The Effect Of An Anti-Inflammatory Homeopathic Product On Cytokine Status In Venous Blood Following 90 Minutes Of Downhill Running

Dr A. Docrat

SUPERVISOR: Prof. E. M. Peters - Futre

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December 2008
Declaration

I, Aadil Docrat, declare the work on which this project is based is original and my own work (except where acknowledgements indicate to the contrary) and that neither the whole work nor part thereof has been, is presently or is to be submitted for another degree at this or any other university.

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Durban
December 2008

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Dedication

In the service of Almighty God, I dedicate this research to the following people who have made an indelible impression on my life:

My dear father, Mohammed Farouk, my dear mother Facia (1952-1996), my dear stepmother Amina
My dear wife Fatima
My children Zahraa, Salmaa, Husnaa and Muaaz
My wife’s parents, Ahmed and Zaheda Jadwat, who accepted me as a son
My siblings, Tahir and Sadiyya, and their families
I would like to express my humble, heartfelt and sincere gratitude to the following people:

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2. Mr Fanie Blignaut and Heel (Pty) Ltd South Africa for the financial contribution received towards the expenses of this work and his liaison with Heel GmbH, Germany regarding the arrangement of the Insurance Policy required by the University and the strictly “blinded” provision of the Traumeel and placebo tablets.

3. Ms. Megan Smith, for her contribution to this project in terms of recruitment of subjects, assisting with laboratory and field testing and capturing of data.

4. Mr Norrie Williamson for the important contribution he made in conducting the field tests for the athletes.

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9. My dear wife, Fatima and children for the unseen sacrifices that were made so that this study could be completed.

10. All the participants of this study without whom it would not have materialised.
Abstract

Background: Downhill running involves eccentric contractions of the gluteal, quadriceps, hamstring and calf muscles and the lengthening of muscle fibres as they contract. Several studies have demonstrated that this type of prolonged eccentrically biased exercise induces tissue damage and subsequent enhancement of an inflammatory response.

Traumeel® S (Heel GmbH, Baden-Baden, Germany) is a homeopathic-complex used to treat trauma and inflammatory processes that is sold as an over the counter remedy in pharmacies. Although the anti-inflammatory and analgesic effects of Traumeel® S have been demonstrated in selected clinical trials as well as in in vitro experimental models, little is known of its scientific mechanisms of action.

Aim: The aim of this study was to establish whether administration of Traumeel® S five days before and three days after a 90-minute downhill treadmill run at 75% VO_2 peak significantly changes systemic markers of the inflammatory response. These are to include blood-borne concentrations of cortisol and examples of selected T-helper_1-cell cytokines, T-helper_2-cell cytokines, chemokines and pro-inflammatory cytokines during the three days following the 90-minute downhill run.

Method: This study was designed as a double-blinded, placebo-controlled clinical trial in which matched subjects were randomised to Traumeel (TRAU) and Placebo (PLAC) pairs and exposed to two 90-minute downhill running trials.

Twenty subjects (12 men, 8 women) aged between 20 and 50 years, fully complied with all inclusion criteria set for the study. Following baseline laboratory and field testing, they were matched according to gender, body mass index (BMI), training age, training status, peak running performance and foot-strike patterns and randomized into TRAU and PLAC groups. One Traumeel® S tablet was ingested three times per day for five days prior to and three days following a 90-minute downhill run on a treadmill at a -6% gradient and at a speed eliciting 75% VO_2 peak on a level gradient. Blood samples were obtained immediately before the 90-minute trial (PRE), immediately after the trial (IPE) and 24 hours (24 PE), 48 hours (48 PE) and 72 hours (72 PE) following the trial. Each subject was also requested to complete a training record prior to the trial and keep a record of the daily symptoms of delayed onset muscle soreness (DOMS) both at rest (general pain) and while walking (daily living).

Full blood counts, serum creatine kinase (CK) and cortisol concentrations were determined using standard haematological laboratory procedures. A sandwich ELISA was used to determine plasma interleukin-6 (IL-6) concentrations. A commercial bead-array kit was used to conduct flow cytometric analysis of
Interleukin-8 (IL-8), Interleukin-10 (IL-10), Tumour Necrosis Factor alpha (TNFα), and Interleukin-12p70 (IL-12p70) concentrations.

**Results:** Paired student t-tests indicate that the mean ± SEM of the two groups was not significantly different ($p < 0.05$) in terms of age, BMI, percentage body fat, training age, foot strike patterns, running performance, FVC, FEV1, baseline heart rate and blood pressure, RERmax, VO2 peak, VEmax, or training status. Although the TRAU group completed the 90-minute downhill running trial at a significantly faster speed ($13.3 ± 2.1$ vs. $12.8 ± 0.3$ km.hr; $p = 0.02$) and covered a greater distance ($20.1 ± 0.3$ vs. $19.34 ± 0.4$; $p = 0.03$), mean and maximum heart rate and RPE did not differ between trials in the TRAU and PLAC groups. The downhill running protocol resulted in significant increases in neutrophil counts and creatine kinase, Cortisol, IL-6, IL-8 and IL-10 concentrations in the circulation ($n = 20$; $p < 0.001$). When comparing the TRAU ($n = 10$) and PLAC ($n = 10$) groups, blood neutrophil counts, creatine kinase, cortisol, and IL-6 concentrations over the 5 time points and PRE, IPE and 24 PE plasma TNF, IL-8, IL-10 and IL-12p70 concentrations did not differ significantly ($p > 0.05$). Blood creatine kinase was, however, significantly higher in the TRAU group at 24PE ($p < 0.05$). The post-trial DOMS scores reported by the TRAU group over the 3-day post-exercise recovery period were also significantly lower in the TRAU group at 24PE ($p = 0.03$).

