THE INFLUENCE OF CAFFEINE ON LYMPHOCYTE ACTIVATION AFTER PROLONGED HIGH INTENSITY EXERCISE

By

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ABSTRACT

Many athletes consume caffeine for its known ergogenic effects. Since being legitimised by its 2004 removal from the World Anti-Doping Agency prohibited list of substances, caffeine's ability to enhance performance has led to its widespread use amongst the athletic population. However, despite caffeine's prevalence, little research has focused on the effect of caffeine ingestion on immune function both at rest and in response to exercise in humans. Therefore, the aim of this thesis was to investigate the influence of typically-used doses of caffeine (typical daily intake in training and competition doses) on aspects of innate and acquired immunity in response to prolonged exercise.

At rest both a high (6 $mg \cdot kg^{-1}$) and low dose (2 $mg \cdot kg^{-1}$) of caffeine had little effect on antigen-stimulated T ($CD4^+$ and $CD8^+$) or natural killer (NK) lymphocyte activation, while a high dose of caffeine only increased the number of antigen-stimulated NK cells expressing CD69 1 h following caffeine ingestion (Chapter 4). In response to prolonged high intensity continuous cycling both high and low doses of caffeine increased the natural state of activation as well as the antigen-stimulated activation of NK cells 1 h after exercise cessation (Chapters 5 and 6). However, at the same time-point a high dose of caffeine decreased $CD4^+$ and $CD8^+$ cell activation (Chapter 5). One hour after high intensity intermittent shuttle running, a high dose of caffeine attenuated the exercise-induced increase in NK cell activation both in terms of the number of cells expressing CD69 and their geometric mean fluorescence intensity expression of CD69 (Chapter 7). These effects did not occur in response to intermittent exercise when 2 $mg \cdot kg^{-1}$ caffeine was instead ingested in 3 repeated doses throughout the day (Chapter 7).

In conclusion, the findings of this thesis demonstrate the complex actions of caffeine on antigen-stimulated T and NK lymphocyte activation 1 h after prolonged intensive exercise. However, the biological significance of these findings in terms of caffeine's potential to alter an individuals' susceptibility to infection following prolonged high intensity exercise are yet to be determined.

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LIST OF PAPERS

This thesis incorporates the following four papers;

Fletcher DK & Bishop NC. The effect of a high and low dose of caffeine on antigen-stimulated human lymphocyte activation at rest. *Int. Immunopharmacol.* Currently under review.

Fletcher DK & Bishop NC. Caffeine ingestion and antigen-stimulated human lymphocyte activation after prolonged cycling. *Scand. J. Med. Sci. Sports*. In press.

Fletcher DK & Bishop NC. Effect of a high and low dose of caffeine on antigen-stimulated human natural killer cell activation after prolonged cycling. *Int. J. Sports Nutr. Exerc. Metab.* In press.

Fletcher DK & Bishop NC. Effect of a single and repeated dose of caffeine on antigen-stimulated human natural killer cell activation after high intensity intermittent exercise. *Eur. J. Appl. Physiol.* In press.

In addition the following presentations arose from some of the findings from this thesis;

Fletcher DK, Deighton K, Walsh D & Bishop NC. (2009). The effect of different doses of caffeine on antigen-stimulated natural killer cell activation following prolonged cycling. 9th Symposium of the International Society of Exercise and Immunology. 9; 62 (Abstract). *Oral communication presented at the 9th Symposium of The International Society of Exercise and Immunology, Germany, 2009.*

Fletcher DK, Bowry P, Noon M & Bishop NC. (2009). The effect of caffeine ingestion on antigen-stimulated human T lymphocyte activation following prolonged cycling. *Proc. Physiol. Soc.* 15; PC111 (Abstract). *Poster communication presented at The Physiological Society Meeting, Dublin, 2009. Poster was selected as an entrant for the Blue Riband competition.*

Fletcher DK, Bowry P, Noon M & Bishop NC. (2008). The effect of caffeine ingestion on natural killer lymphocyte activation following prolonged strenuous cycling. Archivos de Medicina del Deporte review. 128; 515 (Abstract). *Poster communication presented at the XXX World Congress of The International Federation of Sports Medicine, Barcelona, 2008.*

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LIST OF ABBREVIATIONS

ANOVA	analysis of variance
APC	antigen presenting cell
BSA	bovine albumin serum
Ca	calcium
CAF	caffeine
cAMP	adenosine 3', 5'–cyclic monophosphate (cyclic AMP)
CD	cluster of differentiation
$CD3^-CD56^+$	natural killer cell
$CD4^+$	T helper cell
$CD8^+$	T cytotoxic cell
$CD45RA^+$	naïve T cell
CD69	early lymphocyte activation antigen
CHO	carbohydrate
Cl	chloride
CMV	cytomegalovirus
EE	energy expenditure
FITC	fluorescein isothiocyanate
g	gram
GMFI	geometric mean fluorescence intensity
h	hour
Hsp	heat shock protein
IFN	interferon
IL	interlukin
K_3EDTA	tripotassium ethylene diamine tetraacetic acid
kg	kilogram
KJ	kilojoule
km	kilometre
L	litre
LAK	lymphokine activated killer
m	metre
mab	monoclonal antibody
mg	milligram
MHC	major histocompatability complex
min	minute
ml	millilitre
mmol	millimole
NK	natural killer
NKCA	natural killer cell cytotoxic activity
nmol	nanomole
PBMC	peripheral blood mononuclear cell
PBS	phosphate buffered saline
PE	phycoerythrin
PE-Cy5	R-phycoerythrin-Cy5
PHA	phytohaemagglutinin
PKA	protein kinase A
PLA	placebo
RER	respiratory exchange ratio

RPE	ratings of perceived exertion
s	second
SD	standard deviation
sIgA	salivary immunoglobulin A
SNP	single-nucleotide polymorphism
T_C	T cytotoxic
TCR	T cell receptor
TGF	transforming growth factor
T_H	T helper
TNF	tumor necrosis factor
Treg	T regulatory
T_S	T supressor
URTI	upper respiratory tract infection
$\dot{V}O_{2\,\max}$	maximum oxygen uptake
$\dot{V}O_{2\,peak}$	peak oxygen uptake
W	watt
WADA	world anti-doping agency
Wmax	maximal workload
℃	degrees
µg	microgram
µl	microlitre
µmol	micromole

Chapter 1

Introduction

Exercise can have both negative and positive effects on immune function and susceptibility to minor illnesses such as upper respiratory tract infections (URTI). These appear to be dependent upon exercise intensity and duration (Gleeson, 2007). It is suggested that the relationship between exercise intensity and risk of infection is "J" shaped (Nieman, 1994). This model suggests that while exercise of moderate intensity may increase immune function reducing the risk of infection below that of a sedentary individual, prolonged high intensity exercise may suppress immune function increasing the risk of subsequent infection above sedentary levels. More recently an "S" shaped model of infection risk has been proposed, suggesting that elite athletes may have a lower risk of infection than individuals participating in prolonged high intensity exercise, as elite athletes may possess an immune system able to withstand infection even during severe physiological stress (Malm, 2006).

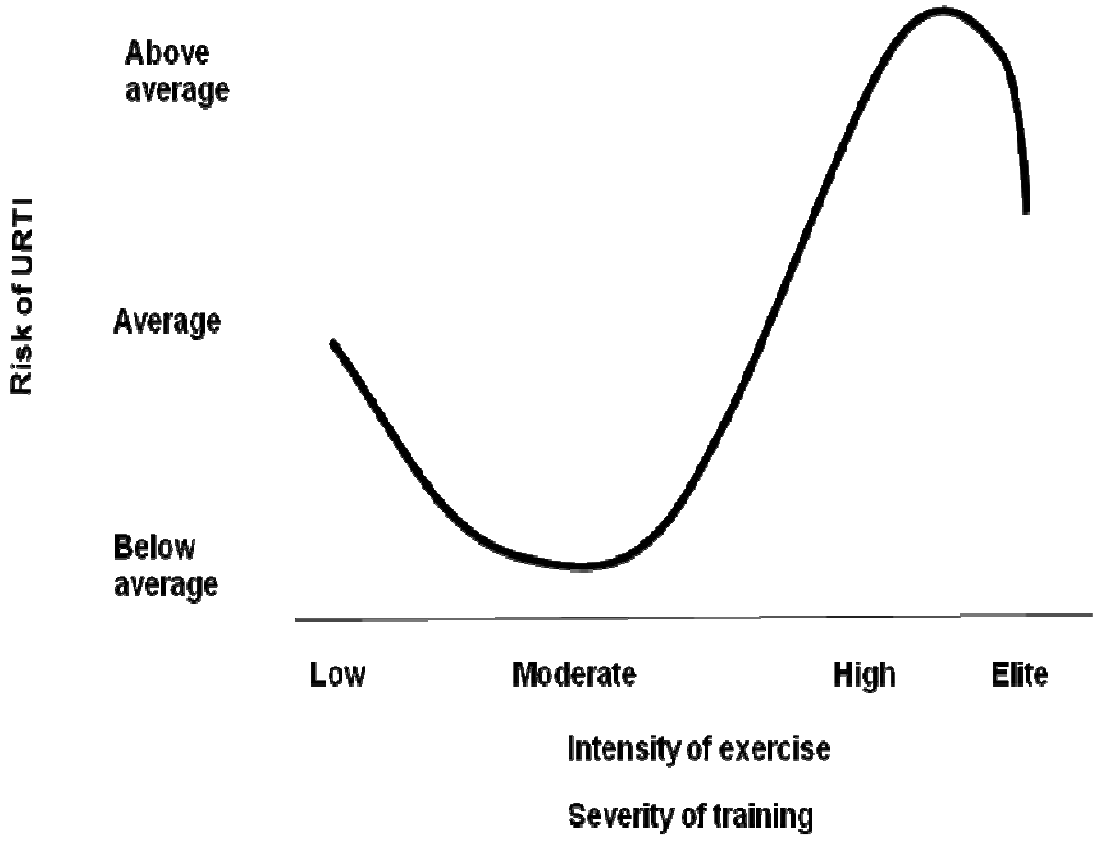


Figure 1.1: S shaped curve of infection risk. Adapted from Malm 2006.

There is general agreement within the exercise immunology literature that an acute bout of high intensity exercise causes a temporary perturbation in various aspects of immune cell function that usually last between ~3 and 24 h (as reviewed in Gleeson, 2007). Investigators have largely attributed these decrements to the hormonal changes that occur in response to intensive exercise (such as increased plasma concentrations of adrenaline and cortisol). It has been hypothesised that during this "open window" of reduced responsiveness, opportunistic bacteria and viruses may gain a foothold, increasing the risk of subsequent infection that may account for the apparent increased susceptibility to URTI of individuals who exercise intensely (Nieman, 2000). However, a direct link between exercise-induced impaired immune function *in vivo* and increased infection risk has yet to be determined.

Caffeine is part of the methylxanthine family of drugs. This drug is legally and socially acceptable and as such is the most commonly used psychoactive substance in the world (Fredholm et al., 1999). A recent large scale survey conducted in the UK found that ~90% of individuals surveyed consumed at least 40 mg of caffeine (equivalent to 1 cup of tea) per day, with a mean daily caffeine intake of 241 ± 173 mg (Heatherley et al., 2006). Daily caffeine intake can vary widely between individuals and from country to country. For example, in the Netherlands mean daily caffeine intake is ~400 mg (Fredholm et al., 1999). Caffeine between the doses of 3 and 6 $mg \cdot kg^{-1}$ body mass has consistently been shown to enhance athletic performance in high intensity continuous (cycling, running etc.) and intermittent (football, rugby etc.) exercise (Goldstein et al., 2010). However, caffeine doses as low as 2 $mg \cdot kg^{-1}$ body mass have also proved ergogenic (Cox et al., 2002). Since being legitimised by its 2004 removal from the World Anti-Doping Agency (WADA) prohibited list of substances, caffeine's ability to enhance performance has led to its widespread use amongst the athletic population (Chester and Wojek, 2008). The main action of caffeine at physiological concentrations is via antagonism of adenosine receptors (Fredholm et al., 1999). Furthermore, caffeine has been consistently shown to increase plasma adrenaline concentration (Graham, 2001; Bishop et al., 2005a; Walker et al., 2006). Despite caffeine's frequent

and prominent use within society, very few studies have investigated the effect of caffeine on immune cell responses in humans, and in particular following exercise. Therefore, this thesis investigated the effect of caffeine ingestion on lymphocyte activation in response to prolonged high intensity exercise.

Firstly the effects of a high (6 mg·kg^{-1}) and low (2 mg·kg^{-1}) dose of caffeine on unstimulated and antigen-stimulated T ($CD4^+$ and $CD8^+$) and natural killer ($CD3^-CD56^+$) lymphocyte activation were determined in participants at rest (Chapter 4). Once it had been ascertained that caffeine had little effect on these parameters at rest, the effect of caffeine ingestion (6 mg·kg^{-1}) on unstimulated and antigen-stimulated $CD4^+$, $CD8^+$ and $CD3^-CD56^+$ lymphocyte activation was determined in response to prolonged intensive exercise (90 min cycle 70% $\dot{V}O_{2peak}$) as described in Chapter 5. As caffeine has been shown to affect lymphocyte function differentially depending on the dose ingested (Kantamala et al., 1990) the effects of a high (6 mg·kg^{-1}) and low (2 mg·kg^{-1}) dose of caffeine on unstimulated and antigen-stimulated $CD3^-CD56^+$ lymphocyte activation in response to prolonged intensive exercise (90 min cycle 70% $\dot{V}O_{2peak}$) was investigated in Chapter 6. It has been suggested that studies using one single bolus dose of caffeine to investigate the effects of caffeine on immune function do not represent normal caffeine consumption patterns (Horrigan et al., 2006), as humans tend to ingest caffeine in several doses throughout the day (Fredholm et al., 1999). Therefore, in Chapter 7 we investigated whether small repeated doses (2 mg·kg^{-1}) of caffeine ingested throughout the day (9:00 h, 12:00 h & 15:00 h) elicited a similar response as one large (6 mg·kg^{-1}) bolus dose ingested 1 h prior to exercise (15:00 h) on unstimulated and antigen-stimulated $CD3^-CD56^+$ lymphocyte activation following 90 min of high intensity intermittent exercise.

Chapter 2

Review of Literature

2.1. Are athletes more susceptible to infection?

It has been proposed within the exercise immunology literature that athletes may be more susceptible to infection, particularly URTI, in the hours following intensive exercise, due to a temporary depression in a number of immune functions (Gleeson, 2007). Findings from survey-based epidemiological research seem to lend support to this notion (reviewed in Moreira et al., 2009), and report that in the weeks following competitive endurance events (such as marathons/ultramarathons) there is a 100-500% increase in risk of picking up an infection (Peters and Bateman, 1983; Nieman et al., 1990). The risk of infection seems to correlate with athletes' race finishing times (Peters and Bateman, 1983) and pre-race training volume (Nieman et al., 1990).

Despite the apparent support from epidemiological research for an association between continuous heavy exercise and increased risk of URTI, one must view this evidence with caution, as a recent study failed to confirm these findings in a large cohort of marathon runners (Ekblom et al., 2006). The authors reported that incidence of post-race infection in the 3 weeks following the race was strongly related to pre-race infection in the 3 weeks leading up to the race. This suggests that the increased rate of infection in athletes may be caused by strenuous exercise too soon after an infection, allowing a reactivation of the virus responsible for the pre-race infection, rather than a depression in immune function.

Apart from epidemiological research, it has been well established within exercise immunology that acute bouts of prolonged intensive exercise are associated with a temporary depression of immune function that appears to last ~3-24 h (Gleeson, 2007). This decline in host defence therefore has been suggested to be responsible for the proposed increased incidence of URTI amongst athletes. Although this research seems to support the notion that intensive exercise may lead to an increased risk of URTI, these studies have tended to investigate the effect of strenuous exercise on isolated immune cells *in vitro*, as such a direct link between immune function *in vivo* and infection risk has yet to be established.

There are only a few studies that have investigated whether the whole immune system's ability to respond to pathogens following acute prolonged exercise is impaired *in vivo* (Bruunsgaard et al., 1997; Harper Smith et al., 2009). Bruunsgaard et al. (1997) reported that triathletes who performed a half ironman event (consisting of 3 km swim, 130 km cycle and 21 km run) showed a significant impairment of *in vivo* cell-mediated immunity in the days following this bout of high intensity exercise. The cellular immune response was evaluated as a skin test response to 7 recall antigens. The skin test response was significantly lower in the group of triathletes who performed the half ironman compared with triathlete controls and moderately trained controls who did not participate in the event. In addition, Harper Smith et al. (2009) have also demonstrated that 2 h treadmill running at 60% $\dot{V}O_{2\,peak}$ significantly decreased *in vivo* cell-mediated immunity 24 h and 48 h post-trial. This study used a novel contact sensitiser diphenylcyclopropenone.

2.2. Lymphocytes and their role in the immune system

2.2.1. T cells

Lymphocytes constitute 20-25% of circulating leukocytes; T cells represent ~60-75% of this total lymphocyte pool (Gleeson and Bishop, 2005). T lymphocytes are part of the acquired immune system and are responsible for orchestrating and participating in a highly specific and long lasting immune response to pathogenic invaders. As such T cells are central elements of the immune system (Fabbri et al., 2003). All T cells selectively express CD3 as well as CD4 (T helper cells) or CD8 (T cytotoxic/suppressor cells). T helper cells constitute around 60-70% of T lymphocytes, while T cytotoxic cells represent 30-40% (Gleeson, 2006).

T helper (T_H, $CD4^+$) cells have a central function in the generation, maintenance and regulation of both the cell-mediated and humoral arm of the immune response (Fabbri et al., 2003). These cells activate other immune cells to kill intracellular targets or to produce antibody. T cytotoxic (T_C, $CD8^+$) cells are

responsible for the cytotoxic killing of virus infected cells and some tumour cells. Antigen-specific cytotoxic T cells kill infected cells via apoptosis, leading to death of the targeted cell via the release of cytotoxic proteins (perforin and granzymes) stored in lytic granules (Janeway, 2001). There is a further subtype of T cell, with immunosuppressive functions and cytokine profiles distinct from other T cells, termed regulatory T (Treg) cells (McGuirk and Mills, 2002). Treg cells express the $CD4^+$ marker (associated with helper function) as well as $CD25^+$, but do not seem to act as classical T helper cells. They produce interleukin (IL)-10 and transforming growth factor (TGF)-β (immunosuppressive cytokines) and their primary function appears to be to suppress inflammatory and cell-mediated immune responses (McGuirk and Mills, 2002).

T_H and T_C lymphocytes can be further subdivided into type 1 (T_{H1} & T_{C1}) or type 2 (T_{H2} & T_{C2}) cells based on their distinct profiles of cytokine production (Lancaster et al., 2005). Type 1 T cells produce interferon (IFN)-γ, IL-2 and tumour necrosis factor (TNF)-α and have been shown to protect against intracellular pathogens. Type 2 T cells produce IL-4, IL-5, IL-6 and IL-13 and defend against extracellular parasites. It is the release of these cytokines by activated T_H cells that determines whether cell-mediated (T_{H1} profile) or humoral (T_{H2} profile) immunity will dominate (Gleeson, 2006). Developed T_{H1} and T_{H2} cells secrete IFN-γ and IL-4 respectively, in an autrocrine and reciprocally inhibitory fashion. IFN-γ promotes growth of T_{H1} cells and inhibits the proliferation of T_{H2} cells, while IL-4 has the opposite influence (Liew, 2002).

During maturation in the thymus and later on in secondary lymphoid organs (spleen, lymph nodes and mucosal-associated lymphoid tissue) T cells acquire the features of specificity of recognition, lack of self reactivity and memory of previous antigen encounters, that are physiological hallmarks of T lymphocytes (Fabbri et al., 2003). Being able to distinguish self from non-self is due to a polymorphic membrane heterodimer, the T cell receptor (TCR) (Fabbri et al., 2003). The TCR recognises foreign antigens that are presented as processed peptides, loaded on a heterodimer called the major histocompatability complex

(MHC), expressed on the plasma membrane of the antigen presenting cell (APC) (Viret and Janeway, 1999). $CD4^+$ cells recognise and respond only to MHC class II proteins, while $CD8^+$ cells are restricted by MHC class I proteins, thus forming the basis for the functional specialisation of the two major T cell subsets (Fabbri et al., 2003).

The primary function of APCs therefore is to stimulate T cells and initiate an immune response. If a mature T cell has not encountered an antigen before, it is said to be naïve. The naïve ($CD45RA^+$) T cell therefore must be introduced to the antigen and MHC by a dendritic APC before it can be initiated into the rites of a primary immune response (Roitt and Delves, 2001). However, binding of the TCR with peptide:MHC complexes (signal 1) on its own will not stimulate T cells to proliferate and differentiate into armed effector T cells. Antigen-specific activation and clonal expansion require a second or co-stimulatory signal, which must be delivered by the same APC before any type of immune response (primary or secondary) can be initiated. Therefore, co-stimulatory molecules on the APC (CD80 and CD86) need to interact with their respective counter-receptors (CD28) on T cells (signal 2), at the same time as TCR mediated recognition [(signal 1) (TCR engagement with its antigen-MHC complex)] (Roitt and Delves, 2001). Engagement of the TCR signal 1 without the accompanying co-stimulatory signal 2 will lead to anergy (unresponsiveness) in resting T cells (Roitt and Delves, 2001). It has been suggested that during initial priming of naïve T cells, interaction of the T cell CD28 receptor with CD80 on the APC tends to favour a T_{H1} differentiation, whereas interaction of the same receptor with CD86 favours a T_{H2} differentiation (Liew, 2002). Once a T cell has differentiated into an effector T cell, a co-stimulatory signal is no longer required in order for this cell to mount an immune response upon encounter with its specific antigen (Janeway, 2001).

2.2.2. Natural killer cells

Natural killer (NK) cells are an essential component of the innate immune system playing a critical role in defence against viral infection and tumour immune surveillance (Cooper et al., 2001). NK cells are distinct from other lymphocytes, as they do not need prior sensitisation or require specific-antigen recognition in order to kill target cells (Moretta et al., 2008). NK cells comprise approximately 5-20% of lymphocytes in peripheral blood (Andoniou et al., 2006) and have the ability to lyse target cells and/or to secrete immunoregulatory cytokines (Cooper et al., 2001). NK cells can be identified through the use of monoclonal antibodies (mab) as they lack expression of CD3, but express CD16 and CD56 on their cell surface (Gleeson, 2006). Two distinct populations of human NK cells have been identified through their surface density expression of CD56. The majority (~90%) of human peripheral blood NK cells express low levels of CD56 ($CD56^{dim}$) and high levels of CD16 ($CD16^{+}$), whereas a minority (~10%) are $CD56^{bright}$ and $CD16^{-}$ (Fehniger et al., 2003). These two subsets appear to have distinct functional characteristics and roles in the human immune response; resting $CD56^{dim}$ NK cells are thought to be more cytotoxic as they contain more perforin, granzymes and cytolyitc granules, while $CD56^{bright}$ NK cells have been shown to be superior in producing abundant immunoregulatory cytokines once stimulated (Jacobs et al., 2001). It has been suggested that following activation with IL-2 or IL-12 *in vitro* $CD56^{bright}$ NK cells and $CD56^{dim}$ cells have similar levels of cytotoxicity (Cooper et al., 2001).

NK cells do not have the ability to rearrange genes encoding for antigen recognition (unlike T cells) but instead have several receptors on their cell surface that can be classified as activating/inhibiting receptors that help to target and kill virally-infected and abnormal cells (Andoniou et al., 2006). Inhibitory receptors engage MHC class I molecules that are found on virtually all healthy cells; while activating receptors bind ligands on the target cell surface thus triggering NK cell activation and lysis of the target cell (Shereck et al., 2007). Cellular ligands for NK cell activating receptors are generally not found on healthy cells but may become induced upon cellular stress such as infection (Andoniou et al., 2006). Engagement of the inhibitory receptors will normally

override any signals from activating receptors and therefore protect healthy cells expressing normal levels of MHC-I from NK cell lysis. However, in some circumstances signals from activating receptors are sufficient to stimulate NK cells despite signalling from MHC-I inhibitory receptors (Chambers et al., 1996) Cells infected by intracellular pathogens tend to express reduced levels of MHC-I; as such infected cells are unlikely to engage NK cell inhibitory receptors and leave themselves open to NK cell attack, especially if activating receptors are bound by appropriate ligands. NK cells are also activated by various pro-inflammatory cytokines, namely IFN-γ, IL-2, IL-12, IL-15 or IL-18, causing an increase in their number and cytotoxic activity resulting in their ability to kill a broader range of targets (Shereck et al., 2007). As such the activation of NK cells is a very complex balance of activating and inhibitory signals that also appears to require the actions of pro-inflammatory cytokines (Andoniou et al., 2006).

Following activation, NK cells secrete cytokines (IFN-γ, TNF-α) that can control the growth and spread of infected cells, as well as alerting the host to the presence of an infectious agent. As such many of the cytokines secreted following NK cell activation can affect the initiation and maintenance of adaptive immune responses. As well as the release of cytokines following NK cell activation, NK cells can directly kill target cells via apoptosis. Target cell apoptosis occurs via exocytosis of granules that contain perforin and granzymes; upon entry into the target cell these molecules induce cell death (Andoniou et al., 2006).

2.3. Lymphocyte responses to acute exercise

2.3.1. T and NK cell number

The circulating number of T and NK lymphocytes tend to show a biphasic pattern in response to acute exercise, with a marked increase during and immediately after exercise (lymphocytosis) and a significant fall during the early stages of recovery (lymphocytopenia) to below baseline values (reviewed in Gleeson and Bishop, 2005); the magnitude of which appears to be more related to the intensity of the exercise than the duration. Low (25% $\dot{V}O_{2\,max}$) and moderate (50% $\dot{V}O_{2\,max}$) intensity exercise tend to elicit few changes in lymphocyte number, the magnitude of which is nowhere near as pronounced as that following high intensity (75% $\dot{V}O_{2\,max}$) exercise (Tvede et al., 1993). For example, Nieman et al. (1994) demonstrated that 45 min of treadmill exercise at 80% $\dot{V}O_{2\,max}$ resulted in a 70% increase in lymphocyte count immediately following exercise, that subsequently fell 48% below pre-exercise levels 1 h later. However, the same duration of exercise at 50% $\dot{V}O_{2\,max}$ had little effect on circulating lymphocyte counts. Tvede et al. (1993) also found similar responses following 1 h cycling at 75% $\dot{V}O_{2\,max}$. Lymphocyte counts increased immediately following exercise but were markedly decreased at 2 h post-exercise. Again in contrast, the same duration of exercise at 25% $\dot{V}O_{2\,max}$ did not change circulating lymphocyte counts.

The biphasic response of T cell number to acute strenuous exercise is also apparent within the T cell subsets $CD4^+$ and $CD8^+$ (Nieman et al., 1994; Steensberg et al., 2001). It appears that when cell number is adjusted and expressed as the percentage change from resting values, the subset of $CD8^+$ T cells exhibit a greater relative increase during and immediately following 45 min treadmill exercise at 80% $\dot{V}O_{2\,max}$, with a greater decline during recovery than $CD4^+$ T cells (Nieman et al., 1994). Evidence suggests that the suppression in both $CD4^+$ and $CD8^+$ cells during recovery from acute intensive exercise is largely due to a decreased percentage of type 1 $CD4^+$ and $CD8^+$ cells (Steensberg et al., 2001; Lancaster et al., 2005). A 50% decrease in the

percentage of circulating $CD4^+$ and $CD8^+$ T cells producing IFN-γ and IL-2 (type 1 cytokines) upon mitogen stimulation has been found immediately and 2 h following a 2.5 h treadmill run at 75% $\dot{V}O_{2\,max}$ compared with pre-exercise values; while for the same time-points the percentage of T cells producing IL-4 (type 2 cytokine) were unchanged (Steensberg et al., 2001). This study suggests that the total decline in $CD4^+$ and $CD8^+$ T cells during recovery from exercise is largely due to a decrease in type 1 T cells. Type 1 T cells are crucial in the defence against intracellular pathogens; therefore a significantly impaired type 1 T cell response following exercise may be partly responsible for the proposed increased risk of URTI after acute strenuous exercise.

NK cells are the most responsive cell type to an acute bout of intensive exercise and large increases (~50-500%) in the number of circulating NK cells have been shown immediately following exercise. However, for several hours following exercise NK cell numbers tend to drop to less than half of normal baseline levels, with normal resting values restored within 24 h (Hoffman-Goetz et al., 1990; Gabriel et al., 1991, 1992; Tvede et al., 1993; Shephard and Shek, 1999). While many studies have investigated how NK cells respond to acute exercise, little research has looked at how different NK cell subsets ($CD56^{bright}$ and $CD56^{dim}$) respond to exercise. Generally this research has suggested that there is a differential mobilisation of NK cell subsets in response to acute exercise; the $CD56^{dim}$ NK subset appears to be more responsive immediately following exercise than its $CD56^{bright}$ counterpart (reviewed in Timmons and Cieslak, 2008). This differential mobilisation appears to exist for high-intensity (~70% $\dot{V}O_{2\,max}$) exercise that lasts between 5 and 60 min (Suzui et al., 2005, 2006; Timmons et al., 2006a,b; Timmons and Bar-Or, 2007). During the post-exercise recovery period (up to 1 h) following 60 min cycling at 70% $\dot{V}O_{2\,max}$, the ratio of $CD56^{bright}$/$CD56^{dim}$ NK cells favours the $CD56^{bright}$ subset (Timmons et al., 2006a,b; Timmons and Bar-Or, 2007). As this subset is naturally less cytotoxic than $CD56^{dim}$ NK cells (Jacobs et al., 2001), any increase in this subset could lead to a dampening of overall killing capacity and potentially leave a host more susceptible to infection following acute intensive exercise. It should

be noted that many of the studies that have investigated the effect of exercise on NK cell subsets have carried out their research using children and adolescents; as such these findings may not be representative of how NK cell subsets respond to acute exercise in adults.

2.3.2. Mechanisms underlying mobilisation during and after acute exercise

Several mechanisms have been proposed to be responsible for the lymphocytosis and lymphocytopenia evident during and following exercise, respectively. It has been suggested that the initial increase in circulating lymphocyte counts is due to a flushing of cells attached to the endothelium from the marginal pool, the spleen and liver (Benschop et al., 1996; Krüger et al., 2008). This action is thought to be mediated via mechanical factors such as an increase in cardiac output, the shear stress associated with an increased pulmonary blood flow as well as a redistribution of blood flow (Foster et al., 1986).

Others have suggested that the increase in lymphocyte counts during and immediately after intensive exercise are due to the effects of catecholamines; which are known to increase in response to strenuous exercise (Hartley et al., 1972; Maisel et al., 1990; Murray et al., 1992; Shephard, 2003). Lymphocytes express a high density of homogenous β_2-adrenoreceptors. Interestingly, lymphocyte subsets appear to have a differential density of β_2-adrenoreceptors (Murray et al., 1992). $CD8^+$ T cells possess 2-3 times more functional β_2-receptors than $CD4^+$ T cells, while NK cells possess nearly 50% more β_2-receptors than $CD8^+$ T cells (Murray et al., 1992). Adrenaline is a β_2-agonist and upon stimulating this receptor induces the formation of intracellular cyclic AMP. Elevations in cAMP concentration via the actions of catecholamines therefore may modify the surface expression of adhesion molecules resulting in the mobilisation of these cells into the circulation (Maisel et al., 1990; Benschop et al., 1997; Nagao et al., 2000; Shephard, 2003). This mechanism along with the differences in the density of β_2-adrenoreceptors amongst lymphocyte subsets helps to explain why NK cell counts appear to be the most responsive

to the affects of exercise and why the relative increase in the number of circulating $CD8^+$ T cells is higher during and after exercise compared with $CD4^+$ T cells.

It has been suggested that the fall in lymphocyte count during recovery from exercise may be due to the trafficking of lymphocytes out of the circulation to sites where their immune functions are needed. In support of this notion, Krüger et al. (2008) have demonstrated that in response to high intensity exercise T cells appear to migrate to sites such as Peyer's Patches and the lungs and have suggested that this accumulation may serve to enhance immune vigilance, as these sites represent the body's major defence barriers. It has been proposed that cortisol may be responsible for the lymphocytopenia that occurs following high intensity exercise, as this stress hormone has been shown to promote lymphocyte movement out of the vascular compartment into the tissues via intravenous administration (Cupps and Fauci, 1982). Plasma cortisol concentrations tend to increase with exercise that is above 60% $\dot{V}O_{2\,max}$ (Bishop, 2006a) and have been shown to stay elevated for some time after exercise cessation (Nieman, 1997; Steensberg et al., 2001). Shinkai et al. (1996) have also suggested that increased cortisol concentrations following a 60 min cycle at 60% $\dot{V}O_{2\,max}$ may have been responsible for the lymphocytopenia that occurred 2.5 h following exercise, as participants whose cortisol concentrations did not appear to respond to the effects of exercise did not demonstrate any such lymphocytopenia. However, in a study by Bishop et al. (2005a), a fall in lymphocyte number during the recovery period following an acute bout of high intensity exercise (90 min cycle, 70% $\dot{V}O_{2\,max}$) was seen without a significant increase in plasma cortisol concentration at this time. As such, these findings suggest that while cortisol may play a significant role in the fall of lymphocyte number seen following strenuous exercise, it does not appear to be the only mechanism responsible.

