The effects of repeated bouts of prolonged cycling and carbohydrate supplementation on immunoendocrine responses in man

THE EFFECTS OF REPEATED BOUTS OF PROLONGED CYCLING AND CARBOHYDRATE SUPPLEMENTATION ON IMMUNOENDOCRINE RESPONSES IN MAN

by

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ABSTRACT

Prolonged strenuous exercise affects the circulating numbers and functions of immune cells. These effects are thought to be largely mediated by the actions of elevated circulating stress hormones and alterations in regulatory cytokines. Although the effects of a single acute bout of exercise on immune system function are quite well established, it is still not clear how time of day and repeated bouts of prolonged exercise on the same day influence immune function. It is of particular interest to understand the effects of nutritional supplementation on immunoendocrine responses. Therefore, the aims of the studies described in this thesis were to determine the effects of two bouts of prolonged cycling and carbohydrate supplementation on immunoendocrine responses.

The saliva collection study showed that the use of a swab for collecting saliva is not an ideal method because it affects the results of saliva composition (Chapter 4). The comparison of the effects of exercise at different times of day on immunoendocrine responses showed that a single bout of prolonged exercise performed in the afternoon induces a larger perturbation in the redistribution of leukocytes into the circulation than an identical bout of morning exercise, which may be due to higher hypothalamic-pituitary-adrenal (HPA) activation and circadian rhythms. However, in terms of oral mucosal immunity, performing prolonged cycling at different times of day does not differently affect the salivary responses. The second compared with the first of two bouts of prolonged exercise on the same day induces a greater HPA activation, a larger leukocyte trafficking into the circulation, a decreased neutrophil degranulation response to lipopolysaccharide (LPS) on per cell basis and a lower saliva flow rate, but does not increase plasma interleukin-6 (IL-6), or change saliva immunoglobulin A (sIgA) secretion rate (Chapter 5). Furthermore, carbohydrate (CHO) ingestion during any period of two bouts of prolonged exercise shows limited beneficial effect in blunting these higher responses in the second exercise bout compared with the first identical exercise bout on the same day (Chapter 6, 7 and 8). The determination of the effects of CHO ingestion on exercise-induced immunoendocrine responses showed that when two bouts of exercise are performed on the same day, the greater benefit in terms of circulating immunoendocrine responses is obtained by feeding CHO at the earliest opportunity (Chapter 6, 7 and 8). A 3-h interval is

insufficient for recovery of leukocyte mobilisation and neutrophil function from the impact of previous exercise whether subjects consumed placebo or CHO during exercise or recovery (Chapter 5, 6, 7 and 8). However, an 18-h interval is sufficient for full recovery of all immunoendocrine variables that were measured in this thesis from the impact of two bouts of prolonged exercise (Chapter 8).

Key words: leukocyte redistribution, neutrophil function, stress hormones, interleukin-6, circadian rhythms, saliva flow rate, immunoglobulin A, carbohydrate, repeated exercise.

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ACTH	Adrenocorticotrophic hormone
ATP	Adenosine triphosphate
cAMP	Adenosine 3', 5'-cyclic monophosphate (cyclic AMP)
CD	Cluster of differentiation
$CD4^+$ cells	Helper T lymphocytes
$CD8^+$ cells	Cytotoxic T lymphocytes
CHO	Carbohydrate
CL	Chemiluminescence
CNS	Central nervous system
CRH	Corticotrophin-releasing hormone
ELISA	Enzyme linked immunosorbant assay
FFAs	Free fatty acids
fMLP	N-formylated methionylpeptides
GH	Growth hormone
H_2O_2	Hydrogen peroxide
HOCl	Hypochlorous acid
HPA	Hypothalamic-pituitary-adrenal
IAUC	Incremental area under curve
IFN	Interferon
IgA	Immunoglobulin A
IL	Interleukin
IL-6	Interleukin-6
K_3EDTA	Tripotassium ethylenediamine-tetraacetic acid

kDa	Kilo-Dalton
LPS	Lipopolysaccharide
LTB4	Leukotriene B4
MPO	Myeloperoxidase
NADPH	Nicotinamide adenine dinucleotide phosphate
NBT	Nitro blue tetrazolium
NK cells	Natural killer cells
$^1O_2$	Single oxygen
$O_2^{-\cdot}$	Superoxide anion
$OH^{\cdot}$	Hydroxyl radical
PAF	Platelet activating factor
PBS	Phosphate buffered saline
PKC	Protein kinase C
PMA	Phorbol-12-myristate-13-acetate
rhIL-6	Recombinant human interleukin-6
ROS	Reactive oxygen species
sIgA	Saliva immunoglobulin A
SNS	Sympathetic nervous system
TNF	Tumour necrosis factor
$\dot{V}CO_2$	Rate of carbon dioxide production
$\dot{V}O_2$	Rate of oxygen uptake
$\dot{V}O_{2\,max}$	Maximal oxygen uptake
URTI	Upper respiratory tract infection

CHAPTER ONE

Introduction

The human immune system consists of a complex network of cellular and humoral factors and is functionally divided into innate immunity and adaptive immunity. Immune cell functions are modified by stress. When homeostasis is disturbed or threatened by internal or external challenges, both the sympathetic nervous system (SNS) and the hypothalamic-pituitary-adrenal (HPA) axis become activated, resulting in increased peripheral levels of catecholamines and glucocorticoids acting in concert to maintain the steady state of internal milieu (Elenkov *et al.*, 2000). Exercise has been recognised as a reliable tool to induce reproducible and quantifiable stress responses via manipulation of type, intensity, frequency and duration (Smith and Pyne, 1997). Therefore, in recent years exercise has been applied to investigate the relationships among the endocrine, nervous, and immune systems (Ostrowski *et al.*, 1998, Suzuki *et al.*, 1999). To date, the effects of exercise on infection and immunity are not fully clear, however, it can be stated that exercise transiently alters various immune parameters and prolonged strenuous exercise can elicit reversible immunodepression (Fricker *et al.*, 1999). This may offer an "open window" to microorganisms and place athletes at a higher risk of infection after heavy exertion (Pedersen, 1999).

Many studies have indicated that regular moderate exercise is beneficial in the prevention of infectious diseases (Suzuki and Machida, 1995, Mackinnon, 2000, Matthews *et al.*, 2002). However, epidemiological studies demonstrate that endurance athletes are at increased risk of upper respiratory tract infection (URTI) after heavy training and/or competition and the vulnerable period can last up to 2 weeks (Nieman *et al.*, 1990, Peters *et al.*, 1993, Nieman, 1997). Furthermore, exercise also results in a biphasic mobilisation of total leukocytes and leukocyte subsets in the circulation (Hoffman-Goets and Pedersen, 1994, McCarthy and Dale, 1988), which includes initial lymphocytosis, monocytosis and neutrophilia, followed by a delayed response of neutrophilia and lymphopenia. Further detailed information about responses of stress hormones, leukocyte redistribution, immunodepression and URTI to exercise is presented in Chapter 2.

The purpose of exercise immunological studies is not only to define the impact of exercise on immune system but also to seek means of preventing immunodepression in athletes. Nutritional strategies, particularly carbohydrate supplementation, have been demonstrated to attenuate responses of the immune and neuroendocrine systems to exercise (Gleeson *et al.*, 2001b). Carbohydrate (CHO) ingestion compared with placebo (PLA) better maintains plasma glucose concentration, attenuates HPA activation (Mitchell *et al.*, 1990), plasma cytokines responses and immunological perturbations to an acute single bout of fixed duration exercise (Gleeson and Bishop, 2000b). However, if exercise is continued to exhaustion, ingesting CHO during exercise may enhance performance, but has little effect on minimising immunoendocrine responses (Bishop *et al.*, 2001).

Routine training programmes of elite athletes commonly consist of several bouts of intensive exercise in a day. This is especially so for endurance athletes, such as marathoners, triathletes, road race cyclists, and cross-country skiers whose daily training schedule usually includes repeated bouts of prolonged exercise. It seems likely that the higher incidence of infection in elite athletes is due, at least in part, to the repeated bouts of intensive exercise without sufficient recovery. Failure to fully recover between training sessions has been suggested to evoke chronic fatigue, underperformance, and further depression of immune function (Gleeson *et al.*, 2001b). Recently, several studies have focused on investigating how repeated bouts of exercise affect immunoendocrine responses and have shown that a second exercise bout on the same day evokes more pronounced changes in numbers of circulating leukocyte subsets and production of stress hormones, especially in adrenaline and growth hormone, compared with a single bout of identical exercise at the same time of day (Ronsen *et al.*, 2001a, 2001b). However, there are only few studies to date that have looked at the relationship between daily two bouts of endurance exercise and immunoendocrine responses, especially the impact on neutrophil function (MaCarthy *et al.*, 1992, Rohde *et al.*, 1998. Ronsen *et al.*, 2001a, 2001b, 2002a, 2002b, Boyum *et al.*, 2002, Mcfarlin *et al.*, 2003).

Neutrophils, which represent 50-60% of the total blood leukocytes, act as the first line of defence against infectious agents. The neutrophil is an effective phagocyte and is considered to be an important part of innate immunity, playing a critical role in the host

defence against various bacterial infections (Nieman, 1994). Muns *et al.* (1994) reported that after long-distance running nasal neutrophils were less able to ingest bacteria and the effect lasted for 3 days. An impaired neutrophil microbicidal capacity may increase the susceptibility to infection in stressed athletes (Fukatsu *et al.*, 1996).

The humoral arm of the mucosal immune system is principally composed of locally synthesized polymeric immunoglobulin A (IgA), which functions as a multi-layered mucosal host defence (Lamm, 1998). Hence, salivary IgA (sIgA) has been used to be a key indicator in determining the effect of different forms of stress on mucosal immunity. Previous studies could not provide an agreement of how exercise stress affects sIgA and URTI (Gleeson, 2000b). The inconsistencies in the literature may be due to several factors, which affect saliva composition including the source of saliva, saliva flow rate, saliva collection method, nature of stimulus, circadian rhythm, and the degree of hydration of the subject. Therefore, we carried out two studies to determine the effect of different saliva collection methods on saliva flow rate and sIgA responses (Chapter 4).

In order to further investigate the effects of repeated bouts of exercise and time of day on immunoendocrine responses, we designed a experimental protocol to examine the effect of both single and repeated bouts of prolonged cycling on leukocyte redistribution, neutrophil degranulation, interleukin-6 (IL-6), plasma stress hormone responses, saliva flow rate and sIgA responses (Chapter 5). Elite athletes usually perform exercise two or three times in a day. To maintain immune function and performance in a subsequent exercise bout, it is particularly important for athletes to rapidly recover from any temporary exercise-induced immunodepression (Gleeson and Bishop, 2000b) and restore glycogen (Maughan, 2002) during recovery intervals. Therefore, we investigated the effect of CHO supplementation during the recovery interval between two bouts of prolonged cycling on leukocyte redistribution, neutrophil degranulation, IL-6, plasma stress hormone responses, saliva flow rate and sIgA responses to a second cycling bout (Chapter 6). For various considerations, many athletes wake up in very early morning and train without breakfast. Thus, we planned a study to clarify the influences of CHO supplementation during the first exercise bout on leukocyte redistribution, neutrophil degranulation and oxidative burst, IL-6, plasma stress hormone responses, saliva flow rate and sIgA responses to this and a

subsequent exercise bout on the same day (Chapter 7). During a second exercise bout muscle glycogen content may be compromised by the previous exercise bout. This may induce an energy crisis in the working muscle, affecting SNS and HPA activation. Therefore, we also investigated the effect of CHO supplementation during the second exercise bout on immunoendocrine responses (Chapter 8). This thesis concludes with a general discussion summarising the findings and discussing the issues arising in these experimental studies (Chapter 9).

CHAPTER TWO

Literature Review

2.1 Circadian Variation in Stress Hormones, IL-6, Circulating Numbers of Leukocytes and sIgA

Many components of the immunoendocrine system show rhythmic changes (Petrovsky *et al.*, 1998, Porterfield, 2001). To understand and control for circadian variations is very important when the aim of a study is to compare the effects of exercise performed at different times of day on immunoendocrine responses.

2.1.1 Stress hormones and IL-6

Cortisol is the major circulating human glucocorticoid and functions as a powerful natural immuno-suppressant. Plasma cortisol exhibits a prominent circadian rhythm, maximal in the early morning hours just before awakening and reaching a nadir in the late evening until next early morning, which appears to impose diurnal variation on immune function (Petrovsky *et al.*, 1998). Plasma adrenocorticotropic hormone (ACTH) also shows a pronounced diurnal pattern, which peaks in the early morning and declines to a nadir in the evening (Porterfield, 2001). Growth hormone (GH) shows significant diurnal rhythm, with a peak secretion in the early morning just before awakening and a lower secretion during the rest of day (Porterfield, 2001). There is no circadian variation in catecholamines (Porterfield, 2001) and IL-6 (Crofford *et al.*, 1997).

2.1.2 Circulating numbers of leukocytes

Circulating leukocyte and neutrophil counts demonstrate circadian rhythms increasing from early morning and peaking in the late evening (Haus, 1994), whereas lymphocyte counts are elevated during the night and decline after wakening (Dhabhar *et al.*, 1994). This inverse relationship between cortisol and circulating leukocyte counts suggests that the endocrine system might play an important role in regulating immune cell turnover and/or redistribution between immune compartments (Dhabhar *et al.*, 1994). Previous

studies have also reported that lymphocyte adrenoreceptor density peaked around noon (Pangerl *et al.*, 1986), whereas the glucocorticoid receptor density peaked around midnight (Homo-Delarche, 1984).

2.1.3 Saliva immunoglobulin A

Saliva IgA shows diurnal variation (Dimitriou *et al.*, 2002). Saliva IgA concentration is highest in the early morning, followed by a decline during the morning and then is stable from around noon onwards (Gleeson *et al.*, 2001a).

2.2. Glucose, Immune Response and Glycogen Restoration

It has been suggested that dietary intake of nutrients potentially affects immune system function because most nutrients are involved in the synthesis and regulation of immune factors (Gleeson and Bishop, 2000a). Imbalanced nutrition may result in glycogen depletion and subsequent elevation of stress hormones and compromise the immune system during prolonged exercise, leading to an increased incidence of infection (Venkatraman and Pendergast, 2002).

It is generally accepted that glucose is the primarily energy source of immune cells (Pedersen, 1999) since phagocytes uptake glucose at the rate that is 10-fold greater than that of glutamine (Scharhag *et al.*, 2002). Extracellular glucose concentration is very important to avoid neutrophil apoptosis via maintenance of the intracellular ATP concentration and stabilisation of mitochondrial function (Leist *et al.*, 1997, Healy *et al.*, 2002). Muscle glycogen depletion and hypoglycaemia are potential causes of fatigue during prolonged exercise (Coyle *et al.*, 1983, Costill and Hargreaves, 1992). CHO supplementation before, during and after prolonged exercise has been proven to better maintain euglycaemia and CHO oxidation rate in exercise, which can delay fatigue, improve endurance exercise performance and concurrently attenuate HPA activation, plasma adrenaline, ACTH, cortisol, GH, and IL-6 levels, leading to a less perturbation of the numbers of circulating leukocytes and subsets (Mitchell *et al.*, 1990, Nieman *et al.*, 1997, Bishop *et al.*, 1999b).

For the best performance elite athletes usually have to train two or three times per day. In this situation, it is particularly important for athletes to ensure a rapid restoration of glycogen during recovery intervals to maintain the quality of subsequent exercise bouts. However, if CHO is not provided during recovery, glucose availability will become the limiting factor for glycogen synthesis since gluconeogenesis is inadequate to support the maximal rate of glycogen synthesis (Satabin *et al.*, 1989). For example, consumption of 1.5 g $CHO \cdot kg^{-1}$ body mass immediately after exercise and at 2-h intervals thereafter can optimally stimulate muscle glycogen synthesis at the rate of 6.0 $mmol \cdot kg^{-1}$ wet $wt \cdot h^{-1}$. However, if no CHO is consumed during the hours of post-exercise, the rate of muscle glycogen synthesis is lower than 3.2 $mmol \cdot kg^{-1}$ wet $wt \cdot h^{-1}$ (Ivy *et al.*, 1988).

The glycogen restoration after glycogen-depleting exercise (glycogen concentration < 30 - 40 mmol) has been found to be biphasic (Price *et al.*, 1994). The rapid phase of glycogen synthesis (27 ± mmol·L^{-1}·h^{-1}, determined by ^{13}C-nuclear magnetic resonance spectroscopy) is insulin-independent lasting 30 - 60 min, and is about 10-fold faster than subsequent slower phase (2.9 ± 0.8 mmol·L^{-1}·h^{-1}), insulin-dependent phase (Price *et al.*, 1994). The type of CHO is less crucial than the amount consumed, but there may be more benefit from ingesting high-glycaemic index foods as soon as possible after exercise to ensure a rapid elevation of the blood glucose level (Maughan, 2002).

The most effective and common strategy applied by endurance athletes to support CHO availability is to ingest CHO-rich drinks or foods during prolonged exercise. However, Jeukendrup and Jentjens (2000) found only one-third of ingested CHO was oxidized during submaximal cycling because of the limited rate of gastric emptying, intestinal digestion and absorption, and subsequent glucose transport into blood stream (Jentjens *et al.*, 2001). Therefore, it has been suggested that the upper limit for glucose absorption in humans is about 1-2 g·min^{-1} during exercise (Jeukendrup and Jentjens, 2000). This limits the maximal oxidative rate for exogenous CHO to approximately 1 g·min^{-1}.

2.3 Stress Hormone Responses and Leukocyte Mobilisation to Exercise

The nervous and endocrine systems can interact with the immune system to alter the function and distribution of immune cells (Dhabhar *et al.*, 1994, Glaser *et al.*, 1999) (Figure 2.1). Leukocyte trafficking is crucial to pathogen surveillance. It has been shown that acute exercise results in a temporary, significant, and reversible redistribution of leukocyte subsets between circulation, marginal pools and the bone marrow (Gleeson and Bishop, 1999); and that this exercise-induced mobilisation is related to elevated plasma concentrations of stress hormones (Benschop *et al.*, 1996) (Figure 2.2).

2.3.1 Catecholamines and leukocyte mobilisation

During exercise, adrenaline is released from the adrenal medulla and noradrenaline is released from sympathetic nerve terminals. Arterial plasma concentrations of adrenaline and noradrenaline increase almost linearly with duration of dynamic exercise and exponentially with intensity (Kjaer, 1989). The effects of catecholamines on target cells are mediated via adrenoreceptors, which can be generally classified to four categories (α_1, α_2, β_1, and β_2) based on their different sensitivities to certain agonists. Adrenaline is a strong stimulator of β-adrenoreceptors, whereas noradrenaline predominately activates α- and β_1-adrenoreceptors (Motulsky and Insel, 1982). Since β-adrenoreceptors have been identified on T cells, B cells, NK cells, macrophages and neutrophils (Landmann, 1992), the degree to which these cells can be influenced by catecholamine signalling depends on the numbers of adrenergic receptors on the individual leukocyte subpopulations (Pedersen, 1999). The numerical orders of adrenergic receptors on lymphocyte subpopulations from highest to lowest are NK cells, $CD8^+$ lymphocytes, B cells, and $CD4^+$ lymphocytes (Rabin *et al.*, 1996). Therefore, after injection/infusion of adrenaline the most pronounced changes are observed for NK cells (CD16, CD56, CD57) and with subsequent smaller changes in $CD8^+$ cells, B cells, and $CD4^+$ (Benschop *et al.*, 1996).

As early as 1904, Loeper and Crouszon described a pronounced leukocytosis after a subcutaneous injection of adrenaline in man (Loeper and Crouzon, 1904). This observation was extended by subsequent studies and suggested the notion that the adrenaline response to leukocyte mobilisation was biphasic, consisting of an initial lymphocytosis within 10

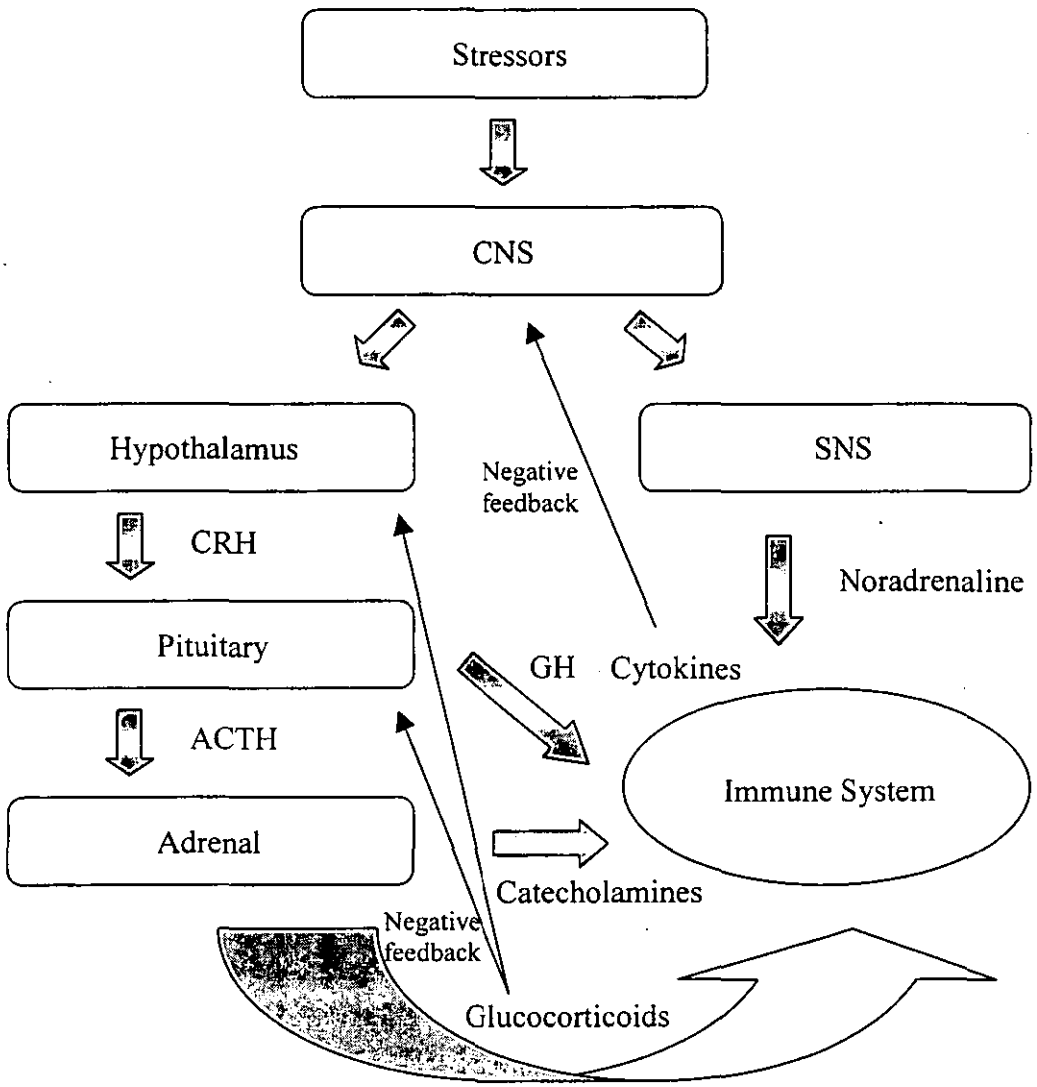


Figure 2.1 Communication between neuroendocrine and immune system. ACTH, Adrenocorticotrophic hormone; CNS, central nervous system; CRH, corticotrophin-releasing hormone; GH, growth hormone; SNS, sympathetic nervous system.

min, peaking at 30 min, and followed by a neutrophilia with relative lymphopenia, peaking at 2 to 4 h (Samuels, 1951). However, adrenaline infusion caused a significantly smaller elevation in neutrophil concentration than that observed following exercise while the plasma concentrations of adrenaline following administration and exercise were similar (Tvede *et al.*, 1994). Although adrenaline is an important hormone in recruiting lymphocytes and neutrophils into the circulation during intensive exercise within 90 min (Ernstrom and Sandberg, 1974, Nieman, 1997), after 90 min during exercise, its effect is lessened by the rising cortisol concentration, which attenuates lymphocytosis with a subsequent decline until exercise is finished (Nieman, 1997). It is likely that the adrenaline exerts a direct effect on neutrophil surface adhesive molecules (e.g., β_2-integrin CD11b/CD18) (Benschop *et al.*, 1996), and this together with exercise-induced haemodynamic shear forces (Foster *et al.*, 1986), work in a synergistic fashion to mobilise neutrophils from the marginal pools into the circulation, inducing the initial neutrophilia during prolonged exercise (Gannon *et al.*, 2001). It has been also demonstrated that the neutrophilia after adrenaline infusion was mainly recruited from spleen (Benschop *et al.*, 1996). Although lung has been suggested to be an important organ of neutrophil storage (Hogg, 1987), it did not seem to contribute significantly to the peripheral neutrophilia induced by exercise (Peters *et al.*, 1992).

2.3.2 Glucocorticoids and leukocyte mobilisation

Increases of plasma cortisol concentrations are associated with exercise intensity and duration of above 1 h. Cortisol is the principal glucocorticoid in humans playing a major role in metabolism and immune function as a potent agent of gluconeogenesis and immunosuppression (Pedersen *et al.*, 1997a). Furthermore, glucocorticoids exert a prominent role in the regulation of leukocyte redistribution (Cupps and Fauci, 1982, Dhabhar *et al.*, 1994). Glucocorticoid administration has been reported to cause neutrophilia together with lymphopenia, monocytopenia, eosinopenia, and a suppression of both NK and T cell function (Fauci, 1976, Cupps and Fauci, 1982). The significant but transient neutrophilia induced by cortisol administration is mainly caused by the influx of neutrophils from spleen and bone marrow (Toft *et al.*, 1994). Further, Nakagawa *et al.* (1998) showed after dexamethasone infusion, the circulating neutrophilia was from bone

marrow (10%), marginated pools (61%) and prolongation of neutrophil intravascular half-life (29%) in rabbits. Cortisol promotes lymphocyte redistribution from the circulation to lymphoid, bone marrow, skin and injured tissue (Wira *et al.*, 1990, Toft *et al.*, 1992), reaching a maximum at 4 to 6 h with a return to baseline within 24 h (Fauci and Dale, 1975, Calvano *et al.*, 1992).

To summarise, the immediate leukocytosis during prolonged exercise is mainly due to elevated plasma catecholamines levels, whereas the delayed neutrophilia is from the influence of elevated plasma cortisol levels. Since plasma cortisol concentration peaks at about 30 min after exercise cessation (Hansen *et al.*, 1991), it is not surprising to observe the development of a significant neutrophilia with lymphopenia within the first hour of recovery (McCarthy and Dale, 1988).

2.3.3 Growth hormone and leukocyte mobilisation

GH is a classical anterior pituitary hormone promoting cell growth and metabolism (Kappel *et al.*, 1993). In terms of immunological development and function, GH promotes lymphocyte maturation and competence, NK cell activity, cytokine production and phagocyte oxidative burst activity (Berczi, 1997, Hattori *et al.*, 2001). Exercise is a powerful stimulant for GH secretion, depending on workload, duration, intensity, prior meal ingestion, and fitness of subjects (Kanaley *et al.*, 2001). Repeated bouts of exercise on the same day appear to augment GH release (Kanaley *et al.*, 1997), whereas glucose ingestion attenuates GH secretion (Smith *et al.*, 1996). Furthermore, GH is probably at least partly responsible for exercise-induced neutrophilia because Kappel *et al.* (1993) have demonstrated that an intravenous GH injection in a physiological dose caused a neutrophilia.

2.3.4 Carbohydrate ingestion and exercise-induced leukocyte mobilisation

Intensive prolonged exercise without nutritional supplementation may cause hypoglycaemia and subsequently evoke the increased secretion of stress hormones. Schwartz *et al.* (1987) reported the thresholds of plasma glucose concentrations for inducing adrenaline, GH, cortisol and hypoglycaemic symptoms were 3.8 ± 0.1 mM, 3.7 ±

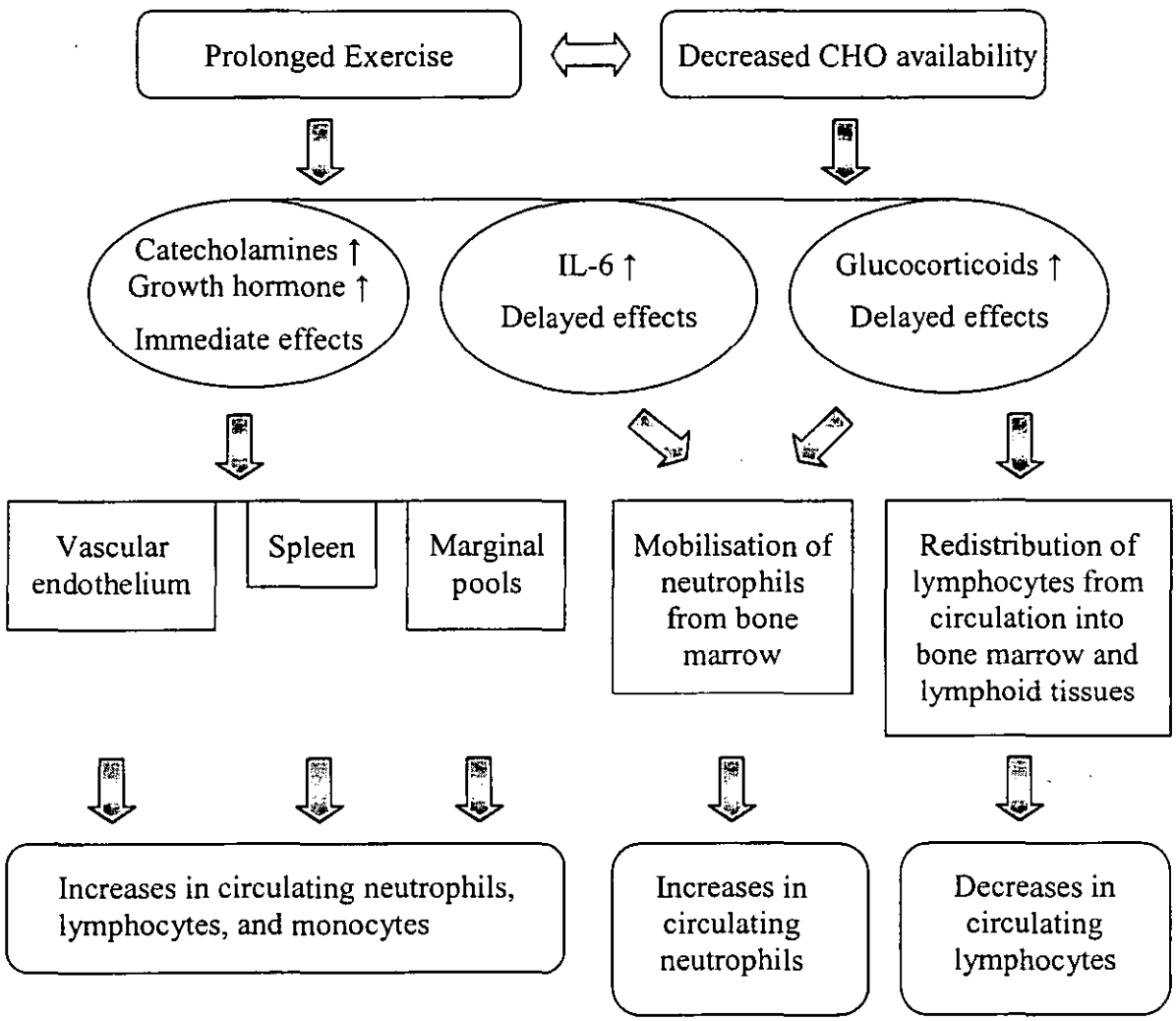


Figure 2.2 Mechanisms of the immediate and delayed leukocytosis induced by prolonged exercise

0.1 mM, 3.2 ± 0.2 mM and 2.9 ± 0.1 mM, respectively. The results of previous studies have consistently demonstrated that ingestion of a CHO drink compared with placebo (PLA) during exercise is beneficial for maintaining plasma glucose concentrations, improving endurance exercise performance and attenuating the elevation of plasma stress hormones and perturbation of circulating counts of total leukocytes and leukocyte subsets (Table 2.1).

Table 2.1 CHO ingestion and exercise-induced leukocyte mobilisation

Reference	Experimental Design	Subject	Main Findings
Bishop *et al.*, 2002	90 min high intensity intermittent running. Subjects ingested 5 mL·kg^{-1} of a 6.4% CHO or PLA before exercise and every 15-min interval during exercise.	6 male footballers	CHO ingestion attenuated the neutrophilia in the circulation compared with PLA.
Bishop *et al.*, 2001a	Subjects cycled at 75% $\dot{V}O_{2\,max}$ until fatigue. Subjects ingested 5 mL·kg^{-1} of a 5% CHO or PLA before exercise and 2 mL·kg^{-1} every 15-min interval during exercise.	9 males	CHO ingestion significantly increased performance but did not affect the circulating leukocytosis, neutrophilia and lymphocytosis compared with PLA.
Henson *et al.*, 2000	2 h rowing at 82.3 % HRmax. Subjects ingested 15 mL·kg^{-1} of a 6% CHO or PLA 15-min before exercise and 4 mL·kg^{-1} every 15-min during exercise.	15 female rowers	CHO ingestion attenuated the increase in blood counts of total leukocytes, neutrophils and monocytes compared with PLA.
Nieman *et al.*, 1997	2.5 h run at 77% $\dot{V}O_{2\,max}$. Subjects ingested 750 mL of a 6% CHO or PLA before exercise and 250 mL every 15-min during exercise.	30 marathoners 24 males & 6 females	CHO ingestion attenuated the increase in blood counts of neutrophils, lymphocytes and monocytes compared with PLA.

2.4 Exercise and Neutrophil Function

Following activation and recognition, neutrophils kill invading pathogens through both oxygen-dependent (release of reactive oxygen species, ROS) and oxygen-independent (release of proteases) mechanisms (Fukatsu *et al.*, 1996, Johnson *et al.*, 1998). It has been recently suggested that neutrophils serves as a last line of defence to block the "open window" during the period of immunodepression after intensive prolonged exercise (Pedersen, 1 999). H owever, s everal s tudies r eported t hat e ndurance t raining t emporarily reduced the activity of neutrophil phagocytosis (Blannin *et al.*, 1996), degranulation (Blannin *et al.*, 1997), and oxidative burst (Gabriel *et al.*, 1994, Pyne *et al.*, 1996).

GH and prolactin are potent neutrophil-priming agents (Fu *et al.*, 1992) promoting superoxide anion production through the tyrosine kinase signalling system in a dose-dependent manner in the concentration range of 10-500 $ng \cdot mL^{-1}$ in GH (Ruy *et al.*, 1997). Glucocorticoids have been shown to depress neutrophil functions, including chemotaxis, adherence to surfaces, phagocytosis, degranulation, oxidative burst, and antibody-dependent cytotoxicity (Liles *et al.*, 1995). However, a recent study reported that physiological levels of cortisol did not affect neutrophil degranulation capacity (Walsh *et al.*, 2000b). The impairment may be further augmented during exercise after the entry of less mature cells with lower capacities from the bone marrow into the circulation (Pyne, 1994). Adrenaline also appears to inhibit neutrophil superoxide production and elastase release in a dose-related manner (Tintinger *et al.*, 2001). Garcia *et al.* (1999) reported that the inhibitory effect of adrenaline on phorbol 12-myristate 13-acetate (PMA)-induced superoxide production was mediated by β_2-adrenergic receptors via adenosine 3', 5'-cyclic monophosphate (cAMP) production. The possible mechanisms by which neutrophil function is inhibited by prolonged exercise are presented in later sections of this review and are illustrated in Figure 2.3.

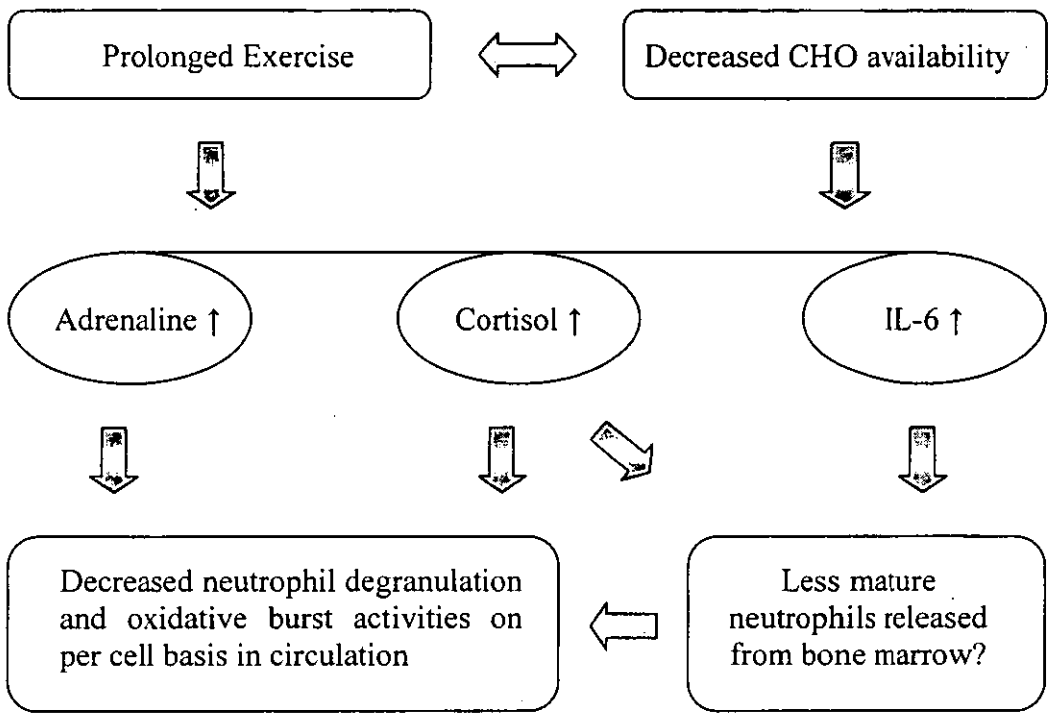


Figure 2.3 Possible mechanisms by which neutrophil function is inhibited by prolonged exercise

2.4.1 Neutrophil degranulation

With the onset of neutrophil antibacterial activity, many different cellular processes including chemotaxis, phagocytosis, oxidative burst and secretion of cytoplasmic enzymes, are initiated by appropriate stimuli. Following the formation of the phagosome, cytoplasmic granules merge with phagosome to form the phagolysosome and then the degradation and dissolving of engulfed materials by stored bactericidal agents and lysosomal enzymes released from the granules takes place (Parslow *et al.*, 2001). Elastase is one of the major degradative enzymes within the azurophilic granules of human neutrophils and functions to degrade a wide variety of extracellular materials (Elsbach, 1980). Several factors seem to affect neutrophil degranulation, including the level of intracellular cAMP (Ottonello *et al.*, 1997), phagocytic activity (Morozov *et al.*, 2003), platelet-neutrophil contacts (Losche *et al.*, 1996), adrenaline (Tintinger *et al.*, 2001), glucocorticoids (Liles *et al.*, 1995) and IL-6 (Johnson *et al.*, 1998).