**Conclusion:** Despite a faster running speed and higher post trial CK concentration in the TRAU group following the 90-minute downhill run, statistically significant differences in circulating stress hormone, and cytokine concentrations (IL-6, IL-8, IL-10, TNFα and IL-12p70) between the TRAU and PLAC groups, were not identified. Delayed onset muscle soreness was also significantly lower in the TRAU group at 24 hours post trial ($p = 0.03$). While these findings would support attenuation of the post-exercise inflammatory response by Traumeel® S, further work is required to verify this possibility.
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>12PE</td>
<td>12 hours post-exercise</td>
</tr>
<tr>
<td>24PE</td>
<td>24 hours post-exercise</td>
</tr>
<tr>
<td>48PE</td>
<td>48 hours post-exercise</td>
</tr>
<tr>
<td>72PE</td>
<td>72 hours post-exercise</td>
</tr>
<tr>
<td>ACTH</td>
<td>Adrenocorticotropic hormone</td>
</tr>
<tr>
<td>ANS</td>
<td>Autonomic nervous system</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>BCAA</td>
<td>Branched chain amino acid</td>
</tr>
<tr>
<td>BMI</td>
<td>Body mass index</td>
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<tr>
<td>CAM</td>
<td>Cell adhesion molecules</td>
</tr>
<tr>
<td>CK</td>
<td>Creatine kinase</td>
</tr>
<tr>
<td>CRH</td>
<td>Corticotropin releasing hormone</td>
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<tr>
<td>CRP</td>
<td>C-reactive protein</td>
</tr>
<tr>
<td>CSIF</td>
<td>Cytokine synthesis inhibitory factor</td>
</tr>
<tr>
<td>CTL</td>
<td>Cytotoxic T Lymphocytes</td>
</tr>
<tr>
<td>DOMS</td>
<td>Delayed onset muscle soreness</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetra acetic acid</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>FBC</td>
<td>Full blood count</td>
</tr>
<tr>
<td>FVC</td>
<td>Forced Vital Capacity; L/min</td>
</tr>
<tr>
<td>$F_{E}V_{1}$</td>
<td>Forced Expiratory Volume in 1 second</td>
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<tr>
<td>GM-CSF</td>
<td>Granulocyte-macrophage colony-stimulating factor</td>
</tr>
<tr>
<td>HPA</td>
<td>Hypothalamic pituitary axis</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>Intercellular adhesion molecule-1</td>
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<tr>
<td>IFN-γ</td>
<td>Interferon-gamma</td>
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<td>Immunoglobulin A</td>
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<td>Interleukin-1 Alpha</td>
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<td>IL-1β</td>
<td>Interleukin-1 Beta</td>
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<tr>
<td>IL1-ra</td>
<td>Interleukin-1 receptor antagonist</td>
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<tr>
<td>IL-5</td>
<td>Interleukin-5</td>
</tr>
</tbody>
</table>
IL-6  Interleukin-6
IL-8  Interleukin-8
IL-10 Interleukin-10
IL-12p40 Interleukin-12p40
IL-12p70 Interleukin-12p70
IPE   Immediately post-exercise
IP-10 Inducible protein-10
LPS   Lippolysaccharide
METS  Metabolic Equivalents
NAP-1 Neutrophil Activating Peptide 1
NF-kB Nuclear Factor Kappa B
NCF   Neutrophil Chemotactic Factor
NK    Natural killer
OTS   Over training syndrome
PGE2  Prostaglandin E2
PLAC  Placebo
PMTS  Peak treadmill speed
RER   Respiratory Exchange Ratio
RPE   Rate of Perceived Exertion
RQ    Respiratory Quotient
SEM   Standard Error of the Mean
Th1   T-Helper cell 1
Th2   T-Helper cell 2
Th3   T-Helper cell 3
TGF-β Transforming growth factor beta
TNF-α Tumor necrosis factor alpha
TRAU  Traumeel
UKZN  University of KwaZulu-Natal
URTI  Upper respiratory tract infection
Vₐ    Pulmonary Ventilation
VCO₂  Carbon dioxide output
VO₂   Oxygen consumption
VO₂peak Peak oxygen consumption
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Chapter 1
Introduction

1.1. Introduction to the Problem
Exercise of an intense and prolonged nature has been known to cause microscopic muscle damage, generating an inflammatory response and causing numerous immune perturbations (Peters, 1997; Nieman, 2000; Pedersen, 2001). This is particularly evident after downhill running, during which the eccentric work of the 'anti-gravity' knee extensor muscles and the anterior and posterior tibial muscles as well as the hip extensors is accentuated and the muscles are forcibly lengthened while contracting (Eston et al., 1995).

Post-exercise leukocytosis has been reported to occur within a few hours after the cessation of intense prolonged exercise (McKinnon, 1999, Nieman, 2000). This is attributed to immediate post-exercise neutrophilia and subsequent monocyte migration to the site of tissue disruption which begins several hours after neutrophil migration (Moshage, 1997). Following eccentric exercise, the debris produced by the muscle damage activates macrophages resident in the muscle tissue, which produce polypeptide messenger molecules known as cytokines. These play an important role in the inflammatory response and the subsequent clearance of the cell debris from the site of exercise induced muscle damage (Suzuki, 2002).

1.2. The Effect of Traumeel On Inflammatory Markers in the Blood
Traumeel® S (Heel GmbH, Baden-Baden, Germany) is a homeopathic-complex remedy used to treat trauma and inflammatory processes. It is a mixture of highly diluted (10^-1-10^-9) extracts from medicinal plants and minerals that is sold as an over the counter remedy in pharmacies in many countries. Its anti-inflammatory and analgesic effects have been demonstrated in clinical trials as well as in in vivo experimental models, including the carrageenin-induced oedema test and the adjuvant arthritis test (Conforti et al., 1997; Schmotz, 1998).

Traumeel® S has been reported to be non-cytotoxic to granulocytes, lymphocytes, platelets and endothelial cells and the intracellular processes of these cells are not impaired by Traumeel® S (Conforti et al., 1997; Porozov et al., 2004). The anti-inflammatory effect has been proposed to be the result of the activity of single components on the different phases of inflammation (Conforti et al., 1997, Porozov et al., 2004). Its efficacy in attenuating inflammatory processes involving muscles and joints, sprains and bruises has been demonstrated in placebo-controlled experimental and clinical studies (Conforti et al., 1997; Heine and Schmotz, 1998; Porozov et al., 2004). The mechanism by which Traumeel® S exerts its therapeutic effects however, remains to be clarified and its possible effects on the behaviour of immune cells, requires detailed investigation.
In vitro experiments revealed that interleukin-1β (IL-1β) activation was reduced by up to 70%, tumor necrosis factor-α (TNF-α) activation was reduced by up to 65% and interleukin-8 (IL-8) activation was reduced by 50% following the administration of Traumeel® S to in vitro immune cells (Human T cells, monocytes, and gut epithelial cells). Traumeel® S did not appear to affect T-cell and monocyte proliferation and maximal inhibition was maintained with dilution of the Traumeel® S stock material (Conforti et al., 1997; Porozov et al., 2004). In studies conducted on whole blood cultures, elevations in the concentration of Transforming Growth Factor Beta (TGF-β), which is a cytokine synthesized by Type 3 T-Helper (Th3) lymphocytes, were reported after administration of Traumeel® S (Heine & Schmotz, 1998).

