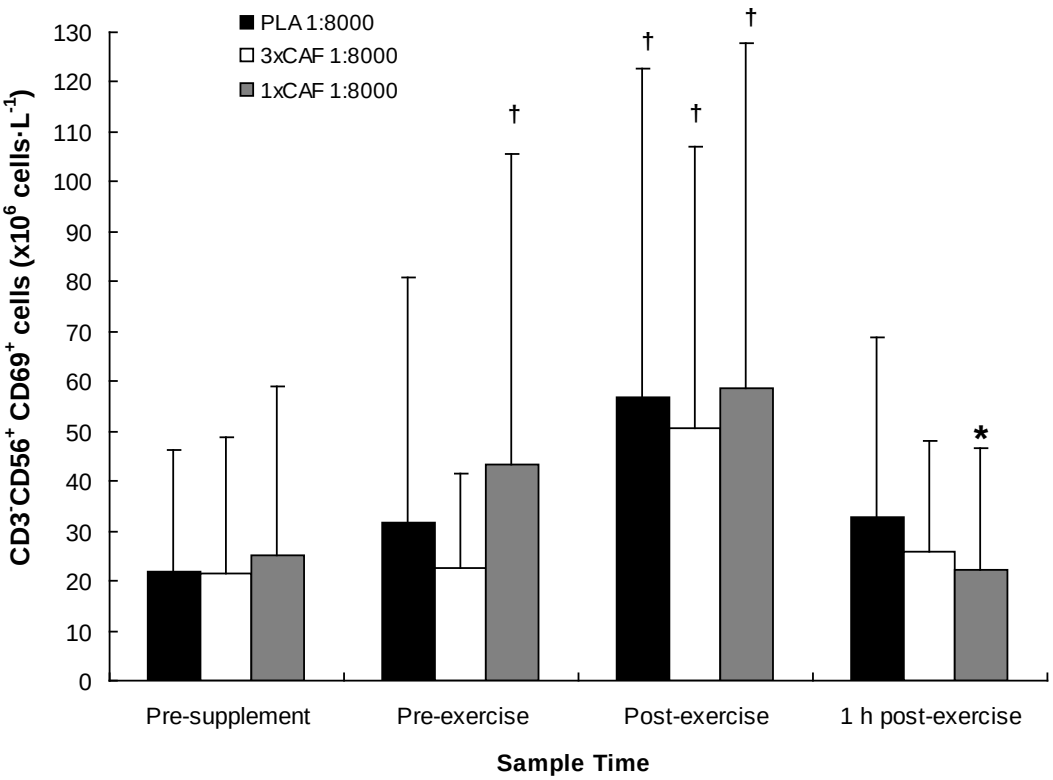
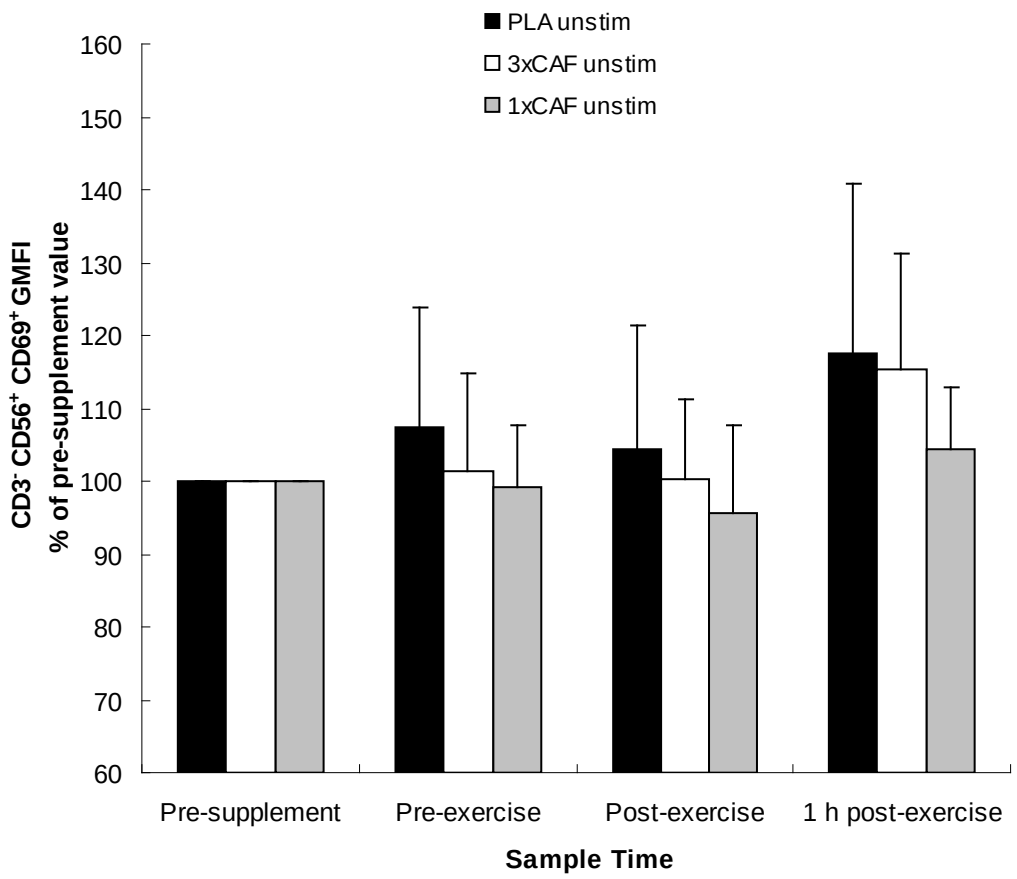
4c