Intense exercise induces neutrophil degranulation, which elevates plasma concentrations of elastase and myeloperoxidase (MPO) (Gleeson *et al.*, 1998). However, the response of neutrophil degranulation to bacterial stimulation *in vitro* declines after exercise on per cell basis and remains depressed for at least 2 h post-exercise and maybe for as long as 24 h post-exercise (Robson *et al.*, 1999, Walsh *et al.*, 2000a). These results may account for the low neutrophil function reported in highly trained individuals (Smith *et al.*, 1990, Blannin *et al.*, 1996). Morozov *et al.* (2003) suggested that elevated glucocorticoid level during exercise might stimulate neutrophil degranulation and result in a decrease of the assayed proteinas content in neutrophils and a concomitant increase in plasma levels of these enzymes. Reduced content of microbicidal proteins in neutrophils could be responsible for the decline in neutrophil phagocytic potency and microbicidal capacity when pathogens are encountered following exercise (Morozov *et al.*, 2003). However, Barnett *et al.* (1997) reported that the stimulation of β-adrenergic receptors attenuates neutrophil superoxide generation, but does not significantly affect degranulation.

2.4.2 Neutrophil oxidative burst

Neutrophils kill invading microorganisms by releasing a group of highly reactive oxidizing agents, including oxidized halogens, oxidizing radicals, and singlet oxygen. The oxidative mechanisms in neutrophils remain inactive until exposed to appropriate stimuli (Reichl *et al.*, 2000) Stimulation can occur via receptor-dependent or receptor-independent mechanisms. Typical receptor-dependent stimuli are activated complement fragment C5a, the chemotactic tripeptide N-formyl-Met-Leu-Phe (fMLP), and bioactive lipids such as platelet activating factor (PAF) and leukotriene B4 (LTB4). Receptor-independent stimuli include long-chain unsaturated fatty acids and direct agonists of protein kinase C (PKC) such as PMA. These two mechanisms for activation of the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase in neutrophils are distinct. Oxidase activation by receptor-dependent stimuli usually lasts less than 5 min, while receptor-independent stimuli activate the enzyme for a much longer period (Chanock *et al.*, 1994 and Meenan *et al.*, 2002).

Activated neutrophils initiate an oxidative burst leading to the production of superoxide anion ($O_2^{\cdot-}$), which is important as the primary substrate for neutrophil metabolism of ROS. However, $O_2^{\cdot-}$ is relatively less toxic than other ROS and is quickly converted to hydrogen peroxide (H_2O_2). The interaction of $O_2^{\cdot-}$ and H_2O_2 can result in the formation of hydroxyl radicals ($\cdot OH$) in the presence of trace metal. On the other hand, neutrophil azurophilic granules contain large quantities of MPO, which works in the presence of H_2O_2 and chloride ions to produce hypochlorous acid (HOCl). Furthermore, the interactions among $O_2^{\cdot-}$, H_2O_2 and HOCl can generate singlet oxygen ($^1O_2$). These oxidants (HOCl, $\cdot OH$ and $^1O_2$) have significantly higher oxidizing potential than the precursors $O_2^{\cdot-}$ and H_2O_2 and contribute to the complexity of the oxygen-dependent antimicrobial systems of neutrophils (Parslow *et al.*, 2001) (Figure 2.4).

PMA-induced luminol-enhanced chemiluminescence (CL) is frequently used for determining neutrophil oxidative burst activity, which is used to measure the MPO-dependent formation (mainly HOCl detection) of hyper responsive ROS (Suzuki *et al.*, 1996). PMA may increase the rate of the oxidative burst by stimulating PKC, which can phosphorylate components of the NADPH oxidase (Garcia *et al.*, 1999). Catecholamines

have been shown to inhibit CL (Weiss 1996). Adrenaline resulted in a dose-related inhibition of the superoxide production in neutrophils (Barnett *et al.*, 1997, Tintinger *et al.*, 2001). The inhibitory effect of adrenaline on neutrophil superoxide generation may be due to the lower production of NADPH via the pentose phosphate pathway. In fact, adrenaline, by stimulating the conversion of glucose into lactate and glucose oxidation, may reduce the flux of substrates through the pentose phosphate pathway (Garcia *et al.*, 1999). A number of studies have also demonstrated that GH primes or stimulates the oxidative burst of human and animal neutrophils and macrophages (Ruy *et al.*, 1997, Smith *et al.*, 1996). ROS release is unaltered by IL-6 alone; however, IL-6 can enhance the priming effect of tumour necrosis factor (TNF) on oxidant generation (Mullen *et al.*, 1995).

Suzuki *et al.* (1999) showed after 90 min cycling at ~53% $\dot{V}O_{2\,max}$ the PMA-induced CL by isolated neutrophils was increased compared with pre-exercise. However, a transient suppression of the oxidative burst after exercise has been also reported (Gabriel *et al.*, 1994, Pyne *et al.*, 1996). After short exhaustive exercise on a treadmill, CL did not immediately change at the first hour of recovery but was reduced by 22% and 28% at 3 and 6 hours post exercise, respectively (Morozov *et al.*, 2003). The slight decrease in neutrophil oxidative burst activity may represent a tendency for reduced killing capacity by circulating neutrophils on a per cell basis after exercise (Pyne, 1994, Smith and Pyne, 1997). The reasons for exercise-induced falls in neutrophil oxidative burst activity may be associated with the influx of less mature band neutrophils and/or nitro blue tetrazolium (NBT)-negative neutrophils from the bone marrow and marginated pools, respectively, into the circulation. The neutrophils in the bone marrow have a lower NADPH-dependent oxidase activity and a lower superoxide response to stimulation with PMA (Berkow and Dodson, 1986), whereas the NBT-negative neutrophils produce less $O_2^{\cdot-}$ in response to *in vitro* stimulation (Suzuki *et al.*, 1996).

2.4.3 Carbohydrate ingestion and exercise-induced alteration of neutrophil function

In neutrophils, most of the glucose and glutamine taken in is not oxidized through the Krebs cycle (Newsholme *et al.*, 1996). The high rates of glycolysis and glutaminolysis in neutrophils provide precursors for the synthesis of common metabolic intermediates, such as pyruvate or NADPH (Newsholme *et al.*, 1996, Healy *et al.*, 2002). Therefore, a fall in

the plasma glucose concentration may directly influence the oxidative burst activity (Scharhag *et al.*, 2002). To date, only few studies have been conducted to investigate the effect of CHO ingestion during exercise on neutrophil function and these results have not shown any difference between CHO and PLA treatments in neutrophil degranulation (Table 2.2) and oxidative burst (Table 2.3) changes during exercise.

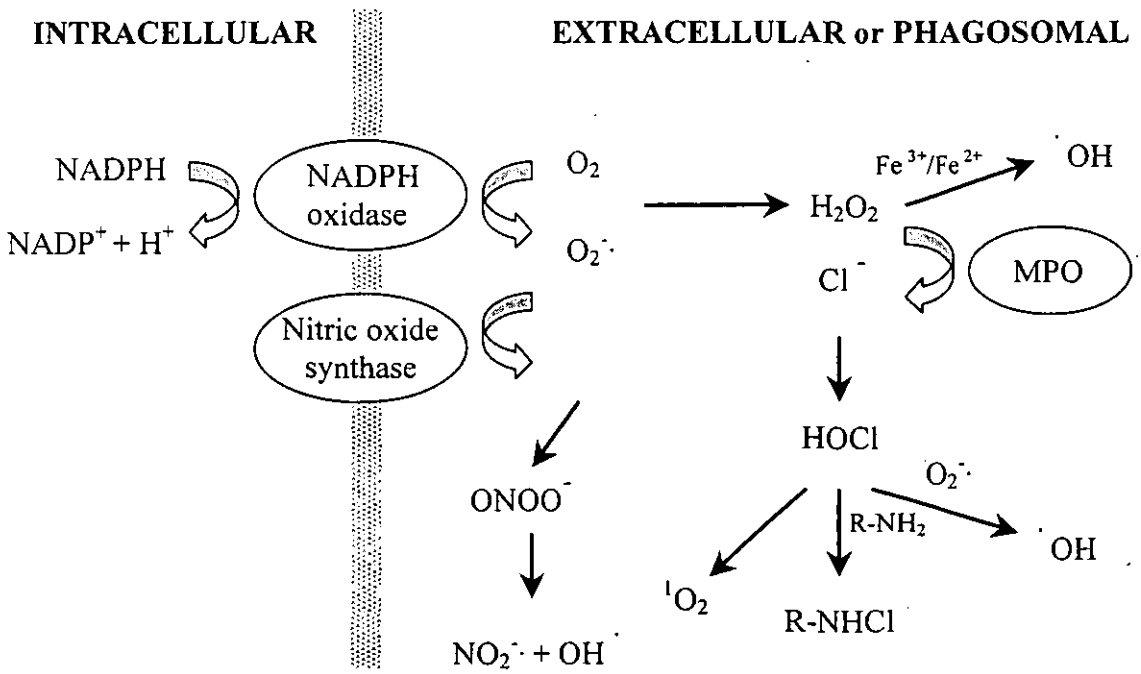


Figure 2.4 The major oxidative generating pathways in stimulated neutrophils. MPO, myeloperoxidase

Table 2.2 Carbohydrate ingestion and exercise-induced alteration in neutrophil degranulation response to lipopolysaccharide *in vitro*

Reference	Experimental Design	Subject	Main Findings
Lancaster *et al.*, 2003	~60 min cycling consisted of 20 min at 65%Wmax and time trial at 80%Wmax (~40 min). Subjects consumed 75 g glucose in 500 mL water at either 15 min or 75 min before exercise.	8 males	Exercise did not affect LPS-stimulated elastase release per neutrophil between pre-EX and post-EX and between CHO and PLA trials.
Bishop *et al.*, 2002	90 min high intensity intermittent running. Subjects ingested 5 $mL \cdot kg^{-1}$ of a 6.4% CHO or PLA before exercise and every 15-min interval during exercise.	6 male footballers	Exercise did not decrease LPS-stimulated elastase release per neutrophil. There was no significant difference between CHO and PLA trials.
Bishop *et al.*, 2001a	Subjects cycled at 75% $\dot{V}O_{2\,max}$ until fatigue. Subjects ingested 5 $mL \cdot kg^{-1}$ of a 5% CHO or PLA before exercise and 2 $mL \cdot kg^{-1}$ every 15-min interval during exercise.	9 males	Exercise decreased LPS-stimulated elastase release per neutrophil in both trials. However, there was no significant difference between CHO and PLA.

Table 2.3 Carbohydrate ingestion and exercise-induced alteration in neutrophil oxidative burst activity

Reference	Experimental Design	Subject	Main Findings
Nieman *et al.*, 1997	2.5 h run at 77% $\dot{V}O_{2\,max}$. Subjects ingested 750 mL of a 6% CHO or PLA before exercise and 250 mL every 15-min during exercise.	30 marathoners 24 males & 6 females	There was no difference between pre-EX and post-EX in both trials and between CHO and PLA in granulocyte oxidative burst activity (FITC mean fluorescence channel).
Smith *et al.*, 1996	60 min cycling at HR 140 bpm. Subjects ingested 250 mL of a 5% CHO or PLA before exercise and every 15-min during exercise.	8 males	Exercise increased PMA-stimulated intracellular H_2O_2 production. However, there was no difference between CHO and PLA.

2.5 Interleukin-6 Responses to Exercise

Cytokines are a group of soluble glycoproteins produced to mediate communication among cells, organs and systems involved in immune responses (Turnbull and Rivier, 1999). Cytokines have been characterized in several categories according to their basic physiological activities, such as pro-inflammatory, anti-inflammatory, immunoregulatory, chemotactic, haemotopoietic, and antiviral (Nieman *et al.*, 2001). IL-1, IL-6, and TNF-α are classified to be the major pro-inflammatory cytokines, whereas IL-1ra, IL-4 and IL-10 are the major anti-inflammatory cytokines (Berczi *et al.*, 1996). Strenuous exercise is associated with increased TNF-α, IL-1β, IL-6 and IL-10 (Ostrowski *et al.*, 1999, Bishop *et al.*, 2001).

IL-6 is a 21- to 28-kDa glycoprotein secreted by various cells including monocytes, macrophages, lymphocytes, epithelial cells, and skeletal muscle cells. Its receptors are also present in a variety of cells including monocytes, macrophages, lymphocytes, neutrophils, epithelial cells, liver cells and adipocytes (Keller *et al.*, 1996). IL-6 plays an important role in the induction of B-cell differentiation, monocyte proliferation, and neutrophil recruitment to inflammatory sites (Keller *et al.*, 1996). Therefore, IL-6 was previously characterized as pro-inflammatory cytokine and one of the major mediators of the acute phase response (Berczi *et al.*, 1996). However, IL-6 was recently suggested to be an anti-inflammatory cytokine because of increasing the plasma IL-1ra, IL-10, and cortisol concentrations after IL-6 infusion in physiological concentrations (Steensberg *et al.*, 2003).

It has well known that the stimulation of β_2 adrenoreceptors increases intracellular cAMP in various cell types. Increased intracellular cAMP subsequently enhances IL-6 gene expression and production. Sondergaard *et al.* (2000) reported an elevated plasma IL-6 was simultaneously elicited with an acute increase in plasma adrenaline (Sondergaard *et al.*, 2000). However, a recent study showed adrenaline exerted a minor role on the rise in the plasma IL-6 level during exercise.

IL-6 has been shown to induce a biphasic increase in circulating leukocyte and neutrophil counts (Suwa *et al.*, 2001). After IL-6 administration, blood leukocyte and neutrophil counts increased 1.3-fold and 1.8-fold at 3 h, a temporary decrease at 6 h (1.2-fold and 1.6-

fold), followed by a second increase (1.3-fold and 1.7-fold) at 9 h compared with baseline, respectively. The band neutrophils significantly increased from 4.0 ± 1% at baseline to 8.5 ± 1.4 % at 9 h (3.8-fold compared to baseline in number) and returned to the baseline by 24 h (Suwa *et al.*, 2001). The first peak was probably induced by mobilization of neutrophils from the marginated pool into the circulation, whereas, the second peak was probably induced by release of neutrophils from the bone marrow (Suwa *et al.*, 2000) since IL-6 decreases the L-selectin levels on neutrophils (Suwa *et al.*, 2002). IL-6 also affects neutrophil functions such as increasing elastase release (Kaplanski *et al.*, 2003) and oxygen-free radical production by stimulating Ca^{2+} signalling in neutrophils (Sitaraman *et al.*, 2001).

It has well known that prolonged strenuous exercise induces a marked elevation in the plasma IL-6 concentration (Nieman, 1997, Ostrowski *et al.*, 1999, Pedersen *et al.*, 2001). Recent studies have demonstrated that a large amount of IL-6 is produced in and released from contracting skeletal muscles into the circulation (Steensberg *et al.*, 2000, Langberg *et al.*, 2002) and may act as hormone-like fashion to mediate hepatic glucose production and/or muscle glucose uptake for maintenance of glucose homeostasis and stimulate lipolysis during exercise (Gleeson, 2000a, Pedersen *et al.*, 2001, Febbraio and Pedersen, 2002). This notion is supported by recent observations in exercise and IL-6 infusion studies in man. Ostrowski *et al.* (1999) observed an up to 100-fold increase in IL-6 concentration immediately after a marathon race. Subsequently, Febbraio and Pedersen (2002) reported that IL-6 did not appear until the later stage of prolonged exercise and that glucose ingestion blunted the plasma IL-6 response. When athletes started exercise in a glycogen-depleted state, plasma IL-6 response was augmented (Gleeson and Bishop, 2000b) and a lower pre-exercise muscle glycogen concentration dramatically enhanced activation of the IL-6 gene in skeletal muscle during prolonged exercise (Keller *et al.*, 2001). Therefore, it has been suggested that muscle glycogen content could be a determining factor for IL-6 production by contracting muscle (Steensberg *et al.*, 2001a). Conversely, IL-6 could also be regarded as a carbohydrate sensor during prolonged exercise (Helge *et al.*, 2003). Injection of recombinant human IL-6 (rhIL-6) into humans increases hepatic glucose production and the fasting blood glucose concentration and the release of free fatty acids (FFAs) and triglyceride in a dose-dependent manner (Pedersen *et al.*, 2001). Although

rhIL-6 has been shown to stimulate the principal glucose counterregulatory hormones, such as cortisol, GH, catecholamines, and glucagon, which would obviously exert an influence on the observed glucose changes (Stouthard *et al.*, 1995, Tsigos *et al.*, 1997), *in vitro* data demonstrated that IL-6 can exert a direct stimulatory effect on hepatic glucose release from glycogen pools by inhibiting glycogen synthase (Ritchie, 1990). However, for initiation of the acute metabolic responses, circulating IL-6 concentration has to be higher than 25-65 $pg \cdot mL^{-1}$ (Tsigos *et al.*, 1997). Resting values of plasma IL-6 are typically 0.2-2.0 $pg \cdot mL^{-1}$ and values of up to about 150 $pg \cdot mL^{-1}$ have been observed after prolonged running.

During resting conditions, adipose tissue would produce 15-25% of the systemic IL-6 around noon and 25-35% in the evening (Mohamed-Ali *et al.*, 1997). IL-6 has been found to increase lipolysis in abdominal subcutaneous adipose tissue within the normal physiological concentration (~30 $pg \cdot mL^{-1}$) (Lyngso *et al.*, 2002). A recent study demonstrated that infusion of rhIL-6 into human subjects to mimic the level observed during strenuous exercise (~140 $pg \cdot mL^{-1}$) resulted in significant lipolysis and fat oxidation without any alterations in plasma adrenaline, insulin, or glucagon levels from 90 min after the start of the infusion (van Hall *et al.*, 2003).

Recently, there are many studies have examined the effect of CHO ingestion on IL-6 responses to exercise. Generally, CHO compared with PLA ingestion appears to attenuate the increase of plasma IL-6 concentrations when exercise duration is above 90 min (Table 2.4).

Table 2.4 Carbohydrate ingestion and exercise-induced IL-6 responses

Reference	Experimental Design	Subject	Main Findings
Febbraio *et al.*, 2003	120 min semi-recumbent cycling consisted of 5 min at 50% $\dot{V}O_{2\,max}$ and 115 min at 65% $\dot{V}O_{2\,max}$. Subjects ingested 250 mL of a 6.4 % CHO or PLA at the onset of and at 15 min interval throughout, exercise.	7 males	Glucose ingestion during exercise attenuates leg IL-6 release ($P < 0.05$) but not decrease in intra-muscular expression of IL-6 mRNA.
Keller *et al.*, 2003	180 min cycling at 60% of maximal workload. Subjects ingested 250 mL of a 6 % CHO or PLA every 15 min throughout exercise.	8 males	At the end of exercise, plasma IL-6 levels were 26.3 ± 3.7 $ng \cdot L^{-1}$ in PLA and 15.6 ± 2.4 $ng \cdot L^{-1}$ in CHO ($P < 0.05$).
Lancaster *et al.*, 2003	~60 min cycling consisted of 20 min at 65%Wmax and time trial at 80%Wmax (~40 min). In timing experiment subjects consumed 75 g glucose in 500 mL water at either 15 min or 75 min before exercise. In amount experiment consumed low-CHO (25 g glucose in 500 mL water), high-CHO (200 g glucose in 500 mL water), or PLA 45 min before exercise.	8 males in timing experiment 10 males in amount experiment	Exercise increased plasma IL-6 levels in all trials in both timing and amount experiments. However, there were no differences between treatments in timing experiment and among treatments in amount experiment.
Nieman *et al.*, 2003	180 min run at treadmill at 70% $\dot{V}O_{2\,max}$. Subjects ingested 12 $mL \cdot kg^{-1}$ of a 6% CHO or PLA 15-30 min before exercise, and 4 $mL \cdot kg^{-1}$ every 15 min during exercise.	16 marathoners	At the end of exercise, plasma IL-6 levels were ~ 14.7 $ng \cdot L^{-1}$ in PLA and ~ 9.5 $ng \cdot L^{-1}$ in CHO ($P < 0.05$).
Bishop *et al.*, 2002	90 min high intensity intermittent running. Subjects ingested 5 $mL \cdot kg^{-1}$ of a 6.4% CHO or PLA before exercise and every 15-min during exercise.	6 male footballers	Exercise increased plasma IL-6 levels in both trials. However, there was no significant difference between trials at post-EX.
Starkie *et al.*, 2001	60 min run or cycling at lactate threshold (4 trials in total). Subjects ingested 8 $mL \cdot kg^{-1}$ of a 6.4% CHO or PLA before exercise, and 2 $mL \cdot kg^{-1}$ at 20 and 40 min during exercise.	7 males	CHO ingestion blunted plasma IL-6 responses. However, there was no effect of exercise mode.

2.6 Saliva Immunoglobulin A Responses to Exercise

Saliva is normally a colourless liquid with a density ranging from 1.002 to 1.012 $g \cdot mL^{-1}$, consisting of inorganic and organic constituents and usually more than 99% water. The secretory volume of saliva each day through the salivary glands approaches 750 mL, which represents a rate of approximately 0.5 $mL \cdot min^{-1}$ arising from the submandibular glands (65%), parotid glands (23%), minor mucous glands (8%) and sublingual glands (4%) (Crawford *et al.*, 1975).

Immunity against microorganisms at remote sites, such as the nasal cavity, oral cavity, respiratory tract, digestive tract and gut, is primarily due to secretory IgA, which has been considered as the first line of defence to infection in the lumen of the respiratory tract and gut (Quan *et al.*, 1997). Secretory IgA is produced in local plasma cells and seems to function as a multi-layered mucosal defence. For example, IgA prevents antigens and microbes from adhering to and penetrating the epithelium (immune exclusion), interrupts replication of intracellular pathogens during transcytosis through epithelial cells (intracellular neutralization), and binds antigens in the lamina propria facilitating their excretion through the epithelium back into the lumen (immune excretion) (Lamm, 1998).

2.6.1 Modulation of saliva IgA secretion

Saliva IgA is produced in the submucosa of salivary glands and then binds to a receptor (polymeric immunoglobulin receptor, pIgR) located on the mucosal epithelium. Subsequently, the complex is transported across the mucosal epithelium and released into the saliva as sIgA (Mostov, 1994, Brandtzaeg, 1998). The modification of sIgA secretion is regulated via the rate of synthesis (days) (Goodrich and McGee, 1998, Toellner *et al.*, 1998) or transcytosis (minutes) (Kugler, 1999). Therefore, the acute alteration induced by exercise is likely through the modulation of the transepithelial secretory process rather than plasma cell (B lymphocyte) activation.

Nervous Control

The salivary glands are innervated by both parasympathetic nerves and sympathetic nerves (Chicharro *et al.*, 1998, Busch *et al.*, 2002). Parasympathetic stimulation induces a marked elevation in regional blood flow to salivary glands by vasodilation, resulting in a higher saliva flow rate with a relatively low protein concentration, whereas the sympathetic stimulation causes vasoconstriction, resulting a lower saliva flow rate that is rich in protein (Garrett, 1987, Anderson, 1998, Chicharro *et al.*, 1998).

Glucocorticoids

Cortisol has been suggested to play an important role in inhibiting sIgA mobilisation (Hucklebridge *et al.*, 1998). Wira *et al.* (1990) reported a decline in sIgA level at 24 h after a single injection of dexamethasone, which preceded a rise in serum IgA concentration detected 24 h after the second hormone treatment and suggested that IgA increased in serum and decreased in salivary secretions due to a redistribution of polymeric IgA from mucosal surfaces to the circulation controlled by glucocorticoids. A subsequent study (Alverdy and Aoys, 1991) showed a fall of 77% in IgA concentration, an augmentation in bacterial adherence (2.4-fold), and an increased incidence of bacterial translocation to the mesenteric lymph nodes (60% *vs* 0%) observed after 2 days in dexamethasone-treated rats. The levels of polymeric IgA and antigen-specific IgA antibody in serum were also reported to be elevated after dexamethasone treatment (Wira and Rossoll, 1991). However, the sIgA level and antigen-specific IgA production after oral antigenic challenge was markedly inhibited. These data suggested that glucocorticoids might impair mucosal IgA synthesis, secretion and function and promote bacterial translocation (Moyer *et al.*, 1981).

2.6.2 Saliva IgA and exercise-induced URTI

Mucosal immunity and susceptibility to URTI are likely related to exercise stress because various aspects of immune function are temporarily changed following exercise (Mackinnon, 1999). Epidemiological studies have indicated that intensive prolonged training or competition is associated with an elevated incidence of URTI, placing athletes at a higher risk of URTI than control groups during and after competition or training (Douglas and Hanson, 1978, Peters and Bateman, 1983, Nieman *et al.*, 1990, Heath *et al.*,

1991). Peters and Bateman (1983) reported the runners who completed an ultramarathon (35 miles) had more than 2-fold incidence of URTI within 2 weeks after the race compared with the matched controls. The running mileage for a year appeared to be an influential factor for developing URTI according to Heath *et al.* (1991), who showed that individuals who ran more than 3.8 miles per day, on average, had a 2-fold higher incidence of URTI than those who ran less than 1.3 miles per day. Nieman *et al.* (1990) also reported that the risk of an infectious episode was 5-fold higher for marathon runners in the week after a race compared with runners who trained but did not compete in the race. Subsequently, Nieman (1994) hypothesised the relationship between susceptibility to URTI and exercise workload as a J-shaped curve. This model predicts that individuals who exercise moderately are at less risk of infection, whereas those who exercise heavily are more at risk compared with sedentary counterparts. If the J-shaped curve is effectively mediated by sIgA, then the alteration in sIgA concentration and/or output should be inversely associated with the incidence of URTI. Preliminary support has been provided by many previous studies (Mackinnon *et al.*, 1993, Gleeson *et al.*, 1999, Reid *et al.*, 2001). Mackinnon *et al.* (1991) described that eleven of twelve URTI episodes were preceded within 2 days by a 22% decrease in sIgA levels. Later a study from the same laboratory supported this idea by reporting that hockey and squash players developed symptoms of URTI had reductions in sIgA of 22% and 23% with 2 days of symptom onset, whereas those players who remained healthy, sIgA either increased slightly or was unchanged (Mackinnon *et al.*, 1993).

Measurement of sIgA is thought to be an indicator of the functional status of the entire mucosal immune system (Mestecky, 1993). Local production of sIgA provides adaptive immunologic protection to mucosal surfaces (Johansen *et al.*, 2001). The low sIgA levels or chronic sIgA deficiency appeared to facilitate the adherence and entrance of pathogens through the epithelial surface (Ostergaard, 1977, Alverdy and Aoys, 1991), increasing frequency of URTI episodes (Gleeson *et al.*, 1999), recurrent URTI (Isaacs *et al.*, 1984), or reduced protection against certain epithelial infections (Asahi *et al.*, 2002). In a meta-analysis of nine studies, Jemmott and McClelland (1989) concluded that low local levels of sIgA could compromise immune resistance to respiratory infections.

2.6.3 Saliva IgA secretion and exercise

Many studies have been done to examine how exercise affects the sIgA concentration and secretion rate. However, the results have been inconsistent. The possible mechanisms involved in the regulation of saliva IgA secretion during prolonged exercise are illustrated in Figure 2.5.

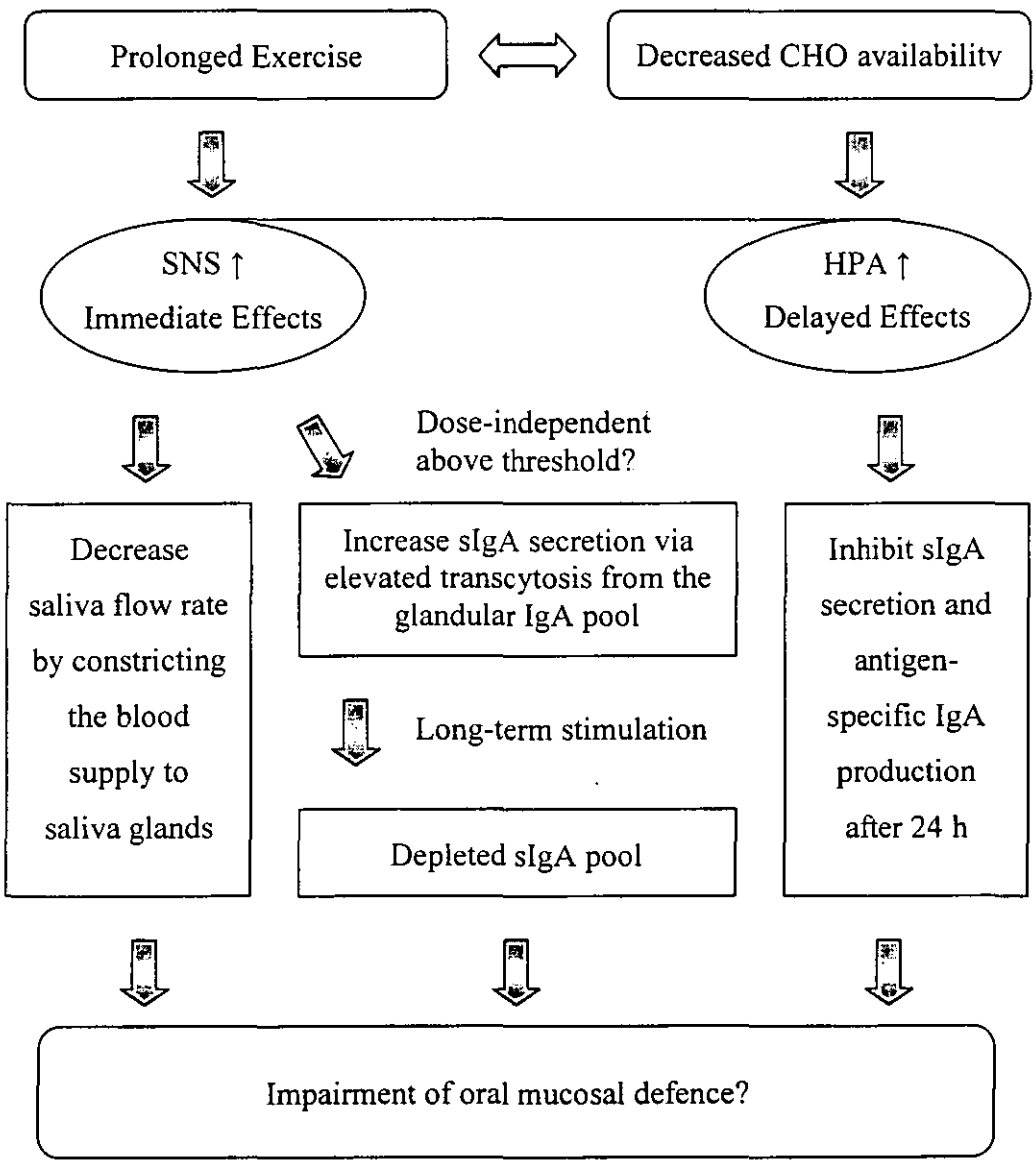


Figure 2.5 The possible mechanisms involved in the regulation of saliva IgA secretion during prolonged exercise

Saliva flow rate

A decreased saliva flow rate has been consistently observed following strenuous exercise (Steerenberg *et al.*, 1997, Blannin *et al.*, 1998, Walsh *et al.*, 2002). A steady blood flow to salivary glands is required for maintenance of adequate salivation because the water of saliva is from the plasma (Smaje, 1998). Anderson and Garrett (1998) demonstrated that the α-adrenergic receptor activation causes vasoconstriction, whereas the β-adrenergic activity induces vasodilation in rat submandibular glands. Further, recent studies have shown that α_1-adrenergic blockade by doxazosin and β-adrenergic blockade by propanolol have no effect on saliva flow rate after 8-min submaximal cycling at 50W (Winzer *et al.*, 1999, Ring *et al.*, 2000). However, α_2-adrenoceptor agonist dexmedetomidine infusion induces vasoconstriction in men (Talke *et al.*, 2003). This suggests that the α_2-adrenergic receptors may play an important role in the exercise-induced decrease of saliva flow rate. Rantonen and Meurman (2000) suggested that the saliva flow rate was likely to be the single salivary defensive factor which significantly affected oral health. This notion was supported by recent studies, which showed the absence of caries in children with familial dysautonomia was associated with a higher saliva flow rate (Mass *et al.*, 2002), and the increased incidence of oral candidal infections in HIV-infected patients (Lin *et al.*, 2001) was related to a lower saliva flow rate. Fox *et al.* (1985) also suggested individuals who suffered from dry mouth syndrome had an increased incidence of URTI.

Saliva IgA concentration

Previous studies have shown paradoxical results in sIgA concentration immediately after exercise; some reported increased (Gleeson *et al.*, 2001a, Dimitriou *et al.*, 2002), unaffected (Housh *et al.*, 1991, McDowell *et al.*, 1991), or decreased sIgA concentration (Tomasi *et al.*, 1982, Mackinnon *et al.*, 1987, Tharp and Barnes, 1990, McDowell *et al.*, 1992, Krzywkowski *et al.*, 2001). A few studies have demonstrated a delayed effect of exercise on the saliva IgA response. Mackinnon and her colleagues reported a significant decrease in sIgA level occurred between 2 to 24 h after intense prolonged exercise (Mackinnon *et al.*, 1987) or on the second and third consecutive days of moderate intensity exercise, but not on the first day (Mackinnon and Hooper, 1994).

Saliva IgA secretion rate

The protective effect of sIgA in the respiratory tract is dependent on both sIgA concentration and saliva flow rate – the total amount of sIgA covering the mucosal surface (Mackinnon and Hooper, 1994). *In vitro*, sIgA is secreted by both acinar and ductal units under the stimulation of α- and β-adrenoreceptors and peptidergic receptor. The secretion rate of sIgA is relatively constant for each agonist across a range of doses (Proctor and Carpenter, 2002). The α-adrenoreceptor agonist phenylephrine has been demonstrated to stimulate the secretion of IgA and protein via β-adrenoreceptor-dependent pathway with a manner of dose-independent above a certain threshold (Proctor *et al.*, 2003). However, Ring *et al.* (2000) suggested that the acute decrease in sIgA secretion rate was mediated by α_1-adrenergic mechanisms. Furthermore, prolonged stimulation of β-adrenoreceptor agonist isoprenaline appeared to reduce the replenishment of IgA into the glandular pool (Proctor *et al.*, 2003).

Several studies showed no alteration in sIgA secretion rate after tennis drill (Nieman *et al.*, 2000), soccer play (Bishop *et al.*, 1999a) or cycling (Blannin *et al.*, 1998). On the other hand, other studies reported a decrease in sIgA secretion rate following Olympic-distance triathlon race (Steerenberg *et al.*, 1997), competitive marathon race (Nieman *et al.*, 2002) and 2 h cycling (Krzywkowski *et al.*, 2001). Recovery of saliva immunoglobulins to pre-exercise levels usually occurs within 24 h. However, in elite athletes undertaking multiple exercise sessions in a single day and/or habitual intensive training, the recovery will be affected by the intensity of the training sessions. The recovery rate may prove to be a key indicator of the long-term consequences of accumulative mucosal immunodepression in high performance athletes (Gleeson, 2000b).

2.6.4 Carbohydrate ingestion and exercise-induced saliva IgA responses

Since exercise influences saliva flow rate and composition via the activation of sympathetic nerves and the HPA-axis, the blunted responses of stress hormones after CHO ingestion may attenuate the effect on oral immunity (Chicharro *et al.*, 1998). However, CHO ingestion appears not to affect sIgA concentration (Nehlsen-Cannarella *et al.*, 2000, Nieman *et al.*, 2002), secretion rate (Nehlsen-Cannarella *et al.*, 2000, Nieman *et al.*, 2002)

and saliva flow rate (Bishop *et al.*, 2000) following a single bout of prolonged exercise compared with a PLA trial (Table 2.5).