In an in vivo study conducted by Jordaan (2006), 50 actively participating marathon runners were divided into a control (n = 24) and an experimental group (n = 26). The subjects used the Traumeel® S or placebo administration for seven days and followed their normal training programme. On the eighth day subjects underwent a downhill run on a treadmill at 75% of peak treadmill speed for 45 minutes at a gradient of -10%. Post exercise blood samples were drawn at the same time of day for four days (24hr, 48hr, 72hr, and 96hr post-exercise). The results of this study did not reveal significant changes in serum creatine kinase concentrations, or neutrophil, monocyte, basophil, eosinophil and lymphocyte counts. It was, however, reported that circulating cortisol concentrations in the Traumeel® S group were higher than in the placebo group at 48hr, 72hr and 96hr post-exercise, although immediate post-exercise concentrations were not reported (Jordaan, 2006).

The aim of the study is therefore to extend upon the work conducted by Jordaan (2006), focusing more deeply on systemic markers of inflammation with specific reference to cytokines.

1.3. Aims and Objectives of the study

1. To determine whether 90-minutes of downhill treadmill running at 75 % VO₂ peak results in an elevation of neutrophils, muscle enzyme (creatine kinase; CK), stress hormone (cortisol), type 1 T-helper (Th1) cytokine (IL-12p70), type 2 T-helper (Th2) cytokine (IL-6, IL-10), chemokine (IL-8) and pro-inflammatory cytokine (TNF-α) concentrations in the circulation.

2. To establish whether administration of Traumeel® S five days before and for three days after 90-minute downhill run significantly attenuates the above-mentioned systemic markers of muscle damage and/or an inflammatory response during the 72 hours following the trial.
1.4. Hypotheses
In keeping with the consistent findings in the literature regarding inflammatory response to downhill running and eccentric exercise (Pedersen et al., 1999; Malm et al., 2004 Peake et al., 2005a; McKune et al., 2006), the following one directional alternate hypothesis was set:

*Downhill running will result in an elevation of neutrophil count, creatine kinase, cortisol and pro/anti-inflammatory cytokine concentrations in the circulation.*

In the absence of consistent findings in *in vivo* exercise intervention studies following administration of Traumeel® S, the following null hypothesis was set prior to the onset of the work:

*Administration of Traumeel® S will not induce changes in post-exercise circulating stress hormone, IL-12p70, IL-6, IL-10, IL-8 and TNF-α concentrations following a 90-minute downhill run at 75% VO₂max.*

1.5. Limitations and Scope of the study
Due to the undisputed evidence which is now available regarding the repeated bout effect viz. the adaptive response to acute repeated exposure to similar bouts of eccentric exercise (Nosaka et al., 2005; McHugh, 2003; Nosaka and Newton, 2002; Stupka et al., 2001), it was not possible to conduct a crossover study. In order to eliminate a training effect as a potential confounder in the study, this double-blind, placebo-controlled study was therefore designed using pairs of runners who were as closely matched as possible to ensure sufficient external validity. As it was also necessary to design this study within the framework of financial constraints as well as difficulty in obtaining full compliance from the subjects, the sample size was restricted to 10 gender-matched couples (*n* = 20).

Two groups of ten (*n* = 20) ultradistance runners, who were matched for gender, Body Mass Index (BMI), training age, training status as well as at least two of the following: running biomechanics (foot-strike pattern), peak oxygen consumption (*VO₂* peak), 1609m running time and/or best performance in endurance running events within six months prior to commencement of the study, were randomised to Traumeel (TRAU) and Placebo (PLAC) trials in a strictly double-blinded protocol.

After field testing on an athletics track and maximal exercise test in a Human Performance Laboratory, each subject completed a -6 % downhill 90-min treadmill run at 75% *VO₂* peak.

Each subject ingested either a Traumeel preparation or a placebo (in tablet form) provided by Heel Pty Ltd., three times a day for five days prior to the 90-minute downhill trial. Circulating concentrations of neutrophils, CK, cortisol, and cytokine concentrations (TNFα, IL-6, IL-8, IL-10, IL-12p70), were
measured from venous blood samples taken pre-, immediately post (IPE), 24 hours post (24PE), 48 hours post (48PE) and 72 hours post (72PE) each exercise-trial, to establish whether a significant difference existed between each of the trials over the 4-day post-exercise recovery time-period. Subjective perceptions of post-exercise delayed onset muscle soreness (DOMS) were also monitored during the 72 hours following each exercise trial.
Chapter 2

Review of the Related Literature

2.1. Eccentric Exercise and Muscle Damage

Prolonged strenuous exercise has been shown to cause perturbations in immune function (Pedersen and Hoffman-Goetz, 2000). For a few hours following heavy exertion, several components of the innate and adaptive immune system are changed; white cell and neutrophil counts are raised, while lymphocyte numbers are reduced (Nieman, 2000). Many theories have been proposed as to the mechanisms behind this temporary alteration in immune function, including hormone-induced trafficking of immune cells and the direct influence of increased concentrations of stress hormones, prostaglandins, cytokines, leukocytes and expression of adhesion molecules (Suzuki et al., 1999, 2000; Smith et al., 2000).

Several studies have demonstrated that unaccustomed eccentrically biased exercise which involves lengthening of muscle while it is contracting, induces tissue damage. Although downhill running is a functional activity involving both concentric and eccentric contraction, there is a longer period during which the knee extensors and ankle flexors are being actively strained and the anterior-posterior braking impulse is almost double that of the propulsive impulse than during level running (Eston et al., 1995). Towards the end of the recovery phase of the downhill running gait, the hamstring muscles contract eccentrically to decelerate the forward swing of the lower limb that was initiated by the quadriceps (Petersen and Holmich, 2005). The knee extensor muscles then contract as they are lengthened after the foot touches the ground and the centre of mass is decelerating. In the lower leg, the ankle plantar flexors also work eccentrically to control the forward movement of the leg over the foot in early stance phase and facilitate heel lift, while ankle dorsiflexors have an eccentric work component as they control the descent of the forefoot. Following downhill running, eccentric muscle contraction therefore occurs in the gluteal, quadriceps, hamstring, and both anterior and posterior tibial muscles (Paul et al., 1978; Eston et al., 1995).