PLA 1:8000
3xCAF 1:8000
1xCAF 1:8000
CD3-CD56+ CD69+ cells (x10^6 cells·L^-1)
0
10
20
30
40
50
60
70
80
90
100
110
120
130
†
†
†
†
*
Pre-supplement
Pre-exercise
Post-exercise
1 h post-exercise
Sample Time

665
666

Fig. 5a



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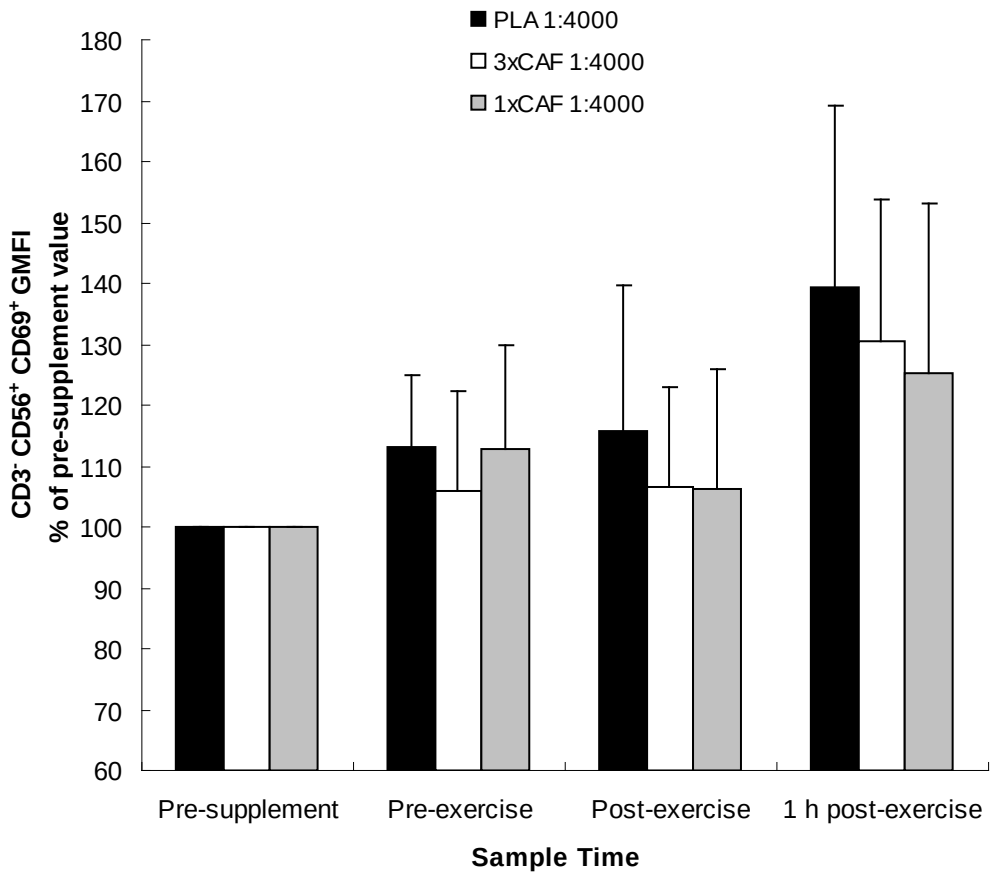
5b