It should be emphasised that the changes described above refer to alterations in T and NK cell number only, not alterations in function. As such the lymphocytosis observed during and immediately following exercise does not necessarily lead to an increase in immune function. Likewise, the lymphocytopenia seen during recovery does not necessarily suggest a suppressed ability of the host to mount an immune response when faced with an antigenic challenge.

2.3.3. Lymphocyte activation

As previously discussed, cell activation is a crucial first stage that cells need to undergo in order to mount a successful immune attack in response to infection. Therefore the ability of lymphocytes to become activated has a direct result on subsequent lymphocyte functions.

Commitment to cellular activation leads to the expression of newly synthesised proteins on the cell surface. CD69 is the first cell surface glycoprotein detected after activation (Ziegler et al., 1994). Resting (unstimulated) peripheral blood lymphocytes do not usually express CD69, with only a small number of circulating lymphocyte cells expressing low levels of this marker (Ziegler et al., 1994; Borrego et al., 1999). However, Borrego et al. (1999) suggest that even the low levels of CD69 expressed on resting lymphocytes are functionally active. Nevertheless, this antigen is rapidly induced on lymphocytes following activation, with transcription of CD69 evident within 30-60 min and surface expression detectable after 2-3 h (Ziegler et al., 1994). As such CD69 has been classed as a marker of early lymphocyte activation. Once expressed on activated lymphocytes, CD69 is believed to act as a co-stimulatory molecule leading to subsequent cytokine production, proliferation and cytotoxicity (Moretta et al., 1991; Borrego et al., 1999).

A number of studies have assessed the effect of acute intensive exercise on lymphocyte activation *in vivo/ex vivo* (unstimulated) and in response to mitogen

stimulation *in vitro*, by assessing the concentration of T and NK cells expressing the early activation marker CD69 (Ronsen et al., 2001; Vider et al., 2001; DuBose et al., 2003; Green et al., 2003; McFarlin et al., 2004; Bishop et al., 2005a; Timmons et al., 2006a, b; Timmons and Bar-Or, 2007). The effect of acute strenuous exercise on the expression of CD69 on T and NK lymphocytes is conflicting.

The *in vivo/ex vivo* unstimulated expression of CD69 on T and NK cells has been shown to be unresponsive (Ronsen et al., 2001; Green et al., 2003; Bishop et al., 2005a) immediately after high intensity exercise (70-80% $\dot{V}O_{2\,max}$) lasting around 60-90 min. However, NK cell CD69 expression has also been shown to increase immediately after 60 min of high intensity exercise (70% $\dot{V}O_{2\,max}$) in both adults and children (McFarlin et al., 2004; Timmons et al., 2006b; Timmons and Bar-Or, 2007). DuBose et al. (2003) have also demonstrated an increase in the percentage of $CD8^+$ T cells expressing CD69 immediately following a ~2 mile run in warm weather in military recruits. During the hours of recovery after 60-90 min of intensive (70-80% $\dot{V}O_{2\,max}$) exercise, expression of CD69 on T and NK cells has been reported to show no change in expression from baseline values (Ronsen et al., 2001; Green et al., 2003; Bishop et al., 2005a; Timmons and Bar-Or, 2007). However, following the same exercise protocol as that described above, the intensity of CD69 expression on NK cells has been shown to increase 1 h post-exercise (Timmons et al., 2006a, b), while the number of NK cells expressing CD69 has been shown to decrease 2 h and 4 h after exercise (McFarlin et al., 2004) when compared to pre-exercise values.

Mitogen-stimulated *in vitro* expression of CD69 on T and NK cells appears to be unresponsive to single bouts of acute intensive exercise (70% $\dot{V}O_{2\,max}$) lasting around 1 h (Ronsen et al., 2001; Green et al., 2003). Vider et al. (2001) have demonstrated a decrease in the percentage of mitogen-stimulated $CD4^+$ and $CD8^+$ T cells expressing CD69 immediately following exhaustive exercise (treadmill run to volitional exhaustion). However, it should be noted that this decrease had already returned to pre-exercise values within 30 min of recovery.

The percentage of $CD4^+$ cells expressing CD69 in response to mitogen stimulation has also been shown to decrease below pre-exercise values in military recruits immediately following a ~2 mile run in warm weather with values returning to baseline within 24 h (DuBose et al., 2003). Moreover, the expression of CD69 on mitogen-stimulated T ($CD4^+$ and $CD8^+$) cells was significantly lower following exercise in military recruits with exertional heat injury, with values still significantly reduced 24 h later (DuBose et al., 2003).

Reasons for the inconsistency in *in vivo/ex vivo* findings are not readily apparent, although differences in exercise duration, overall exercise intensity and/or ambient conditions may be responsible. Alternatively, differences in stimulant concentration, time for culture and whole blood vs. peripheral blood mononuclear cell (PBMC) cultures may also contribute to some of the inconsistency seen amongst *in vitro* studies. Most of the studies above that stimulated lymphocytes did so with the mitogen phytohaemagglutinin (PHA) (Vider et al., 2001; DuBose et al., 2003; Green et al., 2003). However, the amount of PHA used to stimulate cultures varied considerably between studies from 5-20 µg/ml. Reddy et al. (2004) have also demonstrated that the time of culture is also an important consideration when measuring CD69 expression, as this marker has been shown to increase in a time-dependant manner from 3-12 h following stimulation with 5 µg/ml PHA. PHA is also a known T cell stimulant that has little affect upon NK cells in culture (Vargas et al., 1996). However, this mitogen was used by DuBose et al. (2003) to stimulate NK cells as well as T cells and as such may account for why NK cell activation did not appear to change following exercise in this study.

There is debate surrounding the relative merits of mitogen vs. antigen-specific responses. Lymphocyte responses are antigen specific (Roitt and Delves, 2001), therefore the assessment of cell responses to mitogen stimulation *in vitro* may not necessarily provide the most accurate model for the *in vivo* situation (Bishop et al., 2005b). Mitogens such as PHA stimulate a high proportion of circulating lymphocytes that trigger a potent non-specific immune response that may result in a large and unrealistic alteration in lymphocyte function (Bishop et

al., 2005b). Antigens initiate a more specific, subtle immune response that is likely to occur *in vivo* (Bishop et al., 2005b). As such antigen stimulation (rather than mitogen) may give a more realistic insight into the effects of prolonged exercise on lymphocyte activation.

2.4. Mechanisms of exercise induced alterations in lymphocyte activation

2.4.1. Adenosine

Adenosine is a purine nucleoside that is found in free form both in intracellular and extracellular compartments, acting as a regulator of tissue function within almost every cell of the human body (Blay and Hoskin, 2007; Klotz, 2007). The plasma adenosine concentration in humans at rest is approximately 40-50 $nmol \cdot L^{-1}$ (Vizi et al., 2002; Chouker et al., 2005). However, adenosine concentrations dramatically increase when metabolically stressful conditions (i.e. trauma or exercise) occur (Haskó and Cronstein, 2004). It is well known that adenosine is produced during exercise due to the increased dephosphorylation of adenosine triphosphate. Although adenosine is rapidly cleared by erythrocytes (Heptinstall et al., 2005), plasma concentrations of adenosine have been shown to increase in response to exercise (Vizi et al., 2002; Chouker et al., 2005). For example, Chouker et al. (2005) found an increase in plasma adenosine concentration from 40 $nmol \cdot L^{-1}$ at rest to 80 $nmol \cdot L^{-1}$ following a 4-4.5 h strenuous hike at low altitude.

Adenosine binds to specific cell surface adenosine receptors (Haskó and Cronstein, 2004). There are four clearly defined adenosine receptor subtypes; A_1, A_{2A}, A_{2B} and A_3 (Blay and Hoskin, 2007). Both A_1 and A_{2A} receptors have a high affinity for adenosine, while A_{2B} and A_3 receptors have a substantially lower affinity (Haskó and Cronstein, 2004). The primary effector of all four subtypes of adenosine receptors is adenylyl cyclase, whose action can either be stimulated or inhibited depending upon which adenosine receptor is present on a cell. Both A_1 and A_3 receptors can inhibit adenylyl cyclase and thus reduce levels of cAMP, whereas A_{2A} and A_{2B} receptors stimulate adenylyl cyclase leading to an increase in cAMP levels. cAMP is a second messenger that acts

as a negative regulator of T cell activation (Grader-Beck et al., 2003). With few exceptions, the actions of cAMP are mediated via cAMP-dependant protein kinase A type I (PKAI).

Unactivated human T lymphocytes express A_{2A}, A_{2B} and A_3 receptors, all of which exhibit increased expression following T cell activation (Blay and Hoskin, 2007). The capacity for increased signalling by activated T cells suggests a possible feedback role for adenosine in the control of cell-mediated immune responses (Lappas et al., 2005; Blay and Hoskin, 2007). Adenosine receptor expression of NK cells has not yet been formally analysed (Blay and Hoskin, 2007). However, a study conducted with adenosine receptor agonists (selective for each receptor subtype) on human NK cells has led to the conclusion that A_1 and A_2 receptors are present on these cells (Priebe et al., 1990).

Adenosine is a potent endogenous anti-inflammatory agent (Cronstein, 1994) that has been shown to inhibit T cell activation (Apasov and Stikovsky, 1999; Lappas et al., 2005, 2010). Apasov and Stikovsky (1999) demonstrated that adenosine selectively inhibited TCR-induced activation that also blocked the upregulation of CD69 on mouse thymocytes. The authors concluded that the inhibitory effects of adenosine were likely due to general inhibition of TCR-triggered signalling pathways and that this was achieved due to activation of the cAMP-dependant pathway via A_{2A} adenosine receptors; as a selective agonist of A_{2A} adenosine receptors as well as the cAMP analog db-cAMP were able to inhibit TCR-induced activation. However, this study used a pharmacological dose of adenosine (0.1 mM) and as such may not represent the situation *in vivo*. Lappas et al. (2005) demonstrated that under physiological conditions an analog of adenosine (selective A_{2A} agonist ATL146e) at a concentration of 100 $nmol \cdot L^{-1}$ (likely to be achieved following strenuous exercise; Chouker et al., 2005) attenuated the TCR-triggered expression of CD69 on purified murine $CD4^+$ T cells following stimulation with the mab anti-CD3 (responsible for stimulating TCR-mediated activation). As such these findings suggest that at physiological concentrations adenosine appears to inhibit T lymphocyte

activation and subsequent CD69 expression. The likely mechanism responsible for this inhibition involves the binding of adenosine to T cell A_{2A} receptors, that subsequently stimulate adenylyl cyclase leading to an increase in cAMP, which via PKAI interferes with TCR signal transduction (Blay and Hoskin, 2007).

In comparison to T cells, no study to date has investigated the effect of adenosine on NK cell activation and subsequent CD69 expression. Studies have however investigated the effects of adenosine on human NK cytotoxicity (Priebe et al., 1990; Raskovalova et al., 2006). A study by Priebe et al. (1990) on human NK cells demonstrated that the adenosine receptors A_1 and A_2 (suggested to be present on human NK cells) have opposing effects when it comes to cytotoxic activity. A_1 receptors tend to increase cytotoxic activity of NK cells, whereas A_2 receptors tend to suppress cytotoxic activity. The likely explanation for the opposing effects of the adenosine receptors on NK cells is through intracellular cAMP levels, which are known to modulate NK cell function (Goto et al., 1983). It has been suggested that A_1 receptors reduce intracellular levels of cAMP and therefore stimulate NK cell cytotoxic activity (NKCA), while A_2 receptors increase intracellular levels of cAMP and tend to inhibit NK cell lytic activity (Priebe et al., 1990). However, a study using lymphokine activated killer (LAK) cells generated from A_1 and A_3 adenosine-receptor deficient mice have ruled out any involvement of these receptors in the adenosine-mediated inhibition of LAK cytotoxicity (Raskovalova et al., 2005). Raskovalova et al. (2006) have found that the metabolically stable analog of adenosine (2-chloroadenosine) inhibits the cytotoxic activity of human NK cells. However, preincubation of NK cells with an A_{2A} receptor antagonist blocked the inhibitory effects of 2-chloroadenosine. This study demonstrates that the inhibitory actions of adenosine on NK cells are mediated via A_{2A} receptors, which activates adenylyl cyclase and stimulates cAMP production that activates PKAI that transmits a signal leading to an inhibition of NK cytotoxicity.

2.4.2. Adrenaline

Adrenaline is a catecholamine that is derived from the amino acids phenyalanine and tyrosine. Adrenaline concentration at rest is relatively low (0.1 nmol·L^{-1}). However, in response to physical stress such as high intensity exercise adrenaline concentration has been consistently shown to increase significantly from resting values; with values close to 3 nmol·L^{-1} being recorded following exercise to exhaustion (Hartley et al., 1972; Graham and Spriet, 1995; Walker et al., 2008). It should be noted that while increases in adrenaline concentration are also evident at more moderate intensities of exercise (50% $\dot{V}O_{2\,max}$, 0.55 nmol·L^{-1}), once exercise exceeds 70% $\dot{V}O_{2\,max}$ there is an exponential increase in plasma concentrations of adrenaline (Shephard, 2003).

Adrenaline mediates its effects on target cells via stimulation of either α- or β-adrenoreceptors. Lymphocytes have been shown to express a homogenous population of β_2-adrenoreceptors (Murray et al., 1992). As previously described (section 2.3.2) NK, $CD8^+$ and $CD4^+$ T cells differ in their density of β_2-receptors, with NK cells having the greatest number of receptors and $CD4^+$ T cells the lowest (Maisel et al., 1990). Exercise and catecholamine exposure have been shown to increase the density of these receptors (Shephard, 2003). In general, β_2-adrenoreceptors activate adenylate cyclase that increases intracellular cAMP. cAMP executes its effects through activation of PKAI and as such acts to inhibit lymphocyte activation (Grader-Beck et al., 2003). Both NK cells and $CD8^+$ T cells are very responsive to β_2-adrenoreceptor stimulation with regard to cAMP accumulation, while $CD4^+$ cells only appear to show a moderate response (Maisel et al., 1990).

Shimamiya et al. (2003) have shown that at physiological concentrations adrenaline inhibits CD69 expression on mitogen-stimulated T and NK lymphocytes and that this suppression can be blocked by the non-selective β-antagonist propranolol. As β_2-receptor activation via adrenaline increases intracellular cAMP it seems plausible that the mechanisms responsible for the

inhibition of both natural and mitogen-stimulated lymphocyte activation via adrenaline are similar to those of A_{2A} receptor activation via adenosine.

Additionally, increases in natural lymphocyte activation following strenuous exercise (McFarlin et al., 2004; Timmons et al., 2006b) could reflect an adrenaline-mediated mobilisation of a specific population of lymphocytes into the circulation with an activated phenotype. As previously described (section 2.3.2) adrenaline via β_2-adrenoreceptor activation is thought to modulate the expression of adhesion molecules on lymphocytes, leading to an increase in circulating lymphocyte number following either exercise or adrenaline infusion (Kappel et al., 1991). Timmons et al. (2006b) showed a significant increase in adrenaline concentration and number of NK cells expressing CD69 immediately following 60 min cycling at 70% $\dot{V}O_{2\,max}$. The increase in number of NK cells expressing CD69 most likely reflected a recruitment of NK $CD69^+$ cells into the circulation as the increase in activated cells corresponded with post-exercise lymphocytosis. As well as this, the NK cell CD69 expression on a per cell basis did not increase in response to exercise.

2.5. Caffeine

The effect of caffeine ingestion on lymphocyte activation in humans either at rest or following exercise has not been extensively studied. Caffeine ingestion however has been consistently shown to increase plasma adrenaline concentration (Graham and Spriet, 1995; Graham, 2001; Bishop et al., 2005a; Walker et al., 2006). Furthermore, the main action of caffeine at concentrations relevant to normal patterns of consumption is via antagonism of adenosine receptors (Fredholm et al., 1999). Caffeine is very similar in structure to adenosine and as such can bind to cell membrane receptors for adenosine, thus blocking their action (Graham, 2001). Therefore, caffeine has the potential to modulate lymphocyte activation either directly via adenosine receptor antagonism or indirectly via stimulation of adrenaline release.

Caffeine is a member of the methylxanthine family of drugs. It is a trimethyl purine base, methylxathine (1, 3, 7- trimethylxathine) with a molecular weight of 194.2 (Mandel, 2002). Caffeine is a chemical substance that has been identified in around 60 plants and is obtained as a white, odourless, crystalline, bitter tasting powder (Debry, 1994). This drug is legally and socially accepted, and as such is the most widely consumed psychoactive substance in the world (Graham, 2001). 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). It is estimated that the mean daily intake of caffeine in the UK 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 from person to person and country to country. For instance the daily caffeine intake in Sweden and Norway is more like 400 $mg \cdot day^{-1}$ (Fredholm et al., 1999). The main sources of caffeine within a person's diet are in the form of coffee, tea, cocoa beverages, chocolate, soft drinks, energy drinks and several drug preparations (Mandel, 2002). Table 2.1 shows the caffeine content of common caffeine-containing products.

Table 2.1: Caffeine content of common caffeine-containing beverages and foods

Source (serving size)	Caffeine content (mg)
Coffee (150 ml) *	50-120
Tea (150 ml) *	15-50
Coca-cola (330 ml)	30
Energy drinks (250 ml)	80
Hot chocolate (250 ml)	10
Chocolate bar (50 g)	40
Pro Plus (1 capsule)	50

Caffeine content obtained from Maughan, 2002. * Large variation in caffeine content due to source and preparation of product.

Caffeine absorption from the gastrointestinal tract is rapid and reaches 99% in humans in about 45 minutes following ingestion (Fredholm et al., 1999). Peak plasma caffeine concentration is generally reached between 15 and 120 min in humans and as such suggests that there are large interindividual differences in response to caffeine ingestion (Fredholm et al., 1999; Desbrow et al., 2009). The rate of caffeine metabolism is also variable and as such the plasma half-life of caffeine can range from 2-12 h in healthy adults. However, on average the half-life of caffeine in humans is 4-6 h (Benowitz, 1990). Caffeine is extensively metabolised, initially by the liver, which demethylates this trimethylxanthine to three dimethylxanthines: paraxanthine (the major primary metabolite), theophylline and theobromine, which are then further catabolised (Benowitz, 1990). Although paraxanthine and theophylline are also potent adenosine antagonists, these metabolites do not appear to increase in the circulation to a concentration considered active during caffeine metabolism (Graham and Spriet, 1995). It appears that caffeine pharmacokinetics are not affected by exercise or dehydration and is similar between males and females (Graham, 2001; Goldstein et al., 2010).

2.6. Caffeine and its relevance within sport

It is commonly reported in the research literature that caffeine between the doses of 3-6 $mg \cdot kg^{-1}$ body mass enhances athletic performance (Goldstein, 2010), with doses as low as 2 $mg \cdot kg^{-1}$ body mass also proving ergogenic (Cox et al., 2002). This enhancement has been demonstrated in activities that last as little as 60 seconds or as long as 2 hours (Graham, 2001), and helps to explain why caffeine is consumed by a variety of athletes from different sporting disciplines (Chester and Wojek, 2008).

Many athletes consume caffeine for its known ergogenic effects, with its prevalence increasing amongst the athletic population since being legitimised by its 2004 removal from the WADA prohibited list of substances (Chester and Wojek, 2008).

2.7. Caffeine and lymphocyte function at rest

A number of studies (*in vitro* and *in vivo*) conducted have demonstrated that caffeine can modulate various aspects of lymphocyte function (as reviewed in Horrigan et al., 2006). Of the research that has been conducted, very few studies have used physiological caffeine concentrations that are relevant to normal human consumption and even fewer have concentrated on human subjects (Horrigan et al., 2006). The typical plasma concentration of caffeine after ingestion of physiological doses (<10 $mg \cdot kg^{-1}$) appears to increase in a dose-dependant fashion and has consistently been reported to be less than 70 µM (Graham and Spriet, 1995; Fredholm et al., 1999; Mandel, 2002). Knowing the likely plasma concentration following oral caffeine ingestion is particularly useful to *in vitro* studies examining caffeine exposure. Therefore, based on the estimates of plasma caffeine concentration, studies employing 70 µM or less of caffeine may be viewed as relevant to human caffeine consumption (Graham, 2001).

2.7.1. *In vitro* studies

Kantamala et al. (1990) demonstrated that caffeine, at a concentration of 20-100 µM had no effect on NK cell cytotoxicity in rats. Caffeine at a concentration of 50-100 µM has been shown to suppress lymphocyte proliferation in response to mitogen stimulation in both rats and humans, whilst a lower dose of caffeine (20 µM) had no effect (Kantamala et al., 1990; Horrigan et al., 2005).

2.7.2. *In vivo* studies

Kantamala et al. (1990) observed that caffeine consumed consistently for 120 days produced differential effects on NK cytotoxicity and mitogen-stimulated T cell proliferation depending on the dose administered. They found that doses of 2 and 18 $mg \cdot kg^{-1} \cdot day^{-1}$ caffeine had no effect at altering NK cell cytotoxicity, while a dose at 6 $mg \cdot kg^{-1} \cdot day^{-1}$ caffeine tended to reduce NK cell cytotoxicity in rat spleen cells. Likewise, a 2 and 6 $mg \cdot kg^{-1} \cdot day^{-1}$ dose of caffeine had no effect on PHA-P stimulated T cell proliferation within rats, while 18 $mg \cdot kg^{-1} \cdot day^{-1}$ caffeine led to an increase in PHA-P stimulated T cell proliferation. Therefore,

this study demonstrates the equivocal nature of the effects of caffeine on lymphocyte function.

When interpreting results from *in vivo* studies it should be kept in mind that most of the studies investigating the physiological effects of caffeine have been carried out on rodents. Therefore, when trying to extrapolate data from rodents to humans, consideration as to the dose equivalence between species is required. It has been suggested that caffeine doses up to 20 mg·kg^{-1} in rodents are within human physiological concentrations (Horrigan et al., 2006). It should also be taken into account that there are significant differences in caffeine metabolism and the plasma half-life of caffeine (rodents: 0.7-1.2 h vs. humans: 4-6 h) between rodents and humans that may limit extrapolation of findings from non-human models.

2.8. Caffeine and lymphocyte activation following exercise

To the author's knowledge only one study to date has investigated the effect of caffeine ingestion on lymphocyte activation in response to exercise (Bishop et al., 2005a). Bishop et al. (2005a) reported that caffeine ingestion (6 mg·kg^{-1}) 1 h prior to exercise increased the percentage of $CD4^+$ and $CD8^+$ cells expressing CD69 both prior to and following 90 min cycle exercise at 70% $\dot{V}O_{2peak}$. Although this study showed an increase the natural state of activation of $CD4^+$ and $CD8^+$ cells both before and after strenuous prolonged exercise with prior caffeine ingestion; this study did not stimulate cells with an antigen and as such may not represent what actually happens when faced with an antigenic challenge.

2.9. Potential effects of caffeine on lymphocyte activation following exercise

Plasma adenosine concentration dramatically increases during periods of stress, such as exercise (Vizi et al., 2002; Chouker et al., 2005). Caffeine is a non-selective adenosine antagonist, with preferential A_1 and A_{2A} receptor antagonism (Ferre et al., 2008). Following intravenous caffeine infusion, plasma adenosine concentration has been shown to increase in a dose-dependant manner that is thought to be due to receptor blockade (Conlay et al., 1997). Adenosine is known to inhibit T cell activation and CD69 expression as well as NK cell cytotoxicity (see section 2.4.1). Therefore it seems plausible that caffeine could inhibit the occupancy and/or action of adenosine on lymphocyte activation. Caffeine is also known to stimulate the release of adrenaline and as such increase plasma adrenaline concentration (Graham, 2001). Adrenaline is a known β_2-adrenoreceptor agonist and upon stimulating this receptor on lymphocytes has been shown to inhibit expression of CD69 (see section 2.4.2). It is likely that in response to caffeine ingestion and exercise both mechanisms will be involved. However, the overall effect on lymphocyte activation may depend upon which mechanism dominates. A summary of the potential mechanisms by which caffeine may influence lymphocyte activation are outlined in Figure 2.1.

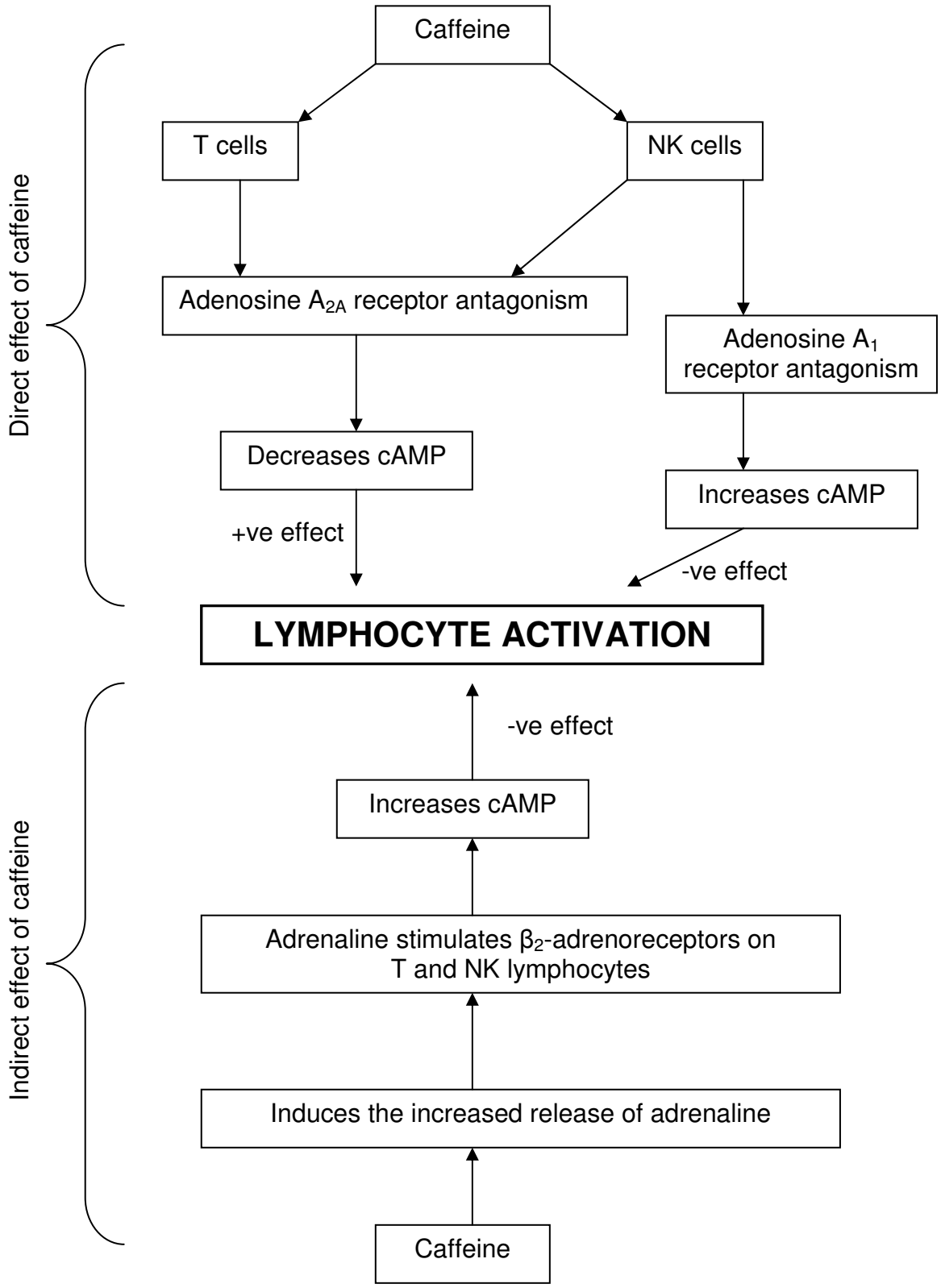


Figure 2.1: A summary of proposed mechanisms by which caffeine influences lymphocyte activation.

2.10. Thesis aims and hypotheses

The aim of this thesis was to investigate the influence of typically-used doses of caffeine (typical daily intake during training and competition doses) on antigen-stimulated lymphocyte (T cells – acquired immunity and NK cells – innate immunity) activation in response to prolonged exercise.

It was hypothesised that:

- A high (6 mg·kg^{-1}) and low (2 mg·kg^{-1}) dose of caffeine would have little effect on T or NK cell activation compared with placebo in response to antigen stimulation during 3.5 h rest.

- A high (6 mg·kg^{-1}) dose of caffeine ingested 1 h prior to exercise would increase T and NK cell activation in response to antigen stimulation compared with placebo following 90 min cycling at 70% $\dot{V}O_{2\,peak}$.

- A high (6 mg·kg^{-1}) dose of caffeine ingested 1 h prior to exercise would increase antigen-stimulated NK cell activation to a greater extent than a low (2 mg·kg^{-1}) dose of caffeine compared with placebo and as such demonstrate a dose-response effect of caffeine following 90 min cycling at 70% $\dot{V}O_{2\,peak}$.

- Caffeine ingested in small (2 mg·kg^{-1}) repeated (3 doses ingested 3 h apart) doses throughout the day would increase antigen-stimulated NK cell activation to a similar extent as one large (6 mg·kg^{-1}) bolus dose of caffeine ingested 1 h prior to exercise compared with placebo following 90 min intermittent shuttle running.

Chapter 3

General Methods

3.1. Ethical Approval

All study protocols presented in this thesis were approved by Loughborough University Ethical Advisory Committee. All participants were fully informed about the rationale for the particular study and the design of the exercise tests and trial protocol, along with their possible risks and discomforts, both verbally and in writing. Following an opportunity to ask any questions, participants provided written informed consent (Appendix A).

Participants completed an extensive health-screening questionnaire (Appendix B) along with a physical activity questionnaire (Appendix C) during their initial visit to the laboratory to ascertain if they were suitable for the study. Any participants that were currently on medication or had reported symptoms of infection in the 4 weeks prior to the study were excluded. Participants' habitual caffeine consumption was also recorded at the beginning of each study using a caffeine consumption questionnaire (Appendix D) to classify participants as low, moderate or high habitual caffeine users. On the day of each main trial, a subsequent health-screening questionnaire (Appendix E) was administered to confirm that participants were not experiencing any symptoms of URTI and were happy to take part in the study.

3.2. Exploratory Methods

Before the main trials of this thesis began, preliminary tests were carried out to ensure that the protocol and methodology of the studies was sound. The Pediacel vaccine (Sanofi Pasteur MSD Ltd, Berkshire, UK) containing diphtheria toxoid, tetanus toxoid, acellular pertussis bacteria, poliovirus and haemophilus influenza type B was chosen as the stimulant for this thesis. The vaccine was initially titrated in order to assess both optimum (1:4000) and sub-optimum (1:8000) doses for cell stimulation in culture (Appendix F). Stimulant concentrations were chosen to elicit a maximal lymphocyte response (optimum dose) as well as a response likely to occur upon initial infection, when small numbers of pathogen enter the body (sub-optimum). Initially during testing of the caffeine (6 $mg \cdot kg^{-1}$ dose) and exercise trials, blood that was being used to set up cell cultures was collected into lithium heparin tubes and put immediately

on a mixer. It soon became apparent that this part of the protocol was flawed, as too few cells were being detected by the flow cytometer. Therefore, cells were dying at some point between blood collection and flow cytometer acquisition.

Analysis of the published literature using similar methodology suggested that a solution to this problem was to change the blood containers from a lithium heparin tube to a tube containing sodium heparin, and to place blood samples immediately on ice after draw (Vider et al., 2001; Green et al., 2003). To ensure that the changes to the protocol had been successful, blood was collected into sterile bijou tubes containing sodium heparin prior to and following 90 min of cycle exercise at 70% $\dot{V}O_{2\,peak}$ without caffeine ingestion. Samples were immediately placed on ice after draw and rolled for 20 min before being assessed for T and NK cell number and their expression of CD69 (for full details of staining and analysis see 3.6.3). Unstimulated, optimum and sub-optimum cultures were also set up as described below (see 3.6.2) and incubated for 20 h at 37°C, 5% CO_2. Following the 20 h incubation, samples were again assessed for T and NK cell number and their expression of CD69. This work demonstrated that the total number of T and NK cells that were viable before incubation were still present the next day when being acquired (Appendix G).

The new protocol was then tested on blood collected at rest with or without prior caffeine ingestion. Blood samples were taken at 8:30 h, 11:00 h and 12:00 h to coincide with some of the time points for the main exercise trials of this thesis (pre-supplement, post-exercise and 1 h post-exercise). The protocol was then tested on blood collected at pre-supplement, post-exercise and 1 h post-exercise from a caffeine and exercise trial identical to the ones in Chapters 5 and 6. This work helped to ensure that regardless of the trial, cells remained viable and could be acquired and that the CD69 marker was detectable (Appendix G). For all trials, participants followed the same standardisation of pre-trial conditions as that described below (see 3.4); and for all of the caffeine trials, a 6 $mg \cdot kg^{-1}$ dose of caffeine was selected to try and elicit a stronger and more identifiable response.