Maclntyre et al. (1995) suggest that eccentric exercise results in a greater amount of tension per active motor unit than concentric contraction during which the muscles shorten while contracting. Peake (2005a) further describes how during repeated eccentric contractions, first the weak and then the stronger sarcomeres, are progressively overstretched and the failure of the sarcomeres to reconnect, results in their disruption. When the muscle lengthens in an eccentric contraction, there may also be shearing of the myofibrils, specifically at the obliquely arranged fibres which occur at the musculotendinous junction (Noonan and Garrett, 1992). Ultrastructural damage occurs to the Z disks that anchor the myofilaments and several intermediate filaments within the sarcomere. Z disks appear to widen or disintegrate. While regional disorganisation and transverse tubule damage have been identified in electron microscopic studies.
(Proske and Morgan, 2001; Grobler et al., 2004), this structural disruption has also been reported to spread to adjacent areas of the muscles and can ultimately lead to damage to the membranes of the sarcoplasmic reticulum or the sarcolemma (Friden and Lieber, 2001). Disturbances of mitochondria, A-band and separation of the myofibrils have been reported (Peake, 2005a). In addition to the loss of cellular integrity which occurs early during heavy eccentric exercise, exercise-induced tissue damage has also been shown to include loss of the non-contractile cytoskeletal proteins, desmin, titin, nebulin and alpha-actin (Stupka et al., 2000; Friden and Lieber, 2001; Amat et al., 2007).

Several theories have been postulated in order to explain the increased muscle stiffness observed following eccentric exercise. It is proposed that the acute decrease in muscle compliance could be due to an increase in the Ca\textsuperscript{2+} ions released following myofilament and sarcolemmal disruptions (Fridén and Lieber, 1992; Jones et al., 1997; Whitehead et al., 2001). Swelling which may occur in and around the muscle and increase the pressure on adjacent structures, may also result in greater stiffness (Ross, 1999).

Damage to the muscle membrane also causes the leakage of muscle proteins including CK, lactate dehydrogenase, troponin-I, alpha-actin and myoglobin into the blood stream (Malm, 1999, Peters et al., 2005, Amat et al., 2007). Abnormally high circulating levels of enzymes such as CK are therefore taken to reflect changes in the integrity of muscle fibre membranes, either as a result of damage to, or increased permeability of the membrane to the enzyme (Eston et al., 1995). Elevated serum concentrations of CK have consistently been measured following eccentric activation of muscle as occurs during downhill running. When compared to the normal reference range for CK of 45-135 µ/L (Hirose et al., 2004), Mckune et al. (2006) found that a 60-minute bout of downhill running at a -13.5% gradient resulted in mean CK concentrations of 1241 (± 134.6) µ/L six hours post-exercise, 1133.7 (± 118.1) µ/L 12hr post-exercise and 826.8 (± 105.3) µ/L 24hr post-exercise. Jordaan (2006) reported pre-exercise CK concentrations in the blood of 251 (± 171) µ/L in the Traumeel® S group and 324 (± 439) µ/L in the placebo group, while 24h post exercise CK concentrations reached 422 (± 424) µ/L in the Traumeel® S group and 599 (± 448) µ/L in the placebo group, returning to pre-exercise levels by 48hrs post-exercise.

The clearance of muscle cell debris from the site of exercise-induced muscle damage is initiated by macrophages that are resident in muscle tissue. They produce cytokines which increase cell membrane permeability and the influx of lymphocytes, neutrophils, monocytes to the damaged tissue, and activate the release of cortisol from the adrenal cortex and acute phase proteins from the liver. This facilitates an inflammatory reaction and results in the clearance of the cell debris and subsequent healing of the tissue (Pedersen, 2000; Pedersen and Hoffman-Goetz, 2000; Pedersen et al., 2001).
2.2. Cytokines and Exercise

2.2.1. Introduction

Cytokines are a diverse family of intracellular polypeptides that have a signaling function and exert important influences on inflammatory and immune processes as well as hematopoiesis (Suzuki et al., 1999). They are divided into several different families including the interleukins, tumor necrosis factors, growth factors and colony stimulating factors (Smith, 2000). These have also been classified according their functions into those that mediate natural immunity, regulate lymphocyte activation, growth and differentiation, activate inflammatory cells, and stimulate hematopoiesis (Suzuki et al., 1999, 2000; Pedersen et al., 2001; Suzuki et al., 2002). Table 2.1 outlines some of the most commonly measured cytokines which fall into these categories, the cells which secrete them, their target cells and their general functions in the immune system.

Table 2.1: Five categories of cytokines commonly measured in exercise studies*

<table>
<thead>
<tr>
<th>Cytokine Classification</th>
<th>Secreted by</th>
<th>Target cells</th>
<th>Selected Primary Functions</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Pro-Inflammatory cytokines</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TNF-α</td>
<td>Macrophages, mast cells, NK cells</td>
<td>Macrophages, tumor cells</td>
<td>Induction and expression of adhesion molecules on leukocytes and induction of inflammatory response which activates vascular endothelium increasing its permeability and expression of IL-β; IL-6; IL-8</td>
</tr>
<tr>
<td></td>
<td>IL-1α</td>
<td>T helper cells, B cells, NK cells</td>
<td>Co-stimulation of Th1 cells, maturation and proliferation of B cells, activation of NK cells, inflammation, acute phase response, fever</td>
</tr>
<tr>
<td></td>
<td>IL-1β</td>
<td>Dendritic cells, B-cells, macrophages</td>
<td>Th cells, B cells, NK cells</td>
</tr>
<tr>
<td><strong>Anti-inflammatory cytokines</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-4</td>
<td>Th2-cells, memory CD4+</td>
<td>Activated B cells, T cells, macrophages</td>
<td>Proliferation and differentiation of Th-cells, IgG1 and IgE synthesis, mediates recruitment and activation of mast cells</td>
</tr>
<tr>
<td>IL-10</td>
<td>Th2 cells</td>
<td>B cells, macrophages</td>
<td>Inhibition of macrophage derived release of TNF-α; IL-2, IL-3, IFN-γ; suppress antigen presentation by macrophages</td>
</tr>
<tr>
<td><strong>Immunomodulatory cytokines</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-2</td>
<td>Th1-cells</td>
<td>Activated T and B cells, NK cells</td>
<td>Stimulation of the growth and proliferation of Th1-cell lymphocytes, facilitate production of immunoglobulins made by B cells and induces the differentiation and proliferation of NK cells</td>
</tr>
<tr>
<td>IL-5</td>
<td>Th2-cells</td>
<td>Activated B cells</td>
<td>Proliferation and differentiation IgA synthesis</td>
</tr>
<tr>
<td>IL-12</td>
<td>Macrophages and B-cells</td>
<td>Activated T cells, NK cells</td>
<td>Differentiation into CTL (with IL-2), activation of NK cells</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>Th1-cells, Tc-cells, NK cells</td>
<td>Activated B cells, Th2 cells</td>
<td>Viral replication MHC I expression, Ig class switch to IgG2a, pathogen elimination</td>
</tr>
</tbody>
</table>
### Multifunctional Cytokines

| **IL-6** | **Activated B cells, adipose cells, muscle cells** | **Plasma cells, Stem cells** | **Differentiation into plasma cells, antibody secretion, activates the acute phase response, stimulates immune response to tissue damage following trauma, involved in regulation of hematopoiesis, stimulation of cortisol release from adrenal medulla, signal in the synthesis of plasma proteins** |
| **Monocytes, Macrophages** | **Th2-cells, Stromal cells, Muscle cells** | **Phagocytes, tumor cells** | **Phagocytosis, cell death** |