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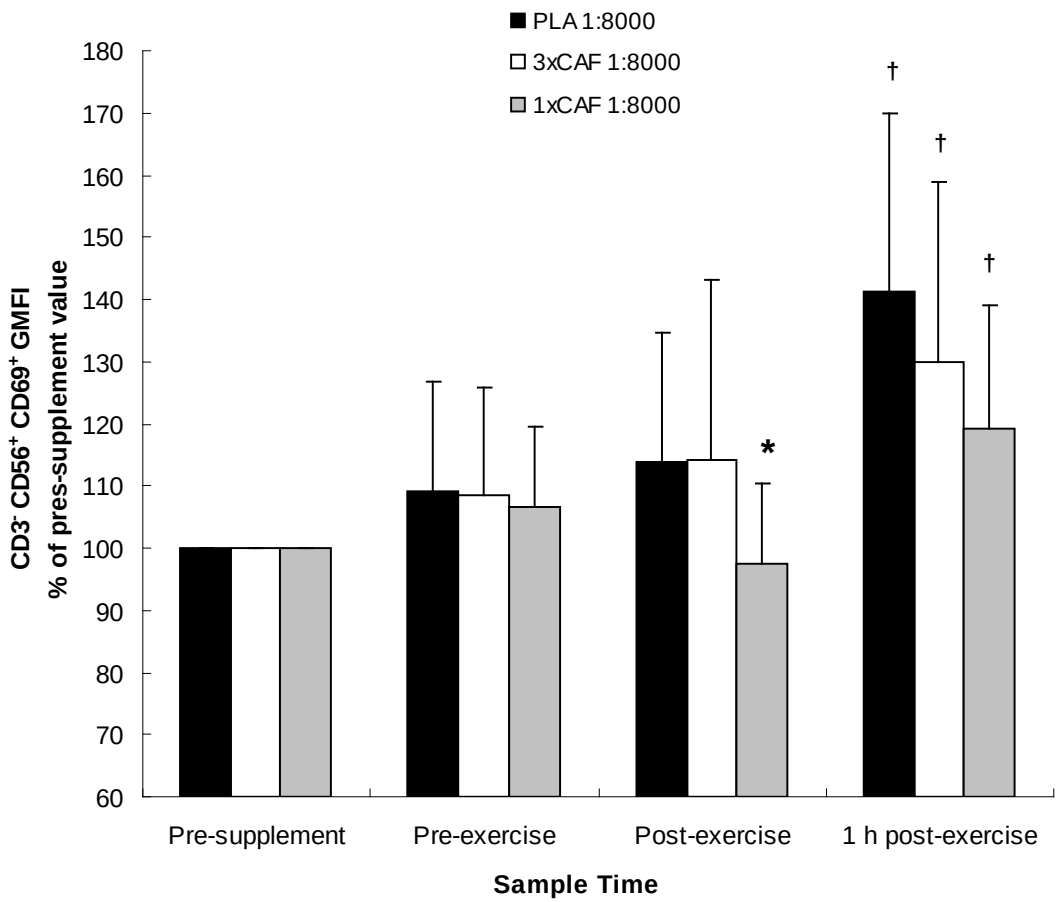
PLA 1:8000
3xCAF 1:8000
1xCAF 1:8000
180
170
160
150
140
130
120
110
100
90
80
70
60
CD3- CD56+ CD69+ GMFI
% of pres-supplement value
†
†
†
*
Pre-supplement
Pre-exercise
Post-exercise
1 h post-exercise
Sample Time