3.3. Preliminary testing

Approximately 2 weeks before the beginning of the main trials, each participant was required to perform a continuous incremental exercise test to volitional fatigue on an electromagnetically braked cycle ergometer (Lode Excalibur, Groningen, Netherlands), to determine their peak oxygen consumption ($\dot{V}O_{2peak}$). Participants started cycling at a work rate of 95 W, with 35 W increments every 3 min, continuing until they reached volitional exhaustion. Verbal encouragement was given to participants to ensure a maximum effort. Maximal work rate (Wmax) was determined according to the following formula: Wmax = Wout + (t/180) * 35, where Wout is the last completed stage and t is the time in seconds in the final stage (Jeukendrup et al., 1996). Expired gas samples were collected into Douglas bags during the 3rd min of each work rate increment and the last min of the exercise test, with heart rates being measured continuously via short-range radio telemetry (Polar FS1, Polar Electro Oy, Kempele, Finland). A paramagnetic O_2 analyser (Servomex 1420B, Crowborough, UK) and an infrared CO_2 analyser (Servomex 1415B) were used to measure the percentages of O_2 and CO_2 within the expired gas samples. The analysers along with a dry gas meter (Harvard Apparatus, Edenbridge, UK) were used to determine minute ventilation, O_2 consumption and CO_2 production. A work rate equivalent of 70% $\dot{V}O_{2peak}$ for each participant was interpolated from the $\dot{V}O_2$ ($l \cdot min^{-1}$) – work rate (W) relationship.

Participants then came back into the lab on a separate occasion to undertake a familiarisation trial. The familiarisation trial required participants to cycle for 90 min at 70% $\dot{V}O_{2peak}$ on an electromagnetically braked cycle ergometer. Heart rate was monitored continuously during the familiarisation trial and 1 min expired gas samples were collected at 20 min intervals throughout to ensure participants were exercising at the correct intensity. If the participant was exercising at an intensity below 65% $\dot{V}O_{2peak}$ or above 75% $\dot{V}O_{2peak}$ the work rate was adjusted accordingly.

3.4. Standardisation of pre-trial conditions

Each participant was given a list of caffeine containing foods and beverages and instructed to abstain from these for 60 h preceding each main trial. Participants were also instructed to avoid alcohol consumption and strenuous physical activity in the 24 h preceding each experimental trial. In order to standardise nutritional status, participants completed a 24 h food diary the day before the first main trial and were asked to follow this during the 24 h preceding the second (Chapters 4, 5, 6) and third (Chapters 4, 6) main trials.

For each trial, participants arrived at the laboratory following an overnight fast of 12 h. Participants were instructed to ingest ~300 ml water upon waking to standardise their hydration status prior to each exercise bout.

3.5. Experimental trial procedures

In the experimental trials of Chapters 4-6, the method of caffeine administration was identical. In Chapter 5, participants ingested 0 or 6 $mg \cdot kg^{-1}$ body mass of caffeine (powder form; 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 (placebo) trial, participants ingested 6 $mg \cdot kg^{-1}$ body mass of dextrose powder (BDH Laboratory Supplies, Poole, UK). In Chapters 4 and 6 participants also ingested 0 or 6 $mg \cdot kg^{-1}$ body mass of caffeine, as well as a 2 $mg \cdot kg^{-1}$ body mass dose of caffeine administered in the same way.

For Chapters 5 and 6 the mode of exercise was cycling performed using an electromagnetically braked cycle ergometer. Participants' favoured saddle height, handle bar distance from saddle and height were all recorded on their first visit to the lab to optimise cycling performance and comfort. All these parameters were then set prior to each additional visit to speed up the transition time from rest to exercise.

For the studies described in Chapters 5 and 6 participants arrived at the laboratory at 08:20 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 collection, participants ingested caffeine or placebo (dextrose) powder taken in the form of cellulose capsules with 300 ml plain water. Participants then rested quietly in the laboratory for 1 h before a further (pre-exercise) venous blood sample was taken, after which participants' pre-exercise body mass (in shorts only) was recorded. Immediately after this, participants began cycling on an electromagnetically braked cycle ergometer for 90 min at a work rate equivalent to 70% $\dot{V}O_{2peak}$. During the 90 min cycle, participants' heart rate and subjective ratings of perceived exertion (RPE) were recorded every 15 min. Participants also consumed 2 ml·kg^{-1} body mass of plain water every 15 min throughout the exercise to standardise fluid intake. At 20, 50 and 80 min of exercise, 1 min expired gas samples were collected to determine $\dot{V}O_2$ and $\dot{V}CO_2$ to ensure that participants were exercising at the required intensity and to allow estimation of fat and carbohydrate (CHO) oxidation as well as energy expenditure using stoichiometric equations (Peronnet and Massicote, 1991). A further venous blood sample (post-exercise) was obtained immediately after cessation of 90 min exercise, before post-exercise body mass (in shorts only) was recorded. Participants then consumed 5 ml·kg^{-1} body mass of plain water and rested quietly in the laboratory for a further hour before a final venous blood sample (1 h post-exercise) was obtained. During this time no additional food or fluid was administered to participants.

3.6. Blood sampling and analysis

All blood samples in this thesis were collected by venepuncture from an antecubital forearm vein with the participant in an upright seated position. For all samples, approximately 20 ml of blood was collected into three evacuated monovette tubes (Starstedt, Leicester, UK), one containing tripotassium ethylene diamine tetraacetic acid (K_3EDTA) (1.6 mg EDTA ml^{-1} blood), one containing lithium heparin (16 IU heparin 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).

3.6.1. Haematological analysis

Blood collected into the K_3EDTA monovette (7.5 ml) was analysed for total and differential leukocyte counts, haematocrit and haemoglobin contents using a haematology analyser ($A^C.T^{TM}$ 5diff analyser, Beckman Coulter, UK). All cell counts were adjusted for plasma volume changes in relation to values from the initial (pre-supplement) blood sample, with changes being estimated from haemoglobin and haematocrit values according to Dill and Costill (1974).

3.6.2. Lymphocyte culture

Five ml of whole blood transferred into the sterile bijou tube containing sodium heparin was immediately placed on ice and mixed (Thermo Denley, Spiramix) for 20 min before setting up cultures as follows: Cells were cultured in Falcon 12 x 75 mm-polystyrene tubes with caps (Becton Dickinson Biosciences, Oxford, UK), and for all samples unstimulated and stimulated cultures were set up. The tubes were stimulated by a 1:400 or 1:800 Pediacel working vaccine. Zero or 20 µl of Pediacel working vaccine (either 1:400 or 1:800) was added to 200 µl of heparinised whole blood, giving a final stimulant concentration of 0 (unstimulated), 1:4000 (optimum) or 1:8000 (sub-optimum), before being incubated for 20 h at 37°C, 5% CO_2.

3.6.3. Assessment of lymphocyte subsets and CD69 expression

After 20 h 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 fluorescein isothiocyanate (FITC) conjugated anti-CD4, 5 µl R-phycoerythrin-Cy5 (PE-Cy5) conjugated anti-CD8 and 10 µl phycoerythrin (PE) conjugated anti-CD69. The other mixture contained 5 µl FITC conjugated anti-CD3, 20 µl PE-Cy5 conjugated anti-CD56 and 10 µl PE conjugated anti-CD69. All stained and unstained 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 then 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 unstimulated samples. This procedure was repeated with unstained stimulated samples for both 1:4000 and 1:8000 stained stimulated samples. All samples were set to collect 30,000 lymphocyte events per analysis.

$CD4^+$ and $CD8^+$ populations were acquired on quadrant dot plots of FL1 (CD4 FITC) and FL3 (CD8 PE-Cy5), along with quadrant dot plots of FL1 (CD4) and FL2 (CD69 PE) and quadrant dot plots of FL3 (CD8) and FL2 (CD69). This data was then displayed as histogram plots and the percentage of T cells expressing CD4 and $CD8^{bright}$ were derived. $CD8^{+bright}$ cells were analysed rather than all $CD8^+$ cells to avoid inclusion of $CD3^-CD8^+$ cells (Campbell et al., 2008). $CD3^+$ (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. $CD4^+$, $CD8^{+bright}$ and $CD3^-CD56^+$ cells were then gated into separate regions and $CD69^+$ histogram plots of the cells within each of these regions was used to calculate the percentage expression and geometric mean fluorescence intensity (GMFI) expression of CD69 of $CD4^+$, $CD8^{+\ bright}$ and $CD3^-CD56^+$ cells. Cell counts of $CD4^+$, $CD8^{+bright}$ and $CD3^-CD56^+$ were calculated by multiplying the percentage of these cells with the absolute lymphocyte count. The number of lymphocyte subsets expressing CD69 were determined by multiplying the percentage of cells expressing CD69 by the total number of $CD4^+$, $CD8^{+\ bright}$ and $CD3^-CD56^+$ 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. (2006a,b).

3.6.4. Serum caffeine and plasma catecholamines

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. Serum was then evenly distributed into labelled eppendorfs and frozen at -80 ºC. Serum caffeine concentration was determined using a commercially available kit (Emit Caffeine Assay, Dade-Behring, Milton Keynes, UK) on an automatic photometric analyser (COBAS Miras Plus, Roche Diagnostic Systems, Switzerland). The intra assay coefficient of variation for serum caffeine was 2.5 %. Heparinised plasma was obtained from blood collected into a lithium heparin monovette (7.5 ml). Samples were spun at 1500 g for 10 min in a refrigerated centrifuge at 4 ºC within 5 min of collection. Two ml of the heparinised plasma obtained was immediately added to chilled tubes, containing 200 µl of antioxidant preservative (pH 6.5) comprising of reduced glutathione (100 $mmol \cdot L^{-1}$) and ethylene glycol tetraacetic acid (100 $mmol \cdot L^{-1}$). Plasma and antioxidant were mixed and frozen at -80 ºC for later determination of plasma adrenaline and noradrenaline concentration by high performance liquid chromatography with electrochemical detection, as previously described (Forster and Macdonald, 1999).

3.7. Statistical analysis

All statistical analysis was conducted using SPSS 16.0 software for Windows (SPSS Inc. Chicago IL, USA). 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. Single comparisons between trials for overall exercise intensity, carbohydrate and fat oxidation rates, rate of energy expenditure and percentage contribution of substrate to energy expenditure were assessed using Student's paired *t*-tests. 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.

Chapter 4

The effect of a high and low dose of caffeine on antigen-stimulated human lymphocyte activation at rest

4.1. Abstract

This study investigated the effect of a high and low dose of caffeine on unstimulated and antigen-stimulated human lymphocyte ($CD4^+$, $CD8^+$ and $CD3^-$ $CD56^+$) activation at rest, as assessed by the early activation molecule CD69. In a randomised cross-over design, 6 healthy males [age: 25 (2) years; body mass: 75 (7) kg, mean ± SD] rested for 3.5 h after ingesting either 0 (PLA), 2 (2CAF) or 6 (6CAF) $mg \cdot kg^{-1}$ body mass of caffeine. Whole blood was stimulated with Pediacel (5 in 1) vaccine. The number of $CD3^-CD56^+$ cells were significantly higher on 6CAF 1 h and 2.5 h following caffeine ingestion compared with both PLA and 2CAF [$P<0.05$; 1 h, PLA: 0.22 (0.07), 2CAF: 0.21 (0.07), 6CAF: 0.41 (0.17); 2.5 h, PLA: 0.28 (0.11), 2CAF: 0.20 (0.10), 6CAF: 0.37 (0.07) $x10^9$ $cells \cdot L^{-1}$]. At these time-points (1 h and 2.5 h) serum caffeine concentrations were significantly higher on 6CAF compared with 2CAF and PLA and significantly higher on 2CAF compared with PLA [$P<0.01$; 1 h, PLA: 0 (0), 2CAF: 16 (8), 6CAF: 48 (11); 2.5 h, PLA: 0 (0), 2CAF: 17 (3), 6CAF: 47 (11) $\mu mol \cdot L^{-1}$] The number of antigen-stimulated $CD3^-CD56^+$ cells expressing CD69 were also higher on 6CAF 1 h following caffeine ingestion compared with both PLA and 2CAF [$P<0.05$; 1:4000 PLA: 31.16 (18.84), 1:4000 6CAF: 68.02 (28.54); 1:8000 2CAF: 29.76 (22.69), 1:8000 6CAF: 47.09 (29.34) $x10^6$ $cells \cdot L^{-1}$]. However, caffeine at a high or low dose had little effect at altering the number of T ($CD4^+$ and $CD8^+$) cells exrpressing CD69 in response to antigen stimulation. Similarly, caffeine at a high or low dose had little effect on the GMFI expression of CD69 on antigen-stimulated $CD4^+$, $CD8^+$ or $CD3^-$ $CD56^+$ cells. It could be suggested that the results of this study may be due to the differences between lymphocyte subsets in their density of β_2-adrenoreceptors. NK cells possess the highest number of β_2-adrenoreceptors of all lymphocytes and as such may explain why NK cells were the only lymphocyte subset responsive to the effects of a large (6 $mg \cdot kg^{-1}$) dose of caffeine in this study.

4.2. Introduction

Caffeine is a legal and socially acceptable drug and as such is commonly found in most people's diets. Caffeine's effects have been determined in a number of physiological systems including the cardiovascular, endocrine and central nervous systems (reviewed by Benowitz, 1990). However, little research has focused on how caffeine may affect various aspects of the immune system (reviewed in Horrigan et al., 2006). Of the research that has been conducted, most have used the rodent model and very few studies have used concentrations of caffeine considered relevant to "normal" human consumption (~70 µM or less; Graham, 2001). Even fewer have used human participants (reviewed in Horrigan et al., 2006).

T and NK lymphocytes are critical components of the acquired and innate immune system, respectively. T cells are responsible for orchestrating and regulating a highly specific and long lasting cell-mediated or humoral immune response to pathogenic invaders (Fabbri et al., 2003), while NK cells play a cruical role in defence against viral infection and tumour immune surveillance (Andoniou et al., 2006). At rest (unstimulated), small numbers of peripheral blood lymphocytes express low levels of CD69 (Testi et al., 1994; Borrego et al., 1999). CD69 is one of the earliest cell surface antigens expressed on lymphocytes following activation (Ziegler et al., 1994) and once expressed, is believed to trigger subsequent cell proliferation, cytotoxicity and secretion of cytokines (Moretta et al., 1991; Borrego et al., 1999; Mueller et al., 2002).

T and NK cells express both β_2-adrenoreceptors and adenosine receptors (T cells, A_{2A}, A_{2B} and A_3; NK cells, A_1, A_{2A} and A_{2B}), with the density of these receptors increased following activation (Shephard, 2003; Blay and Hoskin, 2007). Lymphocytes express a high density of homogenous β_2-adrenoreceptors: $CD8^+$ T cells possess 2-3 times more functional β_2-receptors than $CD4^+$ T cells, while NK cells possess nearly 50% more β_2-receptors than $CD8^+$ T cells (Murray et al., 1992). Both adenosine and adrenaline have been shown to inhibit early lymphocyte activation (Shimamiya et al., 2003; Lappas et al., 2005), via activation of adenylate cyclase and increased intracellular cAMP

(Raskovalova et al., 2006). At physiological concentrations, caffeine's main mechanism of action has been proposed to be adenosine receptor antagonism, with preferential A_1 and A_{2A} antagonism (Fredholm et al., 1999). However, caffeine is also known to stimulate the release of adrenaline, which has also been shown to affect a number of immune functions (Elenkov et al., 2000; Graham, 2001) Therefore, caffeine may have the potential to modulate lymphocyte activation either directly via adenosine receptor antagonism or indirectly via stimulation of catecholamine release.

To the author's knowledge no study to date has investigated the effect of caffeine on human lymphocyte activation at rest either *in vivo/ex vivo* or in response to stimulation *in vitro*. Therefore, the aim of this study was to investigate the effect of a high and low dose of caffeine on unstimulated and antigen-stimulated lymphocyte ($CD4^+$, $CD8^+$ and $CD3^-CD56^+$) activation during 3.5 h rest, as assessed by expression of CD69.

4.3. Methods

Participants and trial protocol

Six healthy participants [mean (SD): age: 25 (2) years; body mass: 75 (7) kg] gave written informed consent to take part in the study, which was approved by Loughborough University Ethical Advisory Committee. Daily caffeine intake amongst participants was varied and ranged from 30 – 400 $mg \cdot day^{-1}$. One participant was characterised as a high user (> 250 $mg \cdot day^{-1}$), 4 as moderate users (50 – 250 $mg \cdot day^{-1}$) and 1 as a light user (< 50 $mg \cdot day^{-1}$). Participants were randomly assigned to either the PLA, 2CAF or 6CAF trial and acted as their own controls in a repeated-measures, single-blind, cross-over design. Each trial was separated by 1 week. Participants arrived at the laboratory at 08:20 h following an overnight fast. Participants then ingested 0, 2 or 6 $mg \cdot kg^{-1}$ body mass of caffeine and rested quietly in the laboratory for 3.5 h, during which time water was consumed *ad libitum*. Venous blood samples were obtained at pre-supplement (0 h) and 1 h (09:30 h), 2.5 h (11:00 h) and 3.5 h (12:00 h) after CAF or PLA ingestion. Laboratory conditions throughout the study were 19.5 (0.4) ℃ and 33.4 (2.9) % relative humidity.

Blood analytical methods

Blood sampling and analysis methods are detailed in Chapter 3.6

Statistical analysis

Statistical methods are detailed in Chapter 3.7

4.4. Results

Serum caffeine concentration at 1 h, 2.5 h and 3.5 h after caffeine ingestion was higher on 6CAF than 2CAF and PLA and higher on 2CAF than PLA (P<0.01; Figure 4.1).

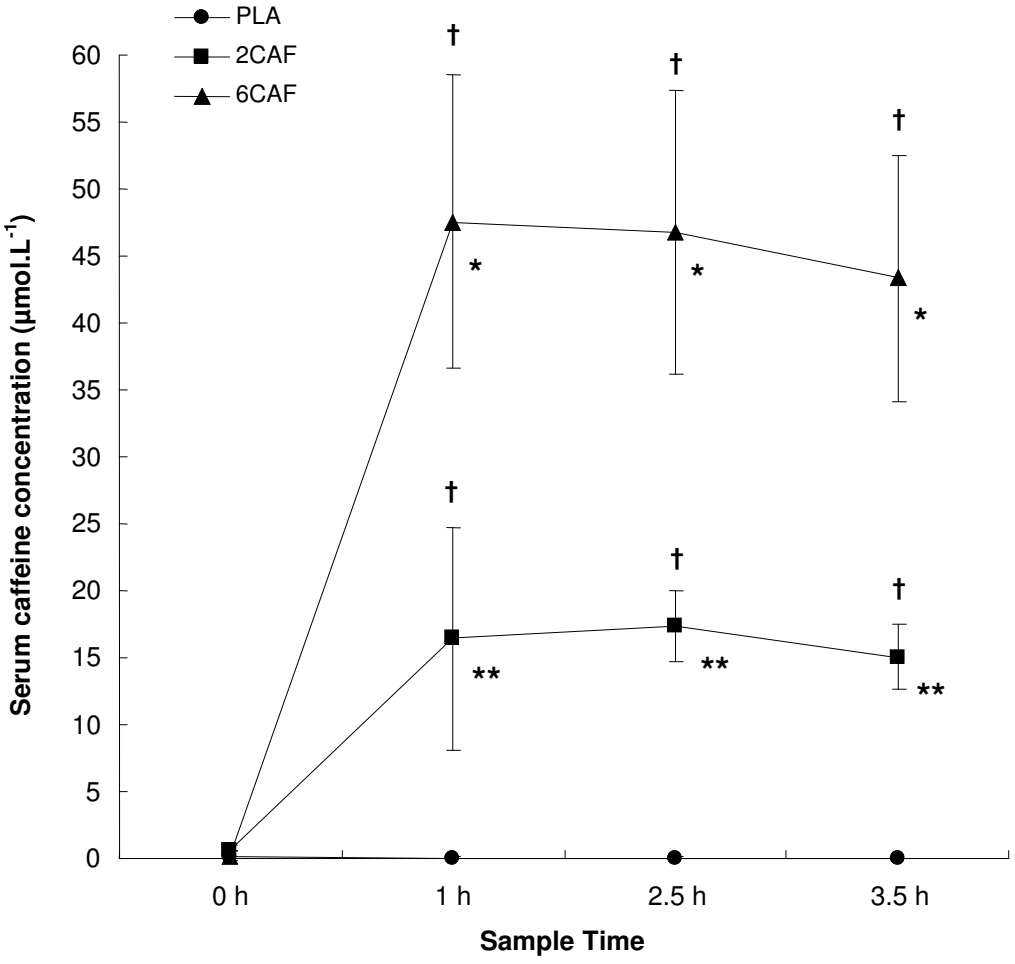


Figure 4.1: Serum caffeine concentrations during PLA, 2CAF and 6CAF trials. * significantly higher on 6CAF than PLA and 2CAF (P<0.01); ** significantly higher on 2CAF than PLA (P<0.01); † significantly higher than pre-supplement within trial (P<0.01). Values are means ± SD.

At 1 h and 2.5 h after caffeine ingestion, plasma adrenaline concentration was significantly higher on 6CAF than PLA (1 h, $P<0.01$; 2.5 h $P<0.05$; Figure 4.2). There were no differences between 2CAF and PLA. Caffeine ingestion did not affect plasma noradrenaline concentrations (Table 4.1). Neither caffeine ingestion nor time of day affected the number of $CD4^+$ and $CD8^+$ cells within the circulating lymphocyte population (Table 4.1). However, the number of $CD3^-$ $CD56^+$ cells was higher 1 h and 2.5 h after caffeine ingestion on 6CAF than on PLA and 2CAF ($P<0.05$; Figure 4.3).

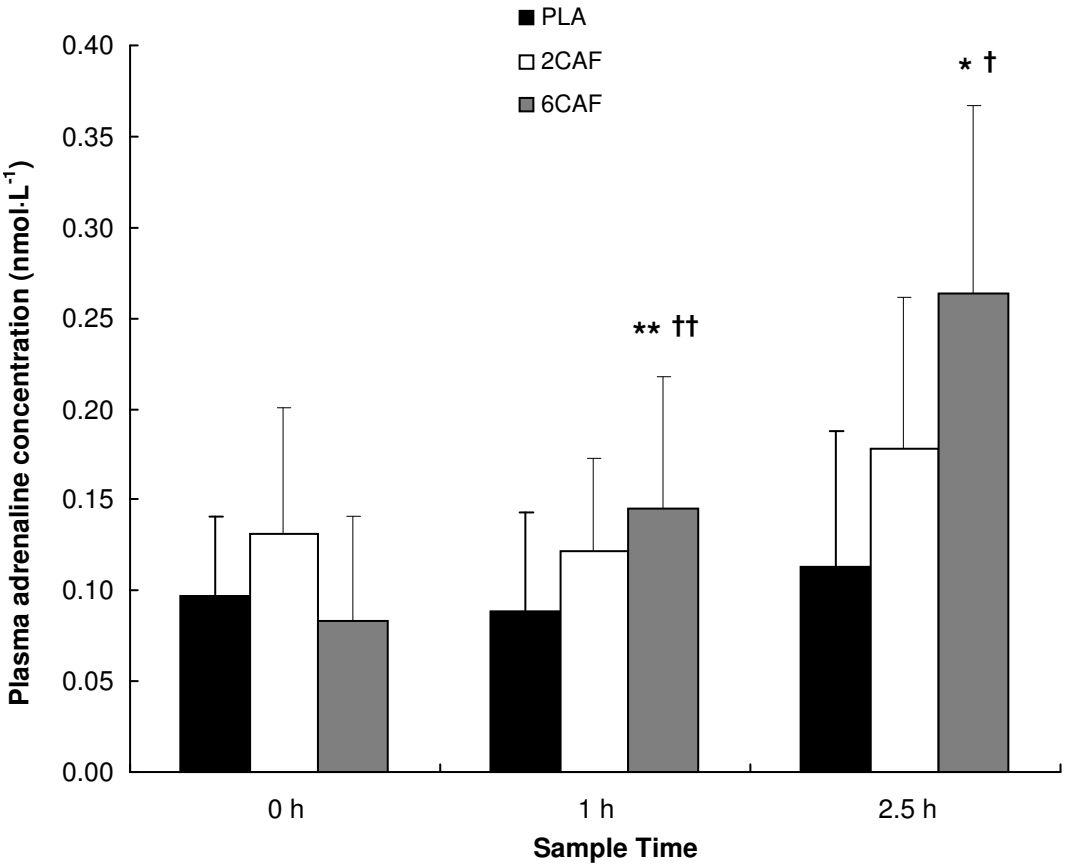


Figure 4.2: Plasma adrenaline concentration during PLA, 2CAF and 6CAF trials. Significantly higher on 6CAF than PLA * $P<0.01$, ** $P<0.05$; Significantly higher than pre-supplement within trial † $P<0.01$, †† $P<0.05$. Values are means + SD.

Table 4.1: Plasma noradrenaline concentration, total lymphocyte count and number of circulating $CD4^+$ and $CD8^+$ cells during PLA, 2CAF and 6CAF trials.

	0 h	1 h	2.5 h	3.5 h
Plasma noradrenaline concentration ($nmol \cdot L^{-1}$) [a]				
PLA	2.01 (0.45)	2.31 (0.47)	2.59 (0.55)	-
2CAF	1.77 (0.30)	2.07 (0.58)	2.29 (0.70)	-
6CAF	1.81 (0.56)	2.27 (0.61)	2.54 (0.69)	-
Total lymphocytes ($x10^9$ $cells \cdot L^{-1}$)				
PLA	1.90 (0.30)	1.70 (0.10)	1.80 (0.20)	1.80 (0.30)
2CAF	1.80 (0.40)	1.60 (0.40)	1.70 (0.30)	1.80 (0.30)
6CAF	1.90 (0.30)	2.00 (0.30)	2.00 (0.30)	1.80 (0.10)
$CD4^+$ cells ($x10^9$ $cells \cdot L^{-1}$)				
PLA	0.77 (0.23)	0.70 (0.11)	0.68 (0.13)	0.67 (0.11)
2CAF	0.66 (0.19)	0.62 (0.16)	0.62 (0.15)	0.71 (0.14)
6CAF	0.66 (0.15)	0.62 (0.13)	0.58 (0.19)	0.64 (0.08)
$CD8^+$ cells ($x10^9$ $cells \cdot L^{-1}$)				
PLA	0.58 (0.10)	0.50 (0.09)	0.51 (0.09)	0.52 (0.13)
2CAF	0.54 (0.13)	0.50 (0.14)	0.50 (0.11)	0.54 (0.10)
6CAF	0.50 (0.10)	0.60 (0.25)	0.50 (0.23)	0.49 (0.13)

Values are mean (SD).
[a] Main effect for time: significantly higher than 0 h at 1 h and 2.5 h, $P<0.01$.

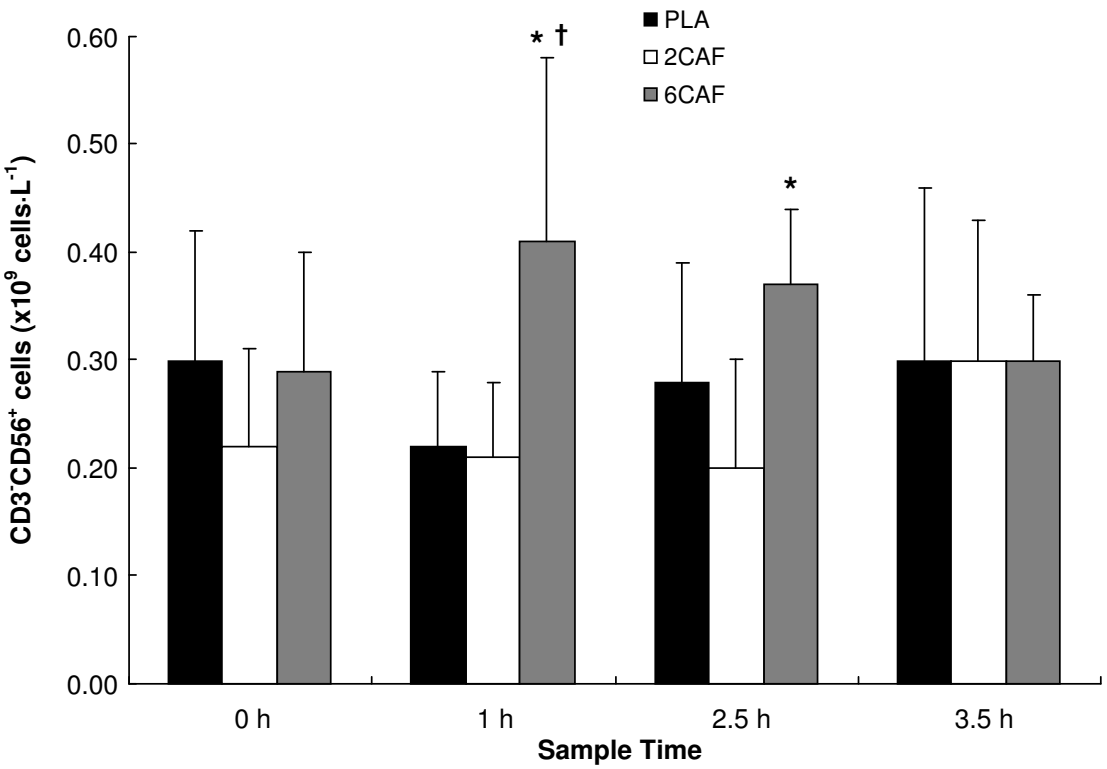


Figure 4.3: Number of circulating $CD3^{-}CD56^{+}$ cells within the lymphocyte population during PLA, 2CAF and 6CAF trials. * significantly higher on 6CAF than PLA and 2CAF ($P<0.05$); † significantly higher than pre-supplement within trial ($P<0.05$). Values are means + SD.

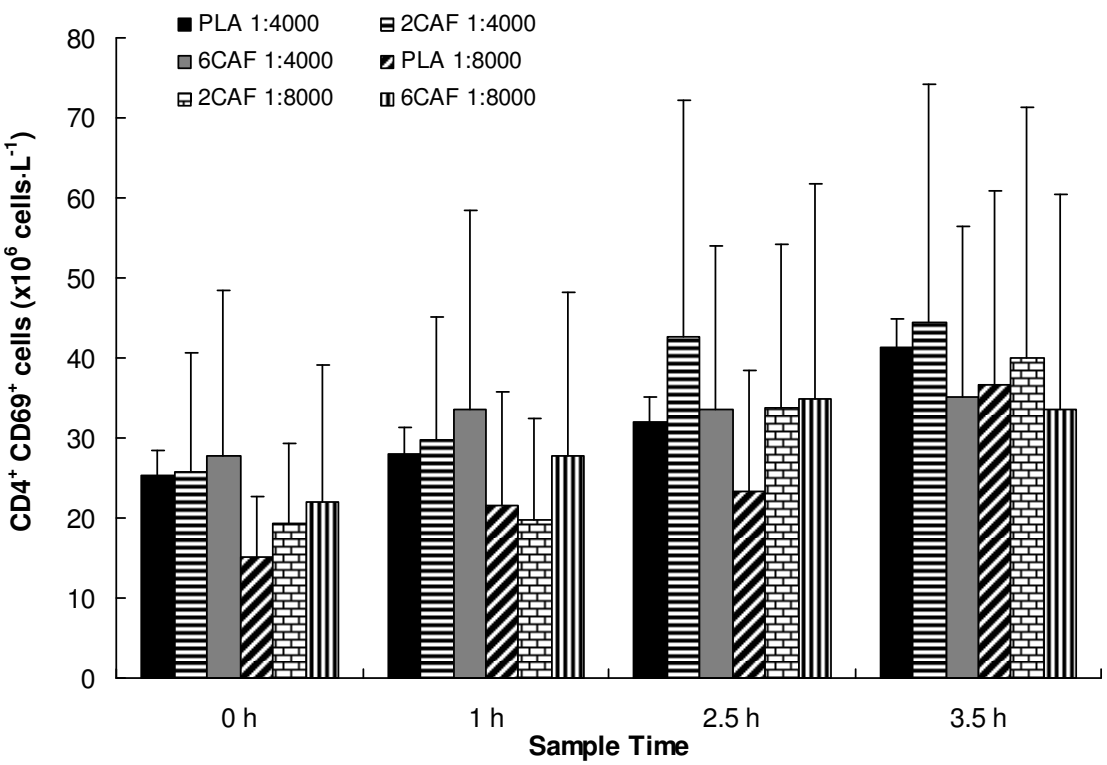
There was no effect of caffeine on the number of unstimulated $CD4^{+}$, $CD8^{+}$ and $CD3^{-}CD56^{+}$ cells or antigen-stimulated (both doses) $CD4^{+}$ and $CD8^{+}$ cells expressing CD69 (unstimulated, Table 4.2; antigen-stimulated, Figure 4.4a & b) Although, diurnal effects were evident for each of these antigen-stimulated (1:8000) measures (main effect for time; $P<0.01$ for all). However, a high dose of caffeine increased the number of antigen-stimulated $CD3^{-}CD56^{+}$ cells expressing CD69 1 h following caffeine ingestion compared with PLA (1:4000, $P<0.05$; Figure 4.4c) and a low dose of caffeine (1:8000, $P<0.05$; Figure 4.4c). Neither caffeine ingestion nor time of day affected the GMFI of CD69 expression on unstimulated (Table 4.2) or antigen-stimulated (both optimal and suboptimal doses) $CD4^{+}$, $CD8^{+}$ and $CD3^{-}$ $CD56^{+}$ cells (Figure 4.5a, b & c).