**TNF-β**

- **Th1 and Tc-cells**
- **Th3-cells, Monocytes**

| **Monocytes, Macrophages** | **Activated B cells** | **Increasing IL-1 production by activated macrophages, inducing a class switch to IgA proliferating B cells** |

| **TGF-β** | **Chemotaxis of neutrophils, basophils, eosinophils, T-lymphocytes** |

### Chemokines

| **IL-8** | **Macrophages, Endothelial cells** | **Neutrophils** | **Chemotaxis of neutrophils, basophils, eosinophils, T-lymphocytes** |

### Colony stimulating factors

| **GM-CSF** | **Th-cells** | **Progenitor cells** | **Growth and differentiation of monocytes and DC** |


2.2.2.2. Pro-Inflammatory cytokines

### 2.2.1. Tumor Necrosis Factor Alpha (TNF-α)

Tumor Necrosis Factor Alpha (TNF-α), also sometimes referred to as cachexin and cachectin, initiates the systemic inflammatory response and is an important component of the acute phase reaction mediating hepatic release of several acute phase proteins. It is secreted mainly, but not exclusively, by macrophages and NK cells (Smirnova et al., 2003). It is also produced by other cells including lymphoid cells, mast cells, endothelial cells, cardiac myocytes, adipose tissue, fibroblasts and some neuronal tissue. It primarily targets macrophages and tumor cells.

The synthesis and release of TNF-α by tissue macrophages that are activated after engulfing and degrading foreign or microbial organisms, has important local and systemic effects. The major local effect of TNF-α is to initiate an inflammatory response. The vascular endothelium is activated resulting in an increase in vascular diameter and leading to increased local blood flow and redness. Vascular permeability is also increased resulting in the accumulation of fluid (swelling), immunoglobulins, complement and other blood proteins (Tracey and Cerami, 1993; Bagby et al., 1996; Ganong, 2005).
TNF-α is also instrumental in inducing the expression of adhesion molecules on the endothelium. These bind to the surface of leukocytes and increase the rate at which these cells (particularly neutrophils), diapedes through small blood vessel walls into the tissues. In this manner large numbers of monocytes and polymorphonuclear leukocytes migrate to the site of infection or inflammation (Nieman, 1997; Malm et al., 1999; Pederson and Toft, 2000).

A further important molecular change induced by TNF-α at the endothelial cell surface is induction of the expression of molecules that trigger blood clotting in small blood vessels. This assists in containing the inflammatory response and preventing its spread into the bloodstream which can be deleterious (Ganong, 2005; Stenvinkel et al., 2005; Juge-Aubry et al., 2005).

TNF-α has various functions including pro-inflammatory cytokine expression, apoptosis (cell death), cellular proliferation and differentiation, tumorigenesis, and viral replication. Its primary function is, however, the regulation and mediation of an inflammatory response. When this function is altered, and over or underproduction of TNF-α occurs, a variety of human diseases and syndromes, ranging from the overtraining syndrome (OTS) experienced by some athletes to malignancy, have been reported to occur (Tracey and Cerami, 1994; Taylor, 2002; Smirnova et al., 2003; Smith, 2004; Stenvinkel et al., 2005; Juge-Aubry et al., 2005).

In conjunction with the IL-1 family and IL-6, TNF-α also has many effects on end organs such as the liver and hypothalamus as a result of their secretion into the circulation. These are summarized in Table 2.2.

**Table 2.2: The effects of TNF-α on end organs**

<table>
<thead>
<tr>
<th>Organ/Tissue</th>
<th>Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>Stimulates and mediates the acute phase response and consequently increases circulating concentrations of C-reactive protein (CRP) and other mediators. It causes insulin resistance by promoting serine-phosphorylation of insulin receptor substrate-1, which inhibits insulin signaling.</td>
</tr>
<tr>
<td>Macrophages</td>
<td>Activates phagocytosis, production of IL-1 and the inflammatory lipid prostaglandin E2 (PGE2).</td>
</tr>
<tr>
<td>Hypothalamus</td>
<td>Activates the hypothalamic-pituitary-adrenal axis causing the release of corticotropin releasing hormone (CRH), appetite suppression and pyrexia.</td>
</tr>
<tr>
<td>Other tissue:</td>
<td>Attracts neutrophils very strongly and aids in their adhesion to endothelial cells. Increasing concentration of TNF will cause the cardinal signs of inflammation to occur: heat, swelling, redness, clotting and pain. High systemic concentrations of TNF may precipitate shock-like symptoms.</td>
</tr>
</tbody>
</table>

*Tracey and Cerami 1994; Taylor, 2002; Smirnova et al., 2003; Juge-Aubry et al., 2005; Smith, 2003; 2004*

Release of TNF-α into the bloodstream can, however, also have damaging effects. These include systemic oedema, hypoproteinemia and neutropenia associated with disseminated intravascular coagulation, depletion of clotting factors, multiple organ failure and death (Stenvinkel et al., 2005; Juge-Aubry et al., 2005).
Several exercise studies have examined concentrations of TNF-α post exercise. These are summarised in Table 2.3. Although some studies revealed a slight mean increase, ranging from 0.1 to 4.3 fold, immediately post exercise (Ostrowski et al., 1998a, 1999; Nieman et al. 2000; Toft et al., 2000; Starkie et al., 2001; Smith et al., 2007), the reported values all remain within physiologically normal ranges (Suzuki et al., 2002).