Table 2.5 Carbohydrate ingestion and exercise-induced saliva IgA responses

Reference	Experimental Design	Subject	Main Findings
Nieman *et al.*, 2002	Marathon race. Exercise volume was ~4.4 h at 83% HR max.	CHO: 48 PLA: 50	Saliva IgA secretion rate decreased 34% compared with pre-race level; however, there was no difference between CHO and PLA.
Bishop *et al.*, 2000b	Three trials: CHO, PLA and restricted fluid intake (RFI). 2 h cycling at 60% $\dot{V}O_{2\,max}$	15 males	CHO feeding better maintained plasma glucose concentration compared with PLA and RFI. Saliva flow rate and sIgA concentration in CHO was higher than RFI but not different to PLA.
Nehlsen-Cannarella *et al.*, 2000	2 h rowing consisted of a 3-min rest every 15 min. Subjects drank a 6% CHO or PLA beverage for 12 and 4 $mL \cdot kg^{-1}$ body mass before and every 15 min during rowing, respectively.	15 female rowers	CHO ingestion had no effect on saliva flow rate and sIgA concentration and secretion rate compared with PLA.
Bishop *et al.*, 1999a	90 min soccer-specific exercise protocol. Subjects drank 400 mL of a 6% CHO or PLA beverage at 10 min before the start of each 45 min of exercise and 150 mL at 14 and 29.5 min into each period of exercise.	8 males	CHO ingestion had no effect on saliva flow rate and sIgA concentration and secretion rate compared with PLA.

The saliva samples of all studies in this table were from the unstimulated whole-mixed saliva and the sIgA concentration was measured using ELISA.

2.7 Effects of Daily Repeated Exercise on Immunoendocrine Responses

Although there have been numerous studies that have examined immune responses to single bout of exercise in recent decades, few investigations have been carried out to determine how repeated bouts of exercise on the same day influence immunoendocrine responses. The studies presented in this thesis were performed to contribute knowledge in this area.

2.7.1 Leukocyte mobilisation, IL-6 and plasma stress hormone responses

Field *et al.* (1991) conducted two bouts of exhaustive cycling for 12.9 and 13.2 min separated by 1 h recovery interval and showed that the plasma concentrations of adrenaline and blood counts of total leukocytes, neutrophils, lymphocytes and monocytes were increased to the same levels during both exercise trials. However, a later study using a protocol of cycling for two 30-min sessions at 70% $\dot{V}O_{2\,max}$ separated by a 3-h recovery interval showed the circulating counts of leukocytes, neutrophils and lymphocytes were 48%, 62% and 6% higher in the second bout (EX2) than in the first bout (EX1), respectively. The plasma concentrations of adrenaline and cortisol were also 21% and 6% higher in EX2 compared with EX1 (McCarthy *et al.*, 1992). Subsequently, Rohde *et al.* (1998) carried out a protocol of three bouts of cycling for 60, 45, and 30 min at 73% $\dot{V}O_{2\,max}$ separated by 2-h recovery intervals. The blood concentrations of neutrophils and monocytes continuously increased at the end of each cycling bout and lasted at least for 2 h. The blood lymphocyte concentration increased with exercise but declined below resting values within 2 h post-exercise.

Recently, Ronsen and colleagues conducted a series of investigations using an identical experimental design: two bouts of 75 min cycling consisting of 10 min at 50% $\dot{V}O_{2\,max}$ and 65 min at 75% $\dot{V}O_{2\,max}$ separated by a 3-h (TWO-EX-SHORT) or a 6-h (TWO-EX-LONG) recovery interval, identical single cycling bout in the afternoon (PMEX) and resting control trial (REST). During each trial, subjects were served 4 standardised meals (1000 kcal·meal^{-1}) at 2.5 h before the morning exercise, 1.8 h before the afternoon exercise, 1.2 h and 5 h after the afternoon exercise. The results showed that: 1) the second exercise bout evoked higher circulating counts of total leukocytes (50% and 25%),

neutrophils (63% and 53%) and lymphocytes (33% and 33%) compared with the first exercise bout and PMEX, respectively (Ronsen *et al.*, 2001b); 2) the second exercise bout induced higher plasma concentrations of adrenaline (336% and 408%), ACTH (131% and 88%), cortisol (32% and 18%) and GH (52% and 39%) compared with the first exercise bout and PMEX, respectively (Ronsen *et al.*, 2001a); 3) during the second bout, there were significant increases in plasma concentrations of adrenaline, ACTH and cortisol, but not in GH, and greater neutrophilia and lymphopenia in TWO-EX-SHORT compared with TWO-EX-LONG (Ronsen *et al.*, 2002a); 4) the second exercise bout in TWO-EX-SHORT induced a significantly higher plasma IL-6 level than PMEX, but not compared with TWO-EX-LONG (Ronsen *et al.*, 2002b).

A very recent study investigating the effect of two bouts of 60 min cycling consisting of three 20-min segments: 5 min at 50% $\dot{V}O_{2\,max}$ and 15 min at 70% $\dot{V}O_{2\,max}$, separated by a 4-h passive recovery. During the final 2 h of the recovery period subjects were given 1.63 g $CHO \cdot kg^{-1}$ body weight. The results showed that after two bouts of exercise the blood counts of leukocytes and neutrophils were higher than the identical single bout completed in the morning (69% and 46%) and in the afternoon (33% and 36%) only. Circulating lymphocyte number after the single bout in the afternoon was 14% higher than the identical single bout in the morning (McFarlin *et al.*, 2003).

2.7.2 Neutrophil function

Pyne *et al.* (1996) reported that after 40 min running on the treadmill at a heart rate 140 $beat \cdot min^{-1}$, PMA-induced CL release by isolated neutrophils declined 41% compared with pre-exercise. However, the values of CL did not change further during the 1-h recovery interval and after the second identical bout of running. Another study using the same protocol as Ronsen's showed that the PMA-induced CL release by isolated granulocytes at post-exercise was not different to pre-exercise in any of the trials (Boyum *et al.*, 2002).

2.8 Summary

In this chapter an attempt was made to present an overview of the current knowledge of the immunoendocrine responses to exercise. In particular, literature concerning the circadian variation in plasma stress hormones, IL-6, circulating numbers of leukocytes and sIgA, plasma glucose and immune responses, plasma stress hormones and leukocyte mobilisation, neutrophil function, IL-6 response, sIgA response, and effects of repeated exercise on immunoendocrine responses was highlighted. The available evidence suggests that exercise affects the immune system via stimulation of SNS and HPA-axis and the secretion of cytokines during exercise. However, limited information exists on the effects of repeated prolonged exercise on immunoendocrine responses. Therefore, the effects of repeated prolonged exercise and CHO supplementation on leukocyte redistribution, neutrophil function, plasma stress hormones and IL-6 responses, saliva flow rate and IgA responses as well as the effect of time of day on these parameters are the main research questions of the present thesis.

CHAPTER THREE

General Methods

3.1 Ethical Approval

Approval for these studies was issued by Loughborough University Ethical Advisory Sub-committee. The nature and purpose of each study was fully explained verbally and in writing to each subject. All subjects completed informed consent forms (Appendix A) and were made fully aware that they were free to withdraw from the study at any time. Subjects were also inspected by a health screen (Appendix B) and a physical activity questionnaire (Appendix C) to determine whether they were suitable for doing exercise trials. On the day of exercise trials, subjects were again completed a health questionnaire (Appendix D) to ensure that they were well and fit to participate in the study. If the subject responded 'yes' to any of the questions on the health screen (Appendix B), he would be asked to withdraw from the study. If the subject responded 'yes' to any of the questions on the health questionnaire (Appendix D), he would be asked to postpone his trial until fully recovery.

3.2 Protocol for Determination of Maximal Oxygen Uptake ($\dot{V}O_{2\,max}$)

For the determination of maximal oxygen uptake and trial workloads subjects performed a continuous incremental exercise test on a cycle ergometer (Monark 874E, Monark Exercise AB, Sweden) to volitional exhaustion. Participants began cycling at 70 W with increments of 35 W every 3 min. The cadence remained at 70 rev·min^{-1} and heart rate was monitored continuously using radiotelemetry (Polar Electro Oy, Finland). During the third min of each work rate increment, expired gas was collected in Douglas bags. An oxygen/carbon dioxide analyser (Servomex 1400B, Crowborough, UK) was used along with a dry gas meter (Harvard Apparatus, Edenbridge, UK) for determination of $\dot{V}O_2$ and $\dot{V}CO_2$. From the $\dot{V}O_2$-work rate relationship, the work rate equivalent to 60% $\dot{V}O_{2\,max}$ was interpolated.

3.3 Blood Sampling and Analysis

3.3.1 Blood collection and treatment

Venous blood samples were taken from an antecubital vein by venepuncture, and were collected into three Vacutainer tubes (Becton Dickinson, Oxford, UK). Blood samples in two K_3EDTA vacutainers (4 mL) were used for haematological analysis and determination of changes in the plasma concentrations of stress hormones and interleukin-6 (IL-6). For neutrophil respiratory burst assay, 0.5 mL K_3EDTA aliquots of whole blood were added to eppendorf tubes (1.5 mL capacity) for later measurement within 7 hours. As for the blood dispensed into a lithium heparin vacutainer (7 mL), 1 mL was immediately added to an eppendorf tube containing 50 μL of 10 $mg \cdot mL^{-1}$ bacterial lipopolysaccharide (LPS) solution (Stimulant, Sigma, Poole, UK). Blood and LPS were mixed by gentle inversion and then incubated for 1 h at 37 °C, being gently mixed again every 20 min. After incubation, the mixture was centrifuged for 2 min at 15000 *g*. The supernatant was immediately stored at −80 °C prior to analysis of elastase concentration to determine the neutrophil degranulation response as described in Section 3.3.4

The remaining K_3EDTA and heparinized whole blood was spun at 1500 *g* for 10 min in a refrigerated centrifuge at 4 °C within 10 min of sampling. The plasma obtained was immediately stored at −80 °C prior to analysis.

To obtain accurate measurements, the whole-blood samples in our laboratory were stored at the ideal conditions: 1) keeping at temperatures of 20 °C to 24 °C; 2) measuring blood composition and cell counts and cellular activities within 10 min and 7 h, respectively; 3) no centrifugation or agitation, but gentle and multi-axle mixing before testing (Li, 2003).

3.3.2 Determination of total and differential leukocyte counts

Haematological analysis including haemoglobin, haematocrit, and total and differential leukocyte counts using a haematology analyser ($A^C \bullet T^{TM}$ 5diff analyzer, Beckman Coulter, UK). The intra-assay coefficient of variation was 0.9%, 1.1% and 1.3% for haemoglobin, haematocrit and leukocyte counts, respectively. Plasma volume changes were calculated from measurements of haemoglobin concentration and haematocrit according to Dill and Costill (1974).

3.3.3 Determination of plasma glucose, stress hormones and IL-6

Plasma aliquots were analysed to determine the concentration of glucose (GOD-PAP method, Randox, UK) using an automatic photometric analyser (Cobas-Mira plus, Roche). Human growth hormone (GH), cortisol (both DRG Instruments GmbH, Germany), adrenaline (IBL GmbH, Hamburg), adrenocorticotropic hormone (ACTH) (Biomerica, Newport Beach, CA) and IL-6 (High Sensitivity Kit, Diaclone Research, France) were determined using enzyme-linked immunosorbant assay (ELISA) kits. The intra-assay coefficient of variation was 1.3%, 2.4%, 6.9%, 12.7%, 5.1% and 1.6% for glucose, GH, cortisol, adrenaline, ACTH and IL-6, respectively, based on duplicate analyses.

3.3.4 Determination of LPS-stimulated neutrophil degranulation

For the neutrophil degranulation assay, elastase concentration in plasma before and after treatment with LPS was determined using an ELISA kit specific for polymorphonuclear cell elastase (Merck, Lutterworth, UK). After thawing, both stimulated and unstimulated plasma samples were initially diluted 20 times by adding 30 μL plasma to 570 μL dilution medium in an eppendorf tube and mixed by shaking. 100 μL of diluted stimulated samples were then further diluted with 1000 μL dilution medium in another eppendorf tube, giving a final dilution of 220×. 1000 μL of wash solution was added to each pre-labelled antibody-coated tube and incubated for 10-20 min at room temperature; then fluid was discarded and tubes tapped on tissue paper. 500 μL of either elastase standard (range from 0.4 to 10.0 $\mu g \cdot L^{-1}$) or plasma was then added to the tubes in duplicate (single for unstimulated samples) and incubated for 60 min at room temperature. After incubation the tubes were tipped upside down over the sink then blotted and washed with 1000 μL wash solution. Tubes were then emptied, blotted and washed with 2000 μL wash solution again. 500 μL antibody-enzyme solution was added to each tube. The tubes were then incubated at room temperature for 30 min. After incubation the tubes were tipped, then blotted and washed with 2000 μL wash solution. Tubes were then tipped, blotted and wash procedure was repeated one more time. 500 μL substrate solution was added to each tube. The tubes were then incubated in the dark at room temperature for 30 min. 100 μL stop solution was then added to every tube. After gentle shaking, 200 μL of solution from each tube was

added to a clear 96-well plate. Then the absorbance was measured at 405 nm. The intra-assay coefficient of variation was 3.9% for the elastase assay.

3.3.5 Determination of PMA-induced neutrophil oxidative burst

A microplate luminometer cell activation kit (Knight Scientific Limited, Plymouth, UK) was used to measure the neutrophil oxidative burst activity. Sample analysis was performed in duplicate as follows: 20 μL of K_3EDTA whole blood sample was added into a dilution tube with 2 mL of blood dilution buffer (HBSS without calcium and magnesium but with 20 mM HEPES, pH 7.4). A 20-μL aliquot of each diluted sample was then added into an opaque white microplate well. 90 μL reconstitution and assay buffer (HBSS with 20 mM HEPES, pH 7.4) was then added into each well followed by the addition of 20 μL reconstituted Adjuvant-K™ and 50 μL Pholasin® (10 $\mu g \cdot mL^{-1}$). The microplate was placed into a luminometer (Anthos Lucy 1 Microplate Luminometer, Anthos Labtec Instrument, Austria) after adding 20 μL PMA (phorbol-12- myristate-13-acetate, 5 $\mu g \cdot mL^{-1}$) into each well. After 1 min shaking and incubation at 37 °C, Pholasin®-enhanced chemiluminescence (CL) was measured at 1-min intervals for 30 min, and the incremental area under the curve (IAUC) was calculated. The oxidative burst activity per cell was calculated by dividing the IAUC by the numbers of neutrophils in each sample. The intra-assay coefficient of variation was 5.7% for the chemiluminescence assay.

3.4 Saliva Sampling and Analysis

3.4.1 Saliva collection and treatment

Participants were seated during all saliva collections. With an initial swallow to empty the mouth, unstimulated whole saliva was collected by expectoration into a pre-weighed vial (7 mL-capacity plastic Bijou tubes with screw top) for 2 min with eyes open, head tilted slightly forward and making minimal orofacial movement. All saliva samples were stored at –20 °C until analysis.

3.4.2 Determination of saliva flow rate

Saliva flow rate ($mL \cdot min^{-1}$) was determined by weighing. The density of saliva was assumed to be 1.0 $g \cdot mL^{-1}$ (Chicharro *et al.*, 1998).

3.4.3 Determination of saliva IgA concentration and secretion rate

The concentration of salivary IgA ($mg \cdot L^{-1}$) was determined by a sandwich-ELISA method similar to that described by Gomez *et al.* (1991). Briefly, flat-bottomed microtitration plates (Costar EIA/RIA plate, Sigma, Poole, UK) were coated with the primary antibody, rabbit anti-human IgA (I-8760, Sigma, Poole, UK), at a dilution of 1 in 800 in carbonate buffer, pH 9.6, and kept at 4 °C over night. After washing with phosphate buffered saline (PBS, pH 7.2) the plates were coated with blocking protein solution ($2\ g \cdot L^{-1}$ bovine serum albumin in PBS). Sample analysis was performed in quadruplicate using saliva samples diluted 1 in 500 with deionised water. A range of standards (Human colostrum IgA, I-2636, Sigma) up to 600 $\mu g \cdot L^{-1}$ was used for calibration. Standards were incorporated into each micro-well plate, and all samples from a single subject were analysed on a single plate. The plates were incubated for 90 min at room temperature. Following a washing step, peroxidase-conjugated goat anti-human IgA (A-4165, Sigma) was added and the plate incubated for a further 90 min at room temperature. Following another washing step, the substrate, ABTS (Boehringer Mannheim, Lewes, UK), was added and after 30 min the absorbance was measured at 405 nm. The intra-assay coefficient of variation was 7.6% for sIgA assay.

The sIgA secretion rate ($\mu g \cdot min^{-1}$) was calculated by multiplying the sIgA concentration by the saliva flow rate.

CHAPTER FOUR

Determination of Saliva Collection Method

Summary

The aims of the two studies described in this chapter were: (1) to determine the effects of different salivary volumes on collection efficiency and sIgA concentration using the Salivette swab collection method (*Study 1*), (2) to investigate the influence of different saliva collection methods on sIgA concentration and saliva flow estimation (*Study 2*). In *Study 1*, eight healthy male subjects were asked to produce 15 mL of unstimulated saliva by dribbling into a tube over a 20 - 30 min period. The samples then were divided into the following volumes: 4, 3, 2, 1, 0.7, 0.4, 0.2, and ~3 mL (control). Swabs were put into each vial (except for the control sample) and placed on a shaker at 500 rpm for 2 min. After shaking the swabs were removed and centrifuged spun at 1500 *g* for 10 min at 18 °C. Samples were then stored frozen at −20 °C prior to analysis, which would estimate the efficiency of saliva collection with the swab and the effect of different saliva volumes on selected salivary biomarkers. In *Study 2*, twelve healthy male subjects completed three 2-min unstimulated saliva sample collection periods in a counterbalanced order; this involved on two occasions putting a Salivette cotton swab under tongue or on the other occasion dribbling into a centrifuge tube. The "DB" sample was collected by dribbling into a collection tube, the "SC" sample was collected with a swab placed under the tongue and centrifuged immediately, and the "SF" sample was collected with a swab without centrifugation before being stored frozen. Saliva samples were analysed for flow rate and sIgA concentration.

Study 1 showed that the Salivette swab became saturated at the saliva volumes of between 2 mL and 3 mL, and this range also produced the best efficiency for collecting saliva samples. The sIgA level was significantly affected by the presence of the Salivette swab. In *Study 2*, the saliva flow rate was not significantly different among the three groups, but the volume of saliva not released from swab in SF was significantly higher than that in SC. Saliva IgA was significantly absorbed by swab in SF and SC compared with DB. The findings from *Study 1* and *Study 2* indicate that the swab collection method is not an ideal

method because it affects the results of sIgA. With regard to previously reported studies that have collected saliva using swabs, our findings suggest that the results may need to be viewed with some caution, particularly of absolute IgA values are key to the findings (rather than patterns of change) and if flow rates exceed 1 $mL \cdot min^{-1}$, increasing the likelihood of swab saturation.

4.1 Introduction

Numerous studies have used a cotton swab placed under the tongue to collect saliva samples (Blannin *et al.*, 1998; Hucklebridge *et al.*, 1998; Bishop *et al.*, 2000b; Shirtcliff *et al.*, 2001) for investigating the changes of salivary biomarkers before and after physical or psychological stress. The cotton-based swab collection methods are definitely convenient and practical, but to our knowledge, there has been few studies inspecting if this collection method affects the concentrations of salivary biomarkers. Therefore, the aim of this chapter was to compare and determine a relatively ideal saliva collection method the *Study 1* was to determine the effects of different saliva volumes on collection efficiency and on sIgA level using a cotton-based swab collection method. The aim of *Study 2* was to investigate the influence of different saliva collection methods on sIgA level and on the estimation of saliva flow rate.

4.2 Methods

Subject

Eight healthy men (mean ± SEM: age 29.1 ± 2.9 years; body mass 74.2 ± 1.3 kg) and twelve healthy men (age 28.3 ± 2.1 years; body mass 74.6 ± 1.7 kg) volunteered to participate in *Study 1* and *Study 2*, respectively.

Experimental Procedures

Study 1: Salivette centrifuge tubes (Sarstedt, Gemany), empty vials and dry swabs were labelled and weighed before subjects' arrival. Subjects visited the Biochemistry Laboratory (this study was carried out at The University of Birmingham) at 9:00 after an overnight fast (from 23:00 the night before experimental trial). Subjects were asked to drink 200 mL water 10 min before saliva collection and then to dribble unstimulated saliva into a Universal tube until tube was half full (about 15 mL). The saliva was divided into aliquots of the following volumes: 4, 3, 2, 1, 0.7, 0.4 and 0.2 mL and placed in separate labelled vials (7 mL-capacity plastic Bijou tubes with screw top). The remaining volume of saliva (~3 mL) was placed in another vial (designated as the control sample). Swabs were put into each vial (except for the control sample) and placed on a shaker at 500 rpm for 2 min. After shaking the swabs were removed with tweezers and placed into the top part of respective Salivette centrifuge tubes, and then were spun at 1500 *g* for 10 min at 18 °C. Afterwards each swab, the bottom part of Salivette centrifuge tube and vial was weighed. Samples were then stored frozen at –20 °C prior to analysis.

Study 2: The empty bottom part of Salivette centrifuge tubes, empty vials and dry swabs were labelled and weighed before subjects' arrival. Subjects visited the Biochemistry Laboratory (this study was carried out at The University of Birmingham) at 13:00 after a 4-h fast (from 9:00 the day of experimental trial). Subjects were asked to drink 150 mL water 5 min before sampling and then were assigned in a counterbalanced order to complete three 2-min unstimulated saliva sample collection periods: two of which involved putting a Salivette cotton swab (SC) under the tongue and the other collection was made by dribbling (DB) into a centrifuge tube. Each saliva collection was separated by a 5-min interval and a drink of water (150 mL), and the mouth was emptied by an initial swallow

before collection. The "DB" saliva sample was weighed to calculate the saliva flow rate. One of the "SC" saliva samples was centrifuged immediately after collection at 1500 *g* for 10 min at 18 °C. The bottom part of the Salivette centrifuge tube and the swab were weighed before being frozen. The other sample collected with a cotton swab, designated "SF" was not centrifuged before being frozen. All samples were stored at –20 °C until analysis. On the day of analysis the SF samples were allowed to thaw at room temperature for 100 min before being spun at 1500 *g* for 10 min at 18 °C and weighed.

Analytical Methods

Saliva flow rate, sIgA concentration, glucose, adrenaline, cortisol, maximal oxygen uptake, and equivalent work rate were determined (as described in Chapter 3)

Statistical analysis

Data in the text and tables are presented as mean values and standard errors of the mean (± SEM). In *Study 1*, the data were examined using repeated measures one-way ANOVA and paired *t*-tests. In *Study 2*, statistical evaluation was carried out using paired *t*-tests and Pearson's product-moment formula. $P < 0.05$ were accepted significant.

4.3 Results

Study 1

The percentage of saliva not absorbed by the Salivette swab was relatively low (~1%) for saliva volumes of 0.2 - 2 mL but increased to 1.5% and 10.7% for saliva volumes of 3 and 4 mL respectively (Table 4.1). The percentage of saliva not released from the swab after centrifugation was higher at lower saliva volumes. The amount of saliva retained by the swab was not a constant amount and not a constant percentage of the saliva sample volume. This implies that the Salivette swab was very effective in absorbing saliva but it became saturated between the saliva volumes of 2 mL and 3 mL, and this range also produced the best efficiency for collecting saliva samples (Table 4.1).

The sIgA level was significantly affected by exposure to the Salivette swab (Figure 4.1A). The sIgA concentration in the control sample (197 ± 14 $mg \cdot L^{-1}$) was significantly higher than the average (165 ± 18 $mg \cdot L^{-1}$) of all the sample volumes that were exposed to the swab ($P < 0.05$). There was no significant difference in sIgA concentration among samples, which were exposed to the swab (Figure 4.1B).

Table 4.1 The efficiency of saliva collection with Salivette swab for sampling volumes of 0.2 - 4.0 mL.

	Saliva not absorbed by swab		Saliva not released from swab		Saliva obtained for analysis	
	Volume (mL)	Percentage	Volume (mL)	Percentage	Volume (mL)	Percentage
4 mL	0.430 (0.074)	10.7	0.277 (0.025)	6.9	3.286 (0.076)	82.1
3 mL	0.044 (0.009)	1.5	0.269 (0.009)	9.0	2.706 (0.012)	90.2
2 mL	0.007 (0.001)	0.4	0.242 (0.011)	12.1	1.775 (0.010)	88.7
1 mL	0.004 (0.001)	0.4	0.163 (0.009)	16.3	0.849 (0.004)	84.9
0.7 mL	0.004 (0.001)	0.6	0.148 (0.008)	21.1	0.582 (0.006)	83.1
0.4 mL	0.003 (0.001)	0.8	0.090 (0.014)	22.3	0.309 (0.006)	77.2
0.2 mL	0.002 (0.001)	1.1	0.091 (0.007)	45.5	0.127 (0.006)	63.4

Mean ± (SEM)

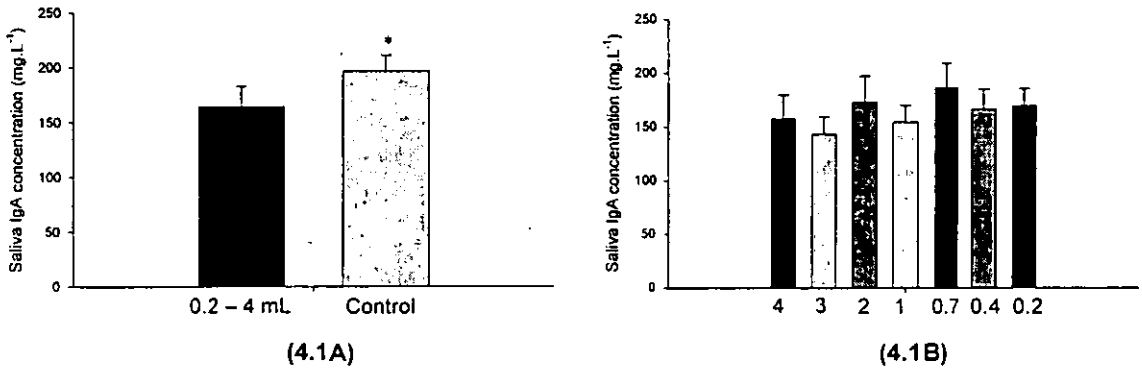
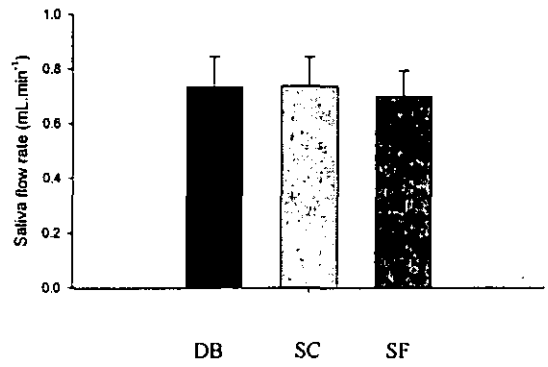


Figure 4.1 The sIgA concentrations (4.1A) in control were significantly higher than the average of all other groups (*$P < 0.05$). There was no significant difference in sIgA concentration among samples, which were exposed to the swab (4.1B).

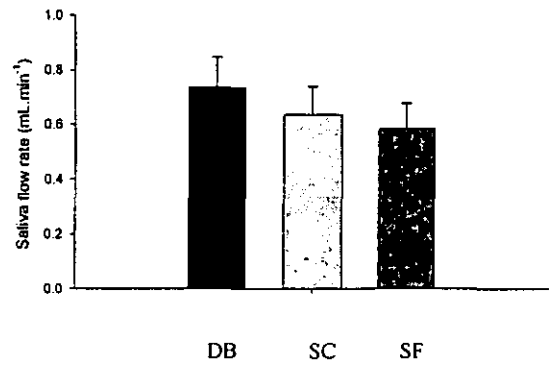
Study 2

The average saliva flow rate was not significantly different among the three groups (Figure 4.2A & B), but the volume of saliva not released from the swab in SF (0.232 ± 0.009 mL) was significantly higher than for SF (0.206 ± 0.007 mL) (Figure 4.2C).

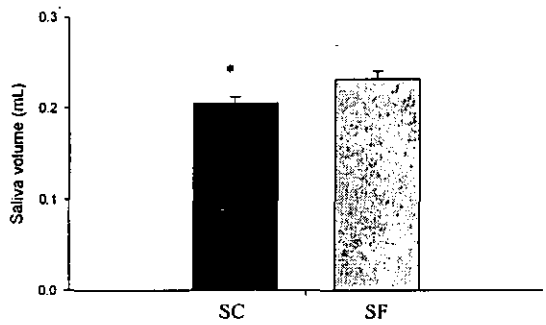
The sIgA concentration was also influenced by swab collection (Figure 4.3). The sIgA concentration of the sample collected by DB (111.1 ± 16.1 $mg \cdot L^{-1}$) was significantly higher than that in SC (55.1 ± 8.9 $mg \cdot L^{-1}$) and SF (33.0 ± 7.7 $mg \cdot L^{-1}$). Furthermore, the sIgA concentration in SC was also significantly higher than in SF.



(4.2A)



(4.2B)



(4.2C)

Figure 4.2 Saliva flow rate with the dribbling and swab collection method including the saliva volume retained in the swab (4.2A), excluding the saliva volume retained in the swab (4.2B). The volume of saliva remaining within the swab (4.2C). There was a notable difference between SC and SF ($*P < 0.05$) in the volume of saliva not released from the swab. SF samples were stored at –20°C for 9 ± 1.1 days (range from 2 to 14 days) before analysis. There was no correlation between the volume of saliva not released from swab and the number of days of storage. There was no significant difference among collection methods.

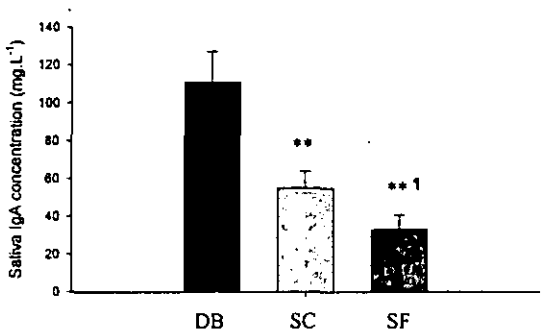


Figure 4.3 The sIgA concentration collected by DB was significantly higher than SC and SF ($**P < 0.01$) and SC was significantly higher than SF ($^{¶}P < 0.05$).

4.4 Discussion

In this Chapter, the results of *Study 1* (Table 4.1) show that the Salivette swab has good efficiency for saliva sample collection, especially for volumes between 1 mL to 3 mL, suggesting that a 4-min sampling period might be better than a 2-min sampling period based on the usual resting unstimulated flow rate of about 0.3 - 0.7 $mL \cdot min^{-1}$. However, the swab became saturated at saliva volumes greater than 3 mL. Furthermore, our finding shows that the smaller the volume of sample, the larger the proportion of sample volume that is retained in the swab. *Study 1* also indicates that exposure of whole saliva to a cotton swab for a 2-min period results in a ~ 15% lower sIgA level.

The use of a cotton swab to collect saliva samples profoundly affected the results of the selected salivary biomarkers that we analysed. For example, the swabs absorbed 51% sIgA immediately and 70% sIgA when stored frozen for an average of 9 days prior to centrifugation. This finding is similar to that reported by Shirtcliff *et al.* (2001) who also used a Salivette swab to collect saliva and then stored samples at –80°C until the day of assay. They showed the sIgA concentration in swab-collected samples was 72% lower than that in expectorated samples (49 $mg \cdot L^{-1}$ *vs* 177 $mg \cdot L^{-1}$). The results of these two studies strongly confirm that swabs absorb a substantial amount of sIgA in a saliva sample. There are different densities of immunoglobulin-producing cells in the salivary glands and most of the saliva appearing in the mouth is secreted from submandibular glands (65%) and parotid glands (23%) (Crawford *et al.*, 1975). The sIgA concentration secreted by submandibular glands has been reported similar to that secreted by the parotids; however, both resting and stimulated IgA outputs are higher in the former (Stuchell and Mandel, 1978). Hence, it might be important to determine the contributions from these two major glands to sIgA in whole saliva at rest and during exercise in the future.

The mechanism by which swab collection results in lower sIgA concentration in this study is still unknown. However, the different sIgA concentration collected by cotton-based swab method may be due to following reasons: 1) absorption of IgA by the swab material, 2) swab (placed under tongue) may absorb higher proportion of submandibular and sublingular saliva than collection by dribbling, 3) whole saliva may contain some cell debris and precipitated protein particles that are filtered out by the swab. It seems likely

that certain molecules in the sample adhere to the cotton fibres. We presume some sIgA molecules and other proteins may adhere and are trapped in the voids in cotton fibres (Misra *et al.*, 1991; Ramasubbu *et al.*, 1996; Parslow *et al.*, 2001; Shirtcliff *et al.*, 2001). In comparison with *Study 1*, the augmentation of decreased sIgA level in *Study 2* might be because the samples were exposed to cotton swab longer than that in *Study 1*. As the described previously, the swab was put under tongue for 2 min to collect saliva in SC and SF, and the SF samples were even put at room temperature for 100 min for thawing before being centrifugation. Therefore the longer exposure to swab possibly gradually increases the absorption of saliva by the swab. Determination of saliva flow and composition depends on accurate sampling procedures; thus, to study the effect of exercise on oral immunity requires the use of a standard procedure of sampling that does not interfere with analytical methods. It has been clearly shown that a cotton-based Salivette swab collection method is not reliable since the swab absorbs some of the salivary constituents, which are not released following centrifugation.

In conclusion, the data from *Study 1* and *Study 2* has shown that the swab collection method is not an ideal method because it affects the result of salivary IgA. With regard to previously reported studies that have collected saliva using swabs, our findings suggest that the results may need to be viewed with some caution, particularly if absolute IgA values are key to the findings (rather than patterns of change) and if flow rates exceed 1 $mL \cdot min^{-1}$, increasing the likelihood of swab saturation. Therefore, the saliva collection method adopted in this thesis was as follows: subjects were seated and with an initial swallow to empty the mouth, unstimulated whole saliva was obtained by expectoration into a pre-weighed vial for 2 min with eyes open, head tilted slightly forward and making minimal orofacial movement.

CHAPTER FIVE

Effects of prolonged cycling performed once or twice and times of day on immunoendocrine responses

Summary

The present study was designed to compare 1) redistribution of leukocyte subsets, 2) stress hormones and IL-6 responses, 3) alteration of neutrophil degranulation capacity and 4) saliva flow rate and sIgA responses to a single bout of prolonged cycling at different times of day and to a second bout of cycling at same intensity on the same day. In a counterbalanced design, eight males participated in three experimental trials separated by at least 4 days. On the afternoon exercise only trial (PMEX), subjects cycled for 2 h at 60% $\dot{V}O_{2\,max}$ starting at 14:00. On the other two trials, subjects performed either two bouts of cycling at 60% $\dot{V}O_{2\,max}$ for 2 h (EX1 started at 09:00 and EX2 started at 14:00) or a separate resting trial. Venous blood samples were taken 5 min before exercise and immediately post-exercise in the exercise trials and were taken at 09:00, 11:00, 14:00 and 16:00 in the resting trial. Unstimulated whole saliva samples were obtained at 10 min before exercise, after 58 - 60 min and during the last 2 min of exercise at 60% $\dot{V}O_{2\,max}$, at 1 h and 2 h post-exercise, and every hour from 9:00 to 18:00 during a resting control trial. Subjects remained fasted throughout.

The main findings were 1) PMEX induced larger increases in circulating numbers of leukocytes, neutrophils, and monocytes than EX1; 2) compared with EX1, EX2 caused greater increases in circulating numbers of leukocytes, lymphocytes, and monocytes and responses of plasma ACTH, cortisol, and GH; 3) plasma glucose concentration and LPS-stimulated neutrophil degranulation on per cell basis were lower after EX2 than after EX1; 4) there were circadian rhythms in the circulating concentrations in ACTH and cortisol, counts of leukocytes, neutrophils and lymphocytes, and sIgA concentration; 5) cycling at 60% $\dot{V}O_{2\,max}$ for 2 h significantly influenced saliva flow rate and sIgA concentration but did not affect sIgA secretion rate; 6) performing prolonged exercise in the morning or in the afternoon had similar effects on salivary variables in the short-term; and 7) in terms of

oral immunity, a 3 h rest was enough to recover from previous strenuous exercise, but was insufficient for circulating numbers of leukocytes, neutrophils, and monocytes, and neutrophil function.

The findings of this study suggest 1) a single bout of prolonged exercise performed in the afternoon induces a larger neutrophilia and monocytosis than an identical bout of morning exercise. This may be due to reduced carbohydrate availability and circadian rhythms in blood neutrophil counts and cortisol levels; 2) a second prolonged exercise bout causes greater immunoendocrine responses but lower plasma glucose levels and neutrophil function compared with the first bout; 3) during such exercise, sympathetic stimulation appears to be strong enough to inhibit saliva flow rate; however, it seems likely that it does not affect sIgA production or transcytosis; and 4) a 3-h recovery period is sufficient for oral immunity but is insufficient for neutrophil function to recover after prolonged exercise.

5.1 Introduction

Routine training programs of elite endurance athletes may be composed of several bouts of intensive exercise in a day. Epidemiological studies have demonstrated that endurance athletes are at increased risk of upper respiratory tract infection (URTI) after heavy training and/or competition and the vulnerable period can last up to 2 weeks (Nieman, 1997). The higher incidence of infection in elite athletes may be due, at least in part, the repetitive intensive exercise bouts without sufficient recovery. Failure to fully recover between training sessions has been suggested to evoke chronic fatigue, underperformance, and more severe immunodepression (Gleeson, 1998, Maughan, 2002).