Table 4.2: Number of circulating unstimulated $CD4^+$, $CD8^+$ and $CD3^-CD56^+$ cells expressing CD69 and the GMFI expression of CD69 during PLA, 2CAF and 6CAF trials.

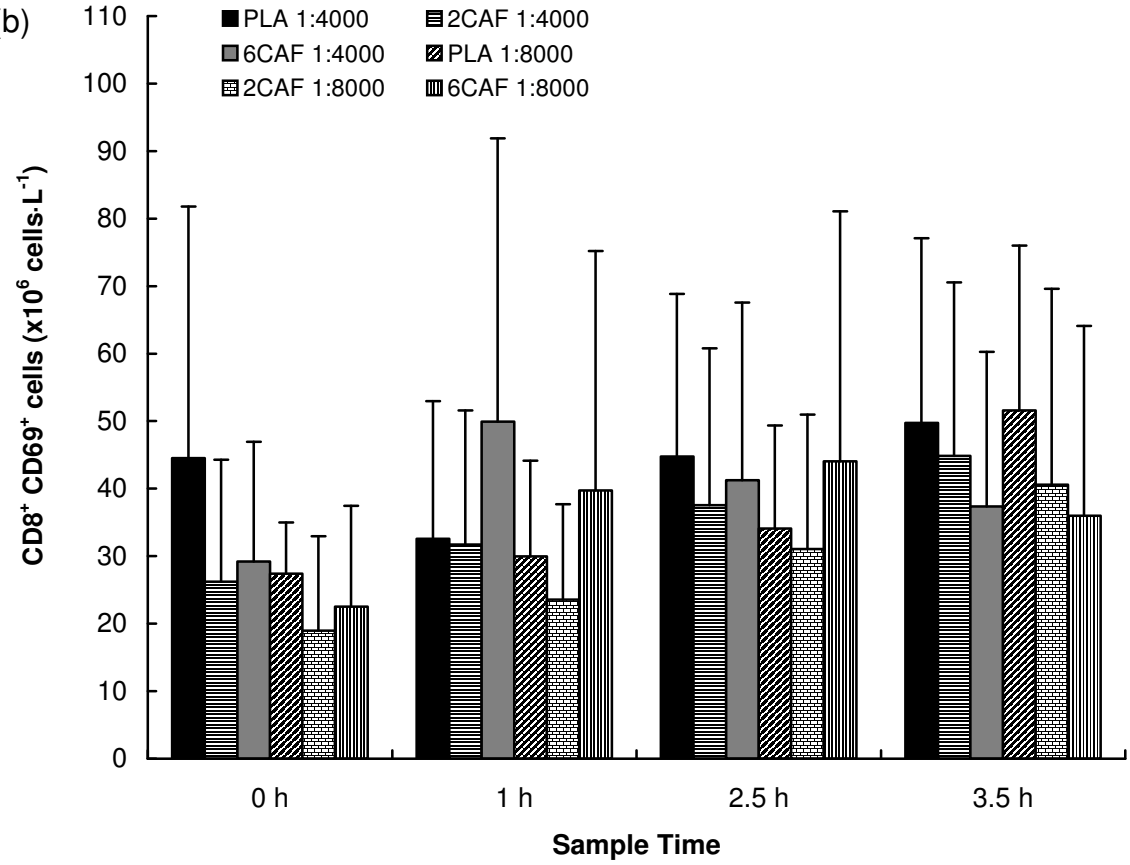
	0 h	1 h	2.5 h	3.5 h
$CD4^+$ $CD69^+$ cells ($x10^6$ cells·L^{-1})				
PLA	5.7 (3.0)	6.1 (2.4)	6.8 (4.3)	11.7 (3.7)
2CAF	9.6 (6.1)	8.9 (6.0)	12.2 (7.0)	13.3 (12.7)
6CAF	13.8 (6.6)	15.9 (8.4)	11.7 (8.6)	13.8 (8.1)
$CD8^+$ $CD69^+$ cells ($x10^6$ cells·L^{-1})				
PLA	20.2 (12.7)	19.2 (15.0)	19.4 (11.2)	28.1 (18.3)
2CAF	13.6 (12.2)	13.7 (11.0)	15.5 (13.1)	16.5 (10.0)
6CAF	19.2 (12.8)	23.6 (16.3)	20.0 (13.6)	18.5 (11.5)
$CD3^-CD56^+$ $CD69^+$ cells ($x10^6$ cells·L^{-1})				
PLA	27.3 (27.4)	16.7 (13.5)	24.0 (19.0)	35.4 (27.2)
2CAF	25.6 (25.9)	28.5 (31.8)	28.7 (36.1)	30.2 (34.9)
6CAF	54.3 (53.5)	23.6 (45.4)	55.2 (45.2)	33.8 (24.7)
$CD4^+$ $CD69^+$ GMFI				
PLA	100 (0)	99 (13)	91 (23)	100 (19)
2CAF	100 (0)	123 (68)	99 (32)	112 (31)
6CAF	100 (0)	90 (16)	98 (18)	80 (18)
$CD8^+$ $CD69^+$ GMFI				
PLA	100 (0)	100 (32)	106 (26)	95 (23)
2CAF	100 (0)	113 (33)	103 (25)	104 (24)
6CAF	100 (0)	100 (24)	104 (31)	108 (15)
$CD3^-CD56^+$ $CD69^+$ GMFI				
PLA	100 (0)	97 (11)	103 (17)	100 (17)
2CAF	100 (0)	115 (30)	104 (22)	111 (28)
6CAF	100 (0)	100 (11)	112 (30)	109 (23)

Values are mean (SD). The GMFI expressions are expressed as a percentage relative to pre-supplement value.

(a)


PLA 1:4000
2CAF 1:4000
6CAF 1:4000
PLA 1:8000
2CAF 1:8000
6CAF 1:8000
CD4+ CD69+ cells (x10^6 cells·L^-1)
0
10
20
30
40
50
60
70
80
0 h
1 h
2.5 h
3.5 h
Sample Time

(b)


PLA 1:4000
2CAF 1:4000
6CAF 1:4000
PLA 1:8000
2CAF 1:8000
6CAF 1:8000
CD8+ CD69+ cells (x10^6 cells·L^-1)
0
10
20
30
40
50
60
70
80
90
100
110
0 h
1 h
2.5 h
3.5 h
Sample Time

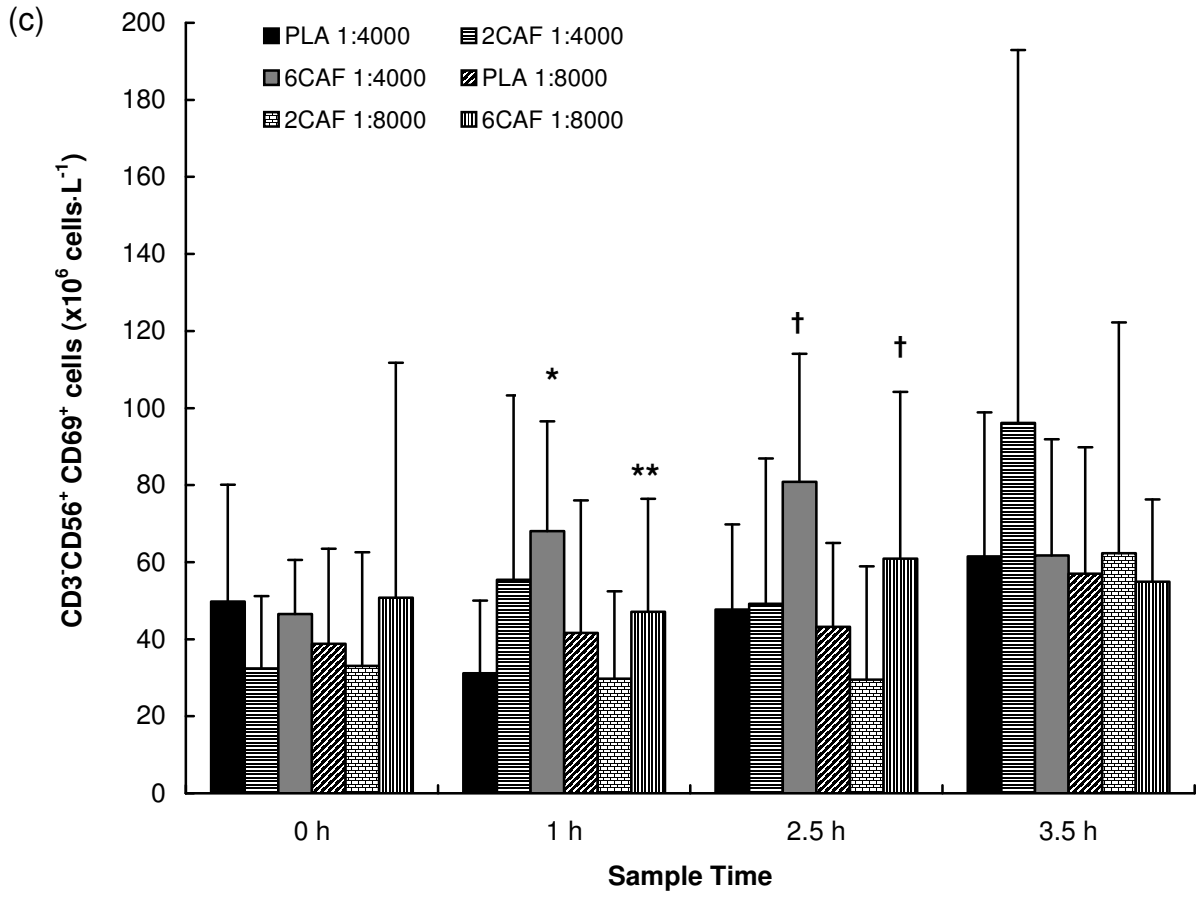
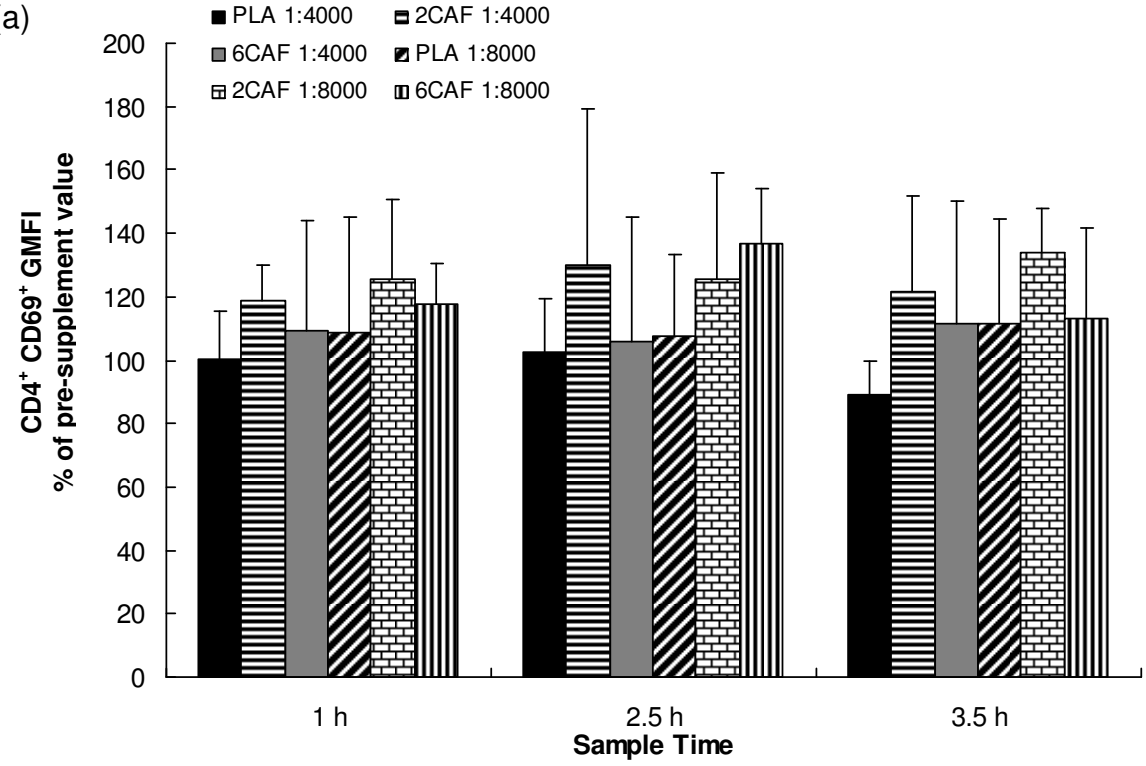
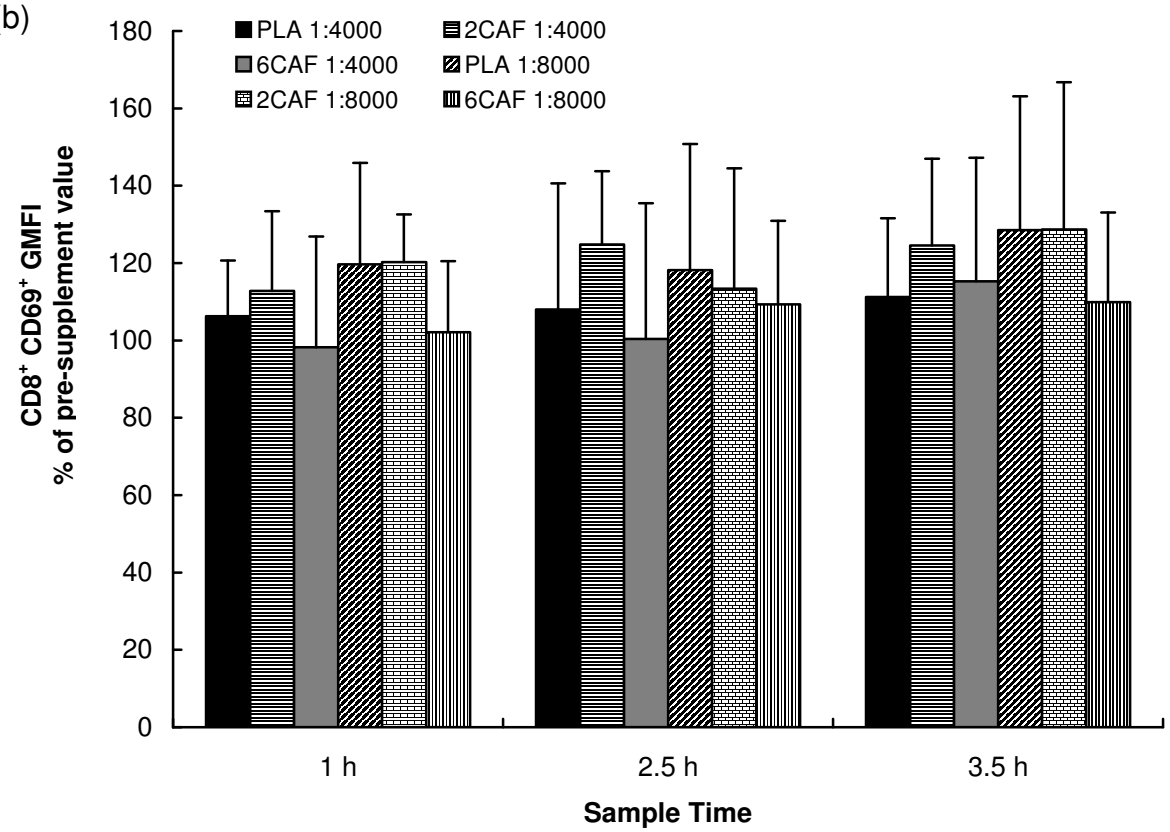


Figure 4.4: Number of antigen-stimulated (**a**) $CD4^+$ T cells, (**b**) $CD8^+$ T cells and (**c**) $CD3^-CD56^+$ NK cells expressing CD69 within the circulating lymphocyte population during PLA, 2CAF and 6CAF trials. * significantly higher on 6CAF than PLA ($P<0.05$); ** significantly higher on 6CAF than 2CAF ($P<0.05$); † significantly higher than pre-supplement within trial ($P<0.05$). Values are means + SD.

(a)


PLA 1:4000
2CAF 1:4000
6CAF 1:4000
PLA 1:8000
2CAF 1:8000
6CAF 1:8000
CD4+ CD69+ GMFI
% of pre-supplement value
200
180
160
140
120
100
80
60
40
20
0
1 h
2.5 h
3.5 h
Sample Time

(b)


PLA 1:4000
2CAF 1:4000
6CAF 1:4000
PLA 1:8000
2CAF 1:8000
6CAF 1:8000
CD8+ CD69+ GMFI
% of pre-supplement value
180
160
140
120
100
80
60
40
20
0
1 h
2.5 h
3.5 h
Sample Time

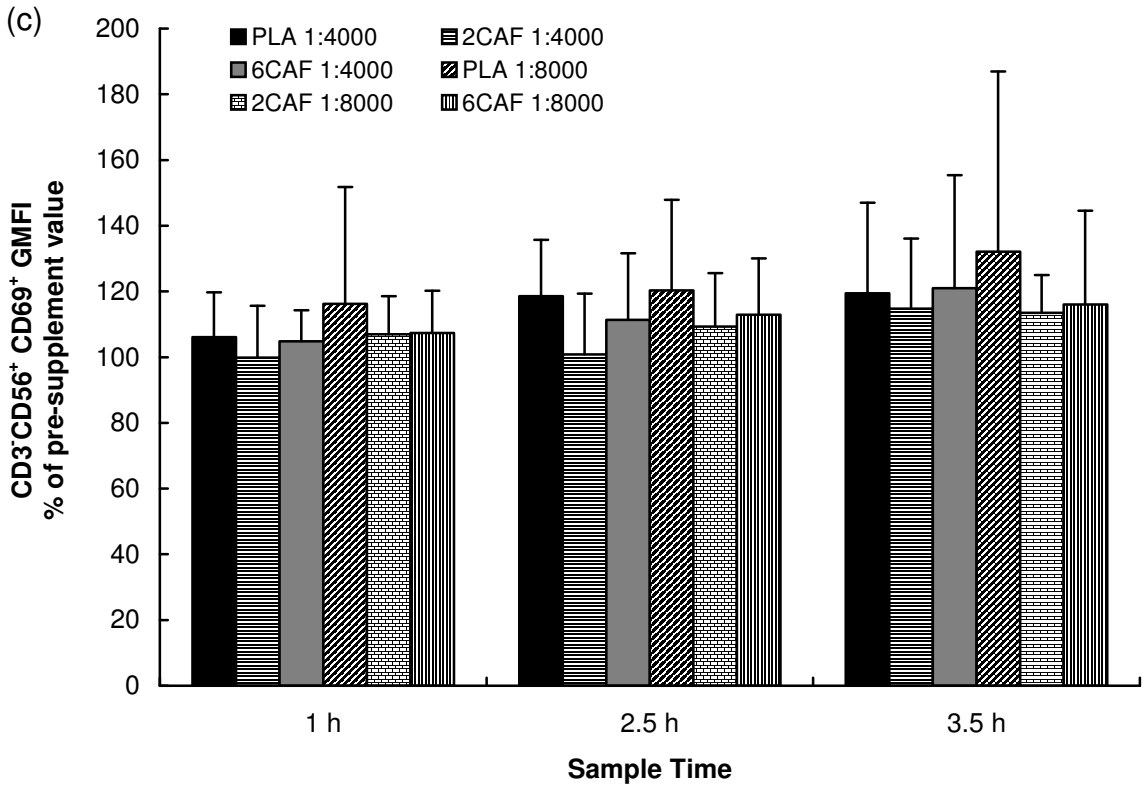


Figure 4.5: Geometric Mean Fluorescence Intensity of CD69 expression on antigen-stimulated (**a**) $CD4^+$ T cells, (**b**) $CD8^+$ T cells and (**c**) $CD3^-CD56^+$ NK cells within the circulating lymphocyte population during PLA, 2CAF and 6CAF trials. Values are means + SD and are expressed as a percentage relative to 0 h (pre-supplement) value.

4.5. Discussion

The main findings of this study suggest that (i) ingesting a high (6 $mg \cdot kg^{-1}$), but not a low (2 $mg \cdot kg^{-1}$) dose of caffeine mobilises $CD3^-CD56^+$ cells into the peripheral circulation 1 h and 2.5 h following caffeine ingestion, when compared with placebo, (ii) a high dose of caffeine also increases the number of antigen-stimulated $CD3^-CD56^+$ cells expressing CD69 1 h following caffeine ingestion compared with both placebo and a low dose of caffeine, (iii) ingesting high and low doses of caffeine has little influence on antigen-stimulated T ($CD4^+$ and $CD8^+$) cell activation, as assessed by the number of cells expressing CD69 or on antigen-stimulated T ($CD4^+$ and $CD8^+$) and NK ($CD3^-CD56^+$) cell activation on a per cell basis, as assessed by the GMFI expression of CD69.

The increased number of NK cells within the circulation 1 h and 2.5 h after ingesting 6 $mg \cdot kg^{-1}$ caffeine (but not after ingesting 2 $mg \cdot kg^{-1}$ caffeine or placebo) may be associated with the increase in plasma adrenaline concentration seen at these time-points. Adrenaline is known to alter adhesion molecules on lymphocytes via stimulation of their β_2-adrenorecptors, leading to an increase in intracellular cAMP (Shephard, 2003), which in turn is thought to be responsible for interfering with signals necessary for adhesion strengthening (Benschop et al., 1997). Lymphocytes express a high density of homogenous β_2-adrenoreceptors: $CD8^+$ T cells possess 2-3 times more functional β_2-receptors than $CD4^+$ T cells, while NK cells possess nearly 50% more β_2-receptors than $CD8^+$ T cells (Murray et al., 1992). This may explain why in the present study only NK cells appeared to be responsive to the effects of a high dose of caffeine. Furthermore, only a high dose of caffeine appeared to increase the number of activated NK cells in response to antigen stimulation compared with placebo or the lower dose of caffeine. Therefore, it may be that increases in plasma adrenaline concentration could have led to a preferential mobilisation of a specific population of NK cells with an increased propensity for activation when stimulated into the peripheral circulation.

In the present study both high and low doses of caffeine had little effect on antigen-stimulated T and NK cell activation on a per cell basis (as assessed by the GMFI expression of CD69). This is in contrast to several reports that caffeine at physiological concentrations decreases mitogen/antigen stimulated lymphocyte proliferation, antibody production and cytokine production (Kantamala et al., 1990; Rosenthal et al., 1992). However, these studies used rodent models to investigate the possible effects of caffeine. This in itself raises issues when trying to compare these results to those from human studies as caffeine metabolism and plasma half-life (plasma half life in rodents: 0.7-1.2 h vs. humans: 4-6 h) between rodents and humans differs significantly (Fredholm et al., 1999). The major metabolic difference between species is that in the rat more than 40% of caffeine metabolites are trimethyl derivatives as compared with less than 6% in humans (Arnaud, 1985).

At physiological concentrations, it is thought that caffeine's main mechanism of action is via adenosine receptor antagonism (Fredholm et al., 1999). However, under physiological conditions, caffeine will only actively compete as an adenosine receptor antagonist when the receptors are engaged by endogenous adenosine (Yang et al., 2009). As such, it could be suggested that in the present study caffeine (regardless of dose) had little effect on antigen-stimulated lymphocyte activation (GMFI expression of CD69) in resting participants due to plasma adenosine concentrations being too low to engage caffeine as an adenosine receptor antagonist. It should be noted that plasma adenosine concentration was not measured in this study and as such this explanation is only speculation. However, support for this speculation comes from the exercise literature where prolonged intensive exercise, which is known to markedly increase plasma adenosine concentrations (Vizi et al., 2002), increased the number of unstimulated $CD4^+$ and $CD8^+$ T cells expressing CD69 (Bishop et al., 2005a).

In summary, this is the first study in humans to determine that a high 6 $mg \cdot kg^{-1}$ (but not a low 2 $mg \cdot kg^{-1}$) dose of caffeine is associated with the mobilisation of $CD3^-CD56^+$ cells into the peripheral circulation 1h and 2.5 h following ingestion, and that in response to antigen stimulation a higher number of these mobilised cells express the early activation marker, CD69. In contrast, caffeine ingestion (regardless of dose) has little effect on T cell trafficking or on antigen-stimulated T and NK cell activation on a per cell basis in humans. Therefore, these findings indicate that caffeine ingestion does not appear to compromise host responses to antigenic challenge and in fact a high dose of caffeine may even be protective 1 h following caffeine ingestion. These findings are in contrast to previously published studies in the animal model and as such suggest that perhaps using animals to investigate the effects of caffeine on human immune functions may not be a reliable model.

Chapter 5

Caffeine ingestion and antigen-stimulated human lymphocyte activation after prolonged cycling

5.1. Abstract

This study investigated the effect of caffeine ingestion on antigen-stimulated T ($CD4^+$ and $CD8^+$) and natural killer ($CD3^-CD56^+$) cell activation after prolonged, strenuous cycling. In a randomised cross-over design, 9 male endurance cyclists [age: 22 (3) years, $\dot{V}O_{2peak}$: 62 (4) ml·kg^{-1}·min^{-1}, mean ± SD] cycled for 90 min at 70% $\dot{V}O_{2peak}$ 60 min after ingesting 6 mg·kg^{-1} body mass of caffeine (CAF) or placebo (PLA). Venous blood samples were obtained before supplementation, pre-exercise, immediately post-exercise and 1 h post-exercise. Whole blood was stimulated with Pediacel (5 in 1) vaccine. At 1 h post-exercise the number of antigen-stimulated $CD4^+$ cells expressing CD69 decreased on CAF compared with PLA [15 (17) x10^6 cells·L^{-1} vs. 23 (22) x10^6 cells·L^{-1}, $P<0.05$]. In addition, the GMFI of CD69 expression on antigen-stimulated $CD8^+$ cells decreased on CAF compared with PLA 1 h post-exercise [78 (10)% vs. 102 (24)%, $P<0.05$]. At the same time-point GMFI of CD69 expression on antigen-stimulated $CD3^-CD56^+$ cells was increased on CAF compared with PLA [103 (9)% vs. 87 (8)%, $P<0.05$]. These findings suggest that caffeine reduces antigen-stimulated CD69 expression on T cells while at the same time increases NK cell activation 1 h after intensive cycling.

5.2. Introduction

Caffeine is a common element in most athletes' diets, yet it is not a typical nutrient nor is it essential for health (Chester and Wojek, 2008). Many athletes consume caffeine for its known ergogenic properties, which have been extensively documented (Goldstein et al., 2010). Since being legitimised by its 2004 removal from the WADA prohibited list of substances, caffeine's ability to enhance athletic performance during training and/or competition has led to its widespread use amongst this population, with use by cyclists particularly common (Chester and Wojek, 2008).

Caffeine is a non-selective adenosine receptor antagonist and stimulates adrenaline release from the adrenal medulla. Increased concentrations of adenosine and adrenaline have been shown to inhibit T and NK cell function *in vitro* and *in vivo* (Kappel et al., 1991; Murray et al., 1992; Huang et al., 1997), via activation of adenylate cyclase and increased intracellular cAMP (Grader-Beck et al., 2003; Raskovalova et al., 2006). Strenuous exercise increases the plasma concentrations of both adenosine and adrenaline (Hartley et al., 1972; Vizi et al., 2002) and adrenaline in particular has been suggested to be an important influence on the temporary perturbations in circulating T and NK cell numbers and functions observed following intensive exercise (Hoffman-Goetz and Pedersen, 1994). These perturbations are thought to partly account for the apparent higher incidence of viral respiratory infection experienced by some elite athletes (Gleeson, 2007). It follows, therefore, that ingesting caffeine before strenuous exercise may exacerbate the exercise-associated cellular influences of adenosine and adrenaline and in this way influence subsequent T and NK cell functions.

Despite the reported widespread use of caffeine by many athletes to enhance performance, few published studies have investigated caffeine's effects on various aspects of the immune system after high intensity exercise (Bishop et al., 2005a, 2006b; Walker et al., 2006, 2007, 2008). Bishop et al. (2005a) have previously demonstrated an increase in numbers of circulating $CD4^+$ and $CD8^+$ cells expressing CD69 1 h after consuming 6 $mg \cdot kg^{-1}$ body mass caffeine and

also immediately after cycling for 90 min at 70% $\dot{V}O_{2peak}$, compared with placebo. However, these findings may simply reflect an adrenaline-mediated redistribution of cells and do not provide information regarding the effect of caffeine on T cell activation to an antigenic challenge. Furthermore, to the author's knowledge no study to date has investigated the effect of caffeine on any aspect of antigen-stimulated NK cell number or function following a bout of intensive exercise. Taking this information together with caffeine's widespread use amongst the athletic population emphasises a need to understand the effect that caffeine may have on an athlete's immune system and subsequent risk of infection following high intensity exercise. Therefore, the aim of this study was to investigate the effects of prior caffeine ingestion on antigen-stimulated T ($CD4^+$ and $CD8^+$) and NK ($CD3^-CD56^+$) cell activation following 90 min cycling at 70% $\dot{V}O_{2peak}$, as assessed by expression of the early activation marker CD69.

5.3. Methods

Participants

Nine endurance trained male cyclists [age 22 (3) years; body mass 75 (4) kg; $\dot{V}O_{2peak}$ 62 (4) ml·kg^{-1}·min^{-1}; peak power output 354 (39) W] 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. Loughborough University Ethical Advisory Committee approved the study. Daily caffeine intake amongst participants was varied and ranged from 30 – 506 mg·day^{-1}. Two participants were characterised as high users (> 250 mg·day^{-1}), 4 as moderate users (50 – 250 mg·day^{-1}) and 3 as light users (< 50 mg·day^{-1}).

Experimental procedures

Approximately 2 weeks before the beginning of the main trials, each participant was required to undertake preliminary measurements including a $\dot{V}O_{2peak}$ test and a familiarisation of the trial protocol. Preliminary measurements and pre-trial standardisation procedures are described in Chapter 3. Participants performed 2 main exercise trials separated by 1 week. Participants were

randomly assigned to either the caffeine (CAF) or placebo (PLA) trial and acted as their own controls in a repeated-measures, single-blind, cross-over design. Participants arrived at the laboratory at 08:20 h following an overnight fast. Participants then ingested 6 mg·kg^{-1} body mass of either CAF or PLA and rested quietly in the laboratory for 1 h. Immediately after this, participants began cycling at a work rate equivalent to 70% $\dot{V}O_{2peak}$ [235 (29) W]. Venous blood samples were obtained at pre-supplement, 1 h after CAF or PLA ingestion (pre-exercise), immediately post-exercise and 1 h post-exercise. Laboratory conditions throughout the study were 20.5 (1.3) ℃ and 40.9 (4.5) % relative humidity.

Blood analytical methods

Blood sampling and analysis methods are detailed in Chapter 3.6

Statistical analysis

Statistical methods are detailed in Chapter 3.7

5.4. Results

Exercise intensity, heart rate and indirect calorimetry

Mean exercise intensity did not differ between trials [CAF: 72.8% (3.1) PLA: 72.5% (2.9)]. Heart rates were comparable between trials during exercise [CAF: 161 (9) beat·min^{-1}, PLA: 156 (11) beat·min^{-1}; mean of all recordings]. Respiratory exchange ratio (RER) and fat and CHO oxidation rates were similar between trials throughout exercise [CAF: 0.95 (0.01), 0.40 (0.20) g·min^{-1}, 3.10 (0.60) g·min^{-1}, PLA: 0.95 (0.01), 0.40 (0.30) g·min^{-1}, 3.00 (0.80) g·min^{-1}, for RER, fat and CHO oxidation rates, respectively]. Likewise, energy expenditure (EE) and the percentage contribution of fat and CHO did not differ between trials [EE: 67 (6) KJ·min^{-1} for both CAF and PLA, contribution of fat: 20.6 (14.1) % and 25.3 (15.1) %, contribution of CHO: 79.4 (14.1) % and 74.7 (15.1) % on CAF and PLA, respectively].

Changes in body mass and plasma volume

After exercise, change in body mass (corrected for fluid intake) was similar for both trials [CAF: -1.7 (0.3) kg, PLA: -1.6 (0.3) 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 9.5 (3.1)% and 9.9 (6.2)% on the CAF and PLA trials, respectively.

Serum caffeine and plasma catecholamines

A significant time x trial interaction was found for serum caffeine concentration ($P<0.01$), with higher concentrations at pre, post and 1 h post-exercise on CAF compared with PLA ($P<0.01$; Table 5.1). A significant time x trial interaction was found for plasma adrenaline concentration, with values significantly higher on CAF than PLA post-exercise ($P<0.05$; Table 5.1). A significant main effect of time was found for plasma noradrenaline concentration, with values elevated above pre-supplement levels at post-exercise ($P<0.01$; Table 5.1).

Table 5.1: Serum caffeine, plasma adrenaline, noradrenaline concentrations, total lymphocyte count and number of circulating $CD4^+$, $CD8^+$ and $CD3^-CD56^+$ cells during CAF and PLA trials.