Numerous reports (Croisier et al., 1999; Toft et al., 2002; Hirose et al., 2004) in particular those following eccentric exercise (Hirose et al., 2004), however, indicate that there is not a significant exercise-induced increase in this pro-inflammatory cytokine concentration in the circulation during exercise. Several factors may account for this. On the one hand, the half-life of circulating TNF-α is short (14 - 18 minutes) and the kidney is a major organ for TNF clearance. A rapid renal clearance from the circulation into the urine has therefore been suggested as one of the reasons for the failure of TNF-α concentrations in venous blood to be elevated post exercise (Suzuki et al., 2002). Exercise has also been shown to stimulate the release of soluble TNF-α receptors which inactivate systemic TNF-α release (Ostrowski et al., 1999b).

2.2.2.2. Interleukin 1β (IL-1β)

Interleukin-1β is a pro-inflammatory cytokine which is involved in the defence against foreign pathogens. It is produced by activated macrophages, dendritic cells and B-cells (Ganong, 2005). It targets T-helper (Th) cells, B-lymphocytes, NK cells and is responsible for the co-stimulation of Th2-cells, the maturation and proliferation of B cells, and the activation of NK cells. (Bagby et al., 1996; Pedersen and Hoffman-Goetz, 2000; Smith, 2000; Suzuki et al., 2002; Peters, 2004).

As does TNF-α, it has been shown to increase the expression of adhesion factors on endothelial cells to enable the movement of leukocytes to the infection site in the tissue (Ganong, 2005, Stenvinkel et al., 2005). It also helps to activate thermoregulatory center in the hypothalamus, leading to an elevation of body temperature (pyrexia) and is therefore referred to as an endogenous pyrogen (Bagby et al., 1996; Huising et al., 2004; Juge-Aubry et al., 2005). Its production in peripheral tissue has also been associated with hyperalgesia (increased sensitivity to pain) associated with pyrexic states (Dinarello, 1994; Dunn et al., 2001; Juge-Aubry et al., 2005). In keeping with these systemic effects, Smith (2003; 2004) reported that increased levels of interleukin 1β may be responsible for the effects of over training syndrome (OTS) in endurance athletes.

Pro-inflammatory cytokines act in concert with specific cytokine inhibitors and soluble anti-inflammatory cytokine receptors (the IL-1 receptor antagonist (IL-1ra), TNF-α receptors, IL-4, and IL-10) to regulate the inflammatory response to exercise induced microtrauma (Richards and Gauldie,
thus their physiologic role in inflammation and pathologic role in systemic inflammatory states are increasingly recognized (Dinarello, 1994; Richards and Gauldie, 1995).

The reported changes in IL-1β concentration in venous blood following exercise longer than 2hrs duration is summarized in Table 2.3. Although a few studies have reported increases in concentration of up to 200% (Nieman et al., 2000; Ostrowski et al., 1999), Ostrowski et al. (1998b) only report increases of 50% and other studies have reported no change (Suzuki et al., 2000).

The inconsistencies in the literature with regard to IL-1β levels following exercise are attributed to different measurement intervals, differing measurement techniques and differences in exercise protocols between studies (Ostrowski et al., 1998b; 1999; Suzuki et al., 2002; Smith et al., 2007). A further factor to which inconsistencies could be attributed, is failure to adjust for the haemoconcentration which so frequently occurs following prolonged intense exercise (Mucci et al., 2000).

It has been reported that the relatively lesser increment in TNF-α and IL-1β concentrations may be due to the release of the above-mentioned cytokine inhibitors in the blood (including IL-1ra, TNF-α antagonists and the anti-inflammatory cytokine IL-10). These may restrict the magnitude and duration of the pro-inflammatory response to exercise (Ostrowski et al., 1998b; 1999; Suzuki et al., 2002; Smith et al., 2007).

Table 2.3 Pro-Inflammatory response to endurance exercise longer than 2hr in duration with specific emphasis on TNF-α and IL-1β concentrations in venous blood

<table>
<thead>
<tr>
<th>Author/s</th>
<th>Methodology</th>
<th>Results/Changes</th>
<th>Assay/ Sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ostrowski et al., 1998b</td>
<td>16 male marathon runners, marathon race, mean: 3h, 17min</td>
<td>TNF→: 2.5 ↑ (Post), 1.9 ↑ (Post 2h), IL - 1β: 50% ↑ (Post), NC (Post 1-4h)</td>
<td>Elisa, P</td>
</tr>
<tr>
<td>Ostrowski et al., 1999</td>
<td>10 male marathon runners, marathon race, mean: 3h, 27min</td>
<td>TNF→: 2 times ↑ (Post-Post 1.5h), Gradually to NC (Post 3.5h), IL - 1β: 1.5 - 2 times ↑ (Post-Post 0.5h), NC (Post 1-4h),</td>
<td>Elisa, P</td>
</tr>
<tr>
<td>Nieman et al., 2000</td>
<td>98 marathon runners, marathon race, mean: 3.8h</td>
<td>TNF→: 10% ↑ (Post), NC (Days 1 and 2), IL - 1β: 2 times ↑ (Post), NC (Days 1 and 2)</td>
<td>Elisa, P</td>
</tr>
<tr>
<td>Peters et al., 2001a</td>
<td>16 runners, 90km ultramarathon, 9-10h</td>
<td>TNF→: NC (Pre, Post, 24h and 48h post)</td>
<td>Elisa, P</td>
</tr>
<tr>
<td>Peters et al., 2001b</td>
<td>29 runners, 90km downhill ultramarathon, 9-10h</td>
<td>TNF→: NC Pre, Post, 24h and 48h post) IL-1β: NC Pre, Post, 24h and 48h post)</td>
<td>Elisa, P</td>
</tr>
<tr>
<td>Starkie et al., 2001</td>
<td>5 male runners, marathon race, 2h-31min - 3h 25min</td>
<td>TNF→: 4.3 times ↑ (Post), 4 times ↑ (Post 2h), NC (Days 1 and 2)</td>
<td>Elisa, P</td>
</tr>
<tr>
<td>Suzuki et al., 2003</td>
<td>10 male endurance athletes, 42 km marathon</td>
<td>TNF→: Pre, Post (ND): &lt;4.3 pg/ml); IL - 1β: ND</td>
<td>Elisa, P</td>
</tr>
</tbody>
</table>

NC: No change; ND: non detectable; P: Plasma
2.2.3. Multifunctional Cytokines

2.2.3.1. Interleukin-6 (IL-6)

IL-6 is one of the most frequently measured cytokines in exercise immunology. Secreted by T-cells, fibroblasts, vascular endothelial cells and macrophages to stimulate the immune response to tissue damage following trauma (Bagby et al., 1996), it is produced in larger amounts than any other cytokine following exercise. It has also been found to be produced by muscle during exercise and has hence been referred to as a myokine (Pedersen et al., 2001). Its level is markedly increased in plasma following activity involving severe eccentric exercise and has been reported to increase by more than 100 fold following marathon and ultra-marathon races. (Ostrowski et al., 1999; Starkie et al., 2001; Table 2.4).