Recently, several studies investigated the effects of repeated exercise bouts in the same day on immunoendocrine responses (McCarthy *et al.*, 1992, Rohde *et al.*, 1998, Ronsen *et al.*, 2001a, 2001b, Boyum *et al.*, 2002, Ronsen *et al.*, 2002a, Ronsen *et al.*, 2002b, McFarlin *et al.*, 2003). In general these studies have shown that a second bout of exercise on the same day induces more pronounced changes in the circulating leukocyte counts and stress hormones compared with a single bout of identical exercise.

Although blood leukocytes represent a small portion of the total number of leukocytes in the body, the blood is a necessary passage by which immune cells travel between different tissues and may be crucial to pathogen surveillance as well as affecting the ability of the immune system to respond to potential or ongoing immune challenge (Dhabhar and McEwen, 1997). It has been showed that acute exercise results in a transient, significant, and reversible redistribution of leukocyte subsets in the circulation, marginal pools and the bone marrow (Gleeson and Bishop, 1999). This exercise-induced mobilisation has been related to elevated secretion of stress hormones (Toft *et al.*, 1994, Benschop *et al.*, 1996). Exercise disturbs homeostasis and, consequently, both the sympathetic nervous system (SNS) and hypothalamic-pituitary-adrenal (HPA) axis become activated, resulting in increased circulating levels of catecholamines and glucocorticoids (Elenkov *et al.*, 2000).

Interleukin-6 (IL-6) is a 21- to 28-kDa glycoprotein secreted by various cells including monocytes, macrophages, lymphocytes, epithelial cells, and myofibres. Its receptors are also present in a variety of cells including most leukocytes, liver, adipose, and epithelial

cells (Keller *et al.*, 1996). It has well known that prolonged strenuous exercise induces marked elevations (up to 100-fold) in the plasma IL-6 level (Nieman, 1997, Ostrowski *et al.*, 1999, Pedersen *et al.*, 2001) and this elevation may induce neutrophil redistribution (Suwa et al., 2001) and affect neutrophil functions (Sitaraman *et al.*, 2001, Kaplanski *et al.*, 2003). The phagocytic neutrophil plays an important role in innate immunity, defending the body against various bacterial infections (Nieman, 1994). Following recognition of pathogens, neutrophils kill them via both oxygen-dependent (release of reactive oxygen species, ROS) and oxygen-independent (release of proteases) mechanisms (Fukatsu *et al.*, 1996, Johnson *et al.*, 1998). It has been recently suggested that neutrophils serve as a last line of defense to block the "open window" during the period of immunodepression after prolonged exercise (Pedersen, 1999). Muns *et al.* (1994) reported that after long-distance running nasal neutrophils were less able to ingest bacteria and the effect was lasted for 3 days. This notion was supported by recent studies, which showed that the endurance training temporarily reduced neutrophil phagocytosis (Blannin *et al.*, 1996), degranulation (Blannin *et al.*, 1997), and oxidative burst (Gabriel *et al.*, 1994, Pyne *et al.*, 1996). An impaired neutrophil microbicidal capacity may increase the susceptibility to infection in stressed athletes (Fukatsu *et al.*, 1996).

Immunity against microorganisms at remote sites, such as the nasal cavity, oral cavity, respiratory tract, digestive tract and gut, is primarily due to secretory immunoglobulin A, which has been considered as the first line of defence to infection in the lumen (Quan *et al.*, 1997). Secretory IgA is produced in local plasma cells and seems to function as a multi-layered mucosal defense. For example, IgA prevents antigens and microbes from adhering to and penetrating the epithelium (immune exclusion), interrupts replication of intracellular pathogens during transcytosis through epithelial cells (intracellular neutralization), and binds antigens in the lamina propria facilitating their excretion through the epithelium back into the lumen (immune excretion) (Lamm, 1998). Mucosal IgA is also a major mediator of nasal immunity (Asahi *et al.*, 2002). Lower levels of salivary IgA (sIgA) or chronic sIgA deficiency have been associated with an increased frequency of URTI episodes (Gleeson *et al.*, 1999), recurrent URTI (Isaacs *et al.*, 1984), or reduced protection against certain epithelial infections (Asahi *et al.*, 2002) because the low sIgA

level may allow easier entry of pathogens into body tissues via the epithelial surface (Ostergaard, 1977).

Numerous studies have examined how the sIgA concentration or secretion rate is affected by exercise. However, the results have been inconsistent to date. Some studies showed sIgA concentration was depressed after strenuous exercise (Tomasi *et al.*, 1982, Mackinnon *et al.*, 1987, Tharp and Barnes, 1990), whereas other studies reported sIgA to be unaffected (McDowell *et al.*, 1991, Walsh *et al.*, 1999, Nieman *et al.*, 2002) or even elevated (Tharp, 1991, Ljungberg *et al.*, 1997, Blannin *et al.*, 1998). Some recent studies reported that saliva flow rate and sIgA secretion rate, but not sIgA concentration, were reduced following a triathlon race (Steerenberg *et al.*, 1997) or tennis drills (Novas *et al.*, 2003). However, several studies have also shown a stable secretion rate of sIgA following tennis drills (Nieman *et al.*, 2000), soccer play (Bishop *et al.*, 1999a), and cycling (Blannin *et al.*, 1998). Jemmott and McClelland (1989) concluded from a meta-analysis of nine studies that the level of IgA secretion might indicate the vulnerability toward URTI. Mackinnon and Hooper (1994) further suggested that the protective effect might not only depend on sIgA concentration but also on saliva flow rate.

Most components of the immune system show rhythmic changes (Shephard and Shek, 1996). Plasma cortisol exhibits a prominent diurnal rhythm, which peaks immediately after awakening and then falls progressively during the morning, stabilising in the afternoon, and reaching a nadir at around midnight (Porterfield, 2001); this could impose diurnal variation on immune function (Petrovsky *et al.*, 1998). Plasma ACTH also shows a pronounced diurnal pattern, which peaks in the early morning and declines to a nadir in the evening (Porterfield, 2001). Circulating leukocyte and neutrophil counts demonstrate circadian rhythms increasing from early morning and peaking in the evening (Haus, 1994) and lymphocyte counts are elevated during the night and decline after wakening (Dhabhar *et al.*, 1994). This inverse relationship between cortisol and peripheral blood leukocyte numbers suggests that the endocrine system might play an important role in regulating the circadian cycle of immune cells (Dhabhar *et al.*, 1994). Gleeson *et al.* (2001a) showed that a diurnal variation in sIgA concentration exists: sIgA concentration was the highest at 08:00 followed by a decline during the morning and then was stable from 12:00 onwards.

This finding was supported by a recent study (Dimitriou *et al.*, 2002), which showed there was a significant difference in sIgA concentration, sIgA secretion rate, and saliva flow rate at 06:00 compared with 18:00: a lower sIgA concentration but a higher saliva flow rate and sIgA secretion rate were observed at 18:00.

Hence, the aims of this present study were to compare changes in redistribution of leukocyte subsets, stress hormones, IL-6, neutrophil function, and saliva flow rate and sIgA responses to a single bout of prolonged cycling at different times of day and to a second bout of cycling at same intensity on the same day.

5.2 Methods

Subjects

Eight male volunteers (age 28.9 ± 1.8 years, body mass 72.2 ± 2.5 kg, $\dot{V}O_{2\,max}$ 56.1 ± 2.8 $mL \cdot kg^{-1} \cdot min^{-1}$; means ± S.E.M.), who were recreationally active and familiar with cycling, participated in the study. After receiving written information about this study and passing a Health Questionnaire screen, subjects gave their written informed consent. Subjects were asked not to perform any strenuous exercise or consume alcohol or medication for 2 days before each trial. The protocol was approved by the Ethics Committee of Loughborough University before the study began.

Experimental Procedures

The subject's workload was determined by a preliminary maximal oxygen uptake testing procedure as described in Chapter 3.2. The subjects completed three experimental trials in a counterbalanced order, each separated by at least 4 days. For the afternoon exercise trial (PMEX), subjects reported to the laboratory at 13:30 after fasting from 23:00 the previous day, then performed 2 h cycling at 60% $\dot{V}O_{2\,max}$ starting at 14:00. On the other two trials, subjects reported to the laboratory at 08:30 after an overnight fast, then either performed two bouts of exercise (EX1 started at 09:00 and EX2 started at 14:00) or a separate resting control trial. EX1 consisted of cycling for 2 h at 60% $\dot{V}O_{2\,max}$, whereas in EX2 subjects cycled at the same exercise intensity to fatigue (75 ± 11 min). Subjects were asked to empty the bladder before measurement of body mass, and performed cycling at 70 $rev \cdot min^{-1}$ on the same ergometer used to determine $\dot{V}O_{2\,max}$. Heart rate was recorded continuously during exercise by radiotelemetry. Ratings of perceived exertion (RPE) were obtained at 15-min intervals. Venous blood samples were taken 5 min before exercise and immediately post-exercise in the exercise trials and were taken at 09:00, 11:00, 14:00 and 16:00 in the resting trial. Unstimulated whole saliva samples were obtained at 10 min before exercise, after 58 - 60 min and during the last 2 min of exercise at 60% $\dot{V}O_{2\,max}$, at 1 h and 2 h post-exercise, and every hour from 9:00 to 18:00 during a resting control trial. Subjects remained fasted throughout. No food was consumed until the trials finished at 18:00 though water ingestion was allowed *ad libitum* during the trials except for 5 min before

each saliva sampling. The laboratory temperature and relative humidity were 21.4 ± 0.4 °C and 54 ± 3%, respectively.

Analytical Methods

Methods of preliminary measurements and blood and saliva collection and analysis are presented in Chapter 3.

Statistical analysis

All results are presented as mean values and standard errors of the mean (± SEM). Data were checked for normality, homogeneity of variance and sphericity before statistical analysis, and where appropriate the Huynh-Feldt method was applied for adjustment of degrees of freedom for the *F*-tests. Data were analysed using a two-factor (trial × time) repeated measures ANOVA with *post hoc* *t*-tests. Physiological variables and RPE were examined using paired *t*-tests. The circadian variations were examined using a one-factor repeated measure ANOVA with *post hoc* Tukey tests. *P*, *t*, and adjusted *F* values are presented and statistical significance was accepted at $P < 0.05$.

5.3 Results

Physiological Variables and RPE

The effects of one or two bouts of exercise on heart rate (HR), rating of perceived exertion (RPE), body mass loss, water intake, and the percentage change in plasma volume are presented in Table 5.1. There were significantly higher HR responses in EX2 than EX1 (t = 3.05, P = 0.019) and higher RPE responses in EX2 and PMEX compared with EX1 (t = 11.23, $P < 0.001$ and t = 3.23, P = 0.011, respectively). Exercise intensity, body mass loss, water intake and percentage change in plasma volume did not differ significantly between trials.

Table 5.1 The exercise intensity and the effect of exercise on HR, RPE, body mass loss, water intake, and percentage change in plasma volume

	EX1	EX2	PMEX
% VO_{2max}	60.7 (0.2)	59.9 (0.2)	61.2 (0.3)
HR (beats·min^{-1}) [a]	146 (2)	151 (3)*	148 (3)
RPE [a]	14.1 (0.2)	17.1 (0.2)**	15.3 (0.4)[¶]
Body mass loss (g·min^{-1}) [b]	12.2 (1.4)	12.2 (1.4)	12.4 (1.4)
Water intake (mL)	935 (129)	934 (268)	989 (189)
Plasma volume change (%) [c]	-4.4 (1.5)	-6.0 (1.4)	-2.4 (1.3)

Values are mean (± SEM, n = 8). Significantly different from EX1 (*$P < 0.05$, **$P < 0.01$). [¶] Significantly different from EX2 ($P < 0.05$). [a] Measurements made in last 15 min of exercise. [b] After correction for water intake. [c] Immediately post-exercise compared with pre-EX.

Leukocyte counts

Circadian Variation

A circadian rhythm was observed for circulating numbers of leukocytes ($F_{3, 18}$ = 21.8, $P <$ 0.001), neutrophils ($F_{3, 18}$ = 18.3, $P < 0.001$), and lymphocytes ($F_{3, 18}$ = 5.8, P = 0.006). Resting blood counts of total leukocytes and neutrophils were increased with time, lower in

the morning and higher in late afternoon (Table 5.2). However, lymphocyte numbers declined during the morning and then increased during the afternoon.

Table 5.2 The circadian rhythms of blood leukocyte subsets and plasma stress hormones

Parameter	09:00	11:00	14:00	16:00
Total Leukocytes ($10^9 \cdot L^{-1}$)	4.57 (0.36)	4.64 (0.24)	5.24 (0.36)**	5.74 (0.42)**
Neutrophils ($10^9 \cdot L^{-1}$)	2.22 (0.28)	2.40 (0.26)	2.75 (0.28)*	3.08 (0.39)**
Lymphocytes ($10^9 \cdot L^{-1}$)	1.80 (0.16)	1.74 (0.17)	1.95 (0.13)	2.08 (0.18)*
ACTH (pM)	7.78 (0.96)	6.04 (0.57)	5.93 (0.58)	5.55 (0.29)*
Cortisol (nM)	387 (80)	242 (37)	199 (39)**	218 (37)*

Values are mean (± SEM, n = 7). Significantly different from 09:00 (*$P < 0.05$, **$P < 0.01$).

The effects of exercise at different times of day (EX1 vs PMEX)

There was a significant main effect of time for the blood counts of leukocytes ($F_{1,7} = 29.4$, $P = 0.001$, Figure 5.1A), neutrophils ($F_{1,7} = 27.6$, $P = 0.001$, Figure 5.1B), lymphocytes ($F_{1,7} = 17.0$, $P = 0.004$, Figure 5.1C), and monocytes ($F_{1,7} = 35.4$, $P = 0.001$, Figure 5.1D), with values higher at post-EX compared with pre-EX. There was also a significant main effect of trial for the blood leukocyte ($F_{1,7} = 8.9$, $P = 0.021$) and neutrophil ($F_{1,7} = 7.8$, $P = 0.027$) counts, which were a higher in PMEX than EX1. Furthermore, there were significant main effects of trial ($F_{1,7} = 7.2$, $P = 0.032$) and interaction between trial and time ($F_{1,7} = 21.1$, $P = 0.003$) for monocytes, with a significantly higher monocyte count at post-PMEX compared with post-EX1.

The effects of the second exercise bout compared with the first bout (EX2 vs EX1)

There was a significant main effect of time for the circulating numbers of leukocytes ($F_{1,7} = 53.7$, $P < 0.001$, Figure 5.1A), lymphocytes ($F_{1,7} = 35.0$, $P = 0.004$, Figure 5.1C), and monocytes ($F_{1,7} = 96.0$, $P < 0.001$, Figure 5.1D), with higher values at post-EX compared with pre-EX. For blood neutrophils, there was a significant main effect of time ($F_{1,7} = 27.9$, $P < 0.001$, Figure 5.1B) and main effect of trial ($F_{1,7} = 25.0$, $P = 0.002$). Both

leukocyte and monocyte counts showed a significant main effect of trial ($F_{1,7} = 31.3$, $P = 0.001$ and $F_{1,7} = 107.2$, $P < 0.001$), with higher values in EX2 compared with EX1. In addition, a significant main effect of trial ($F_{1,7} = 15.8$, $P = 0.005$) and interaction between trial and time ($F_{1,7} = 28.2$, $P = 0.001$) were observed for lymphocyte counts, with higher values at post EX2 compared with post-EX1.

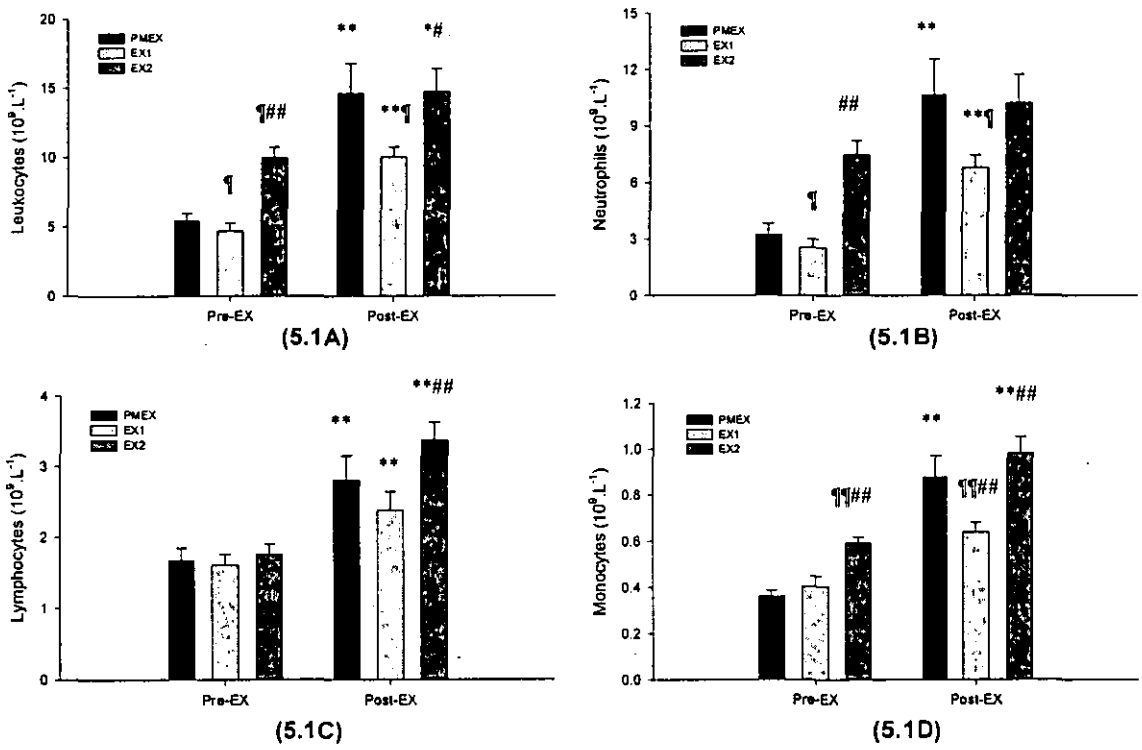


Figure 5.1 Changes in circulating counts of total leukocytes (5.1A), neutrophils (5.1B), lymphocytes (5.1C) and monocytes (5.1D). Values are means ± SEM (n = 8). Significantly different from the pre-EX ($^{*}P < 0.05$, $^{**}P < 0.01$), significantly different from PMEX ($^{¶}P < 0.05$, $^{¶¶}P < 0.01$), significantly different from EX1 ($^{\#}P < 0.05$, $^{\#\#}P < 0.01$).

Comparison between the second bout and the single afternoon bout of prolonged cycling (EX2 vs PMEX)

There was a significant main effect of time for the circulating numbers of leukocytes ($F_{1,7}$ = 5.9, P = 0.046, Figure 5.1A), neutrophils ($F_{1,7}$ = 18.3, P = 0.004, Figure 5.1B), lymphocytes ($F_{1,7}$ = 48.0, P < 0.001, Figure 5.1C), and monocytes ($F_{1,7}$ = 67.1, P < 0.001, Figure 5.1D), with higher values at post-EX compared with pre-EX. There was a significant main effect of trial for leukocyte ($F_{1,7}$ = 5.9, P = 0.046) and monocyte ($F_{1,7}$ = 16.8, P = 0.005) counts.

Stress Hormones

Circadian Variation

A circadian rhythm was observed for plasma concentrations of ACTH ($F_{3,18}$ = 5.1, P = 0.026) and cortisol ($F_{3,18}$ = 6.6, P < 0.006), with higher values in the early morning and a decline with time to lower levels in the late afternoon (Table 5.2).

The effects of exercise at different times of day (EX1 vs PMEX)

There was a significant main effect of time for plasma concentrations of adrenaline ($F_{1,7}$ = 15.7, P = 0.029, Figure 5.2A), ACTH ($F_{1,7}$ = 10.5, P = 0.014, Figure 5.2B), cortisol ($F_{1,7}$ = 7.0, P = 0.033, Figure 5.2C), and GH ($F_{1,7}$ = 31.7, P = 0.001, Figure 5.2D), with higher levels at post-EX than pre-EX. Furthermore, there was a significant interaction between trial and time for plasma cortisol ($F_{1,7}$ = 7.2, P = 0.031). Exercise in the morning did not affect the plasma cortisol concentration, whereas exercise in the afternoon resulted in a 2-fold increase in this variable (Figure 5.2C).

The effects of the second exercise bout compared with the first bout (EX2 vs EX1)

There was a significant main effect of time for plasma concentrations of adrenaline ($F_{1,7}$ = 31.2, P = 0.005, Figure 5.2A), ACTH ($F_{1,7}$ = 15.7, P = 0.005, Figure 5.2B), cortisol ($F_{1,7}$ = 15.7, P = 0.007, Figure 5.2C)and GH ($F_{1,7}$ = 27.7, P = 0.001, Figure 5.2D), with higher levels at post-EX than pre-EX. A significant main effect of trial and an interaction between trial and time were also observed for plasma ACTH ($F_{1,7}$ = 12.4, P = 0.010 and $F_{1,7}$ =

17.7, $P = 0.004$), cortisol ($F_{1,7} = 6.4$, $P = 0.045$ and $F_{1,7} = 21.7$, $P = 0.003$), and GH ($F_{1,7} = 5.9$, $P = 0.045$ and $F_{1,7} = 5.9$, $P = 0.046$), with higher levels at post-EX2 compared with post-EX1.

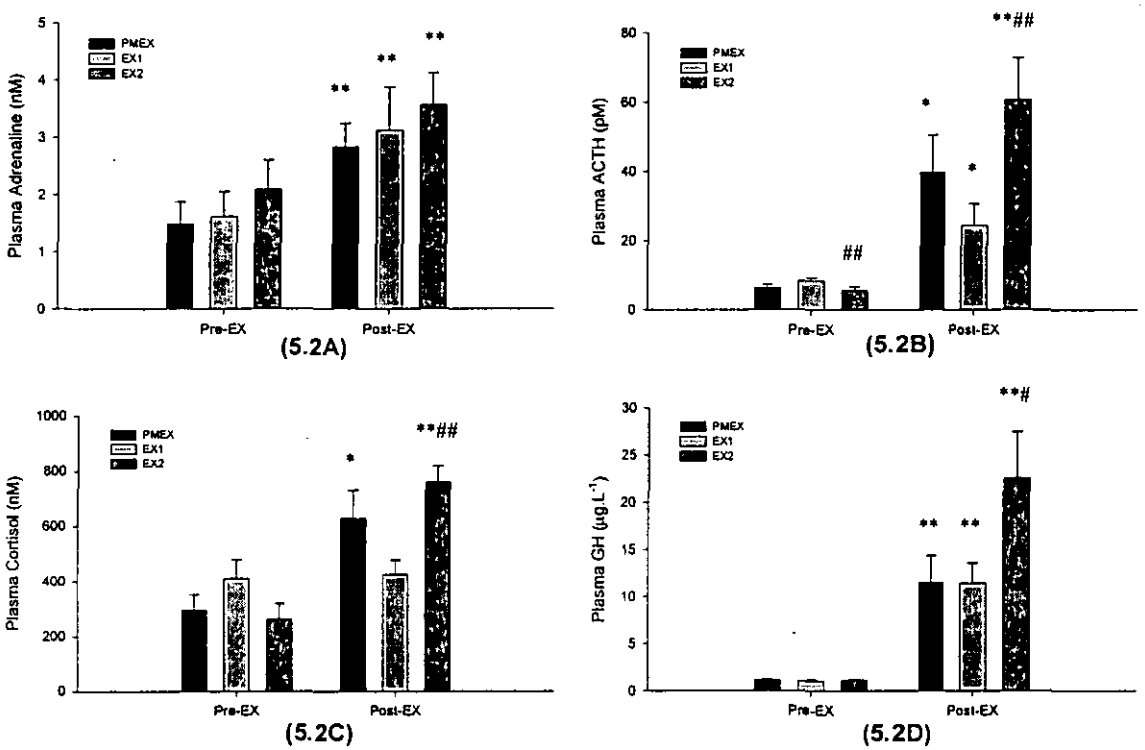


Figure 5.2 Changes in plasma concentrations of adrenaline (5.2A), ACTH (5.2B), cortisol (5.2C) and GH (5.2D). Values are means ± SEM (n = 8). Significantly different from the pre-EX ($^{*}P < 0.05$, $^{**}P < 0.01$) and significantly different from EX1 ($^{\#}P < 0.05$, $^{\#\#}P < 0.01$).

Comparison between the second bout and the single afternoon bout of prolonged cycling (EX2 vs PMEX)

A significant main effect of time was observed for the plasma concentrations of adrenaline ($F_{1,7}$ = 18.0, P = 0.005, Figure 5.2A), ACTH ($F_{1,7}$ = 18.4, P = 0.004, Figure 5.2B), cortisol ($F_{1,7}$ = 22.0, P = 0.003, Figure 5.2C), and GH ($F_{1,7}$ = 24.3, P = 0.002, Figure 5.2D).

Glucose and IL-6

The effects of exercise at different times of day (EX1 vs PMEX)

There was a significant main effect of time ($F_{1,7}$ = 38.9, P < 0.001, Figure 5.3A) and an interaction between trial and time ($F_{1,7}$ = 5.9, P = 0.044) for plasma glucose concentration, which was lower at post-PMEX (but not at post-EX1) compared with pre-EX. Plasma IL-6 concentration showed a significant main effect of time ($F_{1,7}$ = 36.1, P = 0.001, Figure 5.3B), with higher values at post-EX than pre-EX.

The effects of the second exercise bout compared with the first bout (EX2 vs EX1)

There were significant main effects of trial ($F_{1,7}$ = 5.6, P = 0.050) and time ($F_{1,7}$ = 18.8, P = 0.003) and an interaction between trial and time ($F_{1,7}$ = 7.5, P = 0.029) for plasma glucose concentration, with values at post-EX2 significantly lower than at pre-EX2 and post-EX1 (Figure 5.3A). Plasma IL-6 concentration at post-EX was significantly higher than pre-EX (main effect of time: $F_{1,7}$ = 23.4, P = 0.002, Figure 5.3B).

Comparison between the second bout and the single afternoon bout of prolonged cycling (EX2 vs PMEX)

There was a significant main effect of time ($F_{1,7}$ = 25.2, P = 0.002) and an interaction between trial and time ($F_{1,7}$ = 6.0, P = 0.044) for plasma IL-6 (Figure 5.3B), with a higher concentration at post-EX than pre-EX in both EX2 and PMEX.

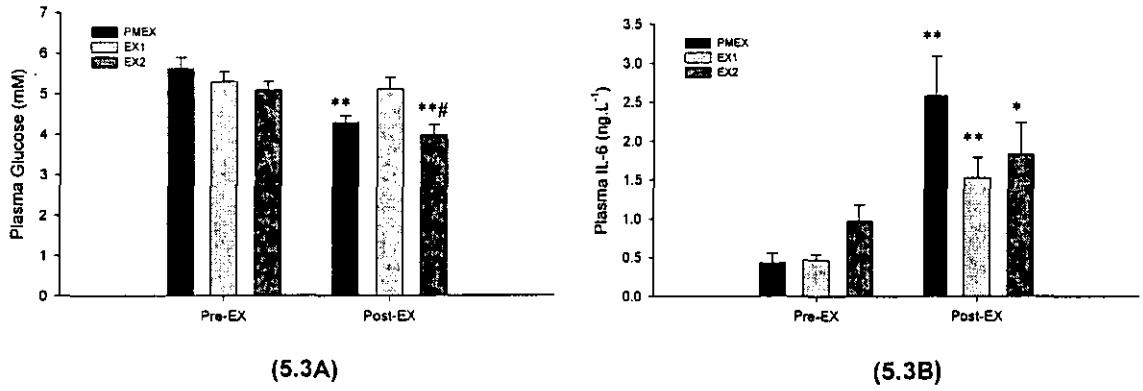


Figure 5.3 Changes in plasma concentrations of glucose (5.3A) and IL-6 (5.3B). Values are means ± SEM (n = 8). Significantly different from the pre-EX ($^{*}P < 0.05$, $^{**}P < 0.01$) and $^{\#}$ significantly different from EX1 ($P < 0.05$).

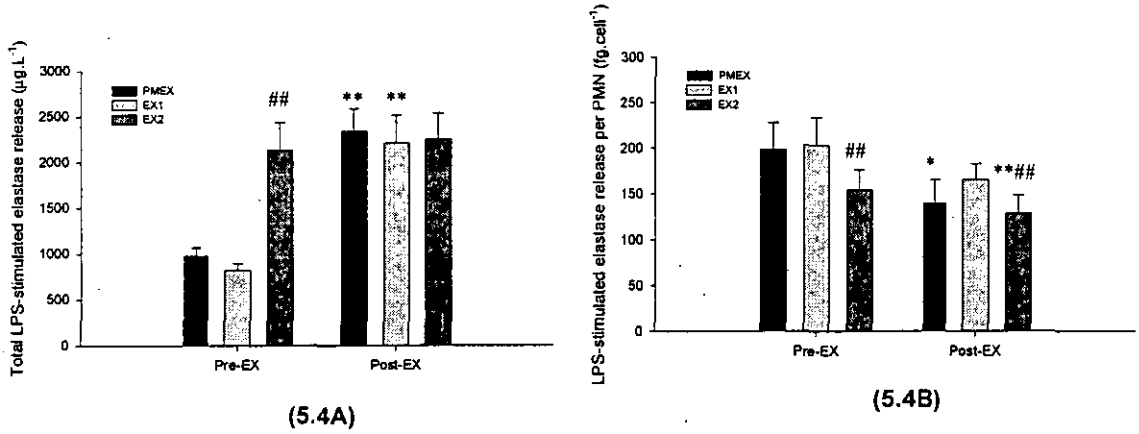


Figure 5.4 Changes in total LPS-stimulated elastase release (5.4A) and LPS-stimulated elastase release per neutrophil (5.4B). Values are means ± SEM (n = 8). Significantly different from the pre-EX ($^{*}P < 0.05$, $^{**}P < 0.01$), ¶¶ significantly different from PMEX ($P < 0.01$), $^{\#\#}$ significantly different from EX1 ($P < 0.01$).

Neutrophil Degranulation

The effects of exercise at different times of day (EX1 vs PMEX)

There was a significant main effect of time ($F_{1,7} = 46.9$, $P < 0.001$) for total LPS-stimulated elastase release, which was higher at post-EX than pre-EX (Figure 5.4A). In contrast, 2 h cycling at 60% $\dot{V}O_{2max}$ caused a significantly lower LPS-stimulated elastase release per neutrophil in post-EX compared with pre-EX (main effect of time: $F_{1,7} = 7.3$, $P = 0.031$, Figure 5.4B).

The effects of the second exercise bout compared with the first bout (EX2 vs EX1)

There were significant main effects of trial ($F_{1,7} = 11.1$, $P = 0.012$) and time ($F_{1,7} = 26.1$, $P = 0.001$) and an interaction between trial and time ($F_{1,7} = 18.0$, $P = 0.004$) for total LPS-stimulated elastase release, which was higher at pre-EX2 than pre-EX1 and at post-EX1 than pre-EX1 (Figure 5.4A). For LPS-stimulated elastase release per neutrophil, there were significant main effects of trial ($F_{1,7} = 32.4$, $P = 0.001$) and time ($F_{1,7} = 8.4$, $P = 0.023$), with higher values at pre-EX than post-EX and a higher values in EX1 compared with EX2 (Figure 5.4B).

Comparison between the second bout and the single afternoon bout of prolonged cycling (EX2 vs PMEX)

There was a significant main effect of time ($F_{1,7} = 37.5$, $P < 0.001$) and an interaction between trial and time ($F_{1,7} = 19.4$, $P = 0.003$) for total LPS-stimulated elastase release (Figure 5.4A) and a main effect of time ($F_{1,7} = 10.6$, $P = 0.014$) for LPS-stimulated elastase release per neutrophil (Figure 5.4B).

Saliva Flow Rate

The effects of exercise at different times of day (EX1 vs PMEX)

There was a significant main effect of time for saliva flow rate ($F_{4,28} = 8.0$, $P < 0.001$), which was decreased following exercise and returned to the levels of pre-EX within 1 h (Figure 5.5).

The effects of the second exercise bout compared with the first bout (EX2 vs EX1)

Similarly, a significant main effect of time for saliva flow rate was observed ($F_{4, 28} = 10.3$, $P = 0.002$), which was decreased following exercise and returned to the levels of pre-EX within 1 h (Figure 5.5).

Comparison between the second bout and the single afternoon bout of prolonged cycling (EX2 vs PMEX)

There was a significant main effect of time ($F_{4, 28} = 11.4$, $P = 0.001$) and an interaction between trial and time ($F_{4, 28} = 3.1$, $P = 0.030$) for saliva flow rate, which was also decreased following exercise and returned to the levels of pre-EX within 1 h in both trials (Figure 5.5).

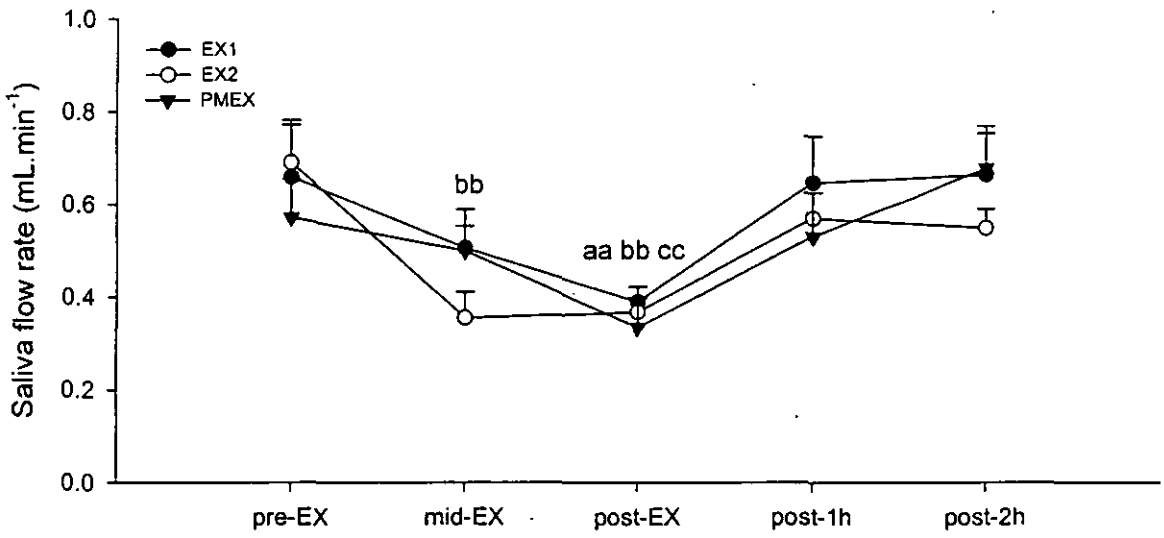


Figure 5.5 Changes in saliva flow rate. Values are means ± SEM (n = 8). [aa] Significantly lower than pre-EX1 ($P < 0.01$), [bb] significantly lower than pre-EX2 ($P < 0.01$), [cc] significantly lower than pre-PMEX ($P < 0.01$).

Salivary IgA Concentration and Secretion Rate

Circadian Variation

Circadian variations were also found in resting sIgA concentration ($F_{9, 63} = 3.6$, $P = 0.009$), which decreased with time from the highest value in the early morning to the lowest value in the evening (Table 5.3).

Table 5.3 The circadian variation of sIgA concentration and sIgA secretion rate at rest

	09:00	10:00	11:00	12:00	13:00	14:00	15:00	16:00	17:00	18:00
sIgA concentration ($mg \cdot L^{-1}$)	125 (26)	128 (18)	107 (20)	125 (24)	119 (27)	115 (200)	110 (13)	93 (18)	74* (11)	75* (10)
sIgA secretion rate ($\mu g \cdot min^{-1}$)	68 (8)	74 (9)	58 (9)	68 (8)	68 (9)	70 (11)	76 (5)	66 (9)	56 (4)	59 (9)

Values are mean (± SEM). * Significantly different from 09:00 ($P < 0.05$).

The effects of exercise at different times of day (EX1 vs PMEX)

There was a significant main effect of time for sIgA concentration ($F_{4, 28} = 5.8$, $P = 0.026$), with an increase with exercise and returned to pre-EX level within 1 h (Figure 5.6). There were no significant differences in sIgA secretion rate in both trials throughout experimental protocol (Figure 5.7).

The effects of the second exercise bout compared with the first bout (EX2 vs EX1)

There were significant main effect of trial ($F_{1, 7} = 7.3$, $P = 0.031$), main effect of time ($F_{4, 28} = 3.0$, $P = 0.035$), and interactions between trial and time ($F_{4, 28} = 3.8$, $P = 0.021$) for sIgA concentration. The sIgA concentration was increased with exercise and remained elevated for at least 2 h in EX2 after exercise cessation. Furthermore, the sIgA concentrations at post-1h and post-2h in EX2 were significantly higher than the same time points in EX1 (Figure 5.6). Only a significant interaction between trial and time was observed for sIgA secretion rate ($F_{4, 28} = 5.0$, $P = 0.004$, Figure 5.7).