	Pre-supplement	Pre-exercise	Post-exercise	1 h post-exercise
Serum caffeine concentration ($\mu mol \cdot L^{-1}$)				
CAF	0 (0)	52 (15) * †	60 (17) * †	48 (12) * †
PLA	0 (0)	0 (0)	0 (0)	0 (0)
Plasma adrenaline concentration ($nmol \cdot L^{-1}$)				
CAF	0.32 (0.23)	0.42 (0.24)	1.49 (0.69) ** ††	-
PLA	0.30 (0.18)	0.24 (0.15)	0.69 (0.25) ††	-
Plasma noradrenaline concentration ($nmol \cdot L^{-1}$) [c]				
CAF	1.97 (0.91)	2.31 (1.28)	8.45 (2.89)	-
PLA	2.28 (1.02)	2.40 (0.87)	6.76 (1.58)	-
Total lymphocytes ($x10^9$ cells$\cdot L^{-1}$) [a,b,d]				
CAF	2.00 (0.60)	1.80 (0.40)	2.50 (1.30)	1.30 (0.50)
PLA	2.00 (0.70)	1.70 (0.50)	2.30 (0.80)	1.40 (0.40)
$CD4^+$ cells ($x10^9$ cells$\cdot L^{-1}$) [a,d]				
CAF	0.68 (0.26)	0.57 (0.20)	0.65 (0.39)	0.45 (0.18)
PLA	0.72 (0.35)	0.57 (0.21)	0.62 (0.27)	0.49 (0.17)
$CD8^+$ cells ($x10^9$ cells$\cdot L^{-1}$) [c,d]				
CAF	0.59 (0.27)	0.56 (0.25)	0.93 (0.66)	0.37 (0.19)
PLA	0.61 (0.31)	0.51 (0.28)	0.80 (0.41)	0.40 (0.18)
$CD3^-CD56^+$ cells ($x10^9$ cells$\cdot L^{-1}$)				
CAF	0.34 (0.16)	0.38 (0.16) **	0.63 (0.43) ** †	0.27 (0.17) ††
PLA	0.36 (0.26)	0.29 (0.19)	0.53 (0.35) ††	0.20 (0.14) †

Values are mean (SD). n = 7 for adrenaline and noradrenaline.
* P<0.01, ** P<0.05: significantly higher on CAF than PLA.
† P<0.01, †† P<0.05: significantly different than pre-supplement within trial.
Main effect for time: significantly lower than pre-supplement at pre-exercise, [a] P<0.01.
Main effect for time: significantly different than pre-supplement at post-exercise,
[b] P<0.05, [c] P<0.01.
Main effect for time: significantly lower than pre-supplement at 1 h post-exercise, [d] P<0.01, [e] P<0.05.

Circulating lymphocyte counts and numbers of $CD4^+$, $CD8^+$ and $CD3^-CD56^+$ cells

No significant time x trial interaction was found for number of circulating lymphocytes but there was a significant main effect for time (P<0.01) with the circulating number of lymphocytes following a biphasic response to exercise (Table 5.1). A significant time x trial interaction was found for the number of

$CD3^-CD56^+$ cells within the circulating lymphocyte population, with values higher at pre-exercise and post-exercise on CAF compared with PLA ($P<0.05$; Table 5.1). Although there were no significant time x trial interactions for the number of $CD4^+$ or $CD8^+$ cells within the circulating lymphocyte population, there was a significant main effect for time (both $P<0.01$; Table 5.1). The number of $CD4^+$ cells decreased at pre-exercise and 1 h post-exercise when compared to pre-supplement numbers ($P<0.01$), while $CD8^+$ cells increased at post-exercise, only to fall significantly below pre-supplement values 1 h later ($P<0.01$).

Number of unstimulated and antigen-stimulated $CD4^+$, $CD8^+$ and $CD3^-CD56^+$ cells expressing CD69

There were no time x trial interaction effects for the number of unstimulated $CD4^+$, $CD8^+$ or $CD3^-CD56^+$ cells expressing CD69. However, the number of unstimulated $CD4^+$ cells expressing CD69 increased significantly above pre-supplement values at immediately post-exercise, while the number of $CD3^-$ $CD56^+$ cells expressing CD69 decreased below pre-supplement values at 1 h post-exercise (main effect for time only; $P<0.01$; Table 5.2). When cells were stimulated, a significant time x trial interaction was found for the number of antigen-stimulated (1:4000) $CD4^+$ cells expressing CD69, with values significantly lower on CAF than PLA at 1 h post-exercise ($P<0.05$; Table 5.2). There were no significant time x trial interaction effects for the number of antigen-stimulated (1:4000 or 1:8000) $CD8^+$ or $CD3^-CD56^+$ cells expressing CD69 (Table 5.2), although the number of antigen-stimulated (both doses) $CD3^-$ $CD56^+$ cells did appear to fall significantly below pre-supplement values 1 h following exercise (main effect for time only; both $P<0.01$; Table 5.2).

Table 5.2: Number of unstimulated and antigen-stimulated $CD4^+$, $CD8^+$ and $CD3^-CD56^+$ cells expressing CD69 during CAF and PLA trials.

	Pre-supplement	Pre-exercise	Post-exercise	1 h post-exercise
$CD4^+$ $CD69^+$ cells ($x10^6$ cells·L^{-1}) [a]				
unstim CAF	11.8 (14.5)	7.7 (8.0)	13.5 (15.0)	6.8 (7.1)
unstim PLA	14.0 (20.1)	12.5 (16.0)	17.3 (17.1)	10.1 (7.4)
1:4000 CAF	35.6 (29.5)	32.0 (20.9)	32.4 (28.0)	15.4 (16.5) * †
1:4000 PLA	40.3 (36.9)	29.5 (21.0)	22.4 (13.2)	23.5 (22.3)
1:8000 CAF	28.7 (22.3)	26.8 (22.8)	31.9 (33.2)	13.1 (14.0)
1:8000 PLA	33.7 (32.2)	27.4 (20.7)	23.1 (15.9)	16.9 (16.4)
$CD8^+$ $CD69^+$ cells ($x10^6$ cells·L^{-1})				
unstim CAF	47.4 (21.3)	52.0 (23.2)	91.8 (69.4)	27.2 (8.7)
unstim PLA	63.7 (60.0)	58.9 (53.9)	91.7 (70.4)	42.4 (39.4)
1:4000 CAF	68.8 (59.7)	75.7 (64.7)	101.2 (65.0)	30.8 (12.3)
1:4000 PLA	87.0 (79.2)	76.6 (67.6)	102.1 (83.5)	51.2 (46.6)
1:8000 CAF	62.5 (48.3)	82.8 (54.7)	115.8 (69.1)	37.1 (21.3)
1:8000 PLA	90.5 (80.6)	70.8 (72.6)	98.9 (89.2)	44.3 (49.4)
$CD3^-CD56^+$ $CD69^+$ cells ($x10^6$ cells·L^{-1}) [b]				
unstim CAF	38.5 (46.1)	21.4 (15.0)	45.5 (57.1)	13.1 (15.1)
unstim PLA	35.9 (30.2)	27.7 (26.6)	38.0 (38.4)	14.7 (17.3)
1:4000 CAF	54.8 (56.2)	61.4 (58.1)	81.9 (28.0)	28.0 (24.2)
1:4000 PLA	75.4 (81.2)	41.0 (27.4)	60.4 (39.4)	30.6 (28.7)
1:8000 CAF	60.0 (53.6)	67.0 (58.6)	61.5 (46.6)	25.0 (26.8)
1:8000 PLA	63.8 (71.1)	45.7 (37.0)	53.6 (47.9)	20.9 (17.7)

Values are mean (SD).
* $P<0.05$, significantly lower on CAF than PLA.
† $P<0.01$: significantly different from pre-supplement within trial.
[a] Main effect for time on unstim cells: significantly higher than pre-supplement at post-exercise, $P<0.01$.
[b] Main effect for time: significantly lower than pre-supplement at 1 h post-exercise, $P<0.01$.

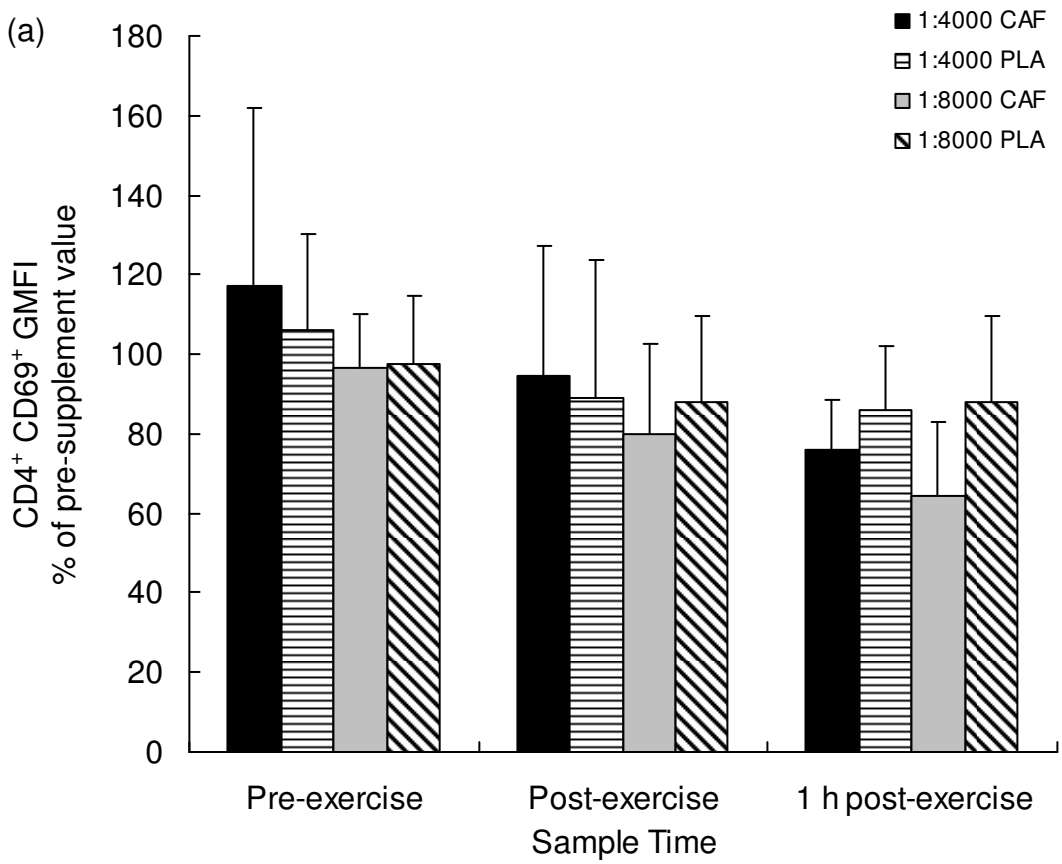
Geometric Mean Fluorescence Intensity of CD69 expression on unstimulated and antigen-stimulated $CD4^+$, $CD8^+$ and $CD3^-CD56^+$ cells

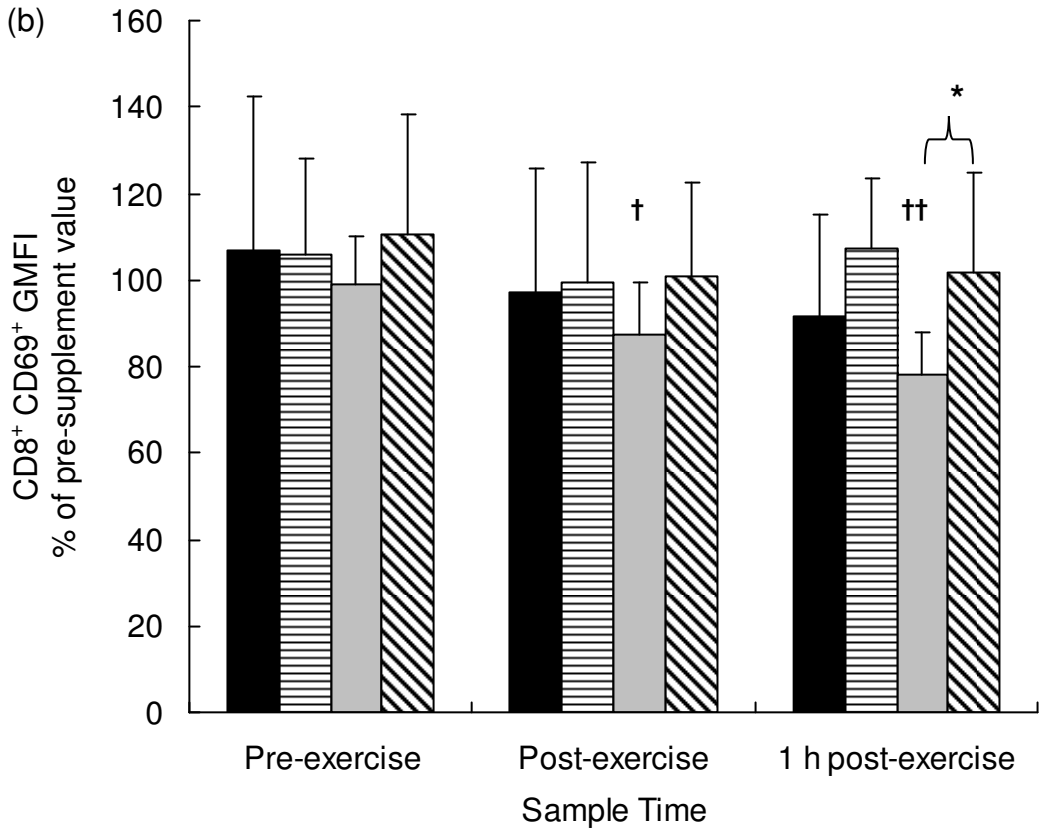
There were no time x trial interaction or main effects for GMFI of CD69 expression on unstimulated $CD4^+$, $CD8^+$ or $CD3^-CD56^+$ cells (Table 5.3). When cells were stimulated, GMFI of CD69 expression on antigen-stimulated (1:8000) $CD4^+$ T cells approached significance (P for interaction=0.054; Figure 5.1a), while both antigen-stimulated (1:8000) $CD8^+$ and $CD3^-CD56^+$ cells showed a significant time x trial interaction (both $P<0.05$; Figure 5.1b & c). The GMFI of CD69 expression on antigen-stimulated (1:8000) $CD8^+$ cells was significantly lower on CAF compared to PLA at 1 h post-exercise ($P<0.05$; Figure 5.1b); for the same time-point the GMFI of CD69 expression on antigen-stimulated (1:8000) $CD3^-CD56^+$ cells was significantly higher on CAF compared to PLA ($P<0.05$; Figure 5.1c).

Table 5.3: Relative GMFI of CD69 expression on unstimulated $CD4^+$, $CD8^+$ and $CD3^-CD56^+$ cells during CAF and PLA trials.

	Pre-supplement	Pre-exercise	Post-exercise	1 h post-exercise
$CD4^+$ $CD69^+$ GMFI				
CAF	100 (0)	109 (39)	105 (28)	101 (29)
PLA	100 (0)	99 (10)	99 (24)	97 (20)
$CD8^+$ $CD69^+$ GMFI				
CAF	100 (0)	110 (20)	111 (15)	109 (22)
PLA	100 (0)	95 (12)	95 (10)	99 (10)
$CD3^-CD56^+$ $CD69^+$ GMFI				
CAF	100 (0)	100 (11)	103 (17)	105 (13)
PLA	100 (0)	96 (16)	95 (13)	91 (14)

Values are mean (SD). GMFI expression of CD69 is expressed as a percentage relative to pre-supplement values.


(a)
1:4000 CAF
1:4000 PLA
1:8000 CAF
1:8000 PLA
CD4+ CD69+ GMFI
% of pre-supplement value
180
160
140
120
100
80
60
40
20
0
Pre-exercise
Post-exercise
1 h post-exercise
Sample Time


(b)
CD8+ CD69+ GMFI
% of pre-supplement value
160
140
120
100
80
60
40
20
0
†
††
*
Pre-exercise
Post-exercise
1 h post-exercise
Sample Time

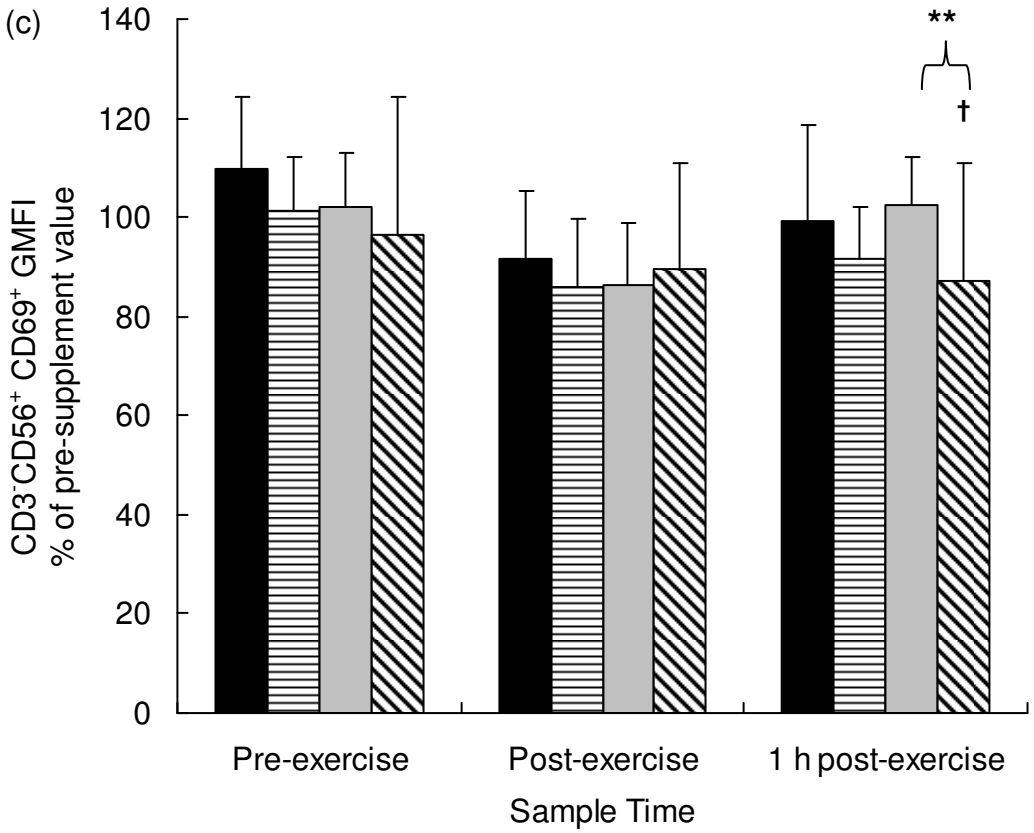


Figure 5.1: Geometric Mean Fluorescence Intensity of CD69 expression on antigen-stimulated (**a**) $CD4^+$ T cells, (**b**) $CD8^+$ T cells and (**c**) $CD3^-$ $CD56^+$ NK cells within the circulating lymphocyte population during CAF and PLA trials. * significantly lower on CAF than PLA ($P<0.05$); ** significantly higher on CAF than PLA ($P<0.05$); significantly lower than pre-supplement within trial (†† $P<0.01$; † $P<0.05$). Values are means + SD and are expressed as a percentage relative to pre-supplement value.

5.5. Discussion

The present study found that ingesting 6 $mg \cdot kg^{-1}$ body mass caffeine 1 hour before performing prolonged, intensive exercise was associated with (i) a reduction in the number of antigen-stimulated $CD4^+$ cells expressing CD69, (ii) a lowering of the intensity of CD69 expression on antigen-stimulated $CD8^+$ cells and (iii) an increase in the intensity of CD69 expression on antigen-stimulated $CD3^-CD56^+$ cells 1 h after exercise compared with placebo. Furthermore, caffeine (compared with placebo) ingestion was also associated with greater

elevations in plasma adrenaline and serum caffeine concentrations. This suggests that ingesting caffeine at a dose of 6 mg·kg^{-1} body mass (compared with placebo) 1 h prior to exercise simultaneously reduced T cell activation and increased NK cell activation, 1 hour after 90 min cycle exercise at 70% $\dot{V}O_{2peak}$.

The present study demonstrates the complex effects of caffeine on peripheral lymphocytes 1 h following 90 min cycle exercise at 70% $\dot{V}O_{2peak}$. As caffeine can act via the same pathways for T and NK cells, it might have been expected that for the same dose of caffeine both cell types would have responded similarly; this was not the case. This apparent immunosuppressive (T cells) and immunostimulatory (NK cells) effect of caffeine on lymphocyte activation may be explained through its ability to alter intracellular cAMP concentration. T and NK cell function have been shown to be inhibited through the actions of adenosine and adrenaline via adenosine and β_2-adrenergic receptors, respectively (Kappel et al., 1991; Huang et al., 1997), via activation of adenylate cyclase and increased intracellular cAMP (Grader-Beck et al., 2003; Raskovalova et al., 2006). Therefore, caffeine may have acted to increase intracellular levels of cAMP in T cells via increased plasma levels of adrenaline, subsequently stimulating T cell β_2-adrenergic receptors, increasing intracellular cAMP (Grader-Beck et al., 2003), thereby reducing cellular activation. Given the amplified adrenaline response on the CAF trial at post-exercise, it seems plausible that the decrease of antigen-stimulated T cell activation reported here with caffeine ingestion at 1 h post-exercise reflects a caffeine-influenced adrenaline-mediated decrease of T cell activation.

While the effects of adrenaline may help to explain the reduction in antigen-stimulated T cell activation, it can not easily explain the apparent stimulatory effect of caffeine ingestion on NK cell activation 1 h following high intensity exercise. Here, it seems more likely that the overriding influence of caffeine is via adenosine receptor antagonism. In this way, caffeine may act to impede the adenosine-mediated increase in intracellular levels of cAMP via antagonism of NK cell A_{2A} adenosine receptors (Raskovalova et al., 2006). It is worth noting that NK cells also possess A_1 adenosine receptors, however blockade of this

subclass leads to increases in cAMP and suppressed NK cell functions and therefore is unlikely to play a principal role here. Although plasma adenosine concentration was not measured in this study it is reported that caffeine doses that approximate to that used in this study increased levels of adenosine by adenosine receptor blockade in the rat model (Conlay et al., 1997). It should be noted that the mechanisms discussed here are unlikely to be exclusive; the observable effect of caffeine ingestion on T and NK cell responses to antigenic challenge most likely reflects the net result of both stimulatory and inhibitory influences. The findings of the present study suggest that enhanced adrenergic stimulation of T cells and A_{2A} adenosine receptor antagonism of NK cells are the foremost pathways by which caffeine at a dose of 6 $mg \cdot kg^{-1}$ is acting here.

It could be suggested that the increase in NK cell activation observed 1 h following high intensity exercise was an attempt by the innate arm of the immune system to compensate for the reduction in T cell activation seen within the acquired arm of the immune system. In support of this notion, other studies using a similar protocol to that of the current study found caffeine to transiently enhance measures of mucosal immune function [salivary immunoglobulin A (s-IgA)] during exercise (Bishop et al., 2006b), and attenuate the post-exercise fall in formyl-methionyl-leucyl-phenylalanine stimulated neutrophil oxidative burst responses (Walker et al., 2006, 2007). Both s-IgA and neutrophils are aspects of the innate immune system that along with NK cells represent a major first line of defence against infectious agents. This highlights the limitation of focussing on specific cellular responses and extrapolating these findings to any potential outcome for overall host immunity and infection risk. Future work should involve using *in vivo* cell-mediated immune function tests to determine the effect of caffeine ingestion on whole body cell-mediated responses following high intensity exercise.

Studies (both *in vitro* and *in vivo*) that have investigated dose-response effects of caffeine on human and rat lymphocyte functions have shown that different doses of caffeine exert different affects over lymphocyte function (Kantamala et al., 1990; Rosenthal et al., 1992). This could help to explain why a 6 $mg \cdot kg^{-1}$

dose of caffeine in this study was beneficial for NK cell function but unbeneficial for T cell function 1 h after intensive exercise. Although a 6 mg·kg^{-1} (~450 g in this study) dose of caffeine has been consistently shown to be ergogenic (Goldstein et al., 2010), athletes tend to ingest much smaller amounts of caffeine (~2-3 mg·kg^{-1} or ~200 mg) to enhance athletic performance (Chester and Wojek, 2008). Therefore, investigating a smaller and possibly more realistic dose of caffeine on lymphocyte function following high intensity exercise will be investigated in Chapter 6.

In summary, the findings of the present study suggest that ingesting 6 mg·kg^{-1} body mass caffeine 1 h before prolonged intensive exercise reduces the antigen-stimulated expression of CD69 on $CD4^+$ and $CD8^+$ T cells, while simultaneously increasing antigen-stimulated $CD3^-CD56^+$ NK cell activation 1 h post-exercise. These findings are not apparent when caffeine is ingested without subsequent exercise (Chapter 4). It is suggested that enhanced adrenergic stimulation and A_{2A} adenosine receptor antagonism are the main pathways by which caffeine reduces and increases T and NK cell activation, respectively. Despite caffeine's immunomodulatory influence on T and NK cell activation the clinical significance of this in terms of subsequent infection risk is yet to be determined.

Chapter 6

Effect of a high and low dose of caffeine on antigen-stimulated human natural killer cell activation after prolonged cycling

6.1. Abstract

In Chapter 5 a high dose of caffeine ingested 1 h prior to exercise increased antigen-stimulated NK cell activation 1 h following 90 min cycling at 70% $\dot{V}O_{2peak}$. However, it was suggested that perhaps different doses of caffeine exert differential effects upon lymphocyte activation in response to prolonged exercise. As such this study investigated the effect of a high and low dose of caffeine on antigen-stimulated natural killer ($CD3^-CD56^+$) cell activation after prolonged, strenuous cycling, as assessed by the early activation molecule CD69. In a randomised cross-over design, 12 healthy male endurance trained cyclists [age: 22 (2) years, $\dot{V}O_{2peak}$: 61 (4) ml·kg^{-1}·min^{-1}, mean ± SD] cycled for 90 min at 70% $\dot{V}O_{2peak}$ 60 min after ingesting either 0 (PLA), 2 (2CAF) or 6 (6CAF) mg·kg^{-1} body mass of caffeine. Whole blood was stimulated with Pediacel (5 in 1) vaccine. A high dose of caffeine increased the number of $CD3^-CD56^+$ cells into the circulation at immediately post-exercise compared with PLA [P<0.05; PLA: 0.62 (0.30) x10^9 cells·L^{-1} vs. 6CAF: 0.79 (0.36) x10^9 cells·L^{-1}]. For both 2CAF and 6CAF, the GMFI of CD69 expression on unstimulated $CD3^-CD56^+$ cells was significantly higher compared with PLA at 1 h post-exercise [both P<0.05; PLA: 93 (13)%, 2CAF: 110 (12)%, 6CAF: 123 (33)%]. When cells were stimulated with antigen, the GMFI of CD69 expression remained significantly higher on 2CAF than on PLA at 1 h post-exercise [P<0.05; 1:8000 PLA: 98 (9)% vs. 1:8000 2CAF: 122 (32)%]. Although not achieving statistical significance, 6CAF also followed a similar trend when stimulated (P=0.09). These results suggest that caffeine at a high (6 mg·kg^{-1}) dose is associated with recruitment of NK cells into the circulation and that both a high and low (2 mg·kg^{-1}) dose of caffeine increases unstimulated and antigen-stimulated natural killer cell activation 1 h following high intensity exercise. As such there does not appear to be a dose-dependent effect of caffeine on natural killer cell activation 1 h following prolonged intensive cycling.

6.2. Introduction

It has been well documented that caffeine between the doses of 3 and 6 mg·kg^{-1} body mass enhances athletic performance (Graham, 2001), however, doses as low as 2 mg·kg^{-1} body mass (~150-200 mg) have also proved ergogenic (Cox et al., 2002).

Many athletes do not consume large amounts of caffeine (~450 mg) in one bolus dose, instead they tend to ingest much smaller amounts (~200 mg) to enhance performance (Chester and Wojek, 2008). A study by Kantamala et al. (1990) demonstrated that different doses of caffeine exert differential influences over NK cell cytotoxic activity in rats at rest. Caffeine doses between 5 and 40 µg·ml^{-1} had little effect on NK cell cytotoxicity *in vitro*. However a 6 mg·kg^{-1}·day^{-1} dose of caffeine ingested consecutively for 120 days *in vivo* significantly decreased NK cell function, while both 2 and 18 mg·kg^{-1}·day^{-1} caffeine doses had no effect. To the author's knowledge no study to date has investigated the effect of different doses of caffeine on human NK cell function following a bout of high intensity exercise. However, data described in Chapter 5 demonstrate that ingesting 6 mg·kg^{-1} body mass caffeine 1 h prior to 90 min cycling at 70% $\dot{V}O_{2peak}$ increases NK cell activation 1 h post-exercise compared with PLA. Therefore, the aim of this study was to determine the effect of a high and low dose of caffeine on NK cell activation, following 90 min cycling at 70% $\dot{V}O_{2peak}$, as assessed by the early activation marker CD69.

6.3. Methods

Participants

Twelve endurance trained male cyclists [age: 22 (2) years; body mass: 71 (6) kg; $\dot{V}O_{2peak}$ 61 (4) ml·kg^{-1}·min^{-1}; peak power output: 330 (25) W] volunteered to participate in this study. All participants were fully informed about the rationale for the study, along with their possible risks and discomforts, before providing written informed consent. Loughborough University Ethical Advisory Committee approved the study. Daily caffeine intake amongst participants was varied and ranged from 30 – 359 mg·day^{-1}. One participant was characterised as a high

user (> 250 mg·day^{-1}), 6 as moderate users (50 – 250 mg·day^{-1}) and 5 as light users (< 50 mg·day^{-1}).

Experimental procedures

Approximately 2 weeks before the beginning of the main trials, each participant was required to undertake preliminary measurements including a $\dot{V}O_{2peak}$ test and a familiarisation of the trial protocol. Preliminary measurements and pre-trial standardisation procedures are described in Chapter 3. Participants performed 3 main exercise trials, each separated by 1 week. Participants were randomly assigned to either the PLA, 2CAF or 6CAF trial and acted as their own controls in a repeated-measures, single-blind, cross-over design. Participants arrived at the laboratory at 08:20 h following an overnight fast. Participants then ingested 0, 2 or 6 mg·kg^{-1} body mass of caffeine and rested quietly in the laboratory for 1 h. Immediately after this, participants began cycling on an electromagnetically braked cycle ergometer for 90 min at a work rate equivalent to 70% $\dot{V}O_{2peak}$ [213 (22) W]. Venous blood samples were obtained at pre-supplement, 1 h after CAF or PLA ingestion (pre-exercise), immediately post-exercise and 1 h post-exercise. Laboratory conditions throughout the study were 19.6 (0.5) ℃ and 35.7 (5.4) % relative humidity.

Blood analytical methods

Blood sampling and analysis methods are detailed in Chapter 3.6

Statistical analysis

Statistical methods are detailed in Chapter 3.7

6.4. Results

Exercise intensity, heart rate and indirect calorimetry

Mean exercise intensity was similar between all trials [PLA: 72.0 (3.2)%, 2CAF: 72.8 (3.3)%, 6CAF: 73.3 (3.3)%]. Heart rates were comparable between trials during exercise [PLA: 155 (9) beat·min^{-1}, 2CAF: 159 (8), 6CAF: 161 (8) beat·min^{-1}; mean of all recordings]. RER and fat and CHO oxidation rates did not differ between trials throughout exercise [PLA: 0.92 (0.05), 0.45 (0.29) g·min^{-1}, 2.67 (0.61) g·min^{-1}; 2CAF: 0.92 (0.06), 0.46 (0.30) g·min^{-1}, 2.69 (0.57) g·min^{-1}; 6CAF: 0.92 (0.06), 0.48 (0.29) g·min^{-1}, 2.69 (0.59) g·min^{-1}, for RER, fat and CHO oxidation rates, respectively]. Likewise, EE and the percentage contribution of fat and CHO were similar on all trials [PLA: 63 (3) KJ·min^{-1}, 27 (17)%, 73 (17)%; 2CAF: 64 (4) KJ·min^{-1}, 28 (18)%, 72 (18)%; 6CAF: 64 (4) KJ·min^{-1}, 29 (18)%, 71 (18)%, for EE and % of fat and CHO, respectively].

Changes in body mass and plasma volume

After exercise, change in body mass (corrected for fluid intake) was similar for all trials [PLA: -1.5 (0.3) kg, 2CAF: -1.6 (0.3) kg, 6CAF: -1.6 (0.3) 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 8.2 (5.2)%, 8.0 (5.0)% and 8.5 (5.4)% on the PLA, 2CAF and 6CAF 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, post and 1 h post-exercise on 6CAF compared with PLA and 2CAF ($P<0.01$; Figure 6.1). At these times, values on 2CAF were significantly higher than on PLA ($P<0.01$; Figure 6.1).