Increased plasma concentrations of IL-6 have been shown to act on the hypothalamic pituitary axis (HPA) at three levels:

1. the hypothalamus by stimulating secretion of corticotropin releasing hormone (CRH)
2. anterior pituitary corticotropes by eliciting adrenocorticotropic hormone (ACTH) release
3. the adrenal gland by enhancing steroid hormone secretion by adrenocortical cells

Stimulation of the adrenal cortex to increase its release of cortisol into the blood stream has in turn been shown to stimulate the release of a number of compensatory anti-inflammatory cytokines, such as IL-10 and IL-1 receptor antagonists (Bagby et al., 1996; Suzuki et al., 1999; Pedersen and Hoffman-Goetz, 2000; Suzuki et al., 2000; Smith, 2000; Pedersen et al., 2001; Peters, 2004).

IL-6 also causes hepatocytes to synthesize plasma proteins such as fibrinogen which contribute significantly to the acute phase response (Tilg et al., 1994; Suzuki et al., 1999). The activation pathway of IL-6 in the production of plasma proteins is different, but contributes to the pathways that are activated by other cytokines such as IL-1 and TNF-α in the production of similar plasma proteins. IL-6 is also a co-stimulator of T-cells and of thymocytes and is a co-factor, together with other cytokines, for the production of haematopoetic cells from the bone marrow and subsequent exercise-induced neutrophilia (Tilg et al., 1994; Suzuki et al., 2002; Smith, 2000; Pedersen et al., 2001).

Although Interleukin-6 has in the past been referred to as an “inflammatory” or “proinflammatory cytokine,” the current trend is to classify it as an “inflammation-responsive” or anti-inflammatory cytokine rather than a pro-inflammatory cytokine, since IL6 does not directly induce inflammation but reacts to it (Pedersen et al., 1998, Ostrowski et al., 1999; Steensberg et al., 2003; Peters, 2004). Smith (2003, 2004) suggests that increased levels of this cytokine in the circulation are also related to the systemic effects associated with over training syndrome (OTS).
It has been shown that contracting skeletal muscles produce large amounts of IL-6 and that muscle contractions per se are a stimulus for the production of muscle-derived IL-6. Many studies have revealed that IL-6 mRNA is markedly elevated in muscle biopsies carried out post exercise (Ullum et al., 1994; Ostrowski et al., 1998; Malm et al., 2000; Keller et al., 2001; Starkie et al., 2001; Steensberg et al., 2001; Malm et al., 2004). The increase has been shown to be greatest when muscle mass recruitment is greatest. Treadmill running has therefore been found to elicit a greater release of IL-6 into the circulation than cycle ergometry. Circulating concentrations of IL-6 have been found to be considerably lower following downhill running than uphill running or running on a level terrain (Ostrowski et al., 1998b).

In addition, it is also evident that in eccentric exercise the IL-6 kinetics differs greatly from that of concentric exercise as shown by Bruunsgaard et al. (1997). Therefore, in concentric exercise, the IL-6 levels increase during exercise and decrease immediately after cessation of the exercise (Steensberg et al., 2001). In contrast, the modest, prolonged increase in IL-6 is found after eccentric exercise is likely to be a sign of repair mechanisms after muscle damage (Bruunsgaard et al., 1997).

Many studies have demonstrated increased levels of IL-6 (up to 128 times) following bouts of severe eccentric exercise such as downhill and marathon running (Ostrowski et al., 1998b; 1999; Singh et al., 1999, Nieman et al., 2001, Starkie et al., 2001; Peters et al., 2001; Smith et al., 2007). These high levels are primarily attributed to the muscle damage associated with severe eccentric exercise (Pedersen and Hoffman-Goetz, 2000) and as a response to the subsequent activation of the pro-inflammatory cytokine cascade (Singh et al., 1999; Nieman et al., 2001; Pedersen, 2000; Pedersen and Hoffman-Goetz, 2000; Pedersen et al., 2001).

### Table 2.4 Inflammatory response to endurance exercise longer than 2hr in duration with specific emphasis on IL-6 concentrations in venous blood

<table>
<thead>
<tr>
<th>Author/s</th>
<th>Methodology</th>
<th>Results/Changes</th>
<th>Assay/Sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ostrowski et al., 1998b</td>
<td>16 male marathon runners, marathon race, mean: 3h, 17min</td>
<td>63 times ↑ (Post), 15 times ↑ (Post 2h)</td>
<td>Elisa, EP</td>
</tr>
<tr>
<td>Ostrowski et al., 1999</td>
<td>10 male marathon runners, marathon race, mean: 3h, 27min</td>
<td>128 times but gradually declined thereafter</td>
<td>Elisa, EP</td>
</tr>
<tr>
<td>Singh et al., 1999</td>
<td>10 female trained runners, Run, 65%-70% max to exhaustion</td>
<td>80% ↑ (During 30min), 5 times ↑ (during 60 min), 7 times ↑ (during 90 min), 11 times ↑ (Post), 10 times (Post 1h), NC (post 24h)</td>
<td>Elisa, EP</td>
</tr>
<tr>
<td>Nieman et al., 2000</td>
<td>98 marathon runners, marathon race, mean: 3.8h</td>
<td>30 times ↑ (Post), NC (post 24h, 48h)</td>
<td>Elisa, P</td>
</tr>
<tr>
<td>Peters et al., 2001b</td>
<td>29 runners, 90km marathon, 9-10h</td>
<td>25 times ↑ (Post), NC (Post 24h and 48h)</td>
<td>Elisa, EP</td>
</tr>
<tr>
<td>Starkie et al., 2001</td>
<td>5 male runners, marathon race, 2h-31min – 3h 25min</td>
<td>about 100 times ↑ (Post) but gradually declined thereafter up to Post 24h</td>
<td>Elisa, EP</td>
</tr>
<tr>
<td>Suzuki et al., 2003</td>
<td>10 male endurance athletes, 42 km marathon</td>
<td>80 times</td>
<td>Elisa, EP</td>
</tr>
</tbody>
</table>
2.2.4. Anti-Inflammatory Cytokines

2.2.4.1. Interleukin-10 (IL-10)

Interleukin-10 (IL-10) is the most important anti-inflammatory cytokine within the human immune response. It is primarily synthesized by CD4+ Th2 cells, monocytes, and B cells and circulates as a homodimer consisting of two tightly packed 160-amino-acid proteins (Opal et al., 1998; Howard and O'Garra, 1992). After engaging its high-affinity 110-kd cellular receptor, IL-10 inhibits monocyte/macrophage-derived TNF-α, IL-1, IL-6, IL-8, IL-12, granulocyte colony-stimulating factor, MIP-1α, and MIP-2α (Clarke et al., 1998; Gerard et al., 1993; Marchant et al., 1994).