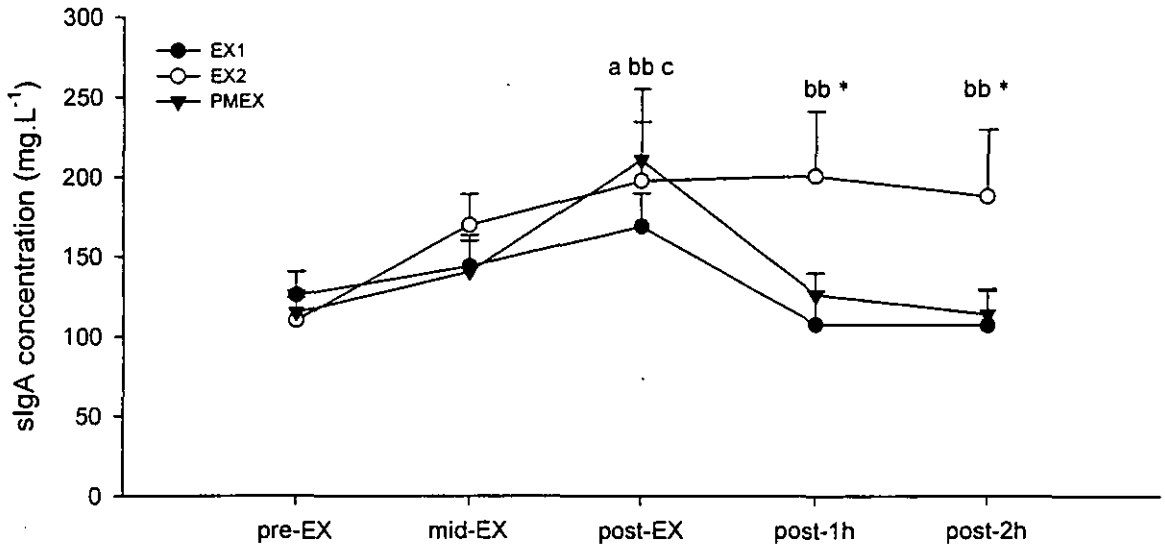


Figure 5.6 Changes in sIgA concentration. Values are means ± SEM (n = 8). [a] Significantly higher than pre-EX1 ($P < 0.05$), [bb] significantly higher than pre-EX2 ($P < 0.01$), [c] significantly higher than pre-PMEX ($P < 0.05$), * significantly higher than EX1 ($P < 0.05$).

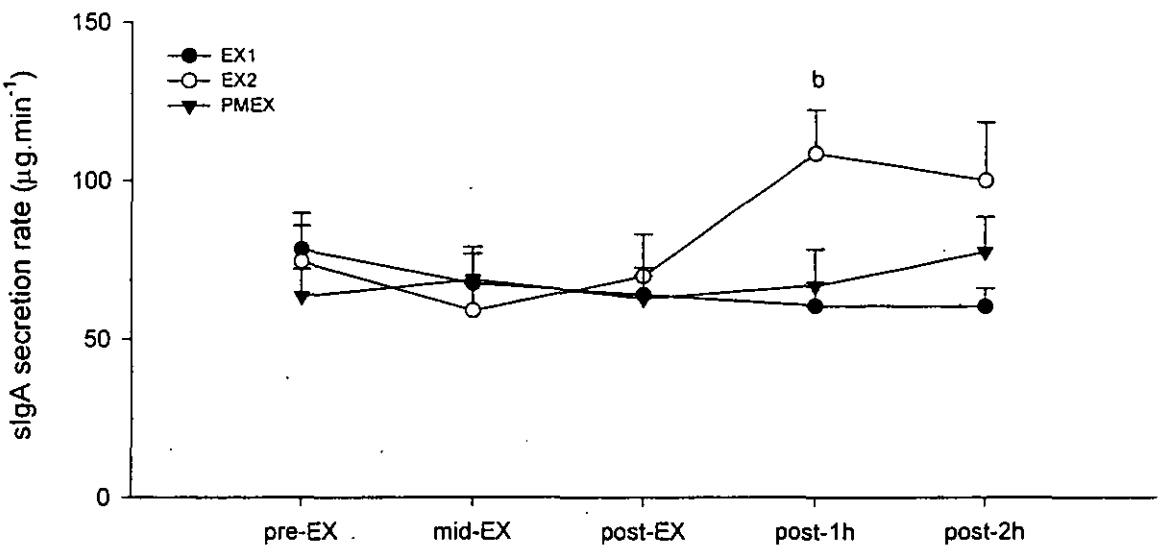


Figure 5.7 Changes in sIgA secretion rate. Values are means ± SEM (n = 8). [b] Significantly higher than pre-EX2 ($P < 0.05$).

Comparison between the second bout and the single afternoon bout of prolonged cycling (EX2 vs PMEX)

There was a significant main effect of time in sIgA concentration ($F_{4, 28} = 4.4$, $P = 0.010$) and sIgA secretion rate ($F_{4, 28} = 3.5$, $P = 0.021$).

5.4 Discussion

The main findings were 1) PMEX induced larger increases in circulating numbers of leukocytes, neutrophils, and monocytes than EX1; 2) compared with EX1, EX2 caused greater increases in circulating numbers of leukocytes, lymphocytes, and monocytes and responses of plasma ACTH, cortisol, and GH; 3) plasma glucose concentration and LPS-stimulated neutrophil degranulation on per cell basis were lower after EX2 than after EX1; 4) cycling at 60% $\dot{V}O_{2\,max}$ for 2 h significantly influenced saliva flow rate and sIgA concentration but did not affect sIgA secretion rate; 5) performing prolonged exercise in the morning or in the afternoon had similar effects on salivary variables in the short-term 6) a 3 h rest was enough for oral immunity, but was insufficient for circulating numbers of leukocytes, neutrophils and monocytes and neutrophil function to recover; and 7) there were circadian rhythms in plasma concentrations of ACTH and cortisol, circulating counts of leukocytes, neutrophils and lymphocytes, and sIgA concentration.

The latter findings were similar to previous studies, which showed that resting blood counts of leukocytes and neutrophils increased from early morning and peaked around 19:00 - 21:00 (Haus, 1994); whereas blood lymphocyte concentrations were elevated during the night time (due to release of cells from lymphoid organs) and declined after wakening (by movement of lymphocytes back to the lymphoid tissues under the influence of cortisol) (Dhabhar *et al.*, 1994, Gatti *et al.*, 1994). A diurnal variation of sIgA concentration was also found supporting the findings of previous studies (Gleeson *et al.*, 2001a, Dimitriou *et al.*, 2002). The findings suggest that the diurnal variation must be considered when the aim of a study is to compare the effect of exercise performed at different times of day on the aforementioned parameters.

A reduction of plasma glucose levels was found at post-PMEX and post-EX2 but not at post-EX1 in the present study. Glucose has been indicated to be the primary energy source of immune cells (Pedersen, 1999). Muscle glycogen depletion and hypoglycaemia are potential causes of fatigue during prolonged exercise (Coyle *et al.*, 1983, Costill and Hargreaves, 1992) and the reduction in plasma glucose levels has been linked to HPA activation, increasing the release of stress hormones (Mitchell *et al.*, 1990). In this study, plasma glucose concentration was unchanged after 2 h cycling at 60% $\dot{V}O_{2\,max}$ in the

morning. However, plasma glucose levels fell markedly during EX2 and subjects were exhausted after 75 ± 11 min. Although there was no direct evidence to prove that subjects' fatigue during EX2 in this study was caused by glycogen depletion, it can be assumed that the subjects' glycogen stores would have been almost fully depleted at the end of EX1. During 2 h of cycling at 60% $\dot{V}O_{2\,max}$, the rate of carbohydrate oxidation was about 1.7 $g \cdot min^{-1}$ (estimated from the respiratory exchange ratio and $\dot{V}O_2$ data during EX1). Thus, after 2 h cycling, around 200 g of carbohydrate would have been oxidised, which probably corresponds to >80% of the total available glycogen stores after an overnight fast. Glycogen resynthesis was probably minimal during the 3 h recovery under fasting conditions (Satabin *et al.*, 1989). Thus, it is not surprising that plasma glucose concentrations at post-EX2 were significantly lower than post-EX1 and below normal fasting values. Plasma glucose levels were also lower at post-PMEX compared with post-EX1 and this may be, at least partly, due to the lower liver and muscle glycogen stores in the afternoon (Clark and Conlee, 1979) and the longer period of fasting than for the morning exercise bout.

Several hormones are involved in the redistribution of immune cells during exercise. GH is likely one of the candidates since it has been shown to cause blood neutrophilia after GH injection (Kappel *et al.*, 1993). However, cortisol appears to have a more wide-reaching effects and has been reported to cause neutrophilia together with lymphopenia, monocytopenia, eosinopenia, and a suppression of NK and T cell function (Fauci, 1976, Cupps and Fauci, 1982) with a time lag of at least 2 h, peaking at 4 h after administration (Pedersen *et al.*, 1997b). Adrenaline seems to be an important hormone in recruiting lymphocytes and neutrophils into circulation during the first 90 min of intensive exercise. However, after 90 min of exercise, its effect is minimised by the rising cortisol, which attenuates lymphocytosis and subsequently induces lymphopenia, which can develop before exercise has finished (Nieman, 1997). In this study, the lower pre-EX blood counts of leukocytes and neutrophils in EX1 compared with PMEX could be due to circadian rhythms. However, the higher circulating numbers of leukocytes, neutrophils and monocytes at post-PMEX compared with post-EX1 could be caused by plasma cortisol because plasma cortisol concentration was significantly higher at post-PMEX than post-EX1. Compared with EX1, EX2 caused larger increases in circulating numbers of

leukocytes, lymphocytes and monocytes and this effect may be attributable to the greater increase in plasma cortisol and GH during EX2. Furthermore, the blood lymphocyte count and plasma stress hormones returned to pre-EX values, whereas the leukocytosis, neutrophilia, and monocytosis induced by EX1 did not return to the values of pre-EX after the 3-h recovery period. This finding was similar to the results of the study conducted by Ronsen *et al.* (Ronsen *et al.*, 2001b), which showed that the elevated counts of leukocytes and neutrophils did not return to resting values within 3 h after a 75 min cycling consisting of a 10-min at 50% $\dot{V}O_{2\,max}$ and a 65-min at 75% $\dot{V}O_{2\,max}$. There were no significant differences in blood counts of total leukocytes, leukocyte subsets or the plasma concentrations of stress hormones between PMEX and EX2 in this study, whereas Ronsen *et al.* (2001a, 2001b) showed that a second bout of cycling induced significantly higher blood counts of leukocytes, neutrophils, and lymphocytes and plasma concentrations of adrenaline, ACTH, cortisol, and GH compared with an identical single bout of cycling performed at the same time of day. The differences between the results of Ronsen *et al.* and the present study may be related to differences in carbohydrate availability. In the studies of Ronsen *et al.* subjects were served 2 standardized meals (2,000 kcal in total) before performing the single afternoon cycling. However, subjects in our study remained fasted from 23:00 the day before trial until 18:00 on the trial day. Furthermore, subjects in our study were unable to complete 2 h of cycling in the second exercise bout.

In the present study, the plasma IL-6 concentration was elevated after exercise in all trials. These results were similar to previous studies, which have shown that prolonged strenuous exercise induces a marked elevation in the plasma IL-6 level (Ostrowski *et al.*, 1999, Pedersen *et al.*, 2001). Recent studies have demonstrated that IL-6 is produced in and released from contracting skeletal muscle into the circulation (Steensberg *et al.*, 2000, Langberg *et al.*, 2002) and that IL-6 may act in a hormone-like fashion to promote the hepatic glucose production and stimulate lipolysis in adipose tissue during exercise (Gleeson, 2000a, Febbraio and Pedersen, 2002). However, in the present study, the absolute plasma concentrations of IL-6 at post-EX were relatively low and there were no significant differences between trials.

Total LPS-stimulated elastase release was significantly increased after exercise. However, this effect was likely due to the blood neutrophilia after exercise. When total LPS-stimulated elastase was divided by the neutrophil count and presented on per cell basis, the capacity of LPS-stimulated elastase release per neutrophil was lower at post-PMEX and post-EX2 compared with pre-EX. The results of this study also showed that 2 h cycling at 60% $\dot{V}O_{2\,max}$ induced a fall in neutrophil degranulation capacity on per cell basis and this decline could not be fully recovered after a 3-h rest period. Thus, the exercise-induced depression of the neutrophil degranulation response may be further deteriorated if athletes begin another exercise bout after insufficient recovery. To our knowledge, this is the first study investigate how repeated bouts of exercise on the same day influence the LPS-stimulated neutrophil degranulation response. Previous studies have shown that the LPS-stimulated neutrophil degranulation response is decreased on per cell basis after a single exercise bout and this decline does not recover within 2 h after exercise (Robson *et al.*, 1999, Walsh *et al.*, 2000a). Exposure to glucocorticoids has been reported to depress neutrophil functions, including chemotaxis, adherence to surfaces, phagocytosis, degranulation, oxidative burst, and antibody-dependent cytotoxicity (Liles *et al.*, 1995) in a dose-related manner (Tintinger *et al.*, 2001). The higher plasma cortisol levels during exercise and the entry into the circulation of less mature neutrophils released from the bone marrow under the influence of cortisol (Pyne, 1994) and IL-6 (Suwa *et al.*, 2000) may be responsible for the observed fall in the LPS-stimulated neutrophil degranulation response after exercise in this study although a recent study suggested that release of less mature cells was not the reason for the fall in LPS-stimulated elastase release after a 2 h cycle at 70% $\dot{V}O_{2\,max}$ (Bishop *et al.*, 2003).

Saliva flow rate significantly decreased at post-EX compared with pre-EX and this fall returned to pre-EX values within 1 h. Salivary glands are innervated by both parasympathetic cholinergic nerves and sympathetic adrenergic nerves. During exercise, the sympathetic stimulation is increased and induces vasoconstriction, which limits saliva secretion rate (Chicharro *et al.*, 1998). Plasma adrenaline concentration was increased during exercise and therefore, it is not surprising to find a significant decrease in saliva flow rate in the present study. Previous studies have also consistently reported falls in saliva flow rate during strenuous exercise (Ljungberg *et al.*, 1997, Steerenberg *et al.*, 1997,

Nieman *et al.*, 2002). Rantonen and Meurman (2000) indicated that the saliva flow rate appeared to be the single salivary defensive factor which affected oral health to a significant degree. This notion was supported by recent studies, which showed the absence of caries in children with familial dysautonomia was associated with a higher salivary flow rate (Mass *et al.*, 2002), and the increased incidence of oral candidal infections in HIV-infected patients (Lin *et al.*, 2001) was associated with a lower saliva flow rate. Fox *et al.* (1985) also suggested individuals who suffered from dry mouth syndrome had an increased incidence of URTI. The results of this Chapter indicate that saliva flow rate responses to prolonged cycling are not affected by time of day or by performing more than one bout of endurance exercise on the same day if the adrenaline responses to exercise are similar.

A significant elevation in sIgA concentration at post-EX compared with pre-EX was found. The alteration seemed to mainly result from the reduction of saliva flow rate since no changes in sIgA secretion rate were observed during this period. Saliva IgA secretion has been shown to be stimulated by α-adrenoceptors (Proctor *et al.*, 2003) and depressed by glucocorticoid 24 h after a single injection (Wira *et al.*, 1990). The observed changes in sIgA concentration and secretion rate in the present study support the notion that cortisol does not affect sIgA level acutely. Since the inhibitory effect of exercise on sIgA level seems to occur on the following day after exercise, further investigations may need to examine longer-term responses to experimental exercise interventions.

In conclusion, the findings of this study suggest that 1) a single bout of prolonged exercise performed in the afternoon induces a larger neutrophilia and monocytosis than an identical bout of morning exercise. This may be due to reduced carbohydrate availability and circadian rhythms in blood neutrophil counts and cortisol levels; 2) a second prolonged exercise bout causes greater immunoendocrine responses but lower plasma glucose levels and neutrophil function compared with the first bout; 3) during such exercise, sympathetic stimulation appears to be strong enough to inhibit saliva flow rate; however, it seems likely that it does not affect sIgA production or transcytosis; and 4) a 3-h recovery period is sufficient for oral immunity but is insufficient for neutrophil function to fully recover after prolonged exercise.

CHAPTER SIX

Effects of carbohydrate supplementation during the recovery interval on immunoendocrine responses to a repeated bout of prolonged cycling

Summary

The purpose of this study was to examine the effect of CHO feeding during the recovery interval separating two 90-min cycling bouts (EX1 started at 09:00 and EX2 started at 13:30) at 60% $\dot{V}O_{2\,max}$ on leukocyte redistribution, neutrophil degranulation response to LPS, plasma IL-6, stress hormones, saliva flow rate and sIgA responses to a 3-h recovery interval and the subsequent EX2. This study consisted of two trials, which were completed in a counterbalanced order and separated by at least 4 days. Subjects (n = 8) consumed a lemon flavoured 10% w/v glucose (CHO) or PLA beverage (22 $mL \cdot kg^{-1}$ body mass) during the first hour of the recovery interval following EX1. Venous blood samples were taken 5 min before exercise and immediately post-exercise and unstimulated whole saliva samples were collected at 10 min before exercise, 48-50 min and 88-90 min of exercise, and 1 h and 2h post-exercise for both trials. The main findings were that ingestion of CHO compared with PLA during the recovery interval did not affect immunoendocrine responses during the recovery interval and the EX2. Compared with EX1, EX2 evoked significantly higher circulating numbers of leukocytes, neutrophils, lymphocytes and monocytes, higher plasma adrenaline concentration and lower plasma glucose concentration. These findings suggest that CHO ingestion during the recovery interval between two bouts of prolonged exercise does not attenuate immunoendocrine responses during a 3-h recovery interval and a subsequent bout of 90 min cycling at 60% $\dot{V}O_{2\,max}$. A second prolonged exercise bout causes a greater disturbance in immunoendocrine responses than the first exercise bout on the same day and this is not substantially influenced by CHO supplementation during the recovery interval.

6.1 Introduction

Numerous studies have been done to examine the influence of an acute single exercise bout on the immune system and the results indicate that after intensive prolonged exercise, such as a marathon race, immunity is depressed for several hours (Mackinnon, 1999). During this so-called "open window" period, pathogens may invade and cause infections in stressed athletes (Pedersen, 1997). The training regimens of many athletes involve several bouts of exercise in a day. Although a few studies have examined the effects of daily repeated exercise bouts on immunoendocrine responses (McCarthy *et al.*, 1992, Rohde *et al.*, 1998, Ronsen *et al.*, 2001a, Ronsen *et al.*, 2001b, Boyum *et al.*, 2002, Ronsen *et al.*, 2002a, Ronsen *et al.*, 2002b, McFarlin *et al.*, 2003), no study has investigated the influence of CHO ingestion during repeated bouts of exercise on immunoendocrine responses.

During stressful periods of training involving multiple exercises with short recovery intervals, it is particularly important for athletes to maintain immunocompetence (Gleeson, 2000c) and rapidly restore glycogen (Maughan, 2002) during recovery if they are to maintain training quality in subsequent bouts of exercise. It has been suggested that inadequate nutrition may result in glycogen depletion and subsequent elevation of stress hormones with impairment of immune cell function during prolonged exercise, leading to an increased incidence of infection (Venkatraman and Pendergast, 2002). The ingestion of CHO compared with PLA in drinks consumed during exercise appears to better maintain plasma glucose concentration, improve endurance exercise performance, and attenuate the elevation of plasma stress hormones and perturbation of circulating counts of total leukocytes and leukocyte subsets (Gleeson *et al.*, 2001b).

Price *et al.* (1994) reported that the recovery of glycogen after glycogen depleting exercise is biphasic: an initial rapid phase (insulin-independent) of glycogen synthesis (27 ± mmol·L^{-1}·h^{-1}, determined by ^{13}C-nuclear magnetic resonance spectroscopy) lasting 30 - 60 min is followed by a slower (insulin-dependent) phase (2.9 ± 0.8 mmol·L^{-1}·h^{-1}). If CHO is not provided during recovery, glucose availability will be the limiting factor for glycogen synthesis since gluconeogenesis would not be able support the maximal rate of glycogen synthesis (Satabin *et al.*, 1989). In the present study subjects were provided with 2.2 g CHO·kg^{-1} body mass during the first hour of the recovery period, which was estimated to

be equivalent to the amount of CHO oxidised during the first exercise bout and is comparable with the recommendation of Ivy *et al.* (1988), who suggested that the optimal strategy to restore glycogen is to consume ~1.5 g CHO·kg^{-1} body mass immediately after exercise and similar amounts at 2 h intervals thereafter.

Therefore, the first aim of the present study was to examine the effect of CHO supplementation during the recovery interval on leukocyte mobilisation, neutrophil degranulation response to LPS, plasma IL-6, stress hormones, saliva flow rate and sIgA responses to a 3-h recovery interval and a subsequent second bout of prolonged exercise. The second aim was to compare the magnitude of the immunoendocrine response between the first and the second exercise bout. We hypothesised that CHO ingestion may be beneficial to blunt the perturbation of immunoendocrine responses during the recovery interval and the subsequent exercise bout and that the second exercise bout would evoke greater immunoendocrine responses compared with the first bout.

6.2 Methods

Subjects

Eight male volunteers (age 29.8 ± 1.6 years, height 175 ± 2 cm, body mass 75.4 ± 2.6 kg, $\dot{V}O_{2\,max}$ 45.5 ± 2.2 $mL \cdot kg^{-1} \cdot min^{-1}$; means ± S.E.M.), who were recreationally active and familiar with cycling, participated in the study. After receiving written information about this study and passing a Health Questionnaire screen, subjects gave their written informed consent. Subjects were requested to complete the dietary record sheet on the day prior to Trial 1 and then repeated it again before Trial 2. Subjects were also asked not to perform any strenuous exercise or consume alcohol or medication for 2 days before each trial. The protocol was approved by the Ethics Committee of Loughborough University before the study began.

Experimental Procedures

The subject's workload was determined by a preliminary maximal oxygen uptake testing procedure as described in Chapter 3.2. The subjects completed two trials in a counterbalanced order, each separated by at least 4 days. Subjects arrived at the laboratory at 08:30 after fasting from 23:00 the previous day and were asked to empty the bladder before body mass was recorded. Subjects then performed two bouts of 90 min cycling (EX1 started at 09:00 and EX2 started at 13:30) at 60% $\dot{V}O_{2\,max}$ at 70 $rev \cdot min^{-1}$ on the same ergometer used to determine $\dot{V}O_{2\,max}$. Subjects consumed a lemon flavoured 10% w/v CHO (glucose) beverage or artificially sweetened placebo (22 $mL \cdot kg^{-1}$ body mass) during the first hour of the recovery period (i.e. 10:30-11:30). The amount of CHO ingested (~165 g) was approximately equivalent to the amount of CHO oxidised during EX1 based on measurements of respiratory gas exchange. Heart rate was recorded continuously during exercise by radiotelemetry. Ratings of perceived exertion (RPE) were obtained at 15-min intervals. Venous blood samples were taken 5 min before exercise and immediately post-exercise and unstimulated whole saliva samples were collected at 10 min before exercise, 48-50 min and 88-90 min of exercise, and 1 h and 2h post-exercise for both trials. Water ingestion was allowed *ad libitum* during the exercise bouts except for 5 min before each saliva sampling. The laboratory temperature and relative humidity were 23.9 ± 0.1 °C and 31 ± 1%, respectively.

Analytical Methods

Methods of preliminary measurements and blood collection and analysis are presented in Chapter 3.

Statistical analysis

All results are presented as mean values and standard errors of the mean (± SEM). Data were checked for normality, homogeneity of variance and sphericity before statistical analysis, and where appropriate the Huynh-Feldt method was applied for adjustment of degrees of freedom for the *F*-tests. Data were analysed using a two-factor (trial × time) repeated measures ANOVA with *post hoc* Tukey tests and paired *t*-tests, where appropriate. For the blood variables the time points used in the ANOVA were post-EX1, pre-EX2 and post-EX2 since the intervention (CHO or PLA) occurred after post-EX1. *P* and adjusted *F* values are presented and statistical significance was accepted at $P < 0.05$.

6.3 Results

Physiological Variables and RPE

There were significant main effects of time for $\%\dot{V}O_{2\,max}$ ($F_{1,7} = 11.7$, $P = 0.013$) and RPE ($F_{1,7} = 21.0$, $P = 0.003$) (Table 6.1).

Table 6.1 The exercise intensity and its effect on HR, RPE, body mass loss, water intake, and percentage change in plasma volume

	CHO		PLA	
	EX1	EX2	EX1	EX2
% VO_{2max}	59.6 (1.3)	62.0 (1.3)	60.2 (1.2)	63.1 (1.0)**
HR (beats·min^{-1}) [a]	140 (3)	146 (3)	142 (4)	148 (3)
RPE [a]	13.1 (0.4)	15.0 (0.5)*	13.4 (0.3)	15.1 (0.2)**
Body mass loss (kg) [b]	1.19 (0.15)	1.23 (0.19)	1.26 (0.18)	1.22 (0.13)
Water intake (mL)	686 (127)	833 (99)	800 (129)	808 (51)
Plasma volume change (%) [c]	- 4.0 (0.5)	- 3.0 (0.4)	- 3.9 (0.7)	- 3.3 (0.5)

Values are mean (±SEM, n = 8). Significantly different from EX1 (*$P < 0.05$, **$P < 0.01$). [a] Measurements made in last 15 min of exercise. [b] After correction for water intake. [c] Immediately post-EX compared with pre-EX.

Leukocyte counts

There was a significant main effect of time and an interaction between trial and time for the blood counts of leukocytes (time: $F_{2,14} = 63.6$, $P < 0.001$; interaction: $F_{2,14} = 6.0$, $P = 0.013$; Figure 6.1A) and neutrophils (time: $F_{2,14} = 26.3$, $P < 0.001$; interaction: $F_{2,14} = 6.6$, $P = 0.025$; Figure 6.1B). There was significant main effect of time for circulating lymphocytes ($F_{2,14} = 63.1$, $P < 0.001$, Figure 6.1C) and monocytes ($F_{2,14} = 36.2$, $P < 0.001$, Figure 6.1D)

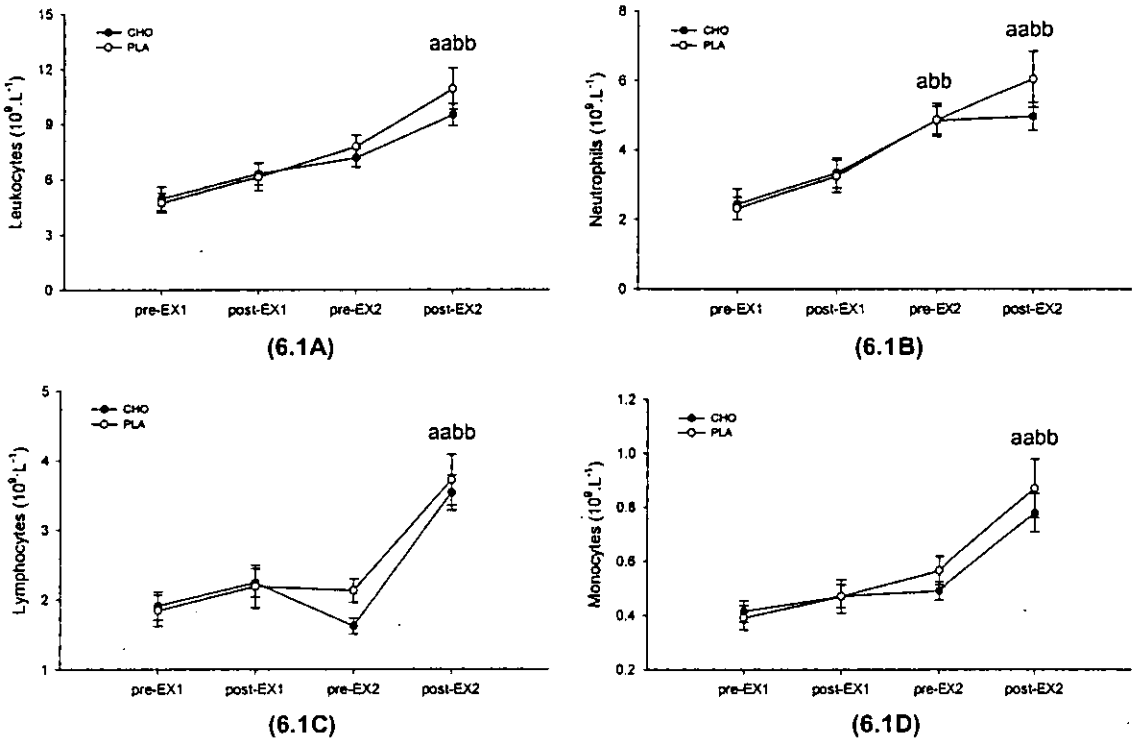


Figure 6.1 Changes in circulating counts of total leukocytes (6.1A), neutrophils (6.1B), lymphocytes (6.1C), and monocytes (6.1D). Values are means ± SEM (n = 8). Significantly different from post-EX1 in CHO ($^{a}P < 0.05$, $^{aa}P < 0.01$) and PLA ($^{bb}P < 0.01$).

Stress Hormones

There was a significant main effect of time for plasma concentrations of adrenaline ($F_{1,7} = 32.8$, $P = 0.001$, Figure 6.2A) and GH ($F_{2,14} = 12.1$, $P = 0.003$, Figure 6.2D). There were significant main effect of trial ($F_{1,7} = 6.1$, $P = 0.043$) and time ($F_{2,14} = 13.1$, $P = 0.008$) and an interaction between trial and time ($F_{2,14} = 7.0$, $P = 0.030$) for plasma ACTH concentration (Figure 6.2B). For plasma cortisol concentration (Figure 6.2 C), there was a significant main effect of time ($F_{2,14} = 32.8$, $P < 0.001$) and an interaction between trial and time ($F_{2,14} = 3.9$, $P = 0.044$).

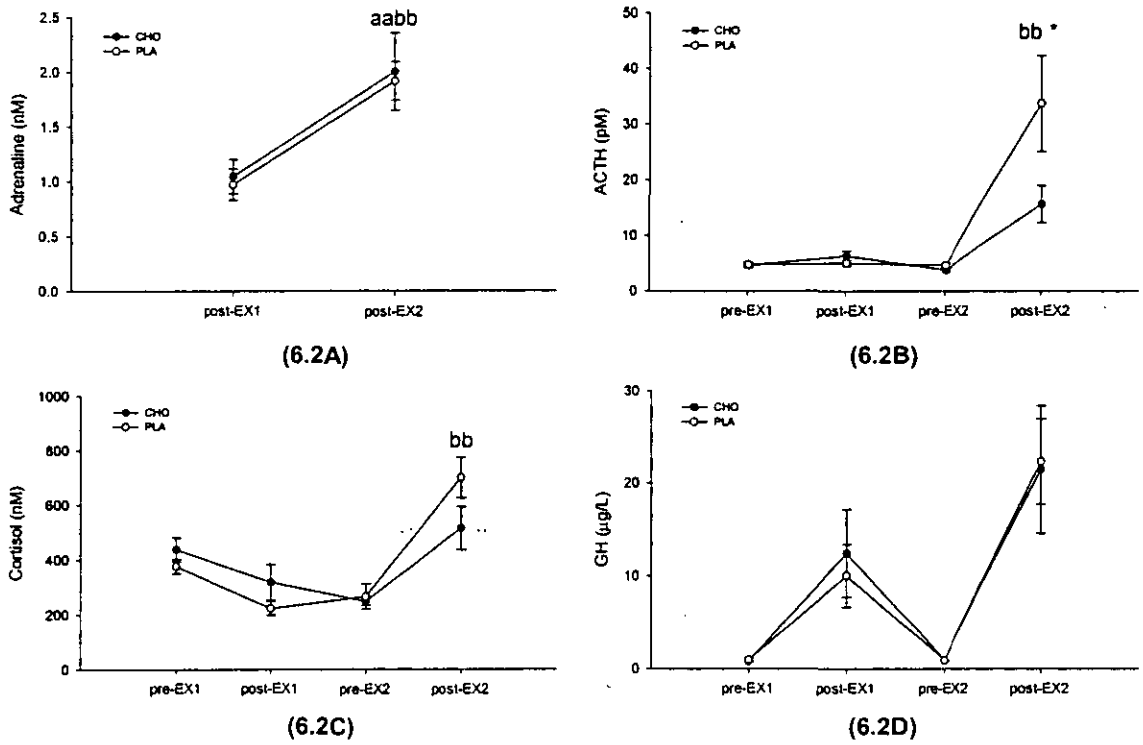


Figure 6.2 Changes in plasma concentrations of adrenaline (6.2A), ACTH (6.2B), cortisol (6.2C), and growth hormone (6.2D). Values are means ± SEM (n = 8). Significantly different from post-EX1 in CHO (^{aa} $P < 0.01$) and PLA (^{bb} $P < 0.01$), significantly different between trials (* $P < 0.05$).

Glucose and IL-6

There was a significant main effect of time for concentrations of plasma glucose ($F_{2, 14} = 21.7$, $P < 0.001$, Figure 6.3A) and IL-6 ($F_{2, 14} = 11.9$, $P = 0.001$, Figure 6.3B).

Neutrophil Degranulation

There was a significant main effect of time ($F_{2, 14} = 12.1$, $P = 0.001$) and an interaction between trial and time ($F_{2, 14} = 9.0$, $P = 0.003$) for total LPS-stimulated elastase release, which was higher at post-EX2 in PLA compared with post-EX1 (Figure 6.4A). There was no main effect and interaction in LPS-stimulated elastase release on a per neutrophil basis (Figure 6.4B).

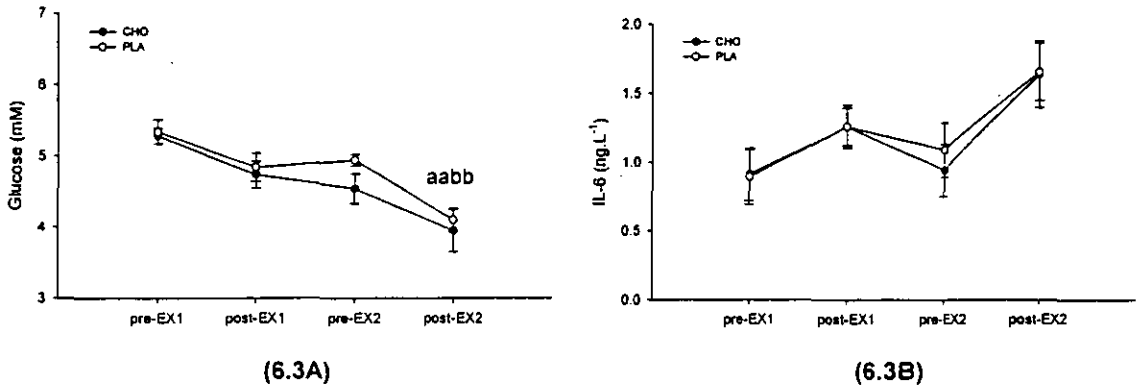


Figure 6.3 Changes in plasma concentrations of glucose (6.3A) and plasma IL-6 (6.3B). Values are means ± SEM (n = 8). Significantly different from post-EX1 in CHO ($^{aa}P < 0.01$) and PLA ($^{bb}P < 0.01$).

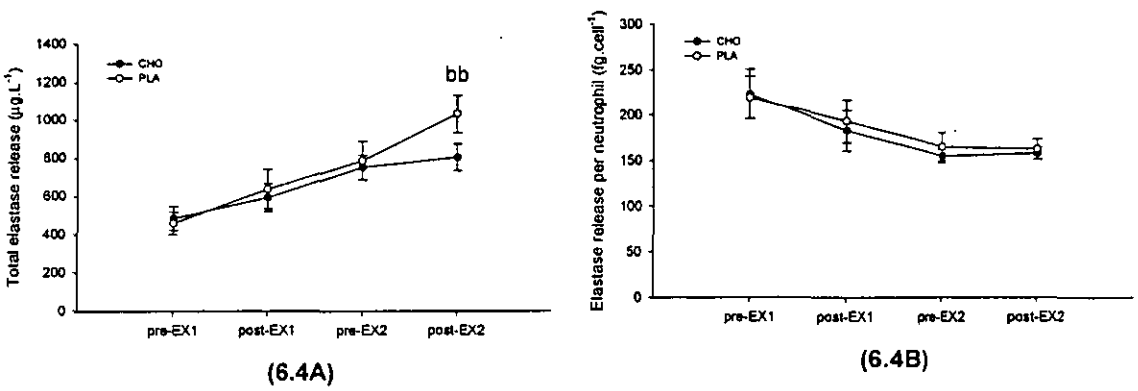


Figure 6.4 Changes in total LPS-stimulated elastase release (6.4A) and LPS-stimulated elastase release per neutrophil (6.4B). Values are means ± SEM (n = 8). Significantly different from post-EX1 in PLA ($^{bb}P < 0.01$).

Saliva Flow Rate

A significant main effect of time was observed for saliva flow rate ($F_{9, 72} = 7.8$, $P < 0.001$), which was decreased at post-EX2 and returned to the values of pre-EX within 1 h after exercise (Figure 6.5).

Figure 6.5 Changes in saliva flow rate. Values are means ± SEM (n = 8). Significantly different from pre-EX1 in the CHO ($^{a}P < 0.05$) and PLA ($^{b}P < 0.05$) trials.