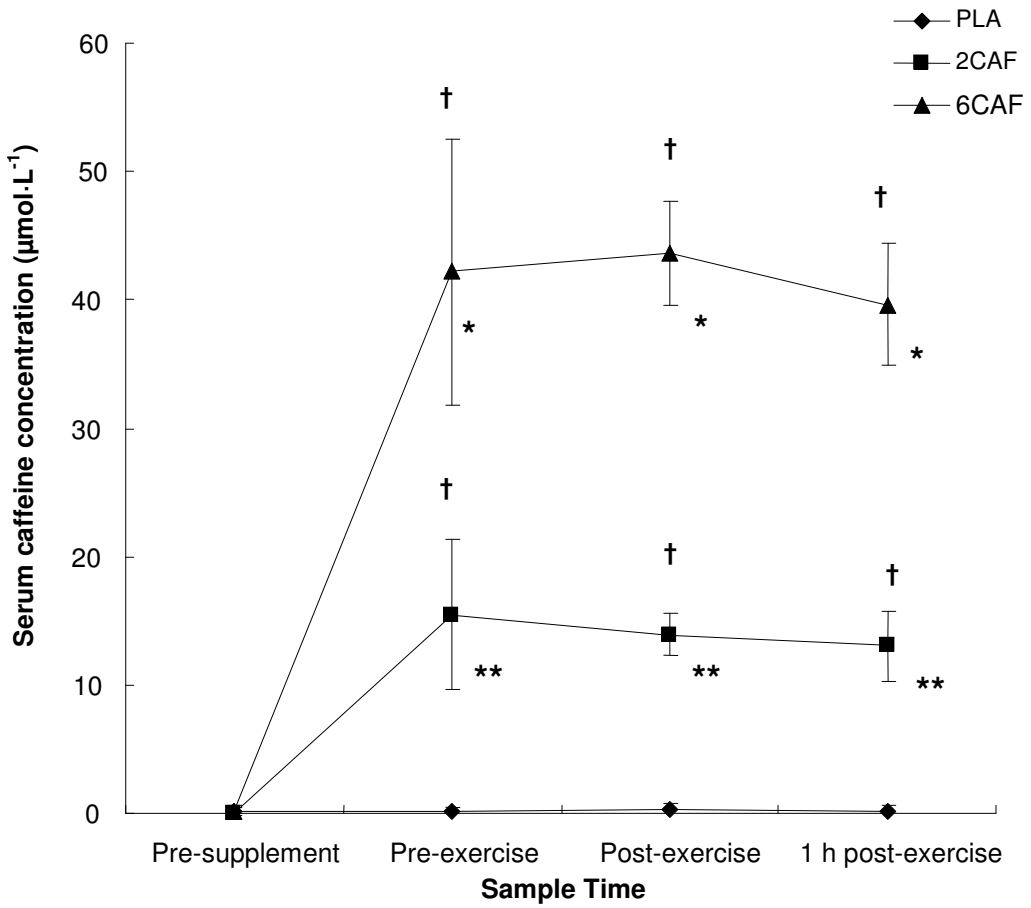


Figure 6.1: Serum caffeine concentrations during PLA, 2CAF and 6CAF trials. * significantly higher on 6CAF than PLA and 2CAF (P<0.01); ** significantly higher on 2CAF than PLA (P<0.01); † significantly higher than pre-supplement within trial (P<0.01). Values are means ± SD.

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

No significant time x trial interaction effect was found for total number of circulating lymphocytes but there was a significant main effect for time (P<0.01) with the circulating number of lymphocytes following a biphasic response to exercise (Table 6.1). A significant time x trial interaction was found for the number of $CD3^-CD56^+$ cells within the circulating lymphocyte population, with values higher on 6CAF at post-exercise compared with PLA (P<0.01; Table 6.1). 6CAF also showed a higher number of $CD3^-CD56^+$ cells within the circulating lymphocyte population than on 2CAF (main effect for trial; P<0.05).

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

There were no significant time x trial interaction effects for the number of unstimulated or antigen-stimulated $CD3^-CD56^+$ cells expressing CD69 for any of the trial comparisons. However, the number of unstimulated and antigen-stimulated $CD3^-CD56^+$ cells expressing CD69 increased above pre-supplement values at post-exercise and decreased below baseline at 1 h post-exercise (main effect for time: P<0.01; Table 6.1).

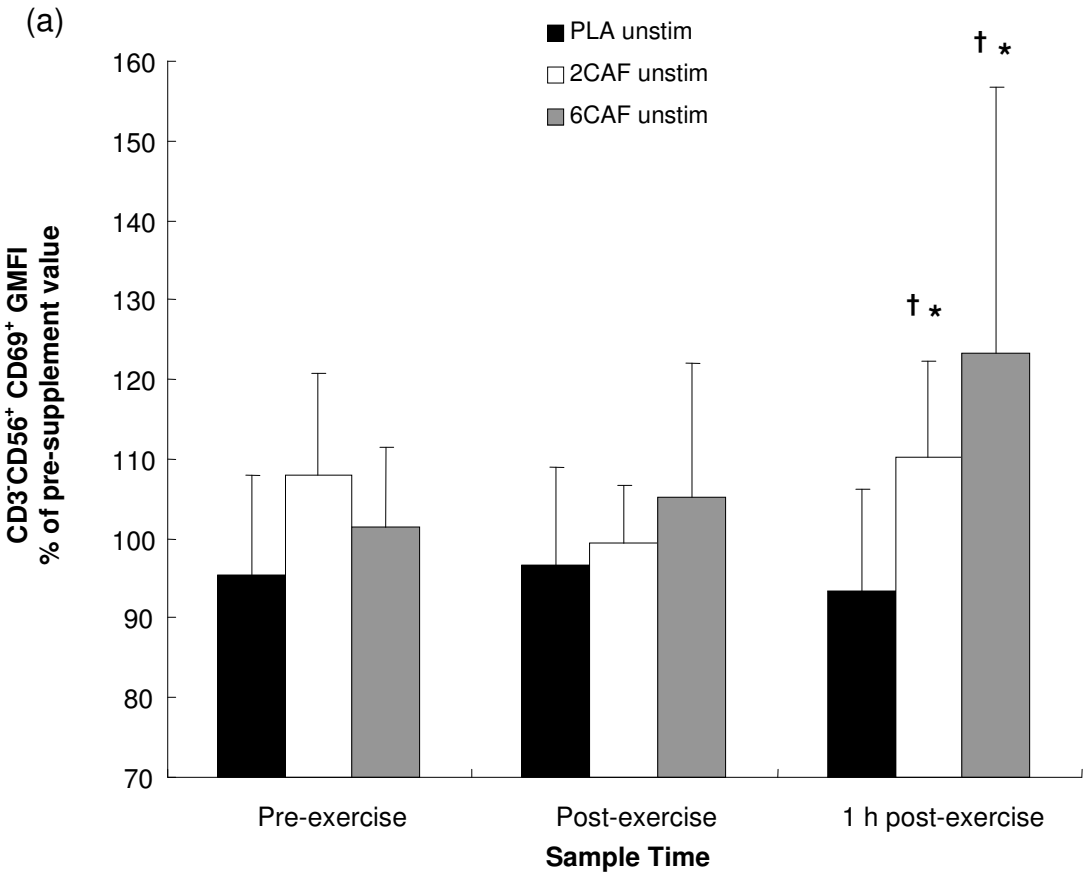
Table 6.1: Total lymphocyte count, number of circulating $CD3^-CD56^+$ cells and number of circulating $CD3^-CD56^+$ cells expressing CD69 during PLA, 2CAF and 6CAF trials.

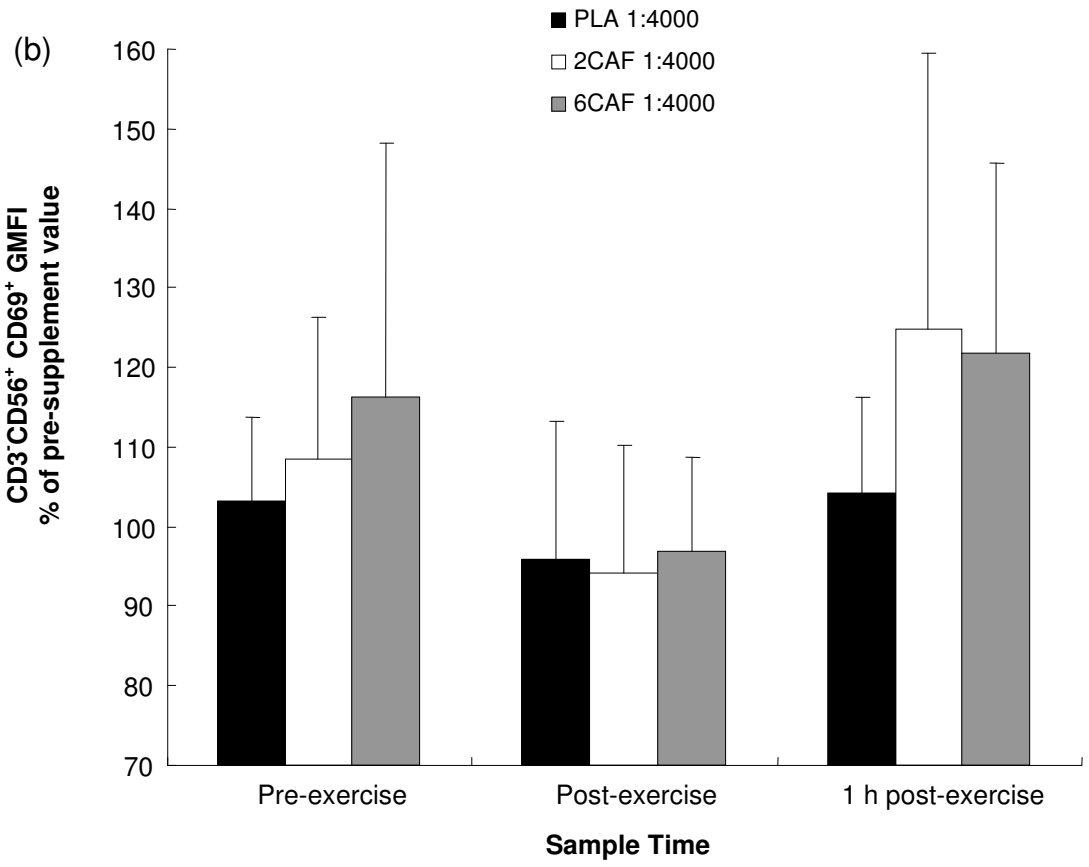
	Pre-supplement	Pre-exercise	Post-exercise	1 h post-exercise
Total Lymphocytes ($x10^9$ cells·L^{-1}) [a]				
PLA	2.10 (0.60)	1.70 (0.40)	2.30 (0.80)	1.50 (0.40)
2CAF	2.10 (0.50)	1.80 (0.40)	2.50 (0.70)	1.50 (0.40)
6CAF	2.10 (0.60)	1.90 (0.40)	2.70 (1.10)	1.50 (0.40)
$CD3^-CD56^+$ cells ($x10^9$ cells·L^{-1}) [b, c]				
PLA	0.39 (0.17)	0.33 (0.16)	0.62 (0.30) ††	0.28 (0.12) ††
2CAF	0.42 (0.16)	0.39 (0.15)	0.68 (0.30)	0.25 (0.11)
6CAF	0.42 (0.24)	0.43 (0.19)	0.79 (0.36) * †	0.31 (0.14)
$CD3^-CD56^+$ $CD69^+$ cells ($x10^6$ cells·L^{-1}) [b, c]				
unstim PLA	39.8 (33.1)	30.2 (23.0)	49.1 (35.0)	20.6 (13.5)
unstim 2CAF	34.2 (22.8)	31.3 (18.0)	55.5 (45.1)	19.3 (15.3)
unstim 6CAF	34.4 (19.7)	39.5 (23.9)	65.1 (32.3)	23.9 (14.8)
1 :4000 PLA	47.7 (22.0)	42.0 (22.2)	82.4 (40.1)	42.1 (24.3)
1 :4000 2CAF	49.5 (24.0)	65.8 (39.6)	95.4 (63.4)	39.3 (26.1)
1 :4000 6CAF	53.6 (25.6)	75.8 (70.3)	98.0 (30.7)	41.8 (24.7)
1 :8000 PLA	37.9 (17.3)	41.5 (23.3)	69.5 (37.4)	26.2 (15.4)
1 :8000 2CAF	40.7 (20.5)	55.6 (31.1)	75.2 (50.9)	28.2 (25.6)
1 :8000 6CAF	40.3 (27.4)	61.8 (63.8)	79.0 (45.5)	32.7 (21.9)

Values are means (SD). * P<0.05: significantly higher on 6CAF than PLA.
† P<0.01, †† P<0.05: significantly different than pre-supplement within trial.
[a] Main effect for time: significantly different from pre-supplement at pre-exercise, post-exercise and 1 h post-exercise, P<0.01.
[b] Main effect for time: significantly different from pre-supplement at post-exercise and 1 h post-exercise, P<0.01.
[c] Main effect for trial: significantly higher on unstim 6CAF than unstim PLA and unstim 2CAF, P<0.05.

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

There was a significant interaction effect for the GMFI of CD69 expression on unstimulated $CD3^-CD56^+$ cells, with both 2CAF and 6CAF demonstrating a significant increase at 1 h post-exercise compared with PLA (both $P<0.05$; Figure 6.2a). Values on 2CAF also appeared to be higher than on PLA at pre-exercise; this approached significance ($P=0.061$). GMFI of CD69 expression on unstimulated $CD3^-CD56^+$ cells was also higher on 2CAF than 6CAF (main effect for trial, $P<0.05$; Figure 6.2a). When cells were stimulated, GMFI of CD69 expression on antigen-stimulated (1:4000) $CD3^-CD56^+$ cells tended to be higher on 6CAF than PLA (P for interaction=0.09; Figure 6.2b), while the GMFI of CD69 expression on antigen-stimulated (1:8000) $CD3^-CD56^+$ cells was significantly higher on 2CAF at 1 h post-exercise compared with PLA ($P<0.05$; Figure 6.2c). There were no differences between 2CAF and 6CAF with either dose of antigen (Figures 6.2b & c, respectively).


(a)
PLA unstim
2CAF unstim
6CAF unstim
† *
† *
160
150
140
130
120
110
100
90
80
70
CD3-CD56+ CD69+ GMFI
% of pre-supplement value
Pre-exercise
Post-exercise
1 h post-exercise
Sample Time


(b)
PLA 1:4000
2CAF 1:4000
6CAF 1:4000
160
150
140
130
120
110
100
90
80
70
CD3-CD56+ CD69+ GMFI
% of pre-supplement value
Pre-exercise
Post-exercise
1 h post-exercise
Sample Time

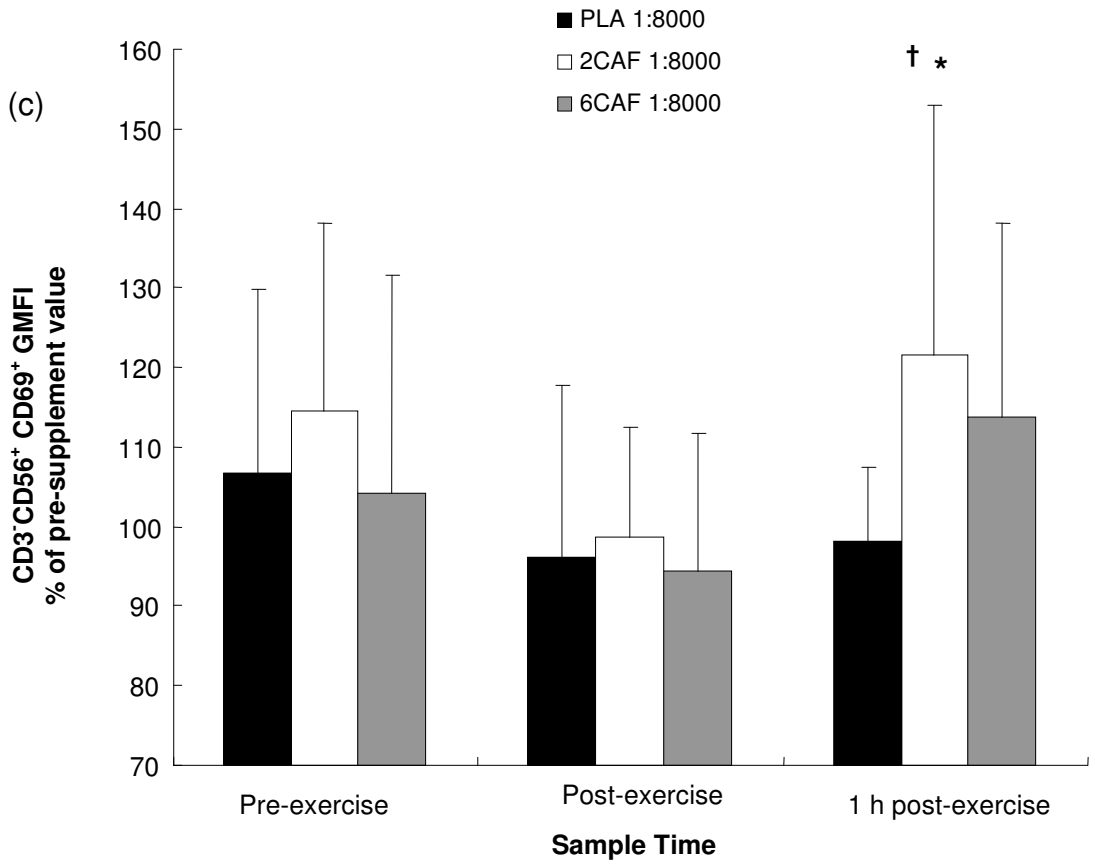


Figure 6.2: Geometric Mean Fluorescence Intensity of CD69 expression on (**a**) unstimulated $CD3^{-}CD56^{+}$ NK cells, (**b**) 1:4000 antigen-stimulated $CD3^{-}CD56^{+}$ NK cells, (**c**) 1:8000 antigen-stimulated $CD3^{-}CD56^{+}$ NK cells within the circulating lymphocyte population during PLA, 2CAF and 6CAF trials. * significantly higher than PLA ($P<0.05$); † significantly higher than pre-supplement within trial ($P<0.05$). Values are means + SD and are expressed as a percentage of pre-supplement value.

6.5. Discussion

The findings of the present study suggest that, compared with placebo, ingesting 2 or 6 $mg \cdot kg^{-1}$ body mass of caffeine 1 h prior to exercise is associated with (i) increases in the natural state of activation of circulating NK cells and (ii) increases in the GMFI of CD69 expression on antigen-stimulated NK cells 1 h following high intensity prolonged cycling. The effects of caffeine

on NK cell activation 1 h after prolonged intensive cycling were not found to be dose-dependent. Also, only a large dose of caffeine (6 mg·kg^{-1}) increased the trafficking of NK cells into the peripheral circulation immediately post-exercise compared with placebo.

In the present study both a high and low dose of caffeine increased unstimulated and antigen-stimulated NK cell activation 1 h following 90 min cycling at 70% $\dot{V}O_{2peak}$. The apparently positive effects of caffeine on NK cell activation may be explained through its ability to alter intracellular cAMP. The actions of adenosine via A_{2A} adenosine receptors (Priebe et al., 1990) have been shown to inhibit NK cell function via activation of adenylate cyclase and increased intracellular cAMP concentration (Goto et al., 1983); while the actions of adenosine via A_1 adenosine receptors have been shown to enhance NK cell function via suppression in cAMP (Goto et al., 1983). It is known that caffeine is a non-selective adenosine receptor antagonist. Therefore, it is suggested that both the high and low dose of caffeine acted to impede the adenosine-mediated increase in intracellular cAMP concentration in NK cells via antagonism of A_{2A} adenosine receptors (Raskovalova et al., 2006). Although plasma adenosine concentration was not measured in this study it has been reported in rats that doses of caffeine corresponding to those used in this study increased plasma concentrations of adenosine via adenosine receptor blockade in a dose-dependent manner (Conlay et al., 1997).

Caffeine also has the potential to reduce NK cell function indirectly via its ability to increase the release of adrenaline (Graham, 2001). Adrenaline stimulates β_2 adrenoreceptors on NK cells and in turn increases the level of intracellular cAMP, leading to an inhibition of NK cell function (Murray et al., 1992). As both caffeine doses in this study increased, rather than decreased, NK cell activation 1 h after exercise it is unlikely that adrenaline played a principal role on NK cell activation here. However, it should be noted that any effect of caffeine on antigen-stimulated NK cell activation is most likely the net result of a number of stimulatory and inhibitory influences. Therefore, in the present study it could be

argued that A_{2A} adenosine receptor antagonism may have exerted a stronger influence on NK cell function than enhanced adrenergic stimulation.

In the present study a high dose of caffeine increased the trafficking of NK cells into the circulation immediately after exercise cessation compared with placebo (this result was also found in Chapter 5). Adrenaline is known to modulate the expression of adhesion molecules on NK cells, leading to increased mobilisation of these cells into the circulation following high intensity exercise (Benschop et al., 1996). With this in mind, an alternative action of caffeine in this study might have been to influence an adrenaline-mediated mobilisation into the peripheral circulation of a specific population of NK cells with an activated phenotype, and/or with increased propensity for activation when stimulated. Although plasma adrenaline concentration was not measured in this study, our participants, exercise protocol and serum caffeine concentration following exercise on the 6 $mg \cdot kg^{-1}$ caffeine dose are comparable with those of Walker et al. (2006); who found a significantly higher plasma adrenaline concentration following exercise with 6 $mg \cdot kg^{-1}$ caffeine compared with placebo (present study: 44 µM; Walker et al.: 46 µM). While high doses of caffeine (6 & 9 $mg \cdot kg^{-1}$) appear to increase plasma concentrations of adrenaline, it has been reported that a low caffeine dose (3 $mg \cdot kg^{-1}$) has little effect at increasing plasma adrenaline concentration in response to high intensity exercise (Graham and Spriet, 1995). This may help to explain why in the present study, NK cell recruitment into the circulation after exercise was higher on 6CAF, with no differences between 2CAF and PLA at this time, and why there was a higher overall number of naturally activated NK cells within the peripheral circulation with 6CAF compared to PLA and 2CAF.

This study is the first to report apparently positive effects of a low dose of caffeine on antigen-stimulated NK cell activation 1 h following high intensity exercise. Furthermore, this study has demonstrated that a low dose of caffeine appears to exert similar effects on NK cell activation 1 h following prolonged intensive cycling as that of a dose 3 times as large. This is a significant finding as many athletes only ingest small amounts of caffeine to enhance athletic

performance (Chester and Wojek, 2008) and suggests that a low dose of caffeine as well as being ergogenic (Cox et al., 2002) may also have the potential to strengthen an athlete's first line of defence against infectious agents following high intensity exercise. However, it is difficult to extrapolate an enhancement in NK cell activation to an enhancement of an athlete's overall immunity 1 h following high intensity exercise; the enhancement in this study relates to one particular cellular response and as such may not accurately represent what is happening to the rest of the athlete's immune system. Future use of *in vivo* cell-mediated immune function tests would help shed light on any effect of caffeine ingestion on whole body cell-mediated immunity.

Although in this study the effects of ingesting 6 $mg \cdot kg^{-1}$ caffeine on antigen-stimulated NK cell activation did not reach statistical significance, it showed a trend for increased cellular responses that was comparable to that observed with ingestion of 2 $mg \cdot kg^{-1}$ caffeine. Data described in Chapter 5 demonstrated that a 6 $mg \cdot kg^{-1}$ body mass dose of caffeine was effective at increasing antigen-stimulated NK cell activation following an identical exercise protocol. The present participants are comparable to those used in Chapter 5 and diet, exercise and caffeine controls before the trial were identical to the former study. However, inter-individual variations in responses were greater here than in Chapter 5. This may relate to individual differences in habitual caffeine intake; as caffeine consumption here was more varied among the participants than in the previous study. Indeed, large inter-individual differences in athlete's physiological and performance responses to the effects of caffeine have been reported (Desbrow et al., 2009; Skinner et al., 2010). Future investigations into the effects of caffeine on specific groups of caffeine users, i.e. light, moderate or high users, would help clarify reasons for such variability.

In conclusion, the findings of the present study suggest that both a high and low dose of caffeine are associated with an increase in the natural state of activation of NK cells, in addition to positive effects on antigen-stimulated NK cell activation 1 h following high intensity prolonged cycling. However, there was no obvious effect of caffeine dose on these responses. It is suggested that

these findings reflect an overriding A_{2A} antagonistic effect of caffeine. Despite both doses of caffeine having a positive influence on NK cell activation 1 h following high intensity exercise the clinical significance of these findings in terms of subsequent infection risk are as yet unclear.

Chapter 7

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

7.1. Abstract

Several studies including those in Chapters 5 and 6 that have investigated the effect of caffeine on immune function following exercise have tended to use 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 compared with placebo on antigen-stimulated NK cell activation following strenuous intermittent exercise. In a randomised cross-over design, 15 healthy male games players [age: 22 (2) years, $\dot{V}O_{2\,max}$: 54 (3) ml·kg^{-1}·min^{-1}, mean ± SD] 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·kg^{-1} body mass caffeine on 3 separate occasions during the day (3x2CAF) or 1 dose of 6 (6CAF) mg·kg^{-1} body mass caffeine, 1 h before exercise. Whole blood was stimulated with Pediacel (5 in 1) vaccine. At 1 h post-exercise the number of antigen-stimulated $CD3^-CD56^+$ cells expressing CD69 was significantly lower on the 6CAF trial than PLA [$P<0.05$; PLA: 41.96 (33.99) x10^6 cells·L^{-1} vs. 6CAF: 26.21 (25.04) x10^6 cells·L^{-1}], with values on 6CAF at this time-point remaining close to pre-supplement. 6CAF also tended to attenuate the exercise-induced increase in the GMFI of CD69 expression on antigen-stimulated $CD3^-CD56^+$ cells 1 h post-exercise [$P=0.055$; PLA: 141 (28)% vs. 6CAF: 119 (20)%]. These findings suggest that one large bolus dose of caffeine attenuates the exercise-induced increase in antigen-stimulated NK cell activation 1 h following strenuous intermittent exercise, while small repeated doses of caffeine ingested throughout the day have little effect on NK cell activation. However, this attenuation at no point fell below pre-supplement values and as such caffeine does not appear to depress NK cell activation.

7.2. Introduction

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., 2005a, 2006b; 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 author's 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. Results in Chapter 6 demonstrate that a small dose of caffeine (2 $mg \cdot kg^{-1}$) ingested 1 h prior to exercise increases antigen-stimulated NK cell activation 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 activation following 90 min of high intensity intermittent exercise, as assessed by the early activation marker CD69.

Caffeine has been shown to increase sympathoadrenal medullary system (SAM) activity and as such plasma adrenaline 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 adrenaline concentration in the present study.

7.3. Methods

Participants

Fifteen healthy male games players [age: 22 (2) years; body mass: 76 (8) kg; $\dot{V}O_{2\,max}$: 54 (3) ml·kg^{-1}·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. Loughborough University Ethical Advisory Committee approved the study. All participants were moderate caffeine users (50 – 250 mg·day^{-1}), with an average daily caffeine intake of 159 (61) mg·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 familiarisation trial, which required them to perform 3x15 min blocks (45 min) of the LIST (Nicholas et al., 2000). Heart rates (Polar FS1, Polar Electro Oy, Kempele, Finland) were monitored continuously during the familiarisation trial.

Experimental trial procedures

Participants were instructed to stop consuming caffeine from 21:00 h the night before each trial. In order to standardise nutritional status, participants completed a 24 h food diary the day before and day of the first main trial and were asked to follow these 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, 6CAF (1 single dose of 6 mg·kg^{-1} caffeine) or 3x2CAF (3 repeated doses of 2 mg·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 & 6CAF) or 2 (3x2CAF) mg·kg^{-1} body mass of caffeine. For the 0 mg·kg^{-1} dose, participants ingested 2 mg·kg^{-1} body mass of dextrose powder. 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 & 6CAF) or 2 (3x2CAF) mg·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 (3x2CAF) or 6 (6CAF) mg·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. The specific timings of PLA or CAF ingestion along with blood and saliva samples are illustrated in Figure 7.1. 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 (Figure 7.2). 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 water *ad libitum* and were asked to rate their perceived exertion on a Borg 6-20 scale (Borg, 1982). 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) were obtained immediately after cessation of exercise, before post-exercise body mass (in shorts only) was recorded. Participants then consumed 5 ml·kg^{-1} body mass of 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 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) ℃ and 42.2 (6.9) % relative humidity.

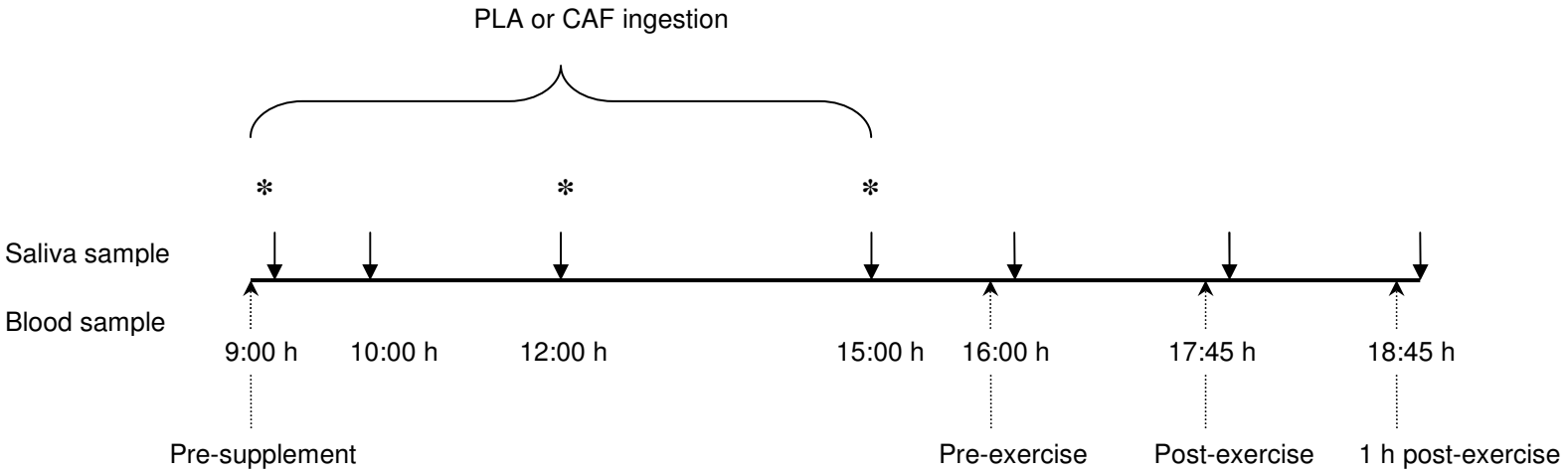


Figure 7.1: Schematic representation of the trial protocol

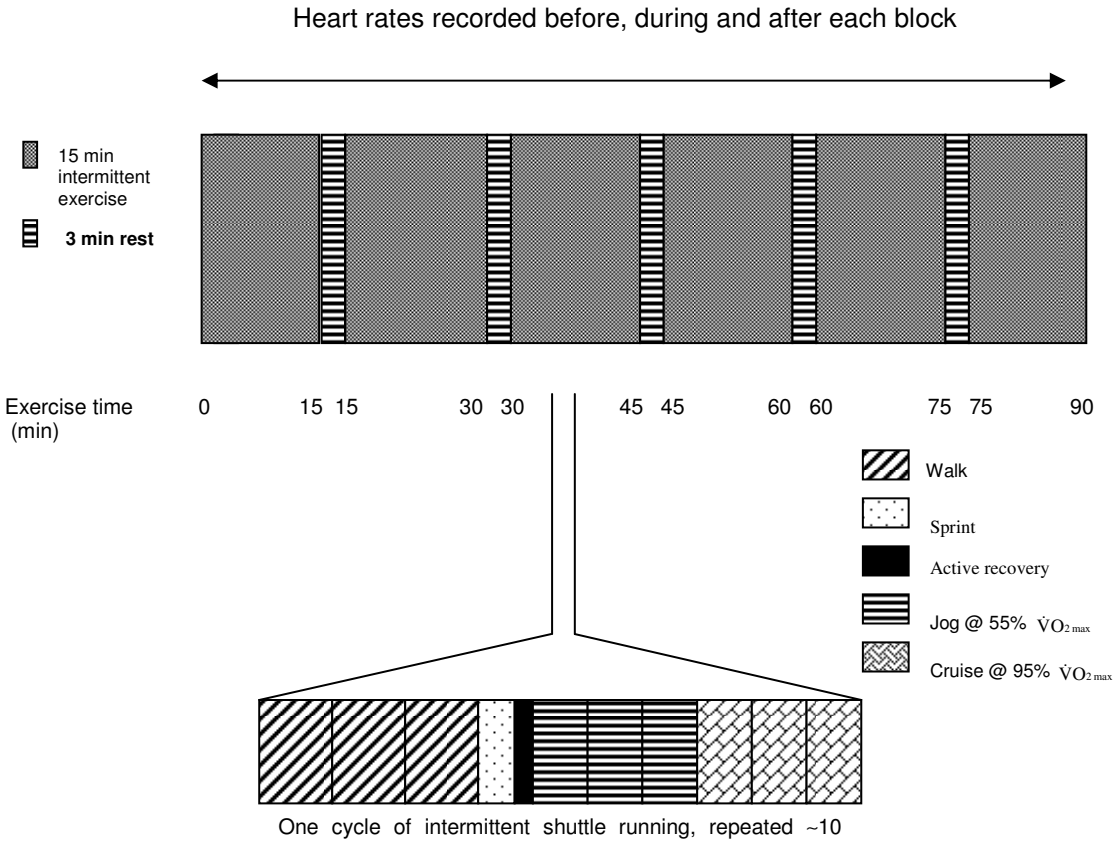


Figure 7.2: Schematic representation of the Loughborough Intermittent Shuttle Test (LIST). Adapted from Nicholas et al. 2000.

Lymphocyte culture

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. All cell culture work was carried out in a class II laminar flow hood (Esco GB Ltd, Salisbury, UK) by using aseptic techniques. Blood sampling and analysis methods are detailed in Chapter 3.6.