It is also sometimes known as human cytokine synthesis inhibitory factor (CSIF) as it is identical to this factor and has the same function viz. it inhibits the synthesis of a number of cytokines such as IFN-γ, IL-2, and TNF-β in the Th1 subpopulations of T-cells (Fiorentino et al., 1989; Howard and O'Garra, 1992). IL-10 is secreted by Th2 cells, targets B cells and macrophages. It is stimulatory towards certain T cells, mast cells, and B cells. It inhibits the synthesis of pro-inflammatory cytokines like INF-γ, IL-2, IL-3, and TNF-α which are secreted by macrophages and Th1 cells. IL-10 also suppresses the antigen presentation capacity of macrophages and lymphocytes and is released by cytotoxic T-cells to inhibit the actions of NK cells in response to viral infection (Suzuki et al., 1999; Smith, 2000; Bagby et al., 1996; Peters, 2004; Pedersen and Hoffman-Goetz, 2000; Trinchieri, 2007).

IL-10 inhibits cell surface expression of major histocompatibility complex class II molecules, B7 accessory molecules, and the lippolysaccharide (LPS) recognition and signaling molecule CD14 (Opal et al., 1998). It also inhibits nuclear factor kappa-B (NF-κ-B) translocation after LPS stimulation (Brandtzaeg et al., 1996; Clarke et al., 1998) and promotes degradation of messenger RNA for the proinflammatory cytokines (Opal et al., 1998). In addition to these activities, IL-10 attenuates surface expression of TNF receptors and promotes the shedding of TNF receptors into the systemic circulation (Dickensheets et al., 1997; Joyce et al., 1994).

Studies have reported between 27 to 100 fold increases in plasma IL-10 following exhaustive endurance exercise (Ostrowski et al., 1999; Nieman et al., 2000, Peters et al., 2001b, Suzuki et al., 2003). Suzuki et al. (2003) also confirmed a positive correlation between the percentage increase of IL—6 (80 fold) and IL-10 (100 fold) following participation in a competitive marathon race by 10 male endurance trained athletes between the ages of 16 and 19 years ($r = 0.78$, $p < 0.01$). This supports previous findings of the induction of IL-10 by the production of IL-6 (Nieman et al., 2003; Steensburg et al., 2001; 2003; Pedersen et al., 2003).
Others have reported no increase in plasma IL-10 levels following short durations of high intensity exercise (Bruunsgaard et al., 1997; Smith et al., 2000; 2007). These findings suggest that high intensity exercise of extended durations only, may induce the release of IL-10 into the circulation (Suzuki et al., 2002; Smith et al., 2007).

2.2.4.2. Interleukin 1-Receptor Antagonist (IL1-ra)

IL1-ra is, as its name suggests, is a polypeptide receptor antagonist that is secreted by macrophages, TH₂ cells and monocytes (Arend, 1993, 2000; Dinarello, 1994). It competes with IL1-α and IL1-β for the same receptor on the cell membrane. This is the only known function of IL1-ra (Hirsch et al., 1996; Arend, 2000). It was initially called IL-inhibitor after its discovery in 1984. As a result of it binding to the same receptor on the cell membrane as the IL1 family of interleukins, it prevents these cytokines from signaling the cell (Dinarello, 1994). It is thus used in the treatment of autoimmune diseases like, rheumatoid arthritis where the role of IL1 is significant in the pathophysiology of the disease. IL1-ra has been reported to be much more similar in structure to IL1-β than it is to IL1-α. Four protein isoforms of IL1-ra have been reported, three of which are intracellular and one is a secreted from. However, all three have the same affinity for the IL1 receptor on the cell membrane (Arend, 1993; 2000; Muzio et al., 1995). As previously mentioned, the interleukin-1 cytokines are pro-inflammatory in nature. Therefore, IL1-ra will have an antagonistic effect on their function. Thus the balance between IL1-α, IL1-β and IL1-ra dictates the dampening or progression of an inflammatory reaction. (Dinarello, 1994; Hirsch et al., 1996; Arend, 2000, Suzuki et al., 2000, Suzuki et al., 2002).

Several studies have demonstrated elevated levels of IL1-ra following exhaustive exercise. These levels range from between 20 fold increases (Peters et al., 2001b) to 40 fold (Tofit et al., 2000) and even 214 fold (Suzuki et al., 2002). The different levels reported have been postulated to be due to different exercise workloads and differing assay techniques (Suzuki et al., 2002). Endurance exercise in itself has also been reported to cause an elevation in IL1-ra levels in several studies (Ostrowski et al., 1999; 2000; Pedersen et al., 2001, Peters et al, 2001b; Tofit et al., 2002, Steensberg et al., 2003; Peake et al., 2005b).

2.2.5. Chemokines

2.2.5.1. Interleukin-8

Interleukin-8 (IL-8) is a chemokine that is produced by muscles, macrophages, endothelial cells and some epithelial cells. Endothelial cells that produce IL-8 store the cytokine in storage vesicles called Weibel-Palade bodies (Utgaard et al., 1998; Wolff et al., 1998). Upon encountering an antigen, macrophages release IL-8 which attracts neutrophils which adhere to and diapadese through the vascular endothelial cells to the region of inflammation (chemotaxis; Utgaard et al., 1998). IL-8 is therefore sometimes referred to as a Neutrophil Chemotactic Factor (NCF) or Neutrophil Activating Peptide 1 (NAP-1). It has also been
shown to display chemotactic activity for basophils, eosinophils and T-lymphocytes (Kishimoto et al., 1995; Van der Poll et al., 1997; Febbraio and Pedersen, 2005; Juge-Aubry et al., 2005).

Some studies have revealed a slight increase in IL-8 levels in the blood following exercise of a moderate intensity (Henson et al., 2000; Suzuki et al., 2002). However, following intense periods of exhaustive exercise, IL-8 levels have been shown to be significantly elevated in the blood (Nieman et al., 2000; Suzuki et al., 2000; Nieman et al., 2001; Ostrowski et al., 2001; Peters et al., 2005; Smith