Salivary IgA Concentration and Secretion Rate

There was a significant main effect of time for sIgA concentration ($F_{9,72} = 9.6$, $P < 0.001$), with an increase with exercise followed by a return to pre-EX levels within 2 h (Figure 6.6). However, there was no main effect and interaction for sIgA secretion rate (Figure 6.7)

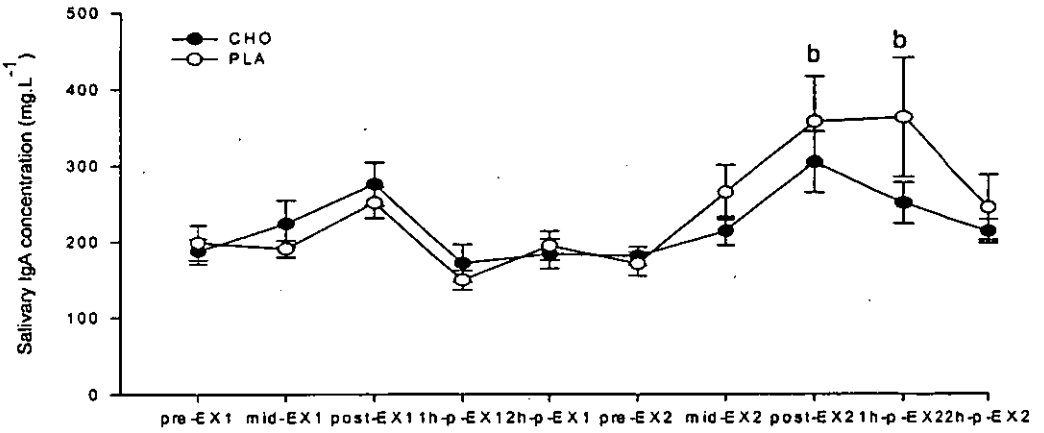


Figure 6.6 Changes in sIgA concentration. Values are means ± SEM (n = 8). Significantly different from pre-EX1 in PLA ($^{b}P < 0.05$) trial.

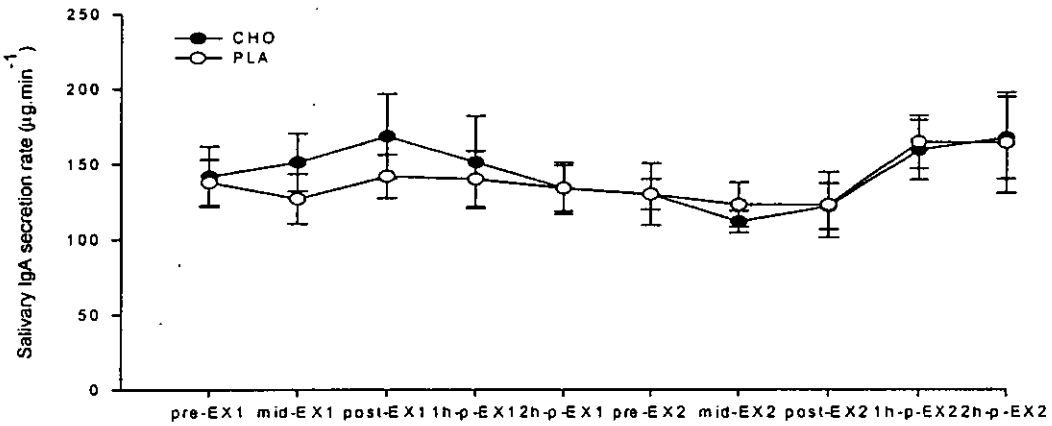


Figure 6.7 Changes in sIgA secretion rate. Values are means ± SEM (n = 8).

6.4 Discussion

The main findings of the present study were that ingestion of CHO compared with PLA during the recovery interval did not affect immunoendocrine responses to a 3-h recovery interval and a subsequent bout of prolonged exercise. Compared with EX1, EX2 evoked significantly higher circulating numbers of leukocytes, neutrophils, lymphocytes and monocytes, higher plasma adrenaline concentration and lower plasma glucose concentration.

CHO supplementation during the first hour of the recovery interval was intended to replace previously depleted hepatic and muscle glycogen stores to enhance the CHO availability during the subsequent 90 min bout of cycling. Although CHO ingestion did not attenuate the decrease of plasma glucose concentration compared with PLA in this study, no hypoglycaemia was found throughout the experimental protocol in either trial. Furthermore, these were no significant differences in plasma concentrations of adrenaline, cortisol and GH between trials. This suggests that endogenous CHO availability was still sufficient in PLA trial throughout two bouts of 90 min cycling at 60% $\dot{V}O_{2\,max}$ and did not further activate the sympathetic nervous system (SNS) and the hypothalamic-pituitary-adrenal (HPA) axis compared with CHO trial.

Circulating counts of total leukocytes, neutrophils, lymphocytes and monocytes were significant increased at post-EX2 compared with post-EX1; however, there were no differences between CHO and PLA trials. The result may be mainly attributable to the higher plasma stress hormone responses during EX2 since previous studies have consistently demonstrated that adrenaline and cortisol mobilise leukocytes into the circulation (Cupps and Fauci, 1982, Dhabhar *et al.*, 1994, Benschop *et al.*, 1996). This observation agrees with the findings of our recent study (Chapter 5) and other previous studies (McCarthy *et al.*, 1992, Ronsen *et al.*, 2001a, 2001b), which showed that the second exercise bout on the same day evokes more pronounced changes in leukocyte subsets and stress hormones compared with the first bout of identical exercise on the same day.

In the present study, we did not find a significant difference in plasma IL-6 levels neither throughout experimental protocol nor between CHO and PLA trials. However, this is not surprising because IL-6 is not produced in large amounts by contracting muscle until glycogen is nearly depleted (Gleeson and Bishop, 2000b, Febbraio and Pedersen, 2002). The low IL-6 concentrations (only about 1.65 $ng \cdot L^{-1}$) at post-EX2 may indicate that subjects did not deplete their muscle glycogen after two bouts of 90 min cycling at 60% $\dot{V}O_{2\,max}$. Although IL-6 has been suggested to maintain glucose homeostasis and stimulate lipolysis during exercise (Gleeson, 2000a), the relatively low plasma concentrations of IL-6 in this study may not exert significant metabolic effects because the criterion level for initiating acute metabolic responses may be as high as 25-65 $ng \cdot L^{-1}$ (Tsigos *et al.*, 1997).

There was no significant difference between trials in total LPS-stimulated elastase release and on a per neutrophil basis. LPS-stimulated elastase release per neutrophil was not significantly decreased until 3 h after EX1 in both trials and there was no further decline during EX2 and no difference between CHO and PLA throughout the experimental protocol. This finding was similar to previous studies of single bouts of prolonged exercise, which have reported a reduced response of neutrophil degranulation to LPS stimulation in vitro on per cell basis (Robson *et al.*, 1999, Walsh *et al.*, 2000a) and found that CHO ingestion did not blunt the decline of LPS-stimulated elastase release per neutrophil during exercise (Bishop *et al.*, 2001, Bishop *et al.*, 2002, Lancaster *et al.*, 2003). The delayed decline of LPS-stimulated elastase release per neutrophil may be due to the influence of plasma cortisol because cortisol is known to induce delayed neutrophilia with less mature neutrophils entering the circulation from the bone marrow (Pyne, 1994). We observed significantly higher counts of circulating neutrophils at pre-EX2 compared with post-EX1, which coincides with the decreased neutrophil degranulation responses to LPS.

Saliva flow rate was decreased at post-EX2 in this study. Since saliva is formed from plasma, a steady blood flow to the salivary glands is required for maintenance of adequate salivation (Smaje, 1998). In the present study, the lower saliva flow rates coincided with the higher plasma adrenaline concentrations, suggesting that the decline of saliva flow rate during exercise may be due to the influence of SNS activity. Noradrenaline is the primary

neurotransmitter of the SNS and may exert its actions via α- and β- adrenergic receptors. Anderson and Garrett (1998) demonstrated that α-adrenergic receptor activation causes vasoconstriction, whereas the β-adrenergic activity induces vasodilation in the submandibular gland of the rat. Recent studies reported that the α_1-adrenergic blocker doxazosin and the β-adrenergic blocker propanolol did not influence saliva flow rate during submaximal cycling at 50W for 8 min (Winzer *et al.*, 1999, Ring *et al.*, 2000). A very recent study reported that infusion of the α_2-adrenoceptor agonist dexmedetomidine caused vasoconstriction in men (Talke *et al.*, 2003). Accordingly, this suggests that the exercise-induced decline in the saliva flow rate may be mainly mediated by the SNS through α_2-adrenergic receptors: effectively, the vasoconstriction limits water supply to salivary glands. Furthermore, Talke *et al.* (2003) reported that the effect of dexmedetomidine on vasoconstriction was dose-dependent with a threshold of 0.15 $pg \cdot L^{-1}$. Therefore, we speculate there may be a threshold level of SNS activity to constrict salivary glandular vessels. However, this assumption needs to be confirmed by further studies.

Regarding the sIgA concentration during exercise, the alterations seemed to mainly result from the reduction of saliva flow rate since no change in sIgA secretion rate was observed. *In vitro*, sIgA is secreted by both acinar and ductal units under the stimulation of α- and β-adrenoceptors and peptidergic receptor. Saliva IgA secretion rate is relatively constant for each agonist across a range of doses (Proctor and Carpenter, 2002). The stimulation of β-adrenoreceptors increased IgA secretion in a dose-independent manner above a certain threshold; however, prolonged β-adrenergic stimulation appeared to reduce the replenishment of IgA into the glandular pool (Proctor *et al.*, 2003). Ring *et al.* (2000) suggested that the acute decrease in sIgA secretion rate during exercise was mediated by α_1-adrenergic mechanisms. Therefore, the previous inconsistencies in responses of sIgA secretion rate to exercise (Blannin *et al.*, 1998, Bishop *et al.*, 1999a, Nieman *et al.*, 2002, Walsh *et al.*, 2002) may be attributable to the interaction between different types of stimulation and their receptors during exercise. For example, when the α_1-adrenergic stimulation is stronger than other types, such as β-adrenergic activity, and is above a certain threshold, sIgA output may be decreased. Conversely, when the β-adrenergic stimulation is stronger than α_1-adrenergic stimulation, sIgA output increases. Cortisol has also been suggested to inhibit sIgA mobilisation (Hucklebridge *et al.*, 1998). However in

this study, we did not find any significant differences in either plasma cortisol concntration or sIgA secretion rate between CHO and PLA. This suggests that two bouts of 90 min cycling at 60% $\dot{V}O_{2\,max}$ separated by 3 h recovery does not evoke sufficient activation of the SNS or HPA-axis to modify sIgA transcytosis.

In conclusion, the findings of the present study suggest that CHO ingestion during the recovery interval between two bouts of prolonged exercise does not attenuate immunoendocrine responses to a 3-h recovery interval and a subsequent bout of 90 min cycling at 60% $\dot{V}O_{2\,max}$. A second prolonged exercise bout causes a greater disturbance in immunoendocrine responses than the first exercise bout on the same day and this is not substantially influenced by CHO supplementation during the recovery interval.

CHAPTER SEVEN

Effects of carbohydrate supplementation during the first of two prolonged cycling bouts on immunoendocrine responses

Summary

The purpose of this study was to examine the effect of CHO feeding during the first of two 90-min cycling bouts (EX1 started at 09:00 and EX2 started at 13:30) at 60% $\dot{V}O_{2\,max}$ on leukocyte redistribution, neutrophil degranulation and oxidative burst, plasma IL-6, plasma stress hormone, saliva flow rate and sIgA responses. This study consisted of two trials, which were completed in a counterbalanced order and separated by at least 4 days. Subjects (n = 9) consumed a lemon flavoured 10% w/v CHO (glucose) or PLA beverage during EX1: 500 mL just before exercise and 250 mL every 20 min during exercise. Venous blood samples were taken 5 min before exercise and immediately post-exercise for both trials and unstimulated whole saliva samples were collected at 10 min before exercise, 48-50 min and 88-90 min of exercise, and 1 h and 2h post-exercise for both trials. The main findings of the present study were that ingestion of CHO compared with PLA during EX1 1) maintained higher plasma glucose concentration throughout the experimental protocol; 2) blunted the responses of plasma adrenaline, ACTH and cortisol during EX2; 3) attenuated circulating leukocytosis and monocytosis throughout the experimental protocol, neutrophilia during the recovery interval, and lymphocytosis during EX2; 4) lessened the decline in LPS-stimulated degranulation and PMA-induced oxidative burst on per neutrophil basis from 3 h post-EX1 onwards; but 5) did not affect changes in plasma IL-6, saliva flow rate and sIgA responses. These findings suggest that ingestion of CHO compared with PLA during the first exercise bout 1) increases CHO availability during both bouts of exercise; 2) has a limited effect on immunoendocrine response during the first exercise bout but attenuates plasma stress hormone responses during the second exercise bout; 3) blunts the delayed neutrophilia and concurrent decline in LPS-stimulated degranulation and PMA-induced oxidative burst on per neutrophil basis after the first bout of prolonged cycling; and 4) however, does not affect oral immunity.

7.1 Introduction

Acute exercise alters immune cell function and modifies leukocyte trafficking between the circulation and tissue compartments and these effects may last for several hours after exercise (Gleeson and Bishop, 1999). During this period, immunity is likely depressed and may open a window to invading pathogens, increasing opportunistic infections in stressed athletes (Pedersen, 1999). This temporarily reversible alteration in immune function is related to elevated plasma concentrations of stress hormones (Benschop *et al.*, 1996).

Nutritional strategies are often used to manipulate exercise-induced immunoendocrine responses. CHO supplementation during exercise is one of the most successful means to attenuate immunoendocrine responses during prolonged exercise (Gleeson *et al.*, 2001b). Ingestion of CHO compared with PLA better maintains plasma glucose concentration, blunts HPA activation (Mitchell *et al.*, 1990) and minimises immunological perturbation to an acute single bout of non-fatiguing, fixed duration exercise (Gleeson and Bishop, 2000b). The most effective and common method applied to increase carbohydrate availability is to ingest CHO-rich drinks during prolonged exercise (Jeukendrup and Jentjens, 2000).

Training programmes of endurance athletes usually involve several bouts of intensive exercise in a day. Therefore maintenance of immune competence during repeated exercise bouts is of crucial importance to prevent athletes from immunodepression and opportunistic pathogen invasion. A few studies have examined the effect of two exercise bouts on the same day on immunoendocrine responses and have shown that the second exercise bout induced a greater hormonal response and a larger leukocyte mobilisation compared with a single identical exercise bout (Ronsen *et al.*, 2001a, 2001b). However, the limited information available is insufficient to fully evaluate the effect of repeated bouts of exercise on immune cell functions and warrants further investigation.

For various considerations, many athletes wake up in early morning and train without breakfast. However, no study has examined the effect of CHO ingestion during the first of two prolonged exercise bouts on immunoendocrine responses. Hence, the aims of the present study were to compare the effect of CHO supplementation during the first of two

prolonged cycling bouts on responses of leukocyte redistribution, LPS-stimulated degranulation and PMA-induced oxidative burst by neutrophils, plasma stress hormones, IL-6, saliva flow rate, and sIgA concentration and secretion rate. We hypothesised that ingestion of CHO compared with PLA during the first exercise bout would be beneficial to attenuate the perturbation of immunoendocrine responses throughout both during the first bout and the subsequent recovery period and second bout of exercise.

7.2 Methods

Subjects

Nine male volunteers (age 29.7 ± 1.6 years, height 177 ± 2 cm, body mass 72.0 ± 1.6 kg, $\dot{V}O_{2\,max}$ 49.4 ± 2.0 mL·kg^{-1}·min^{-1}; means ± S.E.M.), who were recreationally active and familiar with cycling, participated in the study. After receiving written information and passing a Health Questionnaire screen, subjects gave their written informed consent. Subjects were requested to complete the dietary record sheet the day prior to Trial 1 and then repeated it again before Trial 2. Subjects were also asked not to perform any strenuous exercise or consume alcohol or medication for 2 days before each trial. The protocol was approved by the Ethics Committee of Loughborough University before the study began.

Experimental Procedures

Subject's workload was determined by a preliminary maximal oxygen uptake testing procedure as described in Chapter 3.2. The subjects completed two trials in a counterbalanced order, each separated by at least 4 days. Subjects arrived at the laboratory at 08:30 after fasting from 23:00 the previous day and were asked to empty the bladder before body mass was recorded. Subjects then performed two bouts of 90 min cycling (EX1 started at 09:00 and EX2 started at 13:30) at 60% $\dot{V}O_{2\,max}$ at 70 rev·min^{-1} on the same ergometer used to determine $\dot{V}O_{2\,max}$. Subjects were given a lemon flavoured 10% w/v CHO (glucose) or artificially sweetened placebo beverage during the first exercise bout: 500 mL just before exercise and 250 mL every 20 min during exercise. Subjects were asked to consume each drink within 3 min. Heart rate was recorded continuously during exercise by radiotelemetry. Ratings of perceived exertion (RPE) were obtained at 15-min intervals. Venous blood samples were taken 5 min before exercise and immediately post-exercise and unstimulated whole saliva samples were collected at 10 min before exercise, 48-50 min and 88-90 min of exercise, and 1 h and 2h post-exercise for both trials. Water ingestion was allowed *ad libitum* during recovery interval and the second exercise bout except for 5 min before each saliva sampling. The laboratory temperature and relative humidity were 26.3 ± 0.2 °C and 39 ± 1%, respectively.

Analytical Methods

Methods of preliminary measurements and blood collection and analysis are presented in Chapter 3.

Statistical analysis

All results are presented as mean values and standard errors of the mean (± SEM). Data were checked for normality, homogeneity of variance and sphericity before statistical analysis, and where appropriate the Huynh-Feldt method was applied for adjustment of degrees of freedom for the *F*-tests. Data were analysed using a two-factor (trial × time) repeated measures ANOVA with *post hoc* Tukey and paired *t*-tests, where appropriate. *P* and adjusted *F* values are presented and statistical significance was accepted at $P < 0.05$.

7.3 Results

Physiological Variables and RPE

There were significant main effects of time and interactions between trial and time for HR (time: $F_{1,8} = 9.4$, $P = 0.015$ and interaction: $F_{1,8} = 28.0$, $P = 0.001$) and percentage change in plasma volume (time: $F_{1,8} = 16.1$, $P = 0.004$ and interaction: $F_{1,8} = 7.1$, $P = 0.029$). For RPE there were main effects of trial ($F_{1,8} = 8.9$, $P = 0.018$) and time ($F_{1,8} = 42.5$, $P < 0.001$) (Table 7.1).

Table 7.1 The exercise intensity and its effect on HR, RPE, body mass loss, water intake, and percentage change in plasma volume

	CHO		PLA	
	EX1	EX2	EX1	EX2
% VO_{2max}	60.7 (1.8)	59.9 (1.0)	60.9 (1.5)	59.7 (1.4)
HR (beats·min^{-1}) [a]	146 (3)	148 (3)	140 (2)	149 (2)**
RPE [a]	13.1 (0.5)	15.2 (0.4)**	13.4 (0.2)	16.8 (0.5)**¶¶
Body mass loss (kg) [b]	1.32 (0.09)	1.13 (0.09)*	1.43 (0.07)	1.24 (0.14)
Water intake (mL)	CHO	768 (108)**	PLA	854 (129)**
Plasma volume change (%) [c]	-7.5 (1.0)	-2.5 (0.4)**	-5.2 (0.5)	-3.7 (0.6)

Values are mean (±SEM, n = 9). Significantly different from EX1 (*$P < 0.05$, **$P < 0.01$) in same trial; significantly different from the same time point in CHO trial (¶¶ $P < 0.01$). [a] Measurements made in last 15 min of exercise. [b] After correction for water intake. [c] Immediately post-EX compared with pre-EX.

Leukocyte counts

There were significant main effects of trial ($F_{1,8} = 26.7$, $P = 0.001$) and time ($F_{3,24} = 48.7$, $P < 0.001$) for the circulating numbers of leukocytes (Figure 7.1A), with higher values in PLA than CHO and higher values at pre-EX2 and post-EX2 compared with pre-EX1. There were significant main effects of trial ($F_{1,8} = 20.8$, $P = 0.002$) and time ($F_{3,24} = 31.4$, $P < 0.001$) and an interaction of trial and time ($F_{3,24} = 4.0$, $P = 0.033$) for blood

neutrophil counts (Figure 7.1B), with a higher value at pre-EX2 in PLA than CHO and higher values at pre-EX1 and post-EX2 than pre-EX1. There were significant main effects of trial ($F_{1,8} = 30.0$, $P = 0.001$) and time ($F_{3,24} = 31.7$, $P < 0.001$) and an interaction of trial and time ($F_{3,24} = 8.5$, $P = 0.001$) for blood lymphocyte counts (Figure 7.1C), with a higher value at post-EX2 compared with pre-EX1. Furthermore, the value at post-EX2 in PLA was higher than in CHO. For blood monocyte counts (Figure 7.1D), there were also significant main effects of trial ($F_{1,8} = 12.1$, $P = 0.008$) and time ($F_{3,24} = 44.1$, $P < 0.001$) and an interaction between trial and time ($F_{3,24} = 3.3$, $P = 0.037$), with higher values in PLA than CHO. The blood monocyte counts at pre-EX2 and post-EX2 were higher than pre-EX1 in both trials.

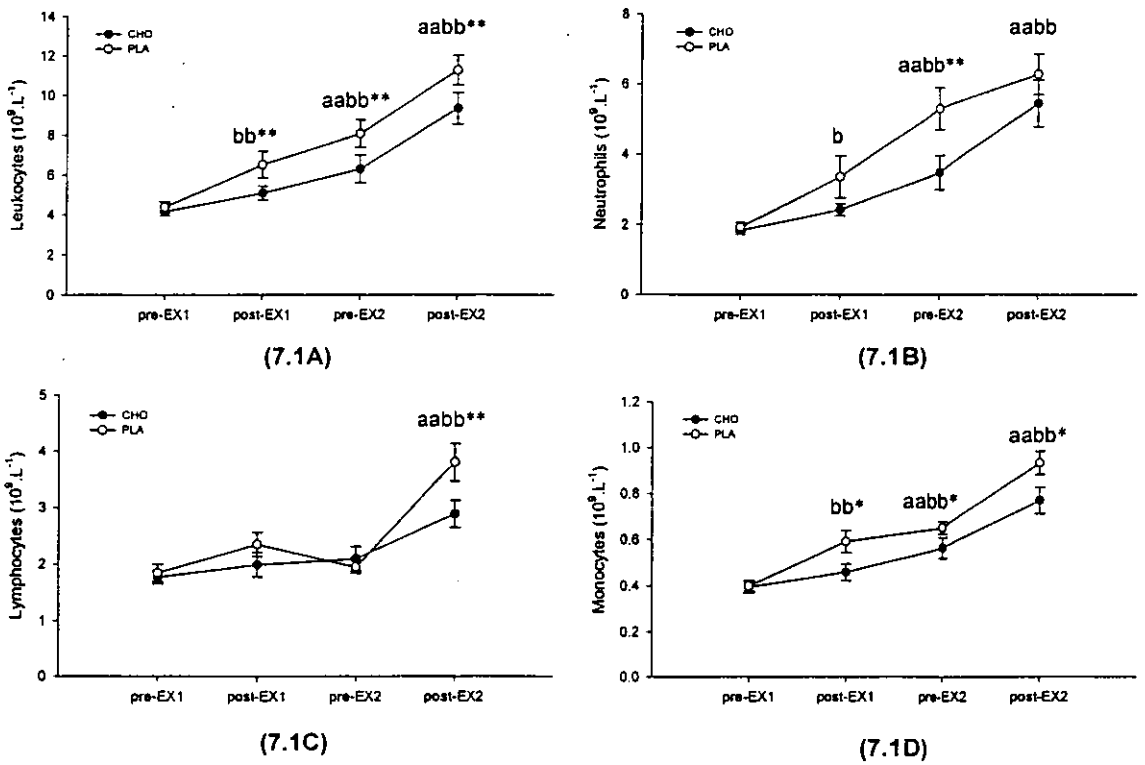


Figure 7.1 Changes in circulating counts of total leukocytes (7.1A), neutrophils (7.1B), lymphocytes (7.1C), and monocytes (7.1D). Values are means ± SEM (n = 9). Significantly different from pre-EX1 in CHO ($^{aa}P < 0.01$) and PLA ($^{bb}P < 0.01$); significantly different between trials ($^{*}P < 0.05$, $^{**}P < 0.01$).

Stress Hormones

There were significant main effects of trial ($F_{1,8} = 7.6$, $P = 0.025$) and time ($F_{2,16} = 20.6$, $P = 0.001$) and an interaction of trial and time ($F_{2,16} = 7.7$, $P = 0.009$) for plasma adrenaline (Figure 7.2A), which showed a higher value at post-EX2 compared with pre-EX1 in both trials and the value at post-EX2 in PLA was higher than in CHO. For plasma ACTH (Figure 7.2B) and cortisol (Figure 7.2C), there were significant main effects of trial ($F_{1,8} = 13.7$, $P = 0.006$ and $F_{1,8} = 10.1$, $P = 0.013$) and time ($F_{3,24} = 25.9$, $P < 0.001$ and $F_{3,24} = 28.9$, $P < 0.001$) and an interaction of trial and time ($F_{3,24} = 16.5$, $P = 0.001$ and $F_{3,24} = 7.3$, $P = 0.002$), with higher values at post-EX2 in PLA compared with pre-EX1 and post-EX2 in CHO. There were significant main effects of trial ($F_{1,8} = 9.8$, $P = 0.014$) and time ($F_{3,24} = 14.7$, $P = 0.002$) and an interaction of trial and time ($F_{3,24} = 5.4$, $P = 0.009$) for plasma GH (Figure 7.2D), with higher values at post-EX2 compared with pre-EX1 in both trials and the value at post-EX1 in PLA was higher than in CHO.

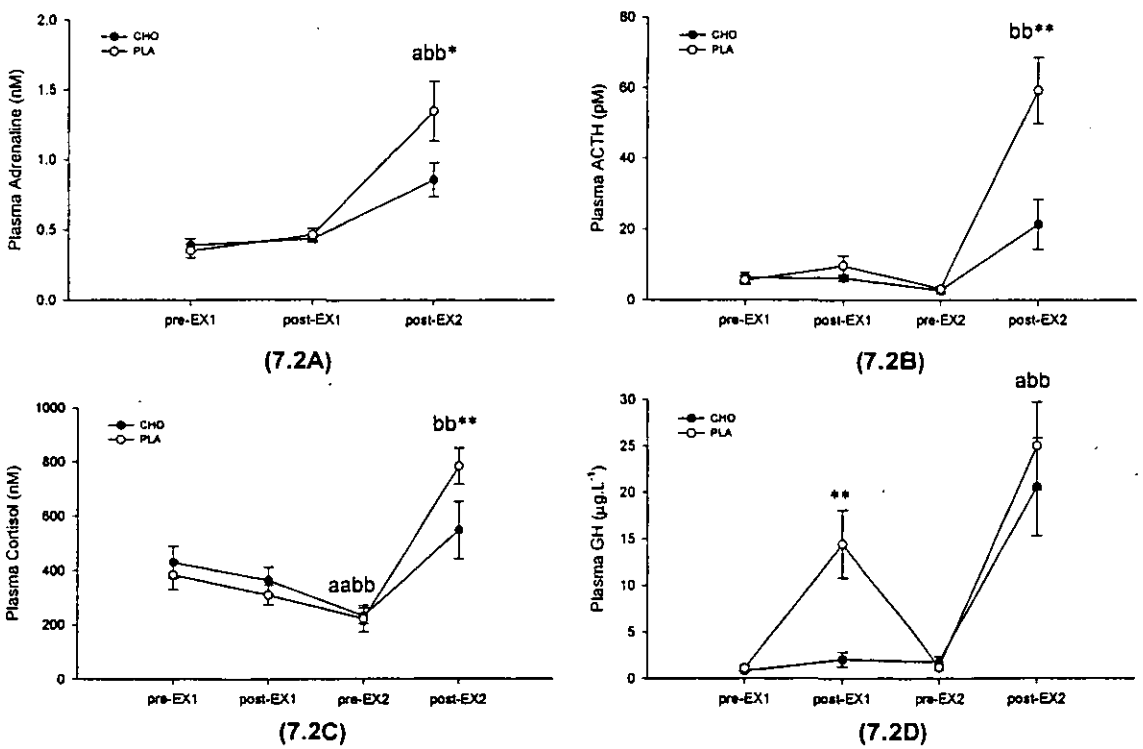


Figure 7.2 Changes in plasma concentrations of adrenaline (7.2A), ACTH (7.2B), cortisol (7.2C), and growth hormone (7.2D). Values are means ± SEM (n = 9). Significantly different from pre-EX1 in CHO ($^{a}P < 0.05$, $^{aa}P < 0.01$) and PLA ($^{bb}P < 0.01$); significantly different between trials ($^{*}P < 0.05$, $^{**}P < 0.01$).

Glucose and IL-6

There were significant main effects of trial ($F_{1, 8} = 23.8$, $P = 0.001$) and time ($F_{3, 24} = 59.7$, $P < 0.001$) and an interaction between trial and time ($F_{3, 24} = 35.4$, $P < 0.001$) for plasma glucose concentration (Figure 7.3A), which showed carbohydrate feeding during EX1 significantly increased plasma glucose concentration compared with pre-EX1. However, the plasma glucose concentration was lower at pre-EX2 and post-EX2 than pre-EX1 in CHO. In PLA, the plasma glucose concentration was significantly decreased after EX1 and remained low throughout the experimental protocol. Furthermore, the plasma glucose concentrations at post-EX in CHO were significantly higher compared with the same time points in PLA. Plasma IL-6 concentrations at post-EX were significantly higher than pre-EX (main effect of time: $F_{3, 24} = 18.7$, $P < 0.001$, Figure 7.3B).

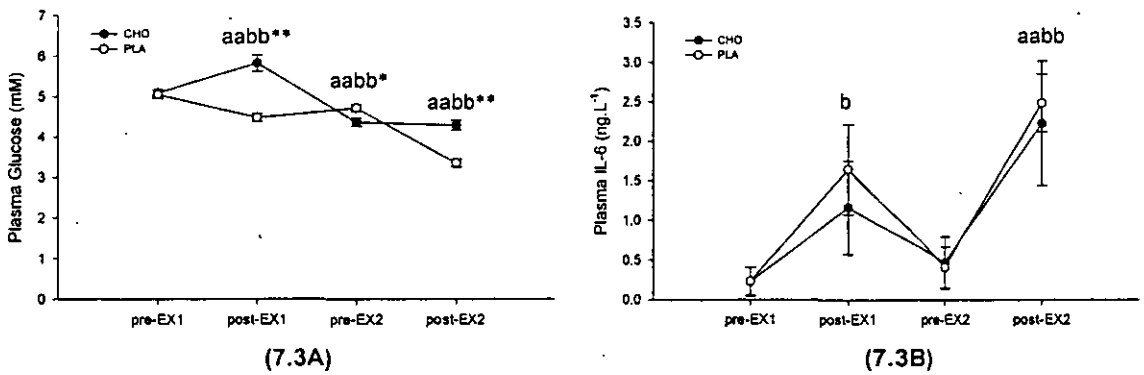


Figure 7.3 Changes in plasma concentrations of glucose (7.3A) and plasma IL-6 (7.3B). Values are means ± SEM (n = 9). Significantly different from pre-EX1 in CHO ($^{aa}P < 0.01$) and PLA ($^{b}P < 0.05$, $^{bb}P < 0.01$); significantly different between trials ($^{*}P < 0.05$, $^{**}P < 0.01$).

Neutrophil Degranulation

There was a significant main effect of time ($F_{3, 24} = 25.4$, $P < 0.001$) and an interaction between trial and time ($F_{3, 24} = 4.1$, $P = 0.018$) for total LPS-stimulated elastase release, which was increased with exercise in both trials (Figure 7.4A). For LPS-stimulated elastase release per neutrophil, there were significant main effects of trial ($F_{1, 8} = 6.2$, $P = 0.038$) and time ($F_{3, 24} = 9.6$, $P = 0.002$), with higher values in CHO compared with PLA and lower values at post-EX2 than pre-EX1 (Figure 7.4B).

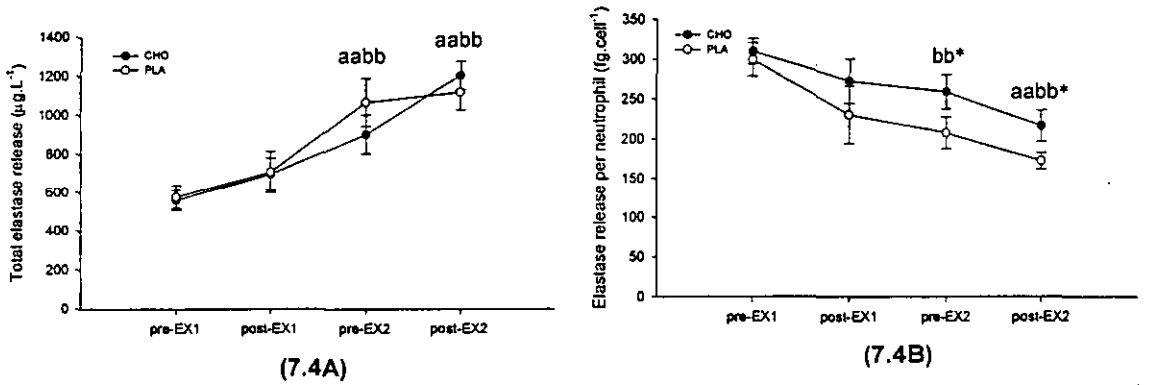


Figure 7.4 Changes in total LPS-stimulated elastase release (7.4A) and LPS-stimulated elastase release per neutrophil (7.4B). Values are means ± SEM (n = 9). Significantly different from pre-EX1 in CHO ($^{aa}P < 0.01$) and PLA ($^{bb}P < 0.01$); significantly different between trials ($^{*}P < 0.05$).

Neutrophil Oxidative Burst

A significant main effect of time was observed for total PMA-induced oxidative burst ($F_{3, 24} = 39.0$, $P < 0.001$, Figure 7.5A), with an increase with exercise. There were significant main effects of trial ($F_{1, 8} = 8.0$, $P = 0.022$) and time ($F_{3, 24} = 18.8$, $P < 0.001$) and an interaction between trial and time ($F_{3, 24} = 5.1$, $P = 0.007$) for PMA-induced oxidative burst per neutrophil (Figure 7.5B), which showed lower levels at pre-EX2 and post-EX2 in PLA compared with pre-EX1 and the same time points in CHO.

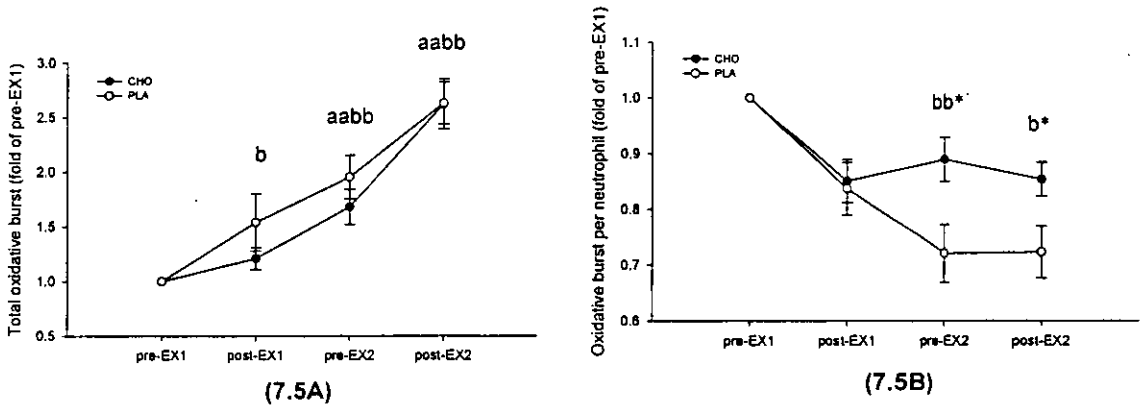


Figure 7.5 Changes in total PMA-induced oxidative burst (7.5A) and PMA-induced oxidative burst per neutrophil (7.5B). Values are means ± SEM (n = 9). Significantly different from pre-EX1 in CHO ($^{aa}P < 0.01$) and PLA ($^{b}P < 0.05$, $^{bb}P < 0.01$); significantly different between trials ($^{*}P < 0.05$).

Saliva Flow Rate

There was a significant main effect of time for saliva flow rate ($F_{9, 72} = 4.4$, $P = 0.013$), with a decrease at post-EX2 and a return within 1 h after exercise (Figure 7.6).

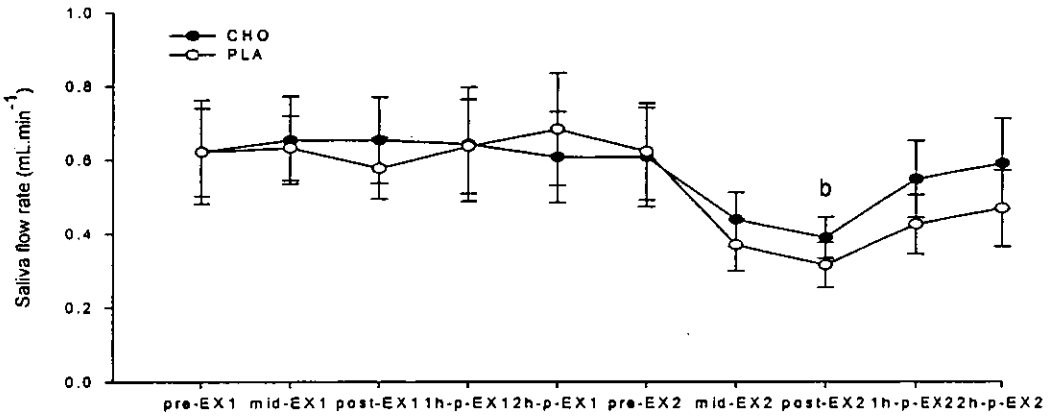


Figure 7.6 Changes in saliva flow rate. Values are means ± SEM (n = 9). [b] Significantly different from pre-EX1 in PLA ($P < 0.05$).

Salivary IgA Concentration and Secretion Rate

There was a significant main effect of time ($F_{9, 72} = 5.0$, $P < 0.001$) for sIgA concentration (Figure 7.7). However, there was no main effect and interaction for sIgA secretion rate (Figure 7.8).