Saliva flow rate

Saliva samples were stored in their plastic containers at –80 ℃ 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^{TM} 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℃ 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%.

Statistical analysis

Statistical methods are detailed in Chapter 3.7.

7.4. 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}, 6CAF: 175 (13) beat·min^{-1}, 3x2CAF: 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, 6CAF: -1.8 (0.3) kg, 3x2CAF: -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, 6CAF and 3x2CAF trials, respectively.

Serum and saliva caffeine concentration

6CAF vs. PLA

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 6CAF compared with PLA ($P<0.01$; Figure 7.3). A significant time x trial interaction was also found for saliva caffeine concentration ($P<0.01$), with higher concentrations at post (17:45 h) and 1 h post-exercise (18:45 h) on 6CAF compared with PLA ($P<0.01$; Table 7.1).

3x2CAF vs. PLA

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 3x2CAF compared with PLA ($P<0.01$; Figure 7.3). A significant time x trial interaction was also 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 3x2CAF compared with PLA ($P<0.01$; Table 7.1).

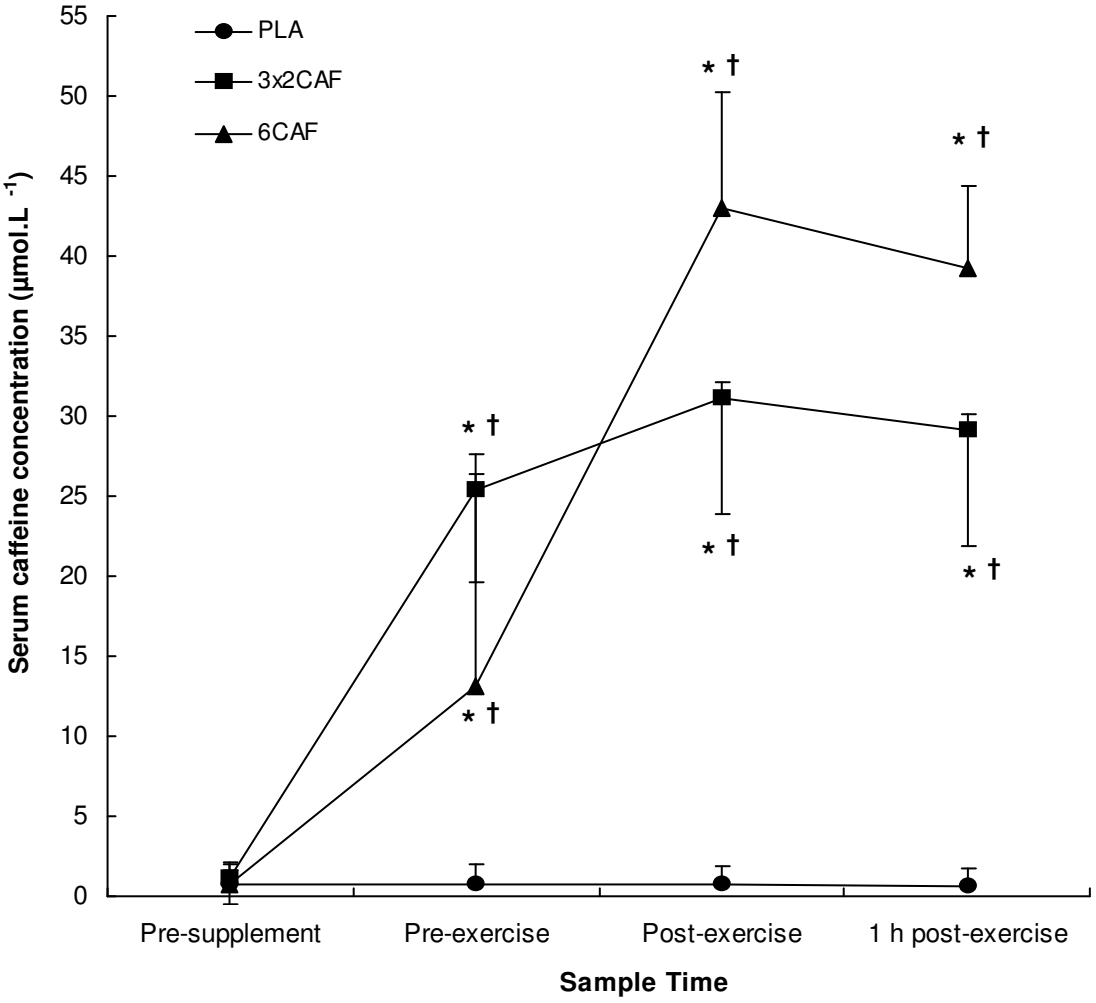


Figure 7.3: Serum caffeine concentrations during PLA, 3x2CAF and 6CAF trials. * significantly higher than PLA (P<0.01). † significantly higher than pre-supplement within trial (P<0.01). Values are means ± SD.

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 7.1). However, when compared against pre-exercise values, saliva flow rate fell significantly at post-exercise (main effect for time: P<0.01; Table 7.1). There were no significant effects of trial/treatment.

Saliva α-amylase

6CAF vs. PLA

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 6CAF compared with PLA ($P<0.05$; Table 7.1). A significant time x trial interaction was also found for saliva α-amylase secretion rate, which was higher at 1 h post-exercise on 6CAF compared with PLA ($P<0.05$; Table 7.1).

3x2CAF vs. PLA

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 3x2CAF compared with PLA ($P<0.05$; Table 7.1). There was no such interaction effect for saliva α-amylase secretion rate.

Table 7.1: Saliva caffeine concentration, flow rate, α-amylase activity and secretion rate during PLA, 3x2CAF and 6CAF 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)	16:45 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)
3x2CAF	1 (1)	10 (4) * †	8 (2) * †	14 (3) * †	15 (3) * †	20 (5) * †	19 (4) * †
6CAF	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)
3x2CAF	431 (216)	571 (254)	586 (185)	591 (294)	585 (252)	467 (209)	656 (286)
6CAF	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) †
3x2CAF	306 (271)	608 (346)	845 (319)	936 (316)	997 (313) * †	1719 (527) †	1098 (430) * †
6CAF	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) †
3x2CAF	138 (151)	239 (291)	526 (353)	570 (357)	587 (357) †	810 (470) †	742 (452) †
6CAF	157 (154)	314 (192)	497 (293)	494 (188)	660 (327) †	812 (338) †	804 (289) ** †

Values are means (SD). n = 6 for saliva caffeine concentration.
* P<0.01, ** P<0.05: significantly higher than PLA,
† 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.

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

6CAF vs. PLA

At immediately post-exercise, the number of circulating lymphocytes was significantly higher compared with pre-supplement on both PLA and 6CAF trials (both P<0.01; Table 7.2). 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 6CAF compared with PLA (P<0.01; Table 7.2).

3x2CAF vs. PLA

There was a main effect for time for the number of circulating lymphocytes, with values increasing above pre-supplement at immediately post-exercise (P<0.01; Table 7.2). 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 3x2CAF compared with PLA (P<0.05; Table 7.2).

Table 7.2: Number of circulating lymphocytes and $CD3^-CD56^+$ cells within the circulating lymphocyte population during PLA, 3x2CAF and 6CAF trials.

	Pre-supplement	Pre-exercise	Post-exercise	1 h post-exercise
Total Lymphocytes ($x10^9$ cells·L^{-1}) [a]				
PLA	2.10 (0.50)	2.10 (0.50)	2.90 (0.80) †	2.10 (0.50)
3x2CAF	2.10 (0.70)	2.00 (0.50)	3.10 (1.00)	1.90 (0.50)
6CAF	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)
3x2CAF	0.28 (0.09)	0.35 (0.10) ††	0.70 (0.22) ** †	0.25 (0.07)
6CAF	0.29 (0.11)	0.36 (0.09) ††	0.80 (0.32) * †	0.27 (0.09)

Values are means (SD).
significantly higher than PLA, * P<0.01 ; ** P<0.05.
significantly higher than pre-supplement within trial, † P<0.01 ; †† P<0.05.
[a] main effect for time; significantly higher than pre-supplement at post-exercise, P<0.01.

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

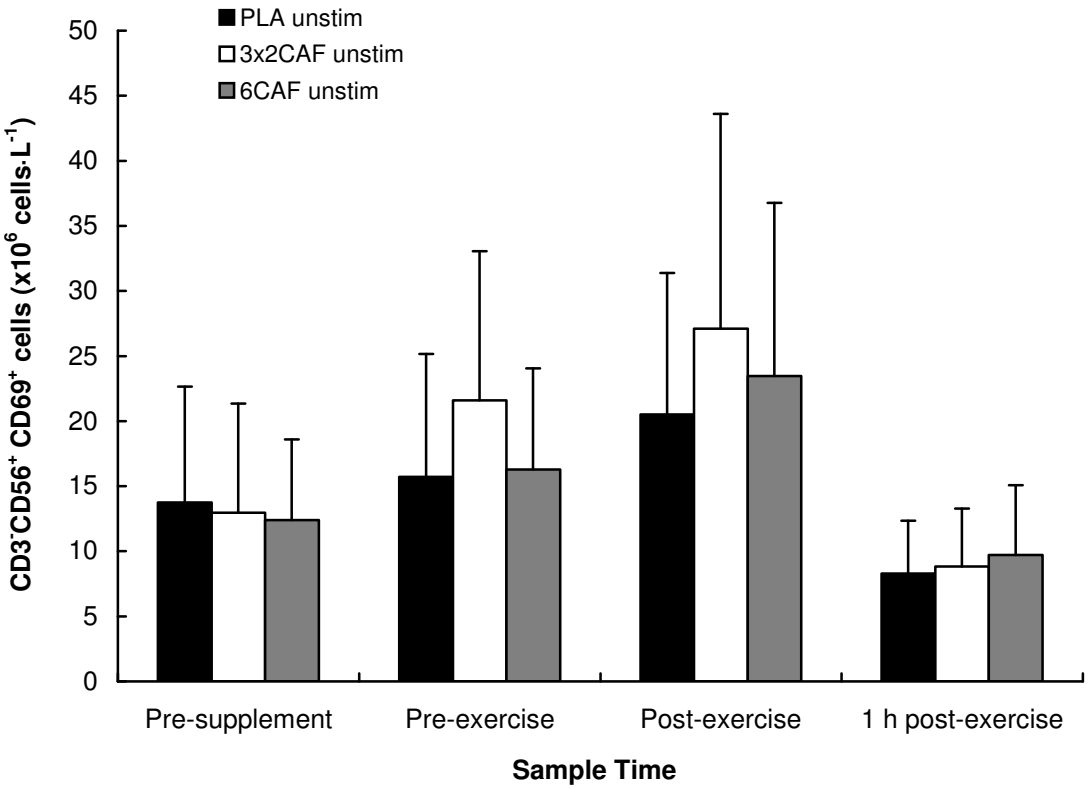
6CAF vs. PLA

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 (pre-exercise $P<0.05$; post & 1 h post-exercise $P<0.01$; Figure 7.4a). When cells were stimulated there was a significant time x trial interaction effect (both doses; $P<0.01$), with the number of antigen-stimulated $CD3^-CD56^+$ cells expressing CD69 lower at 1 h post-exercise on 6CAF compared with PLA; values on 6CAF at this time-point remained close to pre-supplement ($P<0.05$; Figure 7.4b & c).

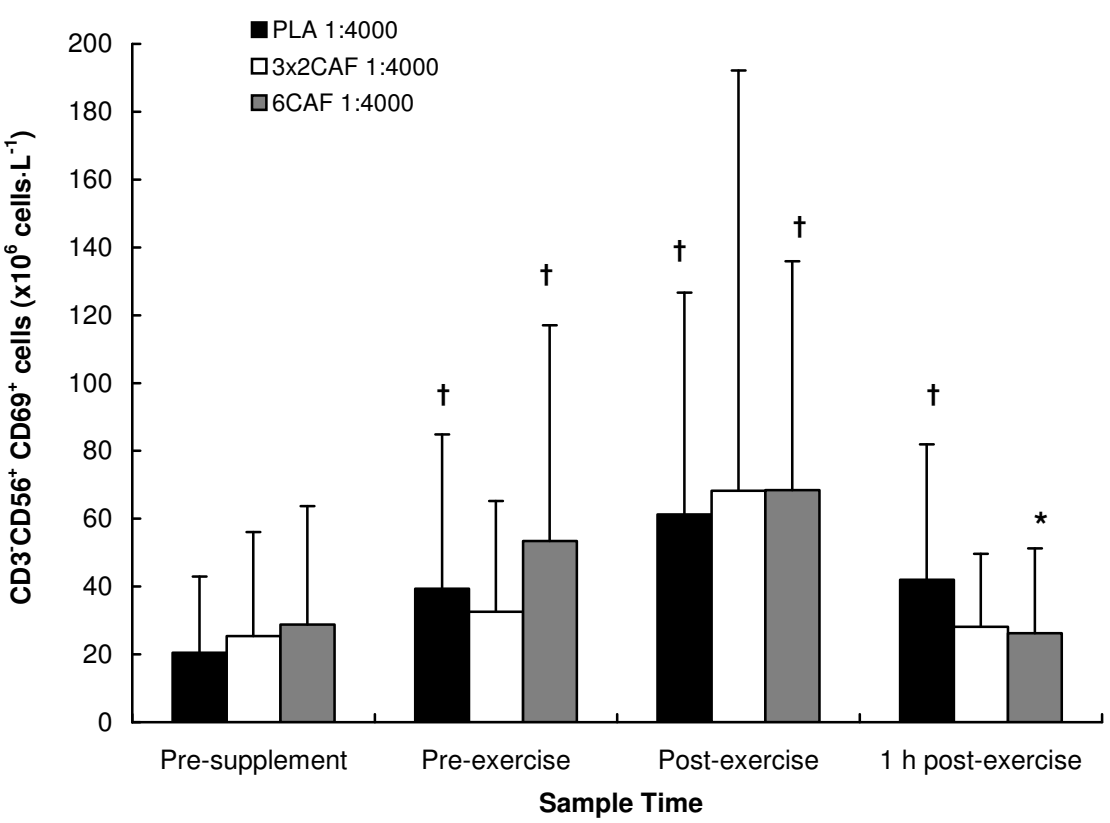
3x2CAF vs. PLA

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 (pre-exercise $P<0.05$; post & 1 h post-exercise $P<0.01$; Figure 7.4a). There were also no interaction effects when cells were stimulated (both doses; Figure 7.4b & c), but 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, post and 1 h post-exercise (all $P<0.01$ except 1:8000 pre-exercise $P<0.05$).

(a)


PLA unstim
3x2CAF unstim
6CAF unstim
CD3-CD56+ CD69+ cells (x10^6 cells·L^-1)
0
5
10
15
20
25
30
35
40
45
50
Pre-supplement
Pre-exercise
Post-exercise
1 h post-exercise
Sample Time

(b)


PLA 1:4000
3x2CAF 1:4000
6CAF 1:4000
CD3-CD56+ CD69+ cells (x10^6 cells·L^-1)
0
20
40
60
80
100
120
140
160
180
200
†
†
†
†
†
*
Pre-supplement
Pre-exercise
Post-exercise
1 h post-exercise
Sample Time

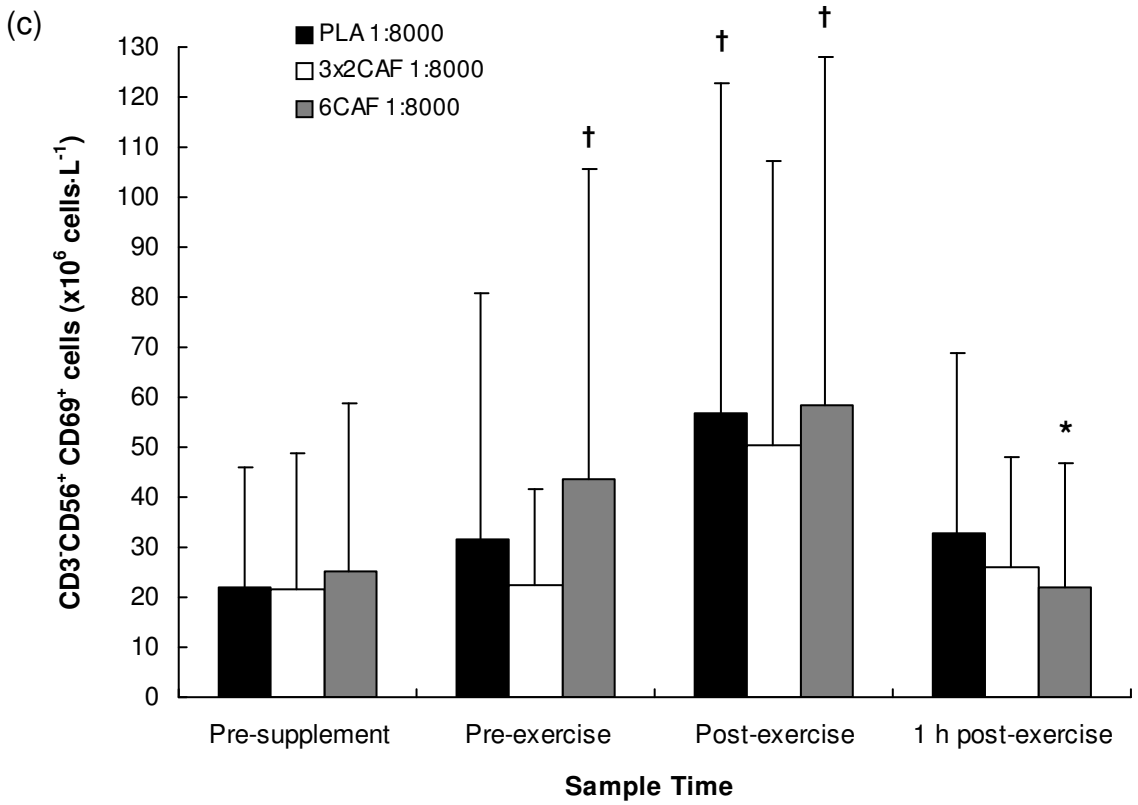


Figure 7.4: Number of (**a**) unstimulated $CD3^-CD56^+$ NK cells, (**b**) 1:4000 antigen-stimulated $CD3^-CD56^+$ NK cells, (**c**) 1:8000 antigen-stimulated $CD3^-CD56^+$ NK cells expressing CD69 within the circulating lymphocyte population during PLA, 3x2CAF and 6CAF trials. * significantly lower than PLA ($P<0.05$); † significantly higher than pre-supplement within trial ($P<0.01$). Values are means + SD.

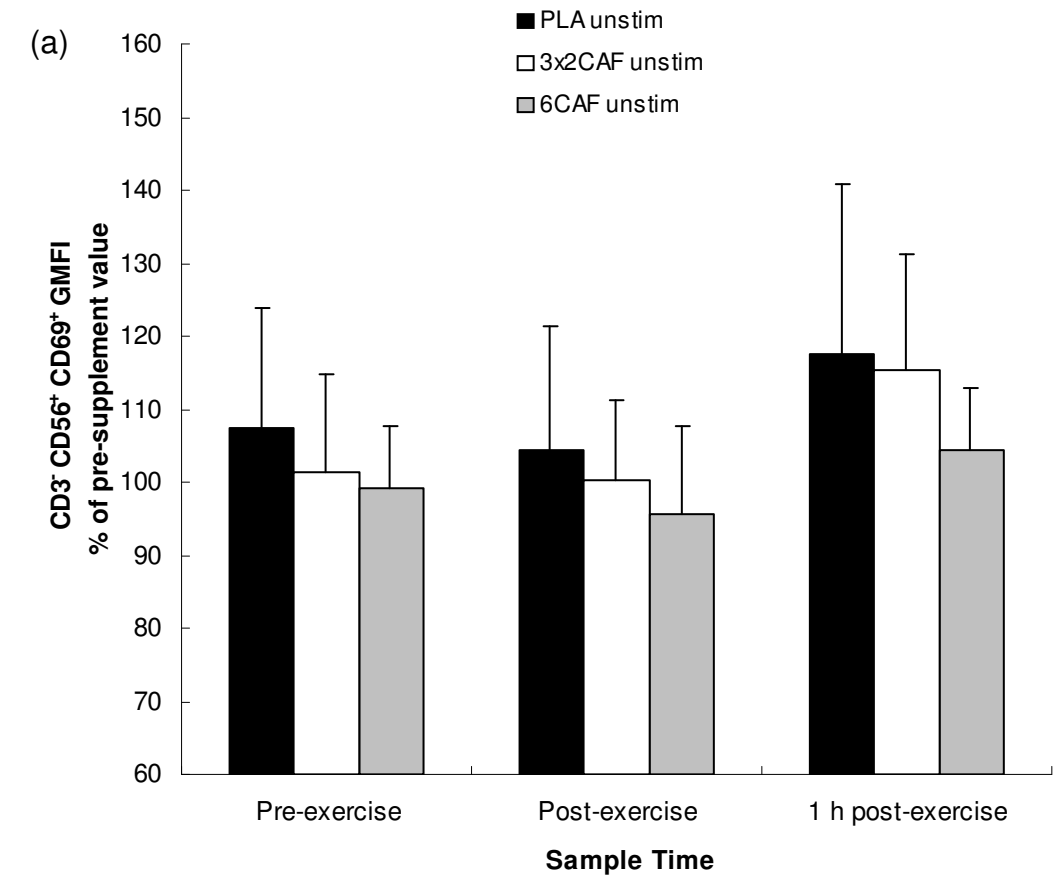
Geometric Mean Fluorescence Intensity of CD69 expression on unstimulated and antigen-stimulated $CD3^-CD56^+$ cells

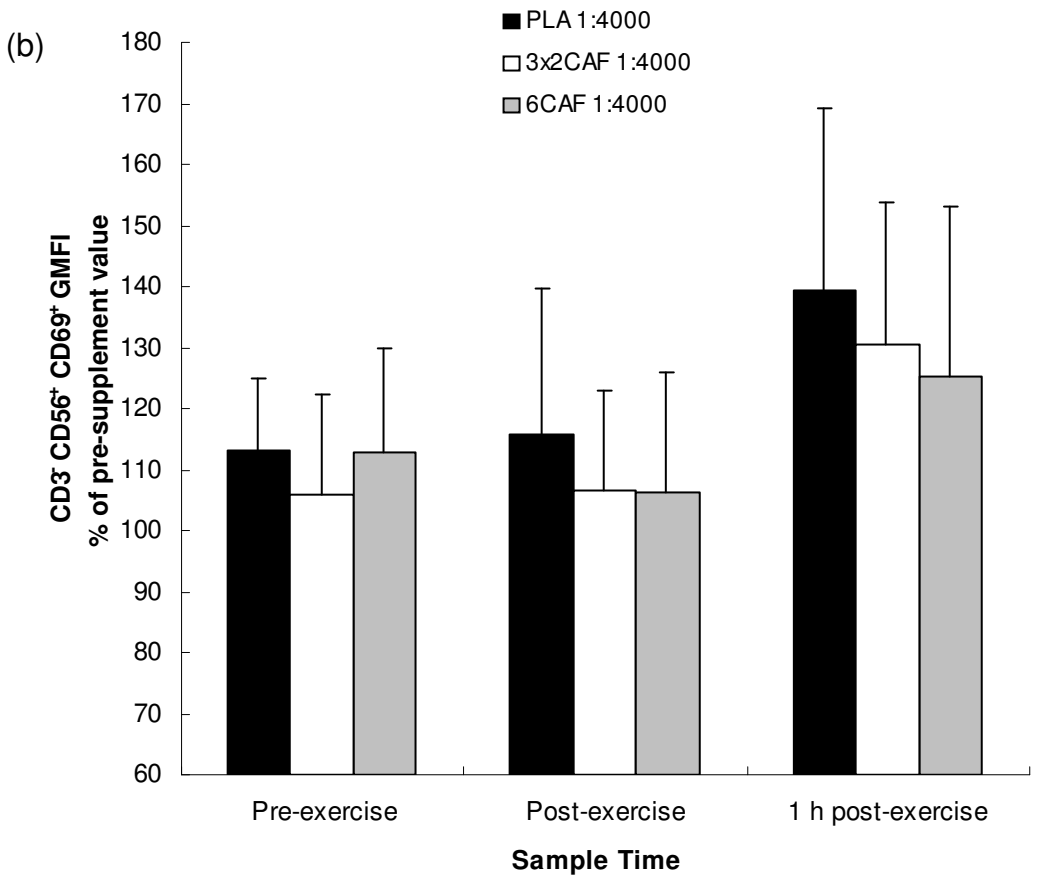
6CAF vs. PLA

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 ($P<0.01$; Figure 7.5a). 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 both trials above pre-supplement values at 1 h post-exercise ($P<0.01$; Figure 7.5c). However, values tended to be lower on 6CAF compared with PLA at this time ($P=0.055$; Figure 7.5c). The GMFI of CD69 expression on antigen-stimulated (1:8000) $CD3^-CD56^+$ cells was also lower at immediately post-exercise on 6CAF compared with PLA, with values on 6CAF at this time-point remaining close to pre-supplement values ($P<0.05$; Figure 7.5c).

3x2CAF vs. PLA

There were no significant interaction effects for the GMFI of CD69 expression on unstimulated or antigen-stimulated (both doses) $CD3^-CD56^+$ 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 ($P<0.01$; Figure 7.5a), and at pre-exercise, post-exercise and 1 h post-exercise on antigen-stimulated (both doses) $CD3^-CD56^+$ cells ($P<0.01$; Figure 7.5b & c).


(a)
PLA unstim
3x2CAF unstim
6CAF unstim
CD3- CD56+ CD69+ GMFI
% of pre-supplement value
160
150
140
130
120
110
100
90
80
70
60
Pre-exercise
Post-exercise
1 h post-exercise
Sample Time


(b)
PLA 1:4000
3x2CAF 1:4000
6CAF 1:4000
CD3- CD56+ CD69+ GMFI
% of pre-supplement value
180
170
160
150
140
130
120
110
100
90
80
70
60
Pre-exercise
Post-exercise
1 h post-exercise
Sample Time

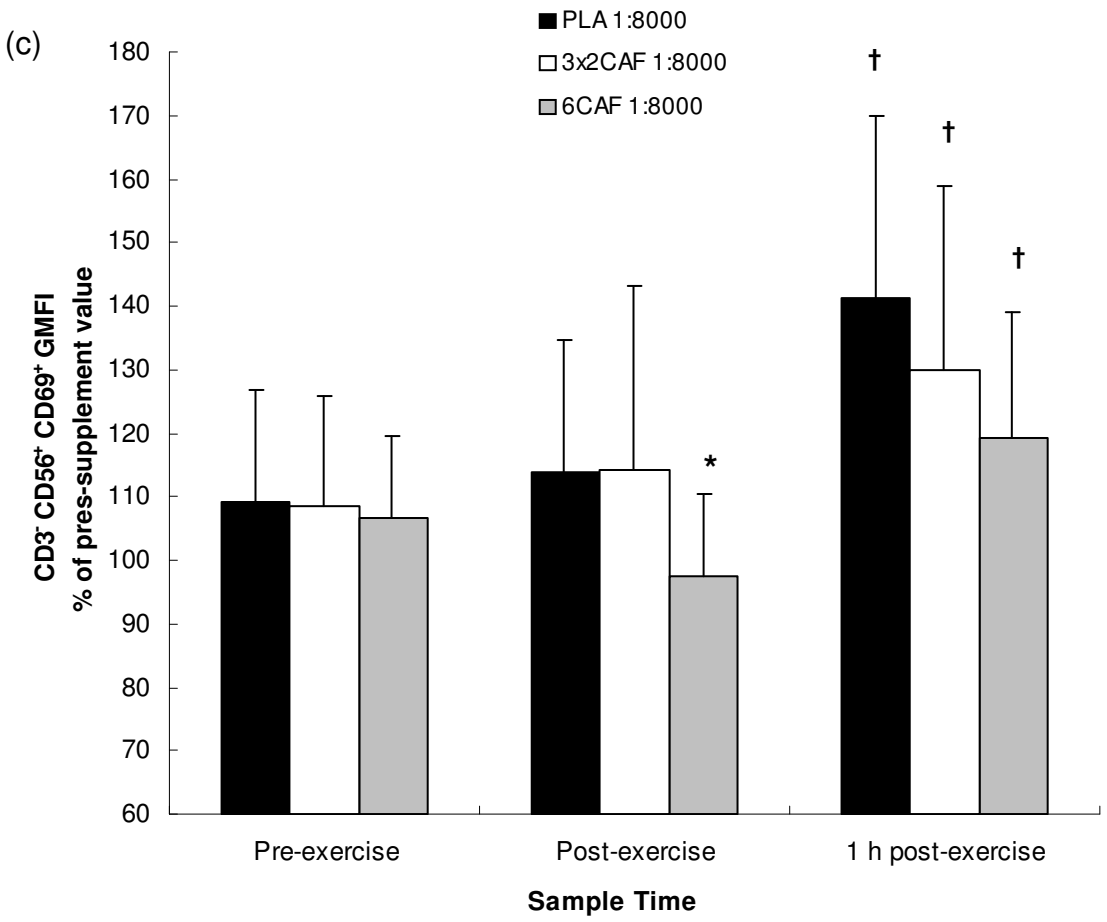


Figure 7.5: Geometric Mean Fluorescence Intensity of CD69 expression on (**a**) unstimulated $CD3^-CD56^+$ NK cells, (**b**) 1:4000 antigen-stimulated $CD3^-CD56^+$ NK cells, (**c**) 1:8000 antigen-stimulated $CD3^-CD56^+$ NK cells within the circulating lymphocyte population during PLA, 3x2CAF and 6CAF trials. * significantly lower than PLA ($P<0.05$); † significantly higher than pre-supplement within trial ($P<0.01$). Values are means + SD and are expressed as a percentage of the pre-supplement value.

7.5. 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; 6CAF). 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; 3x2CAF) had little effect on antigen-stimulated NK cell activation.

It could be suggested that the large bolus dose of caffeine attenuated the exercise-induced increase in NK cell activation following high intensity shuttle running via stimulating the release of adrenaline. Adrenaline 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 mobilised a less active subset of NK cells into the circulation following exercise. Adrenaline 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.

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 activation in response to exercise compared with placebo as previously found in Chapters 5 and 6. 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 antagonised NK cell A_1 receptors (Fredholm et al., 1999), which may have restricted the increase in antigen-stimulated NK cell activation on both caffeine trials in response to high intensity intermittent exercise compared with placebo.

Although plasma adrenaline was not determined in this study, saliva α-amylase activity and secretion rate were measured as a surrogate marker, since elevations in α-amylase concentration are considered a good indicator of

enhanced sympathetic activity (Anderson et al., 1984). Saliva α-amylase activity was significantly higher at 1 h post-exercise on both caffeine trials compared with PLA. However, only the large single dose caffeine trial (6CAF) showed a significantly higher α-amylase secretion rate at 1 h post-exercise compared with PLA. 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 adrenaline 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 adrenaline threshold, above which adrenaline produces inhibitory effects upon NK cell activation. However, as plasma adrenaline 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 activation following exercise is only speculation.

An alternative factor contributing to the contrasting findings reported here and in Chapters 5 and 6 may be the issue of caffeine tolerance. It could be argued that as participants were habitual caffeine users, the effects of caffeine on antigen-stimulated NK cell activation in response to prolonged high intensity exercise in this study may have resulted from participants' tolerance to the effects of caffeine. It has been demonstrated that tolerance to some of the effects of caffeine can develop quite quickly in habitual caffeine users (reviewed in Fredholm et al., 1999), yet resensitisation to caffeine usually occurs within 48-72 h of abstention (Benowitz et al., 1995; Varani et al., 2005). Studies both in rats and humans suggest that following chronic caffeine intake (~14 days) A_1 and A_{2A} adenosine receptors are upregulated leading to an increased sensitivity to adenosine (Johansson et al., 1997; Varani et al., 2005). Therefore, as participants were only required to abstain from caffeine overnight (as opposed to 60 h in Chapters 5 and 6), caffeine habituation may have contributed towards the findings that one large bolus dose of caffeine attenuated the exercise-

induced increase in antigen-stimulated NK cell activation 1 h following high intensity shuttle running yet small repeated doses of caffeine had little effect at all. It should be noted that the mechanisms discussed here are unlikely to occur exclusively; therefore these findings are likely to be the net result of several positive and negative influences.

The mode of exercise in the present study was also different to that used in Chapters 5 and 6 (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 adrenaline concentration between running and cycling to exhaustion. In addition, serum caffeine concentrations immediately following 90 min continuous cycling (Chapter 5) 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). In the present study participants ingested caffeine in a fed state (as opposed to a fasted state that occurred in Chapters 5 and 6). In the present study serum caffeine concentrations were very low 1 h after ingesting 6 $mg \cdot kg^{-1}$ caffeine when compared to the concentrations achieved at the same time-point in Chapters 5 and 6 (present study, 13 µM; Chapter 5, 50 µM and Chapter 6, 43 µM). Skinner et al. (2010) have also reported lower plasma caffeine concentrations 1 h after ingesting 6 $mg \cdot kg^{-1}$ caffeine with pre-exercise feeding (22 µM). At present the effects of consuming a meal on the absorption and appearance of caffeine in serum/plasma are not clear. However, pre-exercise feeding may have the potential to slow gastric emptying, which has been shown to regulate caffeine absorption (Higaki et al., 2008), and as such delay the absorption of caffeine. However at 1 h post-exercise, where the difference in findings between the present study and Chapters 5 and 6 are apparent, serum caffeine concentrations were at comparable values (present study, 40 µM; Chapter 5, 47 µM and Chapter 6, 40 µM), suggesting that differences in caffeine metabolism in a fed vs. fasted state may effect time to peak serum caffeine concentration but have little effect upon overall caffeine metabolism.