669
670

Table 1 Saliva caffeine concentration, flow rate, α-amylase activity and secretion rate during PLA, 3xCAF and 1xCAF trials

	09:00 h (Pre-supplement)	**10:00 h**	**12:00 h**	**15:00 h**	**16:00 h (Pre-exercise)**	**17:45 h (Post-exercise)**	**18:45 h (1 h post-exercise)**
Saliva caffeine concentration (μmol·L^{-1})							
PLA	1 (1)	1 (1)	1 (1)	1 (1)	0 (0)	0 (0)	0 (0)
3xCAF	1 (1)	10 (4)*‡	8 (2)*‡	14 (3)*‡	15 (3)*‡	20 (5)*‡	19 (4)*‡
1xCAF	1 (1)	0 (0)	0 (0)	1 (1)	2 (2)	30 (4)*§	27 (4)*§
Saliva flow rate (μl·min^{-1}) [a,b]							
PLA	441 (268)	549 (252)	543 (180)	567 (203)	572 (200)	484 (171)	683 (248)
3xCAF	431 (216)	571 (254)	586 (185)	591 (294)	585 (252)	467 (209)	656 (286)
1xCAF	423 (241)	575 (240)	546 (203)	566 (165)	642 (223)	520 (203)	698 (225)
Saliva α-amylase activity (U·ml^{-1})							
PLA	392 (257)	439 (243)	793 (416)	967 (373)	827 (373)†	1456 (530)†	867 (494)†
3xCAF	306 (271)	608 (346)	845 (319)	936 (316)	997 (313)**†	1719 (527)†	1098 (430)**†
1xCAF	368 (299)	545 (290)	905 (442)	925 (345)	1023 (369)**†	1615 (457)†	1196 (402)**†
Saliva α-amylase secretion rate (U·min^{-1})							
PLA	174 (167)	234 (150)	444 (284)	569 (433)	487 (323)†	695 (343)†	580 (391)†
3xCAF	138 (151)	239 (291)	526 (353)	570 (357)	587 (357)†	810 (470)†	742 (452)†
1xCAF	157 (154)	314 (192)	497 (293)	494 (188)	660 (327)†	812 (338)†	804 (289)**†

Values are means (SD)
* P<0.01, ** P<0.05: significantly higher than PLA
‡ significantly higher than 1xCAF, P<0.01
§ significantly higher than 3xCAF, P<0.01
† P<0.01: significantly higher than pre-supplement within trial
[a] main effect for time; significantly higher than pre-supplement at all other time-points, all P<0.01 except for post-exercise P<0.05
[b] main effect for time; significantly lower than pre-exercise at post-exercise, P<0.01

Table 2 Number of circulating lymphocytes and $CD3^{-}CD56^{+}$ cells within the circulating lymphocyte population during PLA, 3xCAF and 1xCAF trials

	Pre-supplement	**Pre-exercise**	**Post-exercise**	**1 h post-exercise**
Lymphocytes ($x10^{9}$ cells.L^{-1}) [a]				
PLA	2.10 (0.50)	2.10 (0.50)	2.90 (0.80)	2.10 (0.50)
3xCAF	2.10 (0.70)	2.00 (0.50)	3.10 (1.00)	1.90 (0.50)
1xCAF	2.10 (0.40)	2.10 (0.40)	3.20 (0.80)	1.90 (0.60)
$CD3^{-}CD56^{+}$ cells ($x10^{9}$ cells.L^{-1})				
PLA	0.26 (0.08)	0.30 (0.09)	0.53 (0.16)†	0.25 (0.07)
3xCAF	0.28 (0.09)	0.35 (0.10)††	0.70 (0.22)**†	0.25 (0.07)
1xCAF	0.29 (0.11)	0.36 (0.09)††	0.80 (0.32)*†	0.27 (0.09)

Values are means (SD)

* P<0.01; ** P<0.05, significantly higher than PLA

† P<0.01; †† P<0.05, significantly higher than pre-supplement within trial

[a] main effect for time; significantly higher than pre-supplement at post-exercise, P<0.01