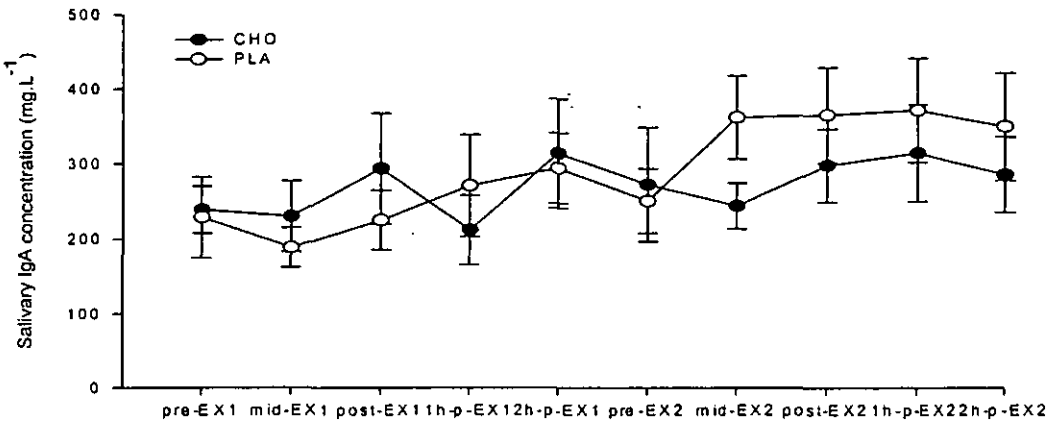


Figure 7.7 Changes in sIgA concentration. Values are means ± SEM (n = 9). [b] Significantly different from pre-EX1 in PLA ($P < 0.05$).

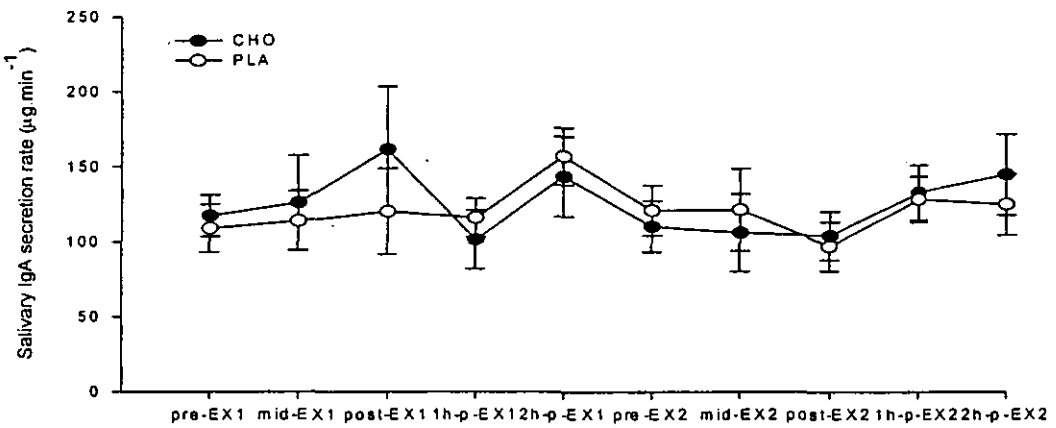


Figure 7.8 Changes in sIgA secretion rate. Values are means ± SEM (n = 9).

7.4 Discussion

The main findings of this study were that ingestion of CHO compared with PLA during EX1 1) maintained higher plasma glucose concentration throughout the experimental protocol; 2) blunted the responses of plasma adrenaline, ACTH and cortisol during EX2; 3) attenuated the leukocytosis and monocytosis throughout the experimental protocol, neutrophilia during the recovery interval, and lymphocytosis during EX2; 4) lessened the decline in LPS-stimulated degranulation and PMA-induced oxidative burst on per neutrophil basis from 3 h post-EX1 onwards and 5) did not affect plasma IL-6 levels and oral immunity.

In the present study, ingestion of CHO compared with PLA during EX1 significantly better maintained plasma glucose concentration during EX1 and EX2. Although the amount of glucose (150g) consumed in the present study was in excess of the upper limit (~1 $g \cdot min^{-1}$) for glucose absorption during exercise (Jeukendrup and Jentjens, 2000) and might not have been completely absorbed during EX1, it helped to maintain CHO availability during EX2 since a mild hypoglycaemia was only observed at post-EX2 in PLA. Plasma glucose concentration at this point was 3.3 ± 0.1 mM, reaching the threshold for evoking adrenaline (3.8 ± 0.1 mM), GH (3.7 ± 0.1 mM) and cortisol (3.2 ± 0.2 mM) secretions (Schwartz *et al.*, 1987). Therefore, it was not surprising to find higher plasma stress hormone responses in PLA compared with CHO during EX2.

Ingesting a CHO beverage compared with PLA in the present study attenuated the responses of plasma stress hormones and circulating leukocytes and subsets. These results were similar to previous studies, which indicated that CHO supplementation during prolonged exercise maintains euglycaemia and attenuates HPA activation, leading to a smaller perturbation of circulating leukocytes and subsets (Mitchell *et al.*, 1990, Nieman *et al.*, 1997, Bishop *et al.*, 1999b). Elevated plasma levels of catecholamines, glucocorticoids and GH during exercise are related to the redistribution (trafficking) of leukocytes (Cupps and Fauci, 1982, Kappel *et al.*, 1993, Benschop *et al.*, 1996) and alteration of neutrophil function (Liles *et al.*, 1995, Ruy *et al.*, 1997, Tintinger *et al.*, 2001). Plasma adrenaline is likely responsible for the recruitment of lymphocytes and neutrophils into the circulation during 90 min intensive exercise, whereas the later rise of plasma cortisol seems to

attenuate adrenaline-induced lymphocytosis and dominate the delayed neutrophilia and lymphopenia that develops in the first few hours after exercise cessation (Nieman, 1997). Furthermore, GH also appears to mobilise neutrophils into the circulation (Kappel *et al.*, 1993). The higher number of circulating monocytes after EX1 in PLA compared with CHO may be due to the effects of GH. Although there is no direct evidence to support this suggestion, we did not observe any differences between CHO and PLA in other possible candidates, such as plasma concentrations of adrenaline and cortisol or haemodynamic factors (heart rate). The higher blood counts of total leukocytes, neutrophils, lymphocytes and monocytes during EX2 and the differences between PLA and CHO are most likely attributable to the effect of elevated plasma concentrations of adrenaline and cortisol in the PLA trial.

In the present study, the responses of LPS-stimulated elastase release per neutrophil during EX1 were similar to previous studies, which reported that during moderate duration exercise (60-90 min) CHO ingestion did not affect neutrophil degranulation on per cell basis (Bishop *et al.*, 2002, Lancaster *et al.*, 2003). However, a significant decline in LPS-stimulated elastase release per neutrophil was found at pre-EX2 and post-EX2. A delayed blood neutrophilia induced by cortisol generally occurs after 90 min of exercise and lasts for a few hours after exercise cessation (Nieman, 1997). Nakagawa *et al.* (1998) reported that about 10% of the circulating neutrophilia was derived from the bone marrow after infusion of the synthetic glucocorticoid dexamethasone and that these neutrophils possessed a lower content of granular digestive enzymes compared with fully mature neutrophils (Pyne, 1994). Recent studies reported that the neutrophil degranulation response to LPS stimulation *in vitro* on per cell basis fell after 2 h cycling at 60% $\dot{V}O_{2\,max}$ (Walsh *et al.*, 2000a) and following cycling to fatigue (98 ± 7 min in the CHO trial) at 75% $\dot{V}O_{2\,max}$ (Bishop *et al.*, 2001). Accordingly, a possible explanation for why the decrease in LPS-stimulated elastase release was not observed until pre-EX2 in the present study is likely that there was an influx of relatively immature and less functionally competent neutrophils from the bone marrow during the recovery period after EX1 although a recent study suggested that release of less mature cells was not the reason for the fall in LPS-stimulated elastase release after a 2 h cycle at 70% $\dot{V}O_{2\,max}$ (Bishop *et al.*, 2003). Regarding the greater decrease in neutrophil degranulation response to LPS on per

cell basis in PLA compared with CHO, this may reflect a higher number of less mature neutrophils entering the circulation from the bone marrow under the influence of higher cortisol levels in PLA.

The negative effect of exercise on PMA-induced oxidative burst activity on per neutrophil basis was blunted by CHO ingestion in the present study. Neutrophil oxidative burst is activated through receptor-dependent mechanisms, such as fMLP (N-formyl-Met-Leu-Phe), which are short-lasting (typically less than 5 min), or receptor-independent mechanisms, such as PMA, which can last for a much longer period (reviewed by Chanock *et al.*, 1994, Meenan *et al.*, 2002). Suzuki *et al.* (1999) showed after 90 min cycling at ~53% $\dot{V}O_{2\,max}$ the PMA-induced CL response of isolated neutrophils was increased. However, a transient suppression of the oxidative burst after exercise has been also reported (Gabriel *et al.*, 1994, Pyne *et al.*, 1996). Pyne *et al.* (1996) showed that the PMA-induced CL response of isolated neutrophils declined 41% after 40 min running at a heart rate of 140 beat·min^{-1}. However, the CL values did not change further during the 1 h recovery interval or after a second identical bout of running. The results from the PLA trial in the present study are similar to a recent study (Morozov *et al.*, 2003), which reported that neutrophil oxidative burst activity (zymosan-induced CL) did not change until 3 h after exercise. Previous studies have shown that ingestion of CHO compared with PLA does not affect granulocyte oxidative burst activity (determined by flow cytometry) (Nieman *et al.*, 1997) or PMA-stimulated intracellular H_2O_2 production (Smith *et al.*, 1996) after a single exercise bout. However, the decrease of PMA-induced oxidative burst activity per neutrophil was blunted by CHO ingestion from 3 h after EX1 onwards in the present study.

Many factors are associated with the regulation of neutrophil oxidative activity. However, the most important factor during exercise may be the extent of neutrophil mobilisation. Berkow and Dodson (1986) reported that neutrophils in the bone marrow have lower NADPH-dependent oxidase activity and superoxide response to PMA stimulation compared to those in the circulation. Moreover, the nitro blue tetrazolium (NBT)-negative neutrophils in marginated pools produce less $O_2^{\cdot-}$ in response to *in vitro* stimulation (Suzuki *et al.*, 1996). Therefore, the greater decline of neutrophil oxidative burst activity

per cell in PLA compared with CHO in the present study may be due to a larger influx of these two types of neutrophils into the circulation in PLA. Another possible cause is the elevated plasma adrenaline concentration, which appears to inhibit neutrophil superoxide production in a dose-related manner (Barnett *et al.*, 1997, Tintinger *et al.*, 2001). Furthermore, neutrophil ROS producing activity is likely to decline with repeated stimulation (Prasad *et al.*, 1991). In contrast, many studies have demonstrated that GH primes and stimulates neutrophils to produce O_2^- via Ca^{2+} signalling (Ruy *et al.*, 1997, Smith *et al.*, 1996) or protein kinase C (Fu *et al.*, 1991) pathways. However, this effect may be opposed by increased levels of other hormones that inhibit neutrophil oxidative burst activity. Laboratory techniques may also influence neutrophil oxidative responses. Many studies have measured neutrophil oxidative burst by isolated neutrophils. However, Fukuda and Schmid-Schonbein (2002) suggested that the cell isolation procedures affect the determination of neutrophil functions. For example, neutrophil ROS production consistently increased after 1 min agitation on a test tube shaker at medium speed.

In the present study, IL-6 levels were not affected by CHO ingestion. IL-6 is a multi-functional cytokine and mediates many physiological functions, such as maintaining glucose homeostasis, stimulating lipolysis (Gleeson, 2000a) and inducing a biphasic neutrophilia (Suwa *et al.*, 2001). However, the post-exercise plasma IL-6 concentration in this study was relatively low (2-3 $ng \cdot L^{-1}$) and may not have exerted significant metabolic effects since the threshold for initiating acute metabolic responses may be as high as 25-65 $ng \cdot L^{-1}$ (Tsigos *et al.*, 1997).

Saliva flow rate was only decreased at post-EX2 in PLA. As described in Chapter 6 the decline of saliva flow rate during exercise may be due to the influence of SNS activity and there may be a threshold level of SNS activity to constrict salivary glandular vessels. In this study plasma adrenaline concentration was 1.34 nM in PLA and 0.85 nM in CHO at post-EX2. Therefore, the minimal concentration to initiate sufficient SNS activity may be between the ranges. Saliva IgA is secreted by both acinar and ductal units under the stimulation of α- and β-adrenoceptors and peptidergic receptor (Proctor and Carpenter, 2002). The insignificant alteration in sIgA response in this study suggests that two bouts of

90 min cycling at 60% $\dot{V}O_{2\,max}$ separated by 3 h recovery may not evoke sufficient activation of the SNS or HPA-axis to modify sIgA transcytosis.

In conclusion, ingestion of CHO compared with PLA during the first exercise bout 1) increased CHO availability during both bouts of exercise; 2) had a limited effect on the immunoendocrine response during the first exercise bout, but attenuated plasma stress hormone responses during the second exercise bout; 3) blunted the delayed neutrophilia and concurrent decline in LPS-stimulated degranulation and PMA-induced oxidative burst on per neutrophil basis following the first bout of 90 min cycling at 60% $\dot{V}O_{2\,max}$ and 4) does not affect oral immunity. The findings of the present study suggest that carbohydrate availability is an important determinant of immunoendocrine responses to repeated bouts of exercise. If athletes need to perform more than one bout of prolonged exercise in a day, regular ingestion of CHO-rich drink providing ~ 1-2 g CHO·min^{-1} before and during the first exercise bout onwards is beneficial to minimise the impact of exercise on immunity.

CHAPTER EIGHT

Effects of carbohydrate supplementation during the second of two prolonged cycling bouts on immunoendocrine responses

Summary

The purpose of this study was to examine the effect of CHO feeding during the second of two 90-min cycling bouts (EX1 started at 09:00 and EX2 started at 13:30) at 60% $\dot{V}O_{2\,max}$ on leukocyte redistribution, neutrophil degranulation and oxidative burst, plasma IL-6, plasma stress hormone, saliva flow rate and sIgA responses in EX2. This study consisted of two trials, which were completed in a counterbalanced order and separated by at least 4 days. Subjects (n = 9) consumed a lemon flavoured 10% w/v CHO (glucose) or PLA beverage during EX2: 500 mL just before exercise and 250 mL every 20 min during exercise. Venous blood samples were taken 5 min before exercise, immediately post-exercise and 18 h post-EX2; unstimulated whole saliva samples were collected at 10 min before exercise, 48-50 min and 88-90 min of exercise, 1 h post-exercise, and 18 h post-EX2 for both trials. The main findings of this study were that ingestion of CHO compared with PLA during EX2 better maintained plasma glucose concentration, blunted the responses of plasma adrenaline, ACTH, cortisol, GH and IL-6, and attenuated the leukocytosis and monocytosis, but had no effect on LPS-stimulated neutrophil degranulation, PMA-induced neutrophil oxidative burst activity, saliva flow rate and sIgA responses. Furthermore, the immunoendocrine disturbances induced by two bouts of prolonged exercise returned to resting values within 18 h. These findings suggest that ingestion of CHO compared with PLA during the second of two bouts of 90 min cycling at 60% $\dot{V}O_{2\,max}$ maintains better CHO availability, blunts hypothalamic-pituitary-adrenal activation, and attenuates leukocyte trafficking, but does not affect neutrophil function and oral immunity. Furthermore, the disturbances of immunoendocrine responses induced by two bouts of prolonged exercise on the same day recover within 18 h.

8.1 Introduction

Prolonged strenuous exercise affects immunoendocrine responses (Chapter 2). Previous chapters of this thesis have demonstrated immunoendocrine responses when subjects ingested CHO during the recovery interval (Chapter 6) and the first exercise bout (Chapter 7). However, during a second exercise bout liver and muscle glycogen content may be compromised by the previous exercise bout. This may induce an energy crisis in the working muscle, affecting SNS and HPA activation. Therefore, we hypothesised that CHO supplementation during the second of two prolonged exercise bouts would be particularly effective in minimising immunoendocrine responses. Furthermore, previous studies demonstrated that the immunodepression induced by intensive strenuous exercise may last for several hours post-exercise (Mackinnon, 1999). Hence, the aims of the present study were to determine the effect of CHO supplementation during the second of two prolonged cycling bouts on leukocyte redistribution, LPS-stimulated neutrophil degranulation and PMA-induced oxidative burst activity *in vitro*, plasma stress hormones, IL-6, saliva flow rate and sIgA responses in EX2. In addition, we also examined if the aforementioned parameters recover from the influence of the two bouts of 90 min cycling at 60% $\dot{V}O_{2\,max}$ within 18 h.

8.2 Methods

Subjects

Nine male volunteers (age 28.7 ± 1.6 years, height 174 ± 2 cm, body mass 74.4 ± 3.2 kg, $\dot{V}O_{2\,max}$ 50.3 ± 2.4 mL·kg^{-1}·min^{-1}; means ± S.E.M.), who were recreationally active and familiar with cycling, participated in the study. After receiving written information and passing a Health Questionnaire screen, subjects gave their written informed consent. Subjects were requested to complete the dietary record sheet on the day prior to Trial 1 and then repeated it again before Trial 2. Subjects were also asked not to perform any strenuous exercise or consume alcohol or medication for 2 days before each trial. The protocol was approved by the Ethics Committee of Loughborough University before the study began.

Experimental Procedures

Subject's workload was determined by a preliminary maximal oxygen uptake testing procedure as described in Chapter 3.2. The subjects completed two trials in a counterbalanced order, each separated by at least 4 days. Subjects arrived at the laboratory at 08:30 after fasting from 23:00 the previous day and were asked to empty the bladder before body mass was recorded. Subjects then performed two bouts of 90 min cycling (EX1 started at 09:00 and EX2 started at 13:30) at 60% $\dot{V}O_{2\,max}$ at 70 rev·min^{-1} on the same ergometer used to determine $\dot{V}O_{2\,max}$. Subjects were given a lemon flavoured 10% w/v CHO (glucose) beverage or artificially sweetened placebo during the second exercise bout: 500 mL just before exercise and 250 mL every 20 min during exercise. Subjects were asked to consume each drink within 3 min. Heart rate was recorded continuously during exercise by radiotelemetry. Ratings of perceived exertion (RPE) were obtained at 15-min intervals. Venous blood samples were taken 5 min before exercise, immediately post-exercise and 18 h post-EX2; unstimulated whole saliva samples were collected at 10 min before exercise, 48-50 min and 88-90 min of exercise, 1 h post-exercise, and 18 h post-EX2 for both trials. Water ingestion was allowed *ad libitum* during the first exercise bout and the recovery interval except for 5 min before each saliva sampling. The laboratory temperature and relative humidity were 24.5 ± 0.2 °C and 33 ± 2%, respectively.

Analytical Methods

Methods of preliminary measurements and blood collection and analysis are presented in Chapter 3.

Statistical analysis

All results are presented as mean values and standard errors of the mean (± SEM). Data were checked for normality, homogeneity of variance and sphericity before statistical analysis, and where appropriate the Huynh-Feldt method was applied for adjustment of degrees of freedom for the *F*-tests. Data were analysed using a two-factor (trial × time) repeated measures ANOVA with *post hoc* Tukey and paired *t*-tests, where appropriate. For the blood variables, the time points used in the ANOVA were pre-EX2 and the post-EX2 since the intervention (CHO or PLA) occurred during EX2. Comparison of immunoendocrine responses between pre-trial and 18 h post-EX2 (both at 09:00) were examined using paired *t*-tests. *P*, *t*, and adjusted *F* values are presented and statistical significance was accepted at $P < 0.05$.

8.3 Results

Physiological Variables and RPE

There were significant main effects of time and interactions between trial and time for RPE (time: $F_{1,8} = 28.0$, $P = 0.001$ and interaction: $F_{1,8} = 14.6$, $P = 0.005$) and body mass loss (time: $F_{1,8} = 14.1$, $P = 0.006$ and interaction: $F_{1,8} = 9.1$, $P = 0.017$). There was a significant main effect of trial ($F_{1,8} = 7.5$, $P = 0.025$) and an interaction between trial and time ($F_{1,8} = 16.3$, $P = 0.004$) for percentage change in plasma volume. A main effect of time was found for $\%\dot{V}O_{2\,max}$ ($F_{1,8} = 7.9$, $P = 0.023$) and HR ($F_{1,8} = 23.3$, $P = 0.001$) (Table 8.1).

Table 8.1 The exercise intensity and its effect on HR, RPE, body mass loss, water intake, and percentage change in plasma volume

	CHO		PLA	
	EX1	EX2	EX1	EX2
% VO_{2max}	60.7 (0.2)	62.4 (1.7)	58.8 (1.5)	63.1 (2.0)**
HR (beats·min^{-1}) [a]	144 (5)	157 (4)**	142 (3)	150 (5)*
RPE [a]	12.8 (0.3)	14.2 (0.4)**	12.8 (0.4)	16.0 (0.8)**¶
Body mass loss (kg) [b]	1.11 (0.09)	1.16 (0.08)	1.03 (0.13)	1.42 (0.13)**
Water intake (mL)	813 (111)	CHO**	989 (189)	PLA**
Plasma volume change (%) [c]	-3.8 (0.4)	-4.7 (0.5)	-3.7 (0.6)	-1.8 (1.0)¶¶

Values are mean (±SEM, n = 9). Significantly different from EX1 (*$P < 0.05$, **$P < 0.01$) in same trial, significantly different from the same time point in CHO trial (¶ $P < 0.05$, ¶¶ $P < 0.01$). [a] Measurements made in last 15 min of exercise. [b] After correction for water intake. [c] Immediately post-EX compared with pre-EX.

Leukocyte counts

There was a significant main effect of trial for the blood counts of leukocytes ($F_{1,8} = 6.8$, $P = 0.031$, Figure 8.1A) and monocytes ($F_{1,8} = 10.7$, $P = 0.011$, Figure 8.1D), with values higher in PLA compared with CHO. There was a significant main effect of time for the blood leukocyte ($F_{1,8} = 44.5$, $P < 0.001$), neutrophil ($F_{1,8} = 14.2$, $P = 0.006$, Figure 8.1B), lymphocyte ($F_{1,8} = 47.7$, $P < 0.001$, Figure 8.1C) and monocyte ($F_{1,8} = 43.7$, $P < 0.001$) counts, with higher values at post-EX2 than pre-EX2. Furthermore, there was a significant interaction between trial and time for blood leukocyte ($F_{1,8} = 16.0$, $P = 0.004$), lymphocyte ($F_{1,8} = 6.4$, $P = 0.035$) and monocyte ($F_{1,8} = 17.1$, $P = 0.003$) counts. No difference was found between pre-EX1 and 18h-p-EX2.

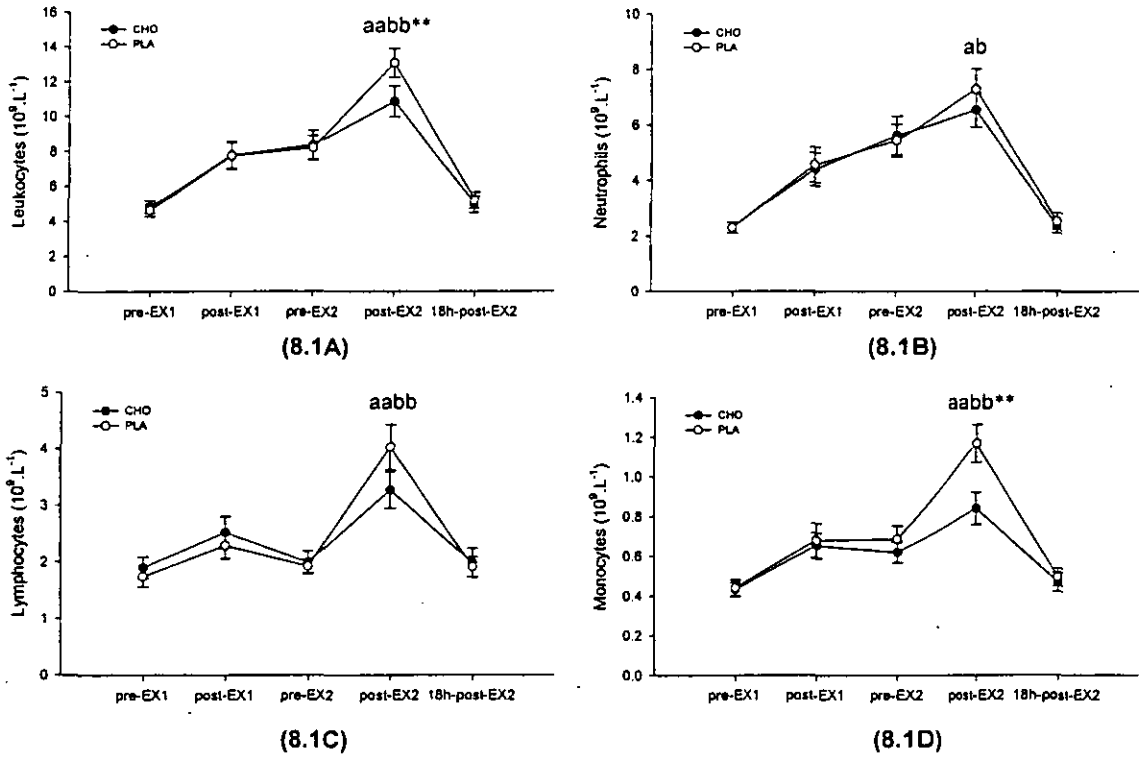


Figure 8.1 Changes in circulating counts of total leukocytes (8.1A), neutrophils (8.1B), lymphocytes (8.1C), and monocytes (8.1D). Values are means ± SEM (n = 9). Significantly different from pre-EX2 in CHO ($^{aa}P < 0.01$) and PLA ($^{bb}P < 0.01$); significantly different between trials ($^{*}P < 0.05$, $^{**}P < 0.01$).

Stress Hormones

There was a significant main effect of trial for plasma concentrations of adrenaline ($F_{1,8}$ = 8.8, P = 0.018, Figure 8.2A), ACTH ($F_{1,8}$ = 10.7, P = 0.011, Figure 8.2B), cortisol ($F_{1,8}$ = 12.8, P = 0.007, Figure 8.2C) and GH ($F_{1,8}$ = 14.3, P = 0.005, Figure 8.2D), with higher values in PLA compared with CHO. A significant main effect of time was observed for adrenaline ($F_{1,8}$ = 11.9, P = 0.009), ACTH ($F_{1,8}$ = 22.2, P = 0.002) and cortisol ($F_{1,8}$ = 70.3, P < 0.001), with higher values at post-EX compared with pre-EX. Furthermore, there was a significant interaction between trial and time for plasma adrenaline ($F_{1,8}$ = 6.9, P = 0.031), ACTH ($F_{1,8}$ = 10.3, P = 0.012), cortisol ($F_{1,8}$ = 11.4, P = 0.010) and GH ($F_{1,8}$ = 14.2, P = 0.006). No difference was found between pre-EX1 and 18h-p-EX2.

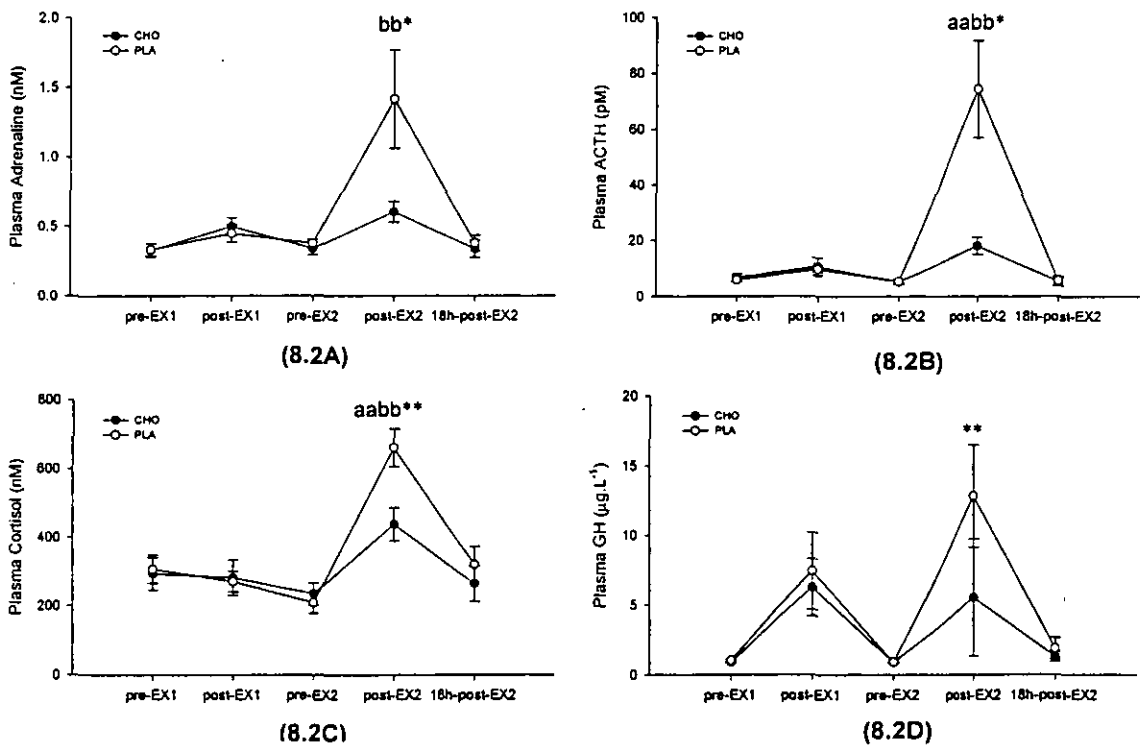


Figure 8.2 Changes in plasma concentrations of adrenaline (8.2A), ACTH (8.2B), cortisol (8.2C), and human growth hormone (8.2D). Values are means ± SEM (n = 9). Significantly different from pre-EX2 in CHO (^{aa} P < 0.01) and PLA (^{bb} P < 0.01); significantly different between trials (* P < 0.05, ** P < 0.01).

Glucose and IL-6

There was a significant main effect of trial and an interaction between trial and time for plasma glucose (trial: $F_{1,8} = 62.2$, $P < 0.001$; interaction: $F_{1,8} = 33.7$, $P < 0.001$; Figure 8.3A) and IL-6 ($F_{1,8} = 6.4$, $P = 0.035$; $F_{1,8} = 7.0$, $P = 0.030$; Figure 8.3B), which were higher at post-EX2 in PLA compared with CHO. A significant main effect of time for plasma IL-6 was observed ($F_{1,8} = 22.5$, $P = 0.001$). No difference was found between pre-EX1 and 18h-p-EX2.

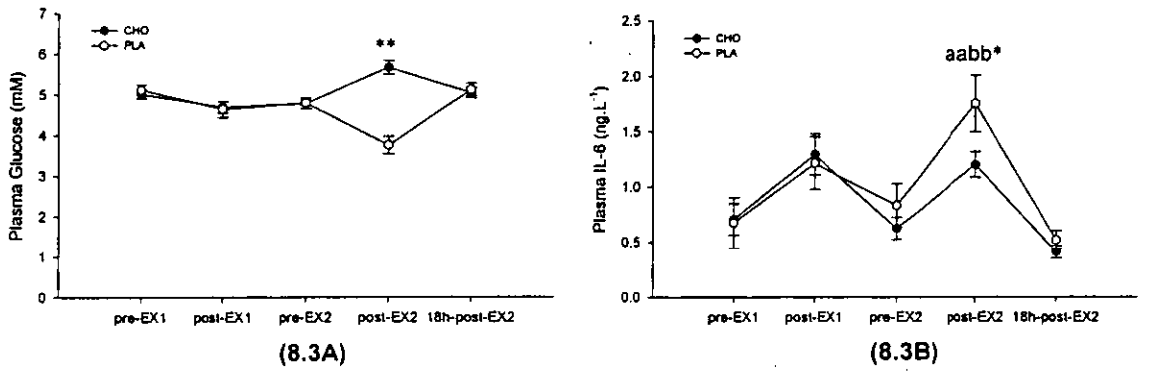


Figure 8.3 Changes in plasma concentrations of glucose (8.3A) and plasma IL-6 (8.3B). Values are means ± SEM (n = 9). Significantly different from pre-EX2 in CHO ($^{aa}P < 0.01$) and PLA ($^{bb}P < 0.01$); significantly different between trials ($^{*}P < 0.05$, $^{**}P < 0.01$).

Neutrophil Degranulation and Oxidative Burst

There was no main effect of trial, time, or interaction between trial and time for LPS-stimulated neutrophil elastase release in total or on per cell basis (Figure 8.4A and 8.4B) and total PMA-induced oxidative burst (Figure 8.5A) from pre-EX2 to post-EX2. However, there was a significant main effect of time for PMA-induced oxidative burst per neutrophil ($F_{1, 5} = 6.6$, $P = 0.050$), which showed a lower value at post-EX2 compared with pre-EX2 (Figure 8.5B). No difference was found between pre-EX1 and 18h-p-EX2.

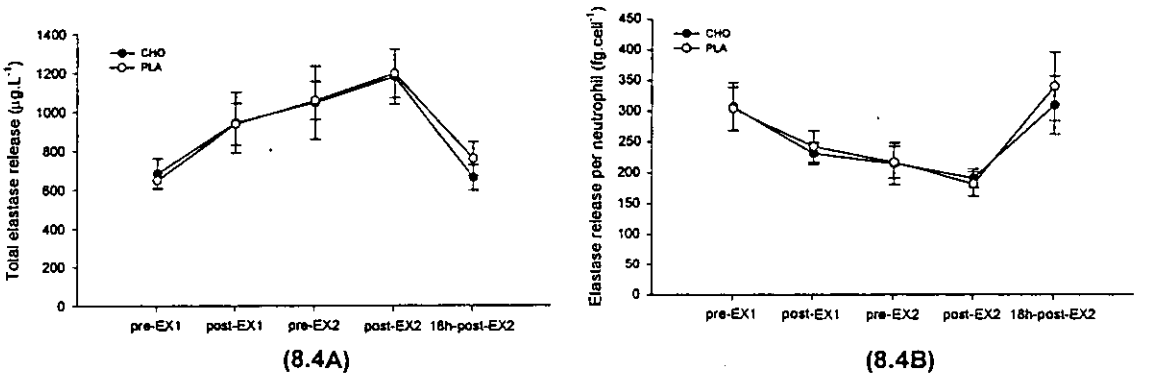


Figure 8.4 Changes in total LPS-stimulated elastase release (8.4A) and LPS-stimulated elastase release per neutrophil (8.4B). Values are means ± SEM (n = 8).

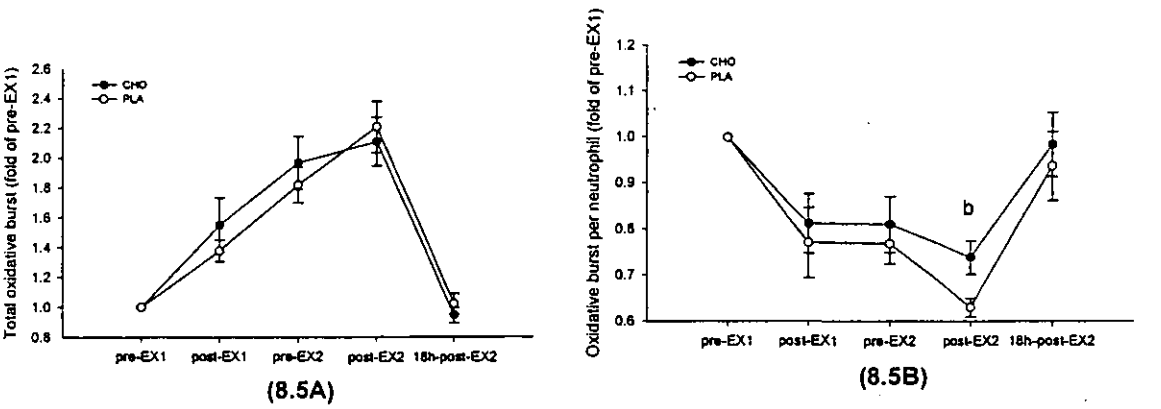


Figure 8.5 Changes in total PMA-induced oxidative burst (8.5A) and PMA-induced oxidative burst per neutrophil (8.5B). Values are means ± SEM (n = 6). Significantly different from pre-EX2 in PLA (^{b} $P < 0.05$).

Saliva Flow Rate

There was a significant main effect of time for saliva flow rate ($F_{3, 21} = 4.6$, $P = 0.014$, Figure 8.6), with a lower value at post-EX2 compared with pre-EX2 and post-EX1 ($t = 3.0$, $P = 0.016$) in PLA. No difference was found between pre-EX1 and 18h-p-EX2.

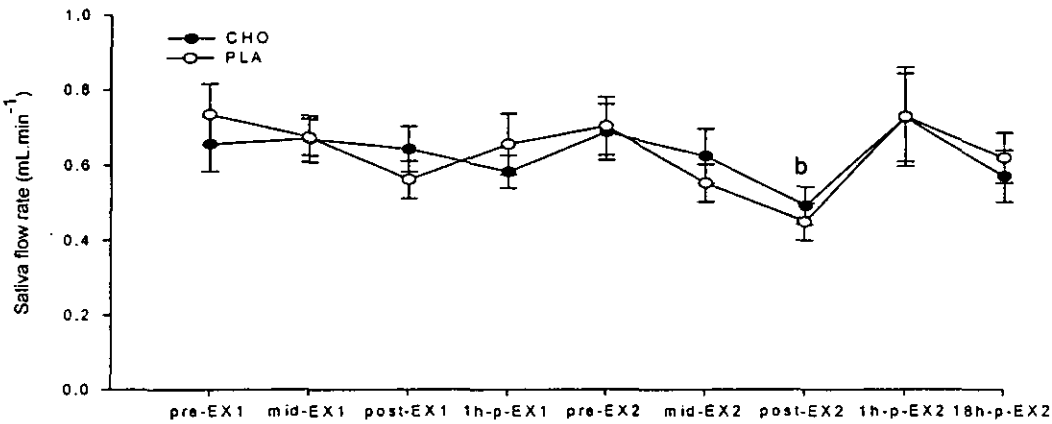


Figure 8.6 Changes in saliva flow rate. Values are means ± SEM (n = 8). [b] Significantly different from pre-EX2 in PLA ($P < 0.05$).

Salivary IgA Concentration and Secretion Rate

There was a significant main effect of trial ($F_{1,7} = 48.6$, $P < 0.001$) for sIgA concentration (Figure 8.7). The sIgA concentrations at mid-EX2 and post-EX2 in PLA were significantly higher compared with the respective time points in CHO. There were no significant changes for sIgA secretion rate in both trials (Figure 8.8). No difference was found between pre-EX1 and 18h-p-EX2.

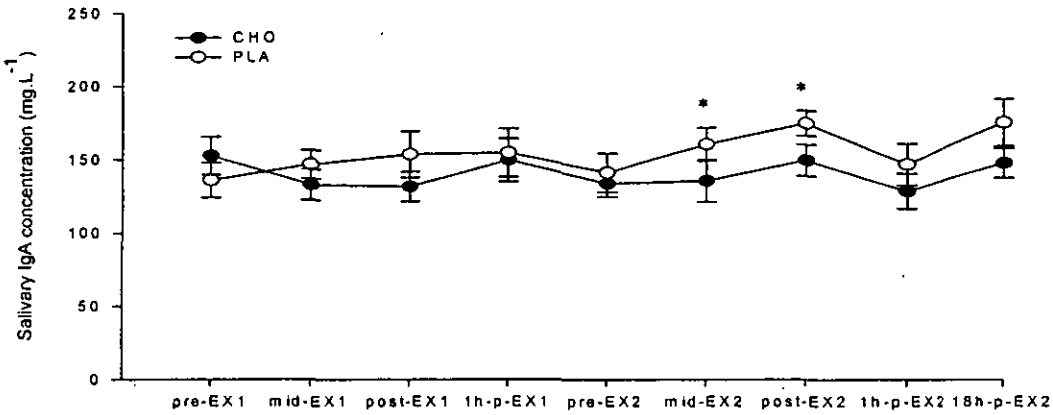


Figure 8.7 Changes in sIgA concentration. Values are means ± SEM (n = 8). * Significantly different between trials ($P < 0.05$).

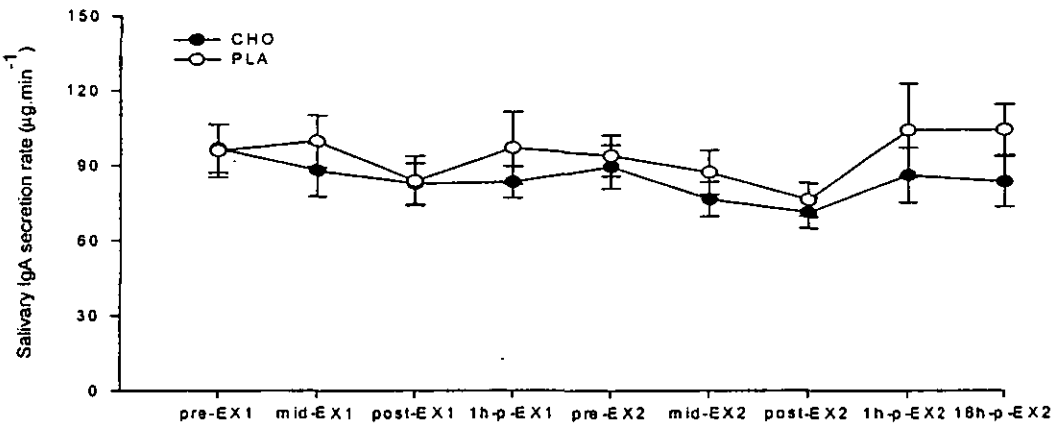


Figure 8.8 Changes in sIgA secretion rate. Values are means ± SEM (n = 8).

8.4 Discussion

The main findings of this study were that ingestion of CHO compared with PLA during EX2 better maintained plasma glucose concentration, blunted the responses of plasma adrenaline, ACTH, cortisol, GH and IL-6, and attenuated the leukocytosis and monocytosis, but had no effect on LPS-stimulated neutrophil degranulation, PMA-induced neutrophil oxidative burst activity, saliva flow rate and sIgA responses. Furthermore, the immunoendocrine disturbances induced by two bouts of prolonged exercise returned to resting values within 18 h.

In the present study, ingestion of CHO compared with PLA better maintained plasma glucose concentration. The plasma glucose level at the end of EX2 was as low as 3.75 ± 0.22 mM, reaching the threshold for inducing elevated plasma adrenaline (3.8 ± 0.1 mM) and GH (3.7 ± 0.1 mM) secretion (Schwartz *et al.*, 1987). Since a low blood glucose level is also associated with HPA activation and stress hormones secretion (Mitchell *et al.*, 1990), it was not surprising to find higher concentrations of plasma adrenaline, ACTH, cortisol and GH at post-EX2 in PLA than CHO in this study.

EX2 elicited significant increases in circulating counts of total leukocytes and subsets, whereas ingestion of CHO compared with PLA blunted the leukocytosis and monocytosis, but not the neutrophilia and lymphocytosis. It is well known that acute exercise leads to a significant but reversible redistribution of leukocyte subsets between the circulation, marginated pools and the bone marrow (Gleeson and Bishop, 1999). This exercise-induced mobilisation of leukocytes is linked to elevated plasma concentrations of stress hormones (Benschop *et al.*, 1996). Catecholamines exert an immediate effect, initiating a lymphocytosis within 10 min and subsequently evoke a neutrophilia and monocytosis with a relative lymphopenia (Benschop *et al.*, 1996). The later rise of plasma cortisol during prolonged strenuous exercise induces a further neutrophilia from the spleen and the bone marrow into the circulation (Toft *et al.*, 1994) but mobilises other leukocyte subsets from the circulation into the bone marrow, lymphoid, skin and injured tissue (Wira *et al.*, 1990, Toft *et al.*, 1992). The leukocyte trafficking during EX2 is likely mediated by the elevated plasma adrenaline rather than cortisol or GH since we did not find a lymphopenia at post-EX2. Moreover, Nieman (1997) has suggested that cortisol does not dominate leukocyte

mobilisation until the duration of exercise is in excess of 90 min and the effect of GH on neutrophil trafficking may not occur until 2 h after infusion (Kappel *et al.*, 1993).

No significant effect of CHO ingestion on neutrophil degranulation and oxidative burst activity was found in the present study. The results support the findings of previous studies, which reported that CHO ingestion during exercise did not affect neutrophil degranulation on per cell basis (Bishop *et al.*, 2002, Lancaster *et al.*, 2003). Although there were no differences in LPS-stimulated elastase release per neutrophil between pre-EX2 and post-EX2 in both trials, a significant decrease was observed at post-EX1 compared with pre-EX1 in both trials. The findings were similar to previous studies, which demonstrated that neutrophil degranulation on per cell basis fell after 2 h cycling at 60% $\dot{V}O_{2\,max}$ (Walsh *et al.*, 2000a) and following cycling to fatigue (98 ± 7 min in the CHO trial) at 75% $\dot{V}O_{2\,max}$ (Bishop *et al.*, 2001). Nakagawa *et al.* (1998) reported that the circulating neutrophilia after dexamethasone infusion was from the bone marrow (10%), marginated pools (61%) and prolongation of neutrophil intravascular half-life (29%) in rabbits. The neutrophils released from the bone marrow appear to have a lower content of granular digestive enzymes compared with fully mature neutrophils (Pyne, 1994). Therefore, an important factor determining the effect of exercise on neutrophil degranulation response to LPS on a per cell basis may be the mobilisation of neutrophils, although Bishop *et al.* (2003) reported that release of less mature cells may be not the reason for the fall in LPS-stimulated elastase release after strenuous exercise and many other factors seem to associated with neutrophil degranulation, such as the level of intracellular cAMP (Ottonello *et al.*, 1997), phagocytic activity (Morozov *et al.*, 2003), platelet-neutrophil contacts (Losche *et al.*, 1996), adrenaline (Tintinger *et al.*, 2001), glucocorticoids (Liles *et al.*, 1995) and IL-6 (Johnson *et al.*, 1998). This suggestion is supported by observations in the present study: a neutrophilia during EX1 coincided with a decline in LPS-stimulated elastase release, whereas the similar neutrophilia in CHO and PLA was associated with similar values of elastase release per neutrophil throughout the experimental protocol.

In the present study, the PMA-induced neutrophil oxidative burst activity (determined by CL) on per cell basis did not decline during EX1 until pre-EX2. The CL was only further

decreased in PLA during EX2. However, there was no significant difference between CHO and PLA throughout the experimental protocol. A few studies have examined the effect of exercise on PMA-induced neutrophil oxidative burst activity and the results were inconsistent. Pyne *et al.* (1996) reported that PMA-induced CL decreased 41% during the first bout of 40 min running at a heart rate ~140 beats·min^{-1} and the CL values did not change further during the 1-h recovery interval or in a second bout of identical exercise. However, Suzuki *et al.* (1999) showed that the PMA-induced CL of isolated neutrophils was increased after 90 min cycling at ~53% $\dot{V}O_{2\,max}$. Moreover, CHO ingestion did not appear to affect PMA-stimulated intracellular H_2O_2 production (Smith *et al.*, 1996). The neutrophils in the bone marrow are less mature with lower NADPH-dependent oxidase activity and superoxide response to PMA stimulation (Berkow and Dodson, 1986), whereas the nitro blue tetrazolium (NBT)-negative neutrophils in the marginated pools are likely to produce less $O_2^{\cdot-}$ in response to *in vitro* stimulation (Suzuki *et al.*, 1996). Therefore, the decline of the PMA-induced CL on per neutrophil basis at pre-EX2 and onwards in the present study may be due to the influx of these two types of neutrophils into the circulation. Other factors also appear to affect neutrophil oxidative burst activity, including adrenaline (Tintinger *et al.*, 2001), GH (Ruy *et al.*, 1997), repeated stimulation (Prasad *et al.*, 1991), and cell isolation procedures (Fukuda and Schmid-Schonbein, 2002).

In the present study, the plasma IL-6 concentration was elevated during exercise and CHO ingestion blunted the IL-6 response compared with PLA. It is accepted that prolonged exercise elicits IL-6 production and release from contracting skeletal muscle into the circulation (Steensberg *et al.*, 2000). The plasma IL-6 level is not markedly elevated until the later stage of prolonged exercise (glycogen depleted state) and CHO ingestion during exercise attenuates the plasma IL-6 response (Febbraio and Pedersen, 2002). The relatively low level of plasma IL-6 in the present study may reflect a sufficient CHO availability during EX2 and may not exert marked metabolic effects on hepatic glucose production, muscle glucose uptake, and lipolysis during exercise (Gleeson, 2000a).

Similar to Chapter 7, saliva flow rate was only decreased in PLA at post-EX2 compared with pre-EX2. Ford *et al.* (1997) reported that regular fluid intake appeared to prevent the decline of saliva flow rate during exercise. However, this notion is not fully supported by

the results of the present study because a significant decrease in saliva flow rate was still observed under sufficient water intake (1.5 L) during EX2. Since a steady blood flow to the salivary glands is required for maintenance of adequate salivation (Smaje, 1998), vasoconstriction may decrease saliva flow rate. Therefore, the lower saliva flow rate at post-EX2 in this study may be due to the higher adrenaline concentration (SNS stimulation). However, the magnitude of SNS stimulation is likely not greater enough to modify sIgA transcytosis.

In conclusion, ingestion of CHO compared with PLA during the second of two bouts of 90 min cycling at 60% $\dot{V}O_{2\,max}$ maintained better CHO availability, blunted HPA activation, and attenuated the leukocytosis and monocytosis; however, had no effect on the neutrophil degranulation response to LPS and oxidative burst activity induced by PMA on per cell basis during the second exercise bout. Oral immunity is likely not affected by exercise itself or CHO ingestion. Furthermore, the disturbances of immunoendocrine responses induced by two bouts of prolonged exercise on the same day recovered within 18 h.

CHAPTER NINE

General Discussion

The aim of this chapter is to attempt to integrate the findings from all studies in this thesis. In order to develop an overall view of the effects of repeated bouts of prolonged exercise and carbohydrate supplementation on immunoendocrine responses, this chapter is divided into three sections. The first section, general discussion, will review and comprehensively discuss the main findings of previous chapters. The second section, conclusions, will present the outcomes of this thesis. Finally, some suggestions derived from the findings of this thesis are given that may help athletes to minimise the impact of repeated bouts of exercise on their immunoendocrine system and may inform researchers of directions that may be worthy of further investigation.

9.1 General Discussion

Repeated training sessions during one day are a common training procedure for many elite athletes nowadays. However, only a few studies have been done on the impact of this type of training programme on immunoendocrine responses (McCarthy *et al.*, 1992, Rohde *et al.*, 1998, Ronsen *et al.*, 2001a, 2001b, Boyum *et al.*, 2002, Ronsen *et al.*, 2002a, 2002b, McFarlin *et al.*, 2003). Thus, the main aims of this thesis were to determine how two bouts of prolonged exercise performed on the same day affect immunoendocrine responses and the influence of nutritional intervention. This section is divided into five parts. Firstly, it was deemed necessary to clarify the effect of different saliva collection methods on measurement of saliva composition in order to determine the optimal method to use in the studies of this thesis. Secondly, the effect of exercise performed at different times of day on immunoendocrine responses was investigated. Thirdly, the difference in the immunoendocrine responses between the first and the second of two bouts of prolonged exercise was compared. Fourthly, the effect of ingestion of CHO compared with PLA on immunoendocrine responses during the second of two bouts of prolonged exercise was determined. Finally, it was established how much recovery time is needed to recover from two bouts of prolonged exercise.

9.1.1 Saliva collection methods

In the literature, the findings regarding the effect of exercise on saliva composition are inconsistent (see 2.6 saliva immunoglobulin A responses to exercise) and the reasons for this may be attributable, at least in part, to the different saliva collection methods, treatment and storage methods, assay techniques, and exercise protocols used. Therefore, we determined the effects of different saliva volumes on collection efficiency and saliva IgA concentration using the Salivette swab collection method (*Study 1* in Chapter 4) and investigated the influence of different saliva collection methods on saliva IgA concentration and saliva flow rate estimation (*Study* 2 in Chapter 4) before the start of exercise studies in this thesis. The findings from these two studies indicated that the swab collection method is not an ideal method because it affects measured values of saliva IgA concentration. Therefore, the optimal saliva collection method used in this thesis is described as the following: with an initial swallow to empty the mouth, unstimulated whole saliva is then expectorated (dribbled) into a pre-weighed vial for 2 min with eyes open, head tilted slightly forward and making minimal orofacial movement.

9.1.2 Different times of day

Many physiological variables exhibit circadian rhythms (see 2.1 Circadian variation in plasma stress hormones IL-6, circulating numbers of leukocytes and sIgA) and the best performance of elite athletes is often observed in the early evening (Atkinson and Reilly, 1996). However, in terms of the immune system, what time of day is best for exercising is still unknown.

In order to compare the effects of exercise performed at different times of day on immunoendocrine responses, we investigated time-dependent changes in plasma stress hormones, IL-6, circulating numbers of leukocyte subsets, neutrophil function, saliva flow rate, and saliva IgA responses. The results showed that there are circadian variations in plasma concentrations of ACTH and cortisol, circulating counts of leukocytes, neutrophils, lymphocytes and saliva IgA concentration (Chapter 5). The observations are similar to previous findings (Dhabhar *et al.*, 1994, Haus, 1994, Gleeson *et al.*, 2001a, Porterfield, 2001) and support the notion that the diurnal variation must be considered when the aim of

a study is to compare the effect of exercise performed at different times of day on the aforementioned parameters.

The main findings regarding the comparison of immunoendocrine responses between a single bout of prolonged exercise in the morning and in the afternoon are presented in Table 9.1 (EX1 *vs.* PMEX).

The plasma concentrations of glucose, adrenaline, ACTH, cortisol, GH and IL-6 and neutrophil degranulation response to LPS on per cell basis in EX1 are similar to PMEX whereas the perturbations of circulating leukocytes and subsets in EX1 are greater compared with PMEX. The larger redistribution of leukocytes and subsets into the circulation at post-PMEX compared with post-EX1 may be caused by higher HPA activation since the IAUC of plasma cortisol was significantly higher in PMEX (83.2 $nM \cdot h^{-1}$) than EX1 (3.5 $nM \cdot h^{-1}$) although no difference in absolute plasma concentration was found under the influence of circadian rhythms. Furthermore, saliva flow rate, sIgA concentration and IgA secretion rate was not altered between EX1 and PMEX.

In summary, a single bout of prolonged exercise performed in the afternoon induces a larger perturbation in redistributing leukocyte into the circulation than an identical bout of morning exercise, which may be due to higher HPA activation and circadian rhythms. However, in terms of oral mucosal immunity, performing prolonged cycling at different times of day does not differently affect the salivary responses.

9.1.3 The first *vs.* the second of two bouts of prolonged exercise

Ronsen and his colleagues reported that the second exercise bout induced greater responses of stress hormones and circulating counts of leukocyte subsets compared with an identical first bout of intensive exercise on the same day (Ronsen *et al.*, 2001a, 2001b). In this thesis we further compared the differences between the first and the second of two bouts of prolonged cycling in plasma glucose, IL-6, stress hormones, leukocyte trafficking, neutrophil function, saliva flow rate and sIgA responses. The main findings about the comparison of immunoendocrine responses between the first and the second of two bouts of prolonged exercise are presented in Table 9.1 (EX1 *vs.* EX2).

Table 9.1 The comparison of immunoendocrine responses between single bout of exercise in the morning and in the afternoon (EX1 *vs.* PMEX) and between the first and the second of two bouts of prolonged exercise (EX1 *vs.* EX2)

	EX1 *vs.* PMEX	EX1 *vs.* EX2			
		NON-CHO	CHO-REC	CHO-EX1	CHO-EX2
Glucose	↔	↓	↓↓	↓↓	↑↑
IL-6	↔	↔	↔	↑	↔
Adrenaline	↔	↔	↑↑	↑	↔
ACTH	↔	↑↑	↑	↔	↔
Cortisol	↔	↑↑	↑	↔	↑↑
GH	↔	↑	↑	↑↑	↔
Leukocyte count	↓	↑	↑↑	↑↑	↑↑
Neutrophil count	↓	↔	↑↑	↑↑	↑↑
Lymphocyte count	↔	↑↑	↑↑	↑↑	↑↑
Monocyte count	↓↓	↑↑	↑↑	↑↑	↑
Degranulation per neutrophil	↔	↓↓	↔	↔	↓↓
Oxidative burst per neutrophil	---	---	---	↔	↔
Saliva flow rate	↔	↔	↔	↓	↔
Saliva IgA concentration	↔	↔	↔	↔	↔
Saliva IgA secretion rate	↔	↔	↔	↔	↔

EX1 *vs.* PMEX and NON-CHO: no CHO supplementation (i.e. PLA or water; Chapter 5); CHO-REC: CHO supplementation during the recovery interval (Chapter 6); CHO-EX1: CHO supplementation during EX1 (Chapter 7); CHO-EX2: CHO supplementation during EX2 (Chapter 8).

Significantly higher at post-EX2 than post-EX1 ($^{\uparrow}$ $P < 0.05$, $^{\uparrow\uparrow}$ $P < 0.01$); significantly lower at post-EX2 than post-EX1 ($^{\downarrow}$ $P < 0.05$, $^{\downarrow\downarrow}$ $P < 0.01$); $^{\leftrightarrow}$ similar between post-EX2 and post-EX1.

Muscle glycogen depletion and hypoglycaemia potentially cause fatigue (Coyle *et al.*, 1983, Costill and Hargreaves, 1992) and elevate HPA and SNS stimulation during prolonged exercise (Mitchell *et al.*, 1990). Low plasma glucose concentration itself may impair neutrophil functions (Leist *et al.*, 1997, Healy *et al.*, 2002). The higher plasma concentrations of adrenaline and cortisol released by elevated HPA and SNS stimulation evoke the redistribution of leukocytes from marginal pools and the bone marrow into the circulation (Gleeson and Bishop, 1999).

Plasma glucose concentration was significantly lower at post-EX2 compared with post-EX1 when subjects ingested water throughout the experimental protocol (Chapter 5) or CHO during the recovery interval (Chapter 6) or EX1 (Chapter 7). Although CHO supplementation increases CHO availability during the experimental protocol, it is likely that the CHO ingestion during any period of two prolonged exercise bouts cannot attenuate the greater activation of HPA and increase in blood counts of leukocyte subsets in EX2 compared with EX1. However, the ingestion of CHO during the recovery interval and EX1 may be beneficial to blunt the decline of neutrophil function and feeding CHO during EX1 seems more effective in this regard. Performing two bouts of prolonged cycling on the same day appears to inhibit saliva flow rate but does not alter sIgA transcytosis in either EX1 or EX2. However, EX2 did not evoke greater changes in sIgA secretion compared with EX1.

In summary, the second compared with the first of two bouts of prolonged exercise on the same day induces a greater HPA activation, a larger leukocyte trafficking into the circulation, and a decreased neutrophil degranulation response to LPS on per cell basis and a lower saliva flow rate; but does not increase plasma IL-6, or change sIgA secretion rate. Furthermore, CHO ingestion during any period of two bouts of prolonged exercise shows limited beneficial effect to blunt these higher responses in the second exercise bout compared with the first identical exercise bout on the same day.

9.1.4 Carbohydrate supplementation during two bouts of prolonged exercise

The main findings concerning the effect of CHO ingestion on immunoendocrine responses during the second of two bouts of prolonged exercise are presented in Table 9.2.

Table 9.2 The effect of CHO ingestion on immunoendocrine responses during the second of two bouts of prolonged exercise

	NON-CHO	CHO-REC	CHO-EX1	CHO-EX2
Glucose	↓↓	↓↓	** ↔	** ↑↑
IL-6	↑	↑	↑	* ↑↑
Adrenaline	↑↑	↑↑	* ---	* ↑↑
ACTH	↑↑	* ↑	** ↑	* ↑↑
Cortisol	↑↑	* ↑	** ↑↑	** ↑↑
GH	↑↑	↑	↑↑	** ↔
Leukocyte count	↑	* ↑↑	** ↑↑	** ↑↑
Neutrophil count	↔	↔	↑↑	↑
Lymphocyte count	↑↑	↑↑	** ↑↑	↑↑
Monocyte count	↑↑	↑↑	* ↑↑	** ↑↑
Degranulation per neutrophil	↓↓	↔	* ↔	↔
Oxidative burst per neutrophil	---	---	* ↔	↔
Saliva flow rate	↓↓	↓↓	↓↓	↔
Saliva IgA concentration	↑↑	↑↑	* ↔	* ↔
Saliva IgA secretion rate	↔	↔	↔	↔

NON-CHO: no CHO supplementation (i.e. PLA or water; Chapter 5); CHO-REC: CHO supplementation during the recovery interval (Chapter 6); CHO-EX1: CHO supplementation during EX1 (Chapter 7); CHO-EX2: CHO supplementation during EX2 (Chapter 8).

Significantly different from PLA at post-EX2 (* $P < 0.05$, ** $P < 0.01$); significantly higher at post-EX2 than pre-EX2 ($^{\uparrow}$ $P < 0.05$, $^{\uparrow\uparrow}$ $P < 0.01$); significantly lower at post-EX2 than pre-EX2 ($^{\downarrow}$ $P < 0.05$, $^{\downarrow\downarrow}$ $P < 0.01$); $^{\leftrightarrow}$ similar between post-EX2 and pre-EX2.

Ingestion of CHO compared with PLA during EX1 or EX2 appears consistently to better maintain the plasma glucose concentration, attenuate the activation of SNS and HPA, blunt the increase in blood numbers of leukocytes and monocytes, and minimise the elevation of sIgA concentration during EX2. CHO supplementation during EX2, moreover, attenuates the decline of neutrophil degranulation response to LPS and oxidative burst activity to PMA on per cell basis compared with PLA. However, CHO ingestion during the recovery interval seems less effective and only blunts the HPA activation and circulating lymphocyte count compared with PLA. Ingesting CHO-rich drinks during prolonged exercise is the most effective and common strategy applied to support CHO availability during exercise (Jeukendrup and Jentjens, 2000), which can attenuate activation of HPA and perturbation of the circulating numbers of leukocytes and subsets (Mitchell *et al.*, 1990, Nieman *et al.*, 1997, Bishop *et al.*, 1999b). In contrast, the CHO ingestion during the recovery interval in Chapter 6 did not appear to attenuate the decline of plasma glucose concentration and was not effective in blunting the immunoendocrine responses during EX2 compared with PLA. The findings suggest that the greatest benefit of CHO supplementation to attenuation of immunoendocrine responses during two bouts of prolonged exercise is obtained when CHO is consumed at the earliest opportunity since it allows more time for absorption and storage of glucose. For example, when CHO drink is consumed during EX1, some ingested CHO is emptied and absorbed (~1 $g \cdot min^{-1}$) to maintain plasma glucose concentration during the first bout of prolonged exercise, and some ingested CHO may provide substrate for glycogen synthesis in the liver and muscle during the recovery interval, and attenuate the decrease in CHO availability and immunoendocrine responses during the subsequent bout of exercise. However, if CHO is consumed during the recovery interval, there is less time available for absorption and glycogen synthesis and may also cause a rebound hypoglycaemia in the early stage of the subsequent exercise bout, inducing the activation of HPA. If CHO is consumed during the second exercise bout, some, but probably not all of the ingested CHO, is absorbed to maintain plasma glucose concentration but it is unlikely to be directed to resynthesis of liver and muscle glycogen during exercise.

In summary, ingestion of CHO compared with PLA during the recovery interval attenuates HPA activation to the second exercise bout to a small extent. If CHO is supplemented during the second exercise bout, the responses of SNS and HPA, plasma glucose, circulating leukocytosis and monocytosis, and sIgA level during the second exercise bout are blunted compared with PLA. Moreover, if CHO is ingested during the first of two bouts of prolonged exercise, the decline in neutrophil function can be prevented (compared with PLA) during the second exercise bout. It seems that when two bouts of exercise are performed on the same day, the greater benefit in terms of circulating immunoendocrine responses is obviously by feeding CHO at the earliest opportunity.

9.1.5 Recovery time

The main findings about the recovery of immunoendocrine variables 3 h after the first and 18 h after the second of two bouts of prolonged exercise are presented in Table 9.3.

It has been suggested that the alteration in immune cell function and leukocyte trafficking may last for several hours after exercise (Gleeson and Bishop, 1999). The results from the studies in this thesis show that the responses of activated SNS and HPA, circulating lymphocyte count, and oral immunity return to pre-EX1 but plasma glucose and IL-6, circulating counts of leukocytes, neutrophils and monocytes, and neutrophil function did not recover to pre-EX values within 3 h after EX1 when subjects only ingest water or placebo during EX1 and the recovery interval (NON-CHO-3h and CHO-EX2-3h). However, if subjects ingest CHO during EX1 or the recovery interval, the decline in neutrophil degranulation response to LPS on per cell basis can be prevented. This may be because the ingestion of CHO compared with PLA attenuates the delayed neutrophilia that arises due to release of cells from the bone marrow into the circulation after EX1. Obviously, a 3-h interval is insufficient for recovery of leukocyte mobilisation and neutrophil function but oral mucosal immunity (sIgA secretion rate) from the impact of previous prolonged exercise. According to Ronsen *et al.* (2002a), a 6-h recovery interval may be better for athletes to recover their cellular immunity for the next training bout.

Table 9.3 The immunoendocrine responses at 3 h after the first and 18 h after the second of two bouts of prolonged exercise

	NON-CHO-3h	CHO-REC-3h	CHO-EX1-3h	CHO-EX2-3h	CHO-EX2-18h
Glucose	↔	↓↓	↓↓	↓	↔
IL-6	↑	↔	↔	↔	↔
Adrenaline	↔	---	---	↔	↔
ACTH	↓↓	↔	↔	↔	↔
Cortisol	↔	↓↓	↓↓	↔	↔
GH	↔	↔	↔	↔	↔
Leukocyte count	↑↑	↑↑	↑↑	↑↑	↔
Neutrophil count	↑↑	↑↑	↑↑	↑↑	↔
Lymphocyte count	↔	↓	↔	↔	↔
Monocyte count	↑↑	↑↑	↑↑	↑↑	↔
Degranulation per neutrophil	↓↓	↔	↔	↓	↔
Oxidative burst per neutrophil	---	---	↓	↓	↔
Saliva flow rate	↔	↔	↔	↔	↔
sIgA concentration	↔	↔	↔	↔	↔
sIgA secretion rate	↔	↔	↔	↔	↔

NON-CHO: no CHO supplementation (i.e. PLA or water; Chapter 5); CHO-REC: CHO supplementation during the recovery interval (Chapter 6); CHO-EX1: CHO supplementation during EX1 (Chapter 7); CHO-EX2: CHO supplementation during EX2 (Chapter 8).

Significantly higher than pre-EX1 ($^{\uparrow}$ $P < 0.05$, $^{\uparrow\uparrow}$ $P < 0.01$); significantly lower than pre-EX1 ($^{\downarrow}$ $P < 0.05$, $^{\downarrow\downarrow}$ $P < 0.01$); $^{\leftrightarrow}$ similar from pre-EX1.

Muns *et al.* (1994) reported that nasal neutrophil function was depressed for 3 days after prolonged running. Moreover, Peters and Bateman (1983) suggested that the depression of sIgA concentration could last up to 18 h after marathon running and that repeated bouts of intense exercise may exert a cumulative effect on mucosal immunity (Mackinnon and Hooper, 1994). However, we did not observe a delayed effect of exercise on immunoendocrine variables 18 h after two bouts of prolonged exercise.

In summary, a 3 h interval is insufficient for recovery of leukocyte mobilisation and neutrophil function from the impact of previous exercise whether subjects consumed placebo or CHO during exercise or recovery. However, an 18 h interval is sufficient for full recovery of all immunoendocrine variables we measured in this thesis from the impact of two bouts of prolonged exercise.

9.2 Conclusions

The major conclusions from this thesis are:

1) The use of a swab for collecting saliva is not an ideal method because it affects the results of saliva composition.

2) A single bout of prolonged exercise performed in the afternoon induces a larger perturbation in the redistribution of leukocytes into the circulation than an identical bout of morning exercise, which may be due to higher HPA activation and circadian rhythms. However, in terms of oral mucosal immunity, performing prolonged cycling at different times of day does not differently affect the salivary responses.

3) The second compared with the first of two bouts of prolonged exercise on the same day induces a greater HPA activation, a larger leukocyte trafficking into the circulation, and a decreased neutrophil degranulation response to LPS on per cell basis and a lower saliva flow rate; but does not increase plasma IL-6, or change sIgA secretion rate. Furthermore, CHO ingestion during any period of two bouts of prolonged exercise shows limited beneficial effect to blunt these higher responses in the second exercise bout compared with the first identical exercise bout on the same day.

4) When two bouts of exercise are performed on the same day, the greatest benefit in terms of circulating immunoendocrine responses is obtained by feeding CHO at the earliest opportunity.

5) A 3 h interval is insufficient for recovery of leukocyte mobilisation and neutrophil function from the impact of previous exercise whether subjects consume placebo or CHO during exercise or recovery. However, an 18 h interval is sufficient for full recovery of all immunoendocrine variables we measured in this thesis from the impact of two bouts of prolonged exercise.

9.3 Suggestions

Some practical suggestions that can be given to athletes based on the findings of this thesis include:

1) If athletes have only a single session of training in a day, performing it in the morning is likely to better minimise the impacts of exercise on immunoendocrine responses compared with afternoon exercise.

2) Maintaining CHO availability during exercise is very important to attenuate immunoendocrine responses. If possible, ingest a CHO-rich drink (~ 1-2 g CHO·min^{-1}) during each exercise session and recovery interval.

3) If a training programme consists of more than one session of exercise, a recovery interval of more than 3 h is essential for maintaining immunocompetence in a subsequent training session.

Possible future studies from the remaining questions in this thesis:

1) Determine how long it takes to fully recover from the impact of two bouts of prolonged exercise.

2) Quantify neutrophil release from the bone marrow and the redistribution of NBT-negative neutrophils from the marginated pools into the circulation following different periods of repeated exercise and measure their function.

3) Compare neutrophil function in isolated cell suspensions and whole blood samples.

4) Examine the balance of T_{h1} and T_{h2} to repeated bouts of exercise on the same day.

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APPENDIX A

Statement of Informed Consent

I have read the subject information sheet, detailing the procedure and requirements which are involved with this study and I fully understand what is required of me. I have had an opportunity to ask for further information and clarification of the demands of each of the procedures.

I am aware that I have the right to withdraw at any time with no obligation to give reasons for my decision.

I agree to take part in the study.

Name: ..... Phone: .....

Age: ..... Date of Birth: .....

Address: .....

Signed: ..... Date: .....

Witnessed by: ..... Date: .....

APPENDIX B

Health Screen for Study Volunteers

Name: ..... **Phone:** .....

Address: .....

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 (e.g. to confirm problem was/is short-lived, insignificant or well controlled).

.....

.....

I have completed the questionnaire to the best of my knowledge and any questions I have been answered to my full satisfaction.

Signed: ..... **Date:** .....

APPENDIX C

Physical Activity Questionnaire

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

Name: .....

Do you practice ENDURANCE TRAINING? Yes ☐ No ☐

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

How may minutes does each session last?

What is your weekly mileage?

Do you practice the following training?

Weight training ☐ Interval training ☐ Skills training ☐.

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

.....

How many minutes does each session last?

.....

Signed: ..... Date: .....

APPENDIX D

Health Questionnaire

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

At present, do you have any health problems for which you are?

1) On medication, prescribed or otherwise Yes ☐ No ☐

2) Attending your general practitioner Yes ☐ No ☐

3) Have you any symptoms of ill health, such as those associated with a cold or other common infection? Yes ☐ No ☐

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

.....

.....

Do you want to take part in today's experiments? Yes ☐ No ☐

Signed: ..... **Date:** .....

APPENDIX E

Food Record Diary

CONFIDENTIAL

Name and Address

.....

.....

.....

.....

Age ..... **Years**

Start Date of Diet: ...../...../.....

Please record everything you eat and drink during the day before first trial, and then repeat the diet as accurately as possible during the day before second trial. Instructions and an example are attached. Information about your diet will be treated in confidence and results will be returned to you as soon as possible.

If you have problem, please contact with:

Professor Mike Gleeson at Sport Hall RR010 or ext. 6345

Mr Tzai-Li Li at Sport Hall RR105 or ext. 6351

Exercise Immunology Laboratory
School of Sport and Exercise Sciences
Loughborough University, Leicestershire, LE11 3TU, UK

INSTRUCTION FOR USING THE FOOD DIARY

Everything that you eat and drink the day before first exercise trial should be weighed, and the weight and type of foods or drinks should be recorded.

The solid foods should be separately placed on a plate or in a container on a scale to be weighed after the scale has been zeroed with the plate or the container; for example: Zero the scale with plate, then place the Roast Beef on plate. If the scale shows 100 g, then the weight of Roast Beef is 100 g.

Drink or liquid food should be added into a cup or a glass on a scale to be weighed after the scale has been zeroed with the cup or the glass. Please remember to record the separate weight of tea, milk and sugar before putting together.

Do not forget to weigh and record second helpings and snacks between meals. The nutrition information (ingredients, weight, and energy) of most snacks has been labelled on the packet. You do not need to weigh it if you eat whole packet once. Any leftovers (e.g. apple cores) should also be weighed and recorded in the leftovers column.

Eating Out – Please carry your diary and scales with you wherever you go. If it is inconvenient, please record the type of foods eaten with an estimated weight – but please note it on the sheet.

Name and description of foods should be as detailed as possible, including the brand and any other available information; e.g. Cheese – is insufficient information, Cheese Cheddar (shape reduced fat) – is sufficient information.

The space provided at the food of each page for general comments is for you to give any further information about your diet and your training/activity for that day; e.g. Steady run, morning, 1 hour. Lunch missed due to stomach pain.

Please record this diary during the day before first exercise trial and then repeated it as accurate as possible during the day before the second exercise trial.

Name:			Date: / /		Please use a separate row for each item		
A	B		C	D	E	F	**Office Use**
Time	Food eaten		Brand name of each item (except fresh food)	Full description of each item including: -whether fresh, frozen, canned -cooked: boiled, grilled, fried, roasted. -what type of fat food fried in	Served	Leftover	**Actual Weight**
am/pm	home	away			(gms)	(gms)	**(gms)**
GENERAL COMMENTS:							

APPENDIX F

Normal Range of Blood Leukocytes

	Numbers ($\times 10^9 \cdot L^{-1}$)	Percentage of Leukocytes
Leukocytes	4.0 – 11.0	
Neutrophils	2.00 – 8.00	50.0 – 80.0
Lymphocytes	1.00 – 5.00	25.0 – 50.0
Monocytes	0.10 – 1.00	2.0 – 10.0
Eosinophils	0.00 – 0.40	0.0 – 5.0
Basophils	0.00 – 0.20	0.0 – 2.0

Data is from the NHS Leicester Hospital.