One limitation of this study was the absence of a resting trial to determine if caffeine acted on antigen-stimulated NK cell activation without the influence of exercise. However, data described in Chapter 4 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 activation up until 12:00 h. McFarlin et al. (2003) have demonstrated that NKCA as measured with a whole blood ^{51}Cr-release assay does not appear to be under any diurnal control, as a 24 h period of observation showed no change in NKCA at any time-point measured. As CD69 expression has been shown to be a comparable measure of NK cell function as that of NKCA (McFarlin et al., 2004), it could be suggested that CD69 expression is also unaffected by diurnal influences.

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 activation. 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 activation following 90 min of high intensity shuttle running.

Chapter 8

General Discussion

8.1. Thesis outline

This thesis set out to determine whether caffeine has any effect on lymphocyte activation in response to prolonged high intensity exercise. In order to answer this a study was first undertaken to ascertain whether caffeine at a high (6 $mg \cdot kg^{-1}$) or low (2 $mg \cdot kg^{-1}$) dose had any effect on T ($CD4^+$ and $CD8^+$) or NK ($CD3^-CD56^+$) cell activation at rest. Once caffeine's effects at rest had been established, subsequent studies went on to investigate:

- The effects of a high (6 $mg \cdot kg^{-1}$) dose of caffeine on T and NK cell activation in response to prolonged high intensity continuous cycling.

- The effects of a high (6 $mg \cdot kg^{-1}$) and low (2 $mg \cdot kg^{-1}$) dose of caffeine on NK cell activation in response to prolonged high intensity continuous cycling.

- The effects of ingesting one large (1x6 $mg \cdot kg^{-1}$) bolus dose of caffeine 1 h prior to exercise and small repeated (3x2 $mg \cdot kg^{-1}$) doses of caffeine throughout the day on NK cell activation in response to prolonged high intensity intermittent shuttle running.

8.2. Main findings of this thesis

- A high dose of caffeine mobilised a greater number of NK cells into the peripheral circulation 1 h and 2.5 h following ingestion at rest. This mobilisation did not occur with a low dose of caffeine.

- A high dose of caffeine had little effect on T cell trafficking at rest or in response to high intensity continuous cycling. A low dose of caffeine also had no effect at rest.

- Regardless of exercise mode (continuous cycling or intermittent running), a high dose of caffeine mobilised a greater number of NK cells into the peripheral circulation immediately following prolonged high intensity exercise.

- A high dose of caffeine is associated with an increased plasma adrenaline concentration but not noradrenaline concentration at rest and following continuous cycling.

- A low dose of caffeine had no effect on unstimulated or antigen-stimulated T or NK cell activation at rest both in terms of the number of cells expressing CD69 or their GMFI expression of CD69.

- A high dose of caffeine had no effect on unstimulated or antigen-stimulated T cell activation at rest, but appeared to increase the number of antigen-stimulated NK cells expressing CD69 1 h following caffeine ingestion while exerting no effect on the GMFI expression of CD69 on NK cells.

- A high dose of caffeine reduced the number of antigen-stimulated $CD4^+$ cells expressing CD69 1 h following continuous exercise, but had little effect on $CD8^+$ or NK cells. A low dose of caffeine also had no effect on the number of NK cells expressing CD69 following continuous exercise. However, 1 h following intermittent running a high dose of caffeine ingested in one bolus dose attenuated the exercise-induced increase in the number of antigen-stimulated NK cells expressing CD69. This effect did not occur in response to intermittent exercise when the high dose of caffeine was instead ingested in small repeated doses throughout the day.

- Both a high and low dose of caffeine increased the GMFI expression of CD69 on unstimulated and antigen-stimulated NK cells 1 h following continuous exercise. However, at 1 h post-exercise a high dose of caffeine also reduced the intensity of CD69 expression on antigen-stimulated $CD8^+$ cells in response to continuous cycling and tended to attenuate the exercise-induced increase in CD69 expression on antigen-stimulated NK cells in response to intermittent exercise. This effect did not occur in response to intermittent exercise when the high dose of caffeine was again ingested in small repeated doses throughout the day.

8.3. Caffeine, exercise and lymphocyte mobilisation

Part of this thesis investigated the effect of caffeine on the mobilisation of lymphocyte subsets into the peripheral circulation in response to prolonged high intensity exercise; with lymphocyte subsets being classified as either $CD4^+$, $CD8^+$ or $CD3^-CD56^+$ cells. Throughout this thesis a high dose of caffeine increased the mobilisation of NK cells into the peripheral circulation immediately following prolonged high intensity exercise. It could be suggested that this mobilisation was likely due to the higher plasma adrenaline concentration seen with this dose of caffeine at this time-point (see previous Chapter discussions)

However, within $CD4^+$, $CD8^+$ and $CD3^-CD56^+$ cells a number of further subsets exist with distinct functional and migratory capabilities (Fabbri et al., 2003; Fehniger et al., 2003; Campbell et al., 2009). As such, distinct lymphocyte subsets may be recruited into the circulation differentially. Indeed, studies have shown that lymphocyte subsets with enhanced cytotoxic functions and tissue migrating potential, such as $CD8^+$ effector memory and NK^{dim} cells are preferentially mobilised into the peripheral circulation in response to acute physiological and psychological stress (Bosch et al., 2005; Campbell et al., 2009). A greater mobilisation of enhanced cytotoxic lymphocytes into the circulation in response to stress has therefore been linked to an enhanced immune surveillance (Bosch et al., 2005). The proposed mechanism responsible for this differential recruitment has been suggested to be via β-adrenergic stimulation (Bosch et al., 2005), and that $CD8^+$ effector memory and NK^{dim} cells appear to express relatively higher levels of β_2-adrenoreceptors than other $CD8^+$ and NK subsets, making these cells the most sensitive to the effects of adrenaline (Bosch et al., 2005; Campbell et al., 2009). Therefore, it could be suggested that in this thesis (Chapters 5, 6 and 7) a high dose of caffeine increased the mobilisation of NK cells capable of promoting enhanced immune function (Bosch et al., 2005) into the peripheral circulation immediately following exercise above that of exercise alone, thereby perhaps leading to a stronger host defence system.

However, these cells also appear to show the largest egress from the blood 1 h following intensive exercise (Timmons and Cieslak, 2008; Turner et al., 2010). Although a fall in effector lymphocyte number could lead to a reduced ability of the host to defend against pathogens, this fall may more simply reflect a trafficking of these cells to sites where they are needed for immune functions with no detriment to overall host defence (Timmons and Cieslak, 2008). Throughout this thesis caffeine ingestion (regardless of dose or dosing strategy) had no effect at altering total lymphocyte counts or lymphocyte subset counts 1 h following intensive exercise when compared to placebo. Interestingly though, only continuous high intensity exercise showed a significant fall below baseline values in both total lymphocyte counts and $CD4^+$ $CD8^+$ and NK cell counts 1 h following exercise. Total lymphocyte and NK cell counts had already returned to baseline values at the same time-point when intermittent high intensity exercise was performed.

8.4. Caffeine, exercise and lymphocyte activation

It could be suggested that a high dose of caffeine in this thesis led to an enhanced first line of defence immediately following prolonged high intensity exercise due to the greater number of mobilised NK cells within the circulation at this time-point (Chapters 5, 6 and 7). However, in order for cells to perform their specific immune functions in response to an antigenic challenge, they first need to become activated. Therefore, one would have expected that the greater number of NK cells immediately following exercise with a high dose of caffeine would have led to a greater number of activated NK cells with a higher intensity of expression of activation markers in response to antigen-stimulation; this was not the case and as such demonstrates that although there was a rise in NK cell number with caffeine ingestion following exercise, this did not translate into an increase in NK cell function at this time-point. Similarly, as there were no differences in $CD4^+$, $CD8^+$ and NK cell number 1 h following exercise with caffeine ingestion when compared to placebo, one might have assumed that caffeine had little effect at altering lymphocyte function at this time-point, if cell number were the only measure used to determine this. However, as seen throughout this thesis caffeine significantly altered

lymphocyte activation in response to antigen stimulation 1 h following high intensity exercise both in terms of the number of antigen-stimulated lymphocyte cells and in the ability of these cells to become activated in response to antigen.

In this thesis a high dose of caffeine reduced the intensity of CD69 expression on $CD8^+$ cells in response to antigen stimulation 1 h following prolonged high intensity exercise. In Chapter 5 it was proposed that the increased plasma adrenaline concentration that occurred with a high dose of caffeine may have stimulated the β_2-adrenoreceptors on these cells, increasing intracellular cAMP concentrations and as such reducing the ability of these cells to become activated in response to antigen stimulation. However, if any of the participants in this study were carrying the cytomegalovirus (CMV), a common herpes virus that is carried by 50-70% of western populations (Turner et al., 2010), their previous infection history with this virus could have confounded the above finding. It has been reported that $CD8^+$ effector memory cells that are specific for CMV are extremely sensitive to the effects of adrenaline due to their high expression of β_2-adrenoreceptors (Bosch, 2010). As such these CMV specific $CD8^+$ cells are mobilised into the circulation following both acute stress (psychological) and β-agonist infusion to a greater extent than any other $CD8^+$ subset (Riddell, 2010). Therefore if any participant were CMV seropositive, these cells could have reduced the ability of the total $CD8^+$ population to respond to antigen stimulation with the ingestion of a high dose of caffeine 1 h following high intensity exercise and may have masked the true effects of caffeine on $CD8^+$ cells in response to high intensity exercise.

This thesis has demonstrated the complex influence of caffeine when it comes to its effects on antigen-stimulated lymphocyte subset activation in response to prolonged high intensity exercise. Caffeine doses and dosing strategies have been shown to exert immunomodulatory effects that have both increased and decreased antigen-stimulated lymphocyte activation mostly 1 h following high intensity exercise, as well as exerting no effect at all depending on the lymphocyte subset measured (T or NK) and the mode of exercise (continuous cycling or intermittent running) performed. However, some of the effects of

caffeine have not been replicated in other studies and so throughout this thesis a number of factors have been considered that may help to explain why caffeine effects are not always replicated and why caffeine appears to effect antigen-stimulated lymphocyte activation differently both in terms of caffeine dose and exercise undertaken. Factors covered have included differences in mechanistic responses, habitual caffeine intake and caffeine tolerance, as well as potential differences between exercise mode and caffeine metabolism.

However, a further factor that needs to be considered is that of genetics. Studies in animals and humans have implicated that genetic make-up appears to play a role in individual variability in caffeine consumption and in the direct effects of caffeine (reviewed in Yang et al., 2010). Both pharmacokinetic and pharmacodynamic polymorphisms have been linked to the differences in individuals' responses to caffeine (Yang et al., 2010). Most interestingly, studies have implicated polymorphisms in adenosine A_1 and A_{2A} receptors in caffeine response (Yang et al., 2010). For instance, the adenosine A_{2A} receptor single-nucleotide polymorphism (SNP) rs5751876, which has three alleles CC, CT and TT, has been implicated in an individual's sensitivity to the effects of caffeine. It was demonstrated that a greater proportion of individuals with the CC genotype who reported themselves as sensitive to the effects of caffeine were also the most at risk of caffeine-induced sleep impairment, while a greater prevalence of those with the TT genotype who reported being insensitive to caffeine's effects showed no risk for caffeine-induced insomnia (Rétey et al., 2007). In light of these studies, it could be suggested that differences in genotype on lymphocyte A_{2A} receptors could have influenced the effects of caffeine on lymphocyte activation in response to exercise in this thesis, and may help to explain why some of the effects of caffeine were not always reproduced despite similarities in caffeine dose and exercise protocol. Indeed, there does appear to be a tentative link in this thesis between the number of unstimulated $CD4^+$ and NK cells expressing CD69 and an individual's habitual caffeine intake. Correlations appear to suggest that the number of naturally active $CD4^+$ ($r= -.714$, $P<0.05$) and NK cells ($r= -.809$, $P<0.01$) increases as the amount of caffeine habitually consumed decreases.

8.5. Other potential mechanisms

Several mechanisms have been explained throughout this thesis as to how caffeine may exert its effects on lymphocyte activation in response to prolonged high intensity exercise. However, these may not be the only mechanisms responsible for caffeine's effects and as such there may be a number of other mechanisms capable of influencing the overall effects of caffeine on lymphocyte activation. Therefore, other potential mechanisms may include the influences of heat shock protein 72 (Hsp72) and β-endorphins. The effects of β-endorphins have been shown to be quite variable on lymphocyte function but generally appear to inhibit T cell proliferation but increase NKCA (Pedersen and Hoffman-Goetz, 2000; Wakao et al., 2000). Recently it has also been suggested that Hsp72 may directly activate NK cells (Multhoff, 2009). Both Hsp72 and β-endorphin plasma concentrations have been shown to increase following intensive exercise (Pedersen and Hoffman-Goetz, 2000; Whitham et al., 2006) and as such may be involved in the temporary modulation of lymphocyte function seen both immediately and in the hours following high intensity exercise (Gleeson, 2007). Following the ingestion of 6 $mg \cdot kg^{-1}$ body mass caffeine both the plasma concentration of Hsp72 and β-endorphins have also been shown to increase significantly in response to high intensity exercise above that of exercise alone (Laurent et al., 2000; Whitham et al., 2006). Therefore, as the ingestion of caffeine appears to increase the plasma concentration of Hsp72 and β-endorphins in response to high intensity exercise, it could be suggested that these increases could have contributed to the increase in NK cell activation and decrease in T cell activation seen in this thesis 1 h following prolonged high intensity exercise.

However, it should be borne in mind that this thesis used whole blood cultures when stimulating cells. Although whole blood cultures are the closest one can get to *in vivo* conditions in terms of retaining the proximity between leukocytes and extracellular milieu (Gleeson, 2007); whole blood cultures make it difficult to ascertain whether caffeine directly affected lymphocyte activation or exerted its effects on other cells and their receptors or soluble factors within the culture (i.e.

monocytes, toll like receptor expression and/or heat shock proteins) that subsequently influenced lymphocyte activation.

8.6. Remaining Issues

The main findings from this thesis have shed light on the effects of caffeine on antigen-stimulated lymphocyte activation in response to prolonged high intensity exercise. However, in doing so this thesis has highlighted a number of issues that require future research.

Throughout this thesis it has been proposed that any differences between caffeine and placebo antigen-stimulated T and NK cell activation in response to exercise have been due to caffeine exerting a direct effect upon these cells. As explained above this may not be the case and as such future research should look to investigate caffeine's effects on isolated T and NK cells *in vitro* in response to antigen stimulation following high intensity exercise in order to determine whether caffeine does directly influence lymphocyte activation.

This thesis has also speculated as to the mechanisms responsible for the effects of caffeine on antigen-stimulated lymphocyte activation in response to prolonged high intensity exercise. In order to determine if these speculated mechanisms presented in this thesis are the main pathways responsible for caffeine's effects, future studies should investigate the effects of caffeine (at different concentrations) combined with adenosine and/or adrenaline (at concentrations likely to occur following high intensity exercise) on antigen-stimulated lymphocyte activation *in vitro*.

The findings from this thesis appear to suggest that a high dose of caffeine ingested in one bolus dose 1 h prior to exercise exerts differential effects on antigen-stimulated NK cell activation depending on whether high intensity continuous cycling or high intensity intermittent shuttle running is undertaken. These findings were unexpected as caffeine metabolism does not appear to differ in response to different exercise modes (Graham and Spriet, 1991). As

such it was suggested that the differences seen between exercise modes could have been influenced by the length of time participants were asked to abstain from caffeine and as such their potential tolerance to caffeine's effects. Therefore, future studies investigating the potential impact of caffeine habituation on the effects of caffeine on antigen-stimulated lymphocyte activation in response to both high intensity continuous and intermittent exercise may help to explain why these differences occurred in this thesis.

8.7. Limitations of this thesis

As with any research study there are always going to be factors that could have been measured that could have added value to your research findings. As such these factors are what I consider to be the main limitations of my thesis and are as follows:

- The effects of a high and low dose of caffeine ingested at 08:30 h on T and NK lymphocyte activation at rest were only measured up until 12:00 h and as such can only be applied to the findings in Chapters 5 and 6. There is no data on the effect of ingesting small repeated doses of caffeine at 09:00 h, 12:00 h and 15:00 h or one bolus dose of caffeine ingested at 15:00 h on NK cell activation at rest up until 18:45 h. Therefore, although the findings in Chapter 7 are more than likely due to the effect of caffeine in response to exercise, it is difficult to ascertain for certain that these effects would not have occurred in response to caffeine ingestion without subsequent exercise.

- The effect of caffeine on T lymphocyte activation was only measured in 1 of the 3 exercise studies in this thesis. Measuring the effect of a low dose of caffeine on T lymphocyte activation in response to continuous cycling as well as the effect of one large bolus dose of caffeine and repeated small doses of caffeine ingested throughout the day on T lymphocyte activation in response to intermittent shuttle running could have strengthened Chapters 6 and 7. These findings may have given an insight into how these caffeine dosing strategies could have affected the adaptive side of the immune

system in response to different modes of exercise and if these were consistent with or contrasted from the finding that a high dose of caffeine reduces T cell activation in response to continuous cycling.

- Plasma catecholamines were only measured in 2 of the 4 studies in this thesis. As such this thesis only has plasma catecholamine concentrations for a high and low dose of caffeine at rest and a high dose of caffeine in response to prolonged high intensity continuous cycling. Measuring the concentration of plasma catecholamines following ingestion of a low dose of caffeine in response to continuous cycling as well as following ingestion of one large bolus dose of caffeine and small repeated doses of caffeine in response to prolonged high intensity intermittent shuttle running could have strengthened Chapters 6 and 7. These measures may have helped to clarify whether caffeine-induced adrenaline-mediated mechanisms had any influence on the effects seen on NK cell activation in response to exercise in Chapters 6 and 7.

- Although CD69 has been shown to be a reliable measure of lymphocyte function (Green et al., 2003; McFarlin et al., 2004), this thesis did not measure any other downstream functional responses of lymphocytes such as T cell proliferation or NKCA. As such any effects of caffeine on the early measure of lymphocyte function (CD69) in response to prolonged high intensity exercise reported in this thesis may not have actually affected subsequent lymphocyte functions.

8.8. Implications of this thesis

This thesis is the first to demonstrate the complex actions of caffeine on antigen-stimulated T and NK lymphocyte activation 1 h after prolonged intensive exercise. A high dose of caffeine decreases antigen-stimulated T cell activation (acquired immunity), while a high and low dose of caffeine increases antigen-stimulated NK cell activation (innate immunity) following continuous exercise. Other studies (Bishop et al., 2006b; Walker et al., 2006, 2007) also report immunostimulatory effects of caffeine on aspects of the innate immune system

during and following high intensity continuous exercise, suggesting that this aspect of immune function is perhaps more susceptible to the beneficial effects of caffeine following continuous exercise. However, a high dose of caffeine ingested 1 h prior to exercise also appears to attenuate the exercise induced increase in NK cell activation 1 h following high intensity intermittent exercise. This attenuation does not occur in response to intermittent exercise when a high dose of caffeine (1x6 mg·kg^{-1}) is broken down into small doses and ingested throughout the day (3x2 mg·kg^{-1}).

Therefore, based on the caffeine habits of athletes and the findings from this thesis, athletes wanting to use caffeine as an ergogenic aid should aim to ingest ~2 mg·kg^{-1}, as this dose of caffeine not only appears to enhance athletic performance (Cox et al., 2002), but also appears to increase the ability of NK cells to respond to infectious agents 1 h following high intensity exercise similar to that of a dose 3 times as large (6 mg·kg^{-1}). In order to maximise the potential benefits of this dose of caffeine both in terms of enhanced performance and improved host defence, athletes should look to abstain from caffeine preferably for a period of 60 h.

One potential strategy to overcome the negative effects of caffeine on post-exercise T cell activation following continuous exercise may be co-ingesting caffeine with carbohydrate, given that carbohydrate ingestion prevents post-exercise falls in antigen-stimulated T cell function (Bishop et al., 2005b). Carbohydrate ingestion has little effect on NK cell responses to exercise (Nieman et al., 1997), therefore combining with caffeine could maximise the benefits of both supplements to immune function, in addition to their ergogenic effects. Another strategy may be to ingest a smaller dose of caffeine (2 mg·kg^{-1}), as small doses (~3 mg·kg^{-1}) appear to have little effect at increasing plasma adrenaline concentrations in response to high intensity exercise (Graham and Spriet, 1995). Therefore, ingesting a small dose of caffeine could limit the negative effects of adrenaline on T cell activation while still allowing caffeine to increase NK cell activation.

Although caffeine has appeared to exert immunomodulatory effects upon antigen-stimulated lymphocyte activation in response to prolonged high intensity exercise throughout this thesis, the biological significance of these findings in terms of their actual potential to alter an individual's susceptibility to infection is still to be determined, yet the widespread use of caffeine within society and the athletic population highlights the need for future investigations in this regard.

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Appendices

INFORMED CONSENT FORM
(to be completed after Participant Information Sheet has been read)

The purpose and details of this study have been explained to me. I understand that this study is designed to further scientific knowledge and that all procedures have been approved by the Loughborough University Ethical Advisory Committee.

I have read and understood the information sheet and this consent form.

I have had an opportunity to ask questions about my participation.

I understand that I am under no obligation to take part in the study.

I understand that I have the right to withdraw from this study at any stage for any reason, and that I will not be required to explain my reasons for withdrawing.

I understand that all the information I provide will be treated in strict confidence.

I agree to participate in this study.

Your name _____

Your signature _____

Signature of investigator _____

Date _____

HEALTH SCREEN FOR STUDY VOLUNTEERS **Name or Number** .....

It is important that volunteers participating in research studies are currently in good health and have had no significant medical problems in the past. This is to ensure (i) their own continuing well-being and (ii) to avoid the possibility of individual health issues confounding study outcomes.

Please complete this brief questionnaire to confirm fitness to participate:

1. **At present,** do you have any health problem for which you are:
   - (a) on medication, prescribed or otherwise ..... Yes ☐ No ☐
   - (b) attending your general practitioner ..... Yes ☐ No ☐
   - (c) on a hospital waiting list ..... Yes ☐ No ☐
2. **In the past two years,** have you had any illness which require you to:
   - (a) consult your GP ..... Yes ☐ No ☐
   - (b) attend a hospital outpatient department ..... Yes ☐ No ☐
   - (c) be admitted to hospital ..... Yes ☐ No ☐
3. **Have you ever** had any of the following:
   - (a) Convulsions/epilepsy ..... Yes ☐ No ☐
   - (b) Asthma ..... Yes ☐ No ☐
   - (c) Eczema ..... Yes ☐ No ☐
   - (d) Diabetes ..... Yes ☐ No ☐
   - (e) A blood disorder ..... Yes ☐ No ☐
   - (f) Head injury ..... Yes ☐ No ☐
   - (g) Digestive problems ..... Yes ☐ No ☐
   - (h) Heart problems ..... Yes ☐ No ☐
   - (i) Problems with bones or joints ..... Yes ☐ No ☐
   - (j) Disturbance of balance/coordination ..... Yes ☐ No ☐
   - (k) Numbness in hands or feet ..... Yes ☐ No ☐
   - (l) Disturbance of vision ..... Yes ☐ No ☐
   - (m) Ear / hearing problems ..... Yes ☐ No ☐
   - (n) Thyroid problems ..... Yes ☐ No ☐
   - (o) Kidney or liver problems ..... Yes ☐ No ☐
   - (p) Allergy to nuts ..... Yes ☐ No ☐
4. **Has any,** otherwise healthy, member of your family under the age of 35 died suddenly during or soon after exercise? ..... Yes ☐ No ☐

If YES to any question, please describe briefly if you wish (eg to confirm problem was/is short-lived, insignificant or well controlled.)

.....

5. **Have you** had a cold or feverish illness in the past month? Yes ☐ No ☐
6. **Are you** accustomed to vigorous exercise (1-3 hours per week)? Yes ☐ No ☐

Thank you for your cooperation

Physical Activity Questionnaire

The following questions are designed to give us an indication of your current level of physical activity.

Name:_____ Date:__/__/__

Are you currently ENDURANCE TRAINING? YES ☐ NO ☐ .

If Yes, how many days each week do you usually train?

…….

How many training sessions do you have a day?

……

How may minutes does each session last? ……..

What is your weekly mileage? …….....

Please break this down according to discipline. E.g. cycle, swim and run

……..

Are you involved in any of the following training programmes?

Weight training ☐ Interval training ☐ Skills training ☐.

If Yes, how many days each week do you usually train?

……

…..

How many minutes does each session last?

……

CAFFEINE CONSUMPTION QUESTIONNAIRE

Please complete the questionnaire concerning your caffeine usage. List the number of times you consume the following substances during a typical day. Please also specify the product you use, E.G. Tea – PG tips.

	Morning	Afternoon	Evening	Night
COFFEE (Regular 5 oz. Mugs)				
Regular Brewed				
Percolated				
Espresso (2 oz. Serving)				
Instant				
Decaffeinated				
TEA (Regular 5 oz mugs)				
Hot Chocolate (Regular 5 oz mugs)				
Chocolate (Regular Bar)				
Chocolate Biscuits / Cakes / Cereals				
Chocolate / Coffee Dairy Products				
SOFT DRINKS (330ml Can Size)				
Coke				
Dr Pepper				
Energy Drinks (Please state which ones)				
OVER THE COUNTER DRUGS				
Pain Killers e.g. Anadin (Please state which ones)				
Pro Plus				

Do you take caffeine prior to exercise? _____

If Yes, How much? _____

What do you take? _____

Why? _____

MANY THANKS

Health Questionnaire

Please complete the following brief questions to confirm your fitness to participate:

1) At present do you have any health problems for which you are:

a) On medication, prescribed or otherwise YES ☐ NO ☐ .

b) Attending your general practitioner YES ☐ NO ☐ .

2) Have you any symptoms of ill health, such as those associated with a cold or other common infection? YES ☐ NO ☐ .

3) Are you presently acting as a subject for any other experiment or research study?

YES ☐ NO ☐ .

If you have answered yes to any of the above questions please give more details below:

……

……

……

…….

Do you want to take part in today's experiments? YES ☐ NO ☐ .

Signature:……. Date:…….

Pediacel Titration Jan 2008

$CD4^+CD69^+$ Gated on R1 (total lymphocyte population)

conc	final conc	% gated	MFI	GMFI
0	0	3.56	46.59	30.52
1:100	1:1000	7.48	187.00	87.52
1:200	1:2000	7.11	273.55	126.29
1:400	1:4000	8.06	223.7	109.40
1:800	1:8000	7.81	272.00	129.02
1:1600	1:16000	7.86	178.28	82.08
1:3200	1:32000	6.96	135.56	66.58
1:6400	1:64000	7.18	103.83	48.96

$CD8^+CD69^+$ Gated on R1 (total lymphocyte population)

conc	final conc	% gated	MFI	GMFI
0	0	3.19	52.14	36.00
1:100	1:1000	3.61	79.34	45.84
1:200	1:2000	5.14	111.24	50.41
1:400	1:4000	5.37	122.49	51.44
1:800	1:8000	4.03	133.08	59.04
1:1600	1:16000	3.36	95.73	50.15
1:3200	1:32000	2.75	81.34	43.45
1:6400	1:64000	3.49	70.5	40.45

$CD3^-CD56^+CD69^+$ Gated on R2 ($CD3^-CD56^+$ lymphocytes)

conc	final conc	% gated	MFI	GMFI
0	0	40.63	21.72	17.19
1:100	1:1000	46.92	40.05	24.78
1:200	1:2000	64.74	55.19	32.36
1:400	1:4000	64.29	56.76	34.34
1:800	1:8000	55.63	46.42	28.25
1:1600	1:16000	47.56	37.06	24.05
1:3200	1:32000	40.18	37.76	24.62
1:6400	1:64000	41.24	26.34	19.9

Exercise trial without caffeine

Time-points	Number of cells on the day (out of 30000 counts)			Number of cells after 20 h incubation (out of 30000 counts)		
	$CD4^+$	$CD8^+$	$CD3^-$ $CD56^+$	$CD4^+$	$CD8^+$	$CD3^-$ $CD56^+$
Pre-exercise	14973 50%	9369 31%	5538 18%	14689 49%	9876 33%	5015 17%
Post-exercise	11724 39%	10803 36%	9861 33%	11074 37%	10266 34%	9349 31%

Time-points	% $CD4^+$ cells expressing CD69				GMFI expression of CD69			
	On the day	0	1:4000	1:8000	On the day	0	1:4000	1:8000
Pre-exercise	0.83	3.16	2.63	4.90	24.53	20.00	45.99	27.59
Post-exercise	0.60	2.12	1.63	4.84	22.81	20.98	19.84	24.87

Time-points	% $CD8^+$ cells expressing CD69				GMFI expression of CD69			
	On the day	0	1:4000	1:8000	On the day	0	1:4000	1:8000
Pre-exercise	2.51	4.59	4.58	7.65	23.81	21.89	31.71	25.00
Post-exercise	2.41	3.91	3.72	6.99	22.19	22.32	20.78	24.76

Time-points	% $CD3^-$ $CD56^+$ cells expressing CD69				GMFI expression of CD69			
	On the day	0	1:4000	1:8000	On the day	0	1:4000	1:8000
Pre-exercise	3.10	4.86	5.79	6.90	49.07	42.66	42.51	41.83
Post-exercise	1.62	3.24	3.38	4.73	33.51	38.05	33.25	36.48

Rest trial without caffeine

Time-points	% $CD4^+$ expressing CD69			GMFI expression of CD69		
	Unstim	1:4000	1:8000	Unstim	1:4000	1:8000
Pre-supplement	0.38	4.53	2.83	42.75	70.66	45.54
Post-exercise	0.52	5.57	3.94	34.39	67.36	64.16
1 h post-exercise	1.54	7.14	5.24	26.73	66.89	58.30

Time-points	% $CD8^+$ expressing CD69			GMFI expression of CD69		
	Unstim	1:4000	1:8000	Unstim	1:4000	1:8000
Pre-supplement	1.44	6.23	2.08	44.96	71.57	35.97
Post-exercise	1.61	8.18	4.39	45.39	73.99	54.05
1 h post-exercise	2.73	9.95	7.04	40.43	78.55	64.52

Time-points	% $CD3^-$ $CD56^+$ cells expressing CD69			GMFI expression of CD69		
	Unstim	1:4000	1:8000	Unstim	1:4000	1:8000
Pre-supplement	3.06	18.60	6.82	40.96	55.01	44.49
Post-exercise	4.23	21.44	16.20	44.15	68.09	64.96
1 h post-exercise	6.59	20.64	16.02	40.54	63.11	52.88

Rest trial with caffeine

Time-points	% $CD4^+$ expressing CD69			GMFI expression of CD69		
	Unstim	1:4000	1:8000	Unstim	1:4000	1:8000
Pre-supplement	0.49	3.34	1.14	29.70	68.72	24.84
Post-exercise	1.56	4.06	1.83	21.68	57.30	48.07
1 h post-exercise	0.67	3.11	2.09	23.96	47.79	33.47

Time-points	% $CD8^+$ expressing CD69			GMFI expression of CD69		
	Unstim	1:4000	1:8000	Unstim	1:4000	1:8000
Pre-supplement	1.22	5.32	2.08	31.65	60.21	39.09
Post-exercise	1.66	6.25	2.18	26.93	55.54	36.24
1 h post-exercise	1.20	3.24	2.53	32.67	40.21	29.96

Time-points	% $CD3^-$ $CD56^+$ cells expressing CD69			GMFI expression of CD69		
	Unstim	1:4000	1:8000	Unstim	1:4000	1:8000
Pre-supplement	4.83	12.64	7.00	39.69	55.07	49.09
Post-exercise	5.01	12.06	9.51	40.66	56.10	49.05
1 h post-exercise	4.89	11.23	8.87	38.51	49.61	44.12

Exercise trial with caffeine

Time-points	% $CD4^+$ expressing CD69			GMFI expression of CD69		
	Unstim	1:4000	1:8000	Unstim	1:4000	1:8000
Pre-supplement	1.71	5.20	3.19	22.04	52.36	40.09
Post-exercise	2.97	5.01	4.12	23.58	39.21	40.38
1 h post-exercise	1.30	4.90	5.90	26.80	52.70	59.91

Time-points	% $CD8^+$ expressing CD69			GMFI expression of CD69		
	Unstim	1:4000	1:8000	Unstim	1:4000	1:8000
Pre-supplement	6.89	10.99	7.94	21.13	28.70	22.28
Post-exercise	8.99	8.32	9.79	21.30	25.14	23.97
1 h post-exercise	6.01	9.89	10.99	23.45	30.75	29.86

Time-points	% $CD3^-$ $CD56^+$ cells expressing CD69			GMFI expression of CD69		
	Unstim	1:4000	1:8000	Unstim	1:4000	1:8000
Pre-supplement	1.89	9.83	6.74	36.37	44.58	39.56
Post-exercise	5.93	7.38	5.35	34.06	40.95	38.08
1 h post-exercise	1.64	9.72	8.55	45.83	42.56	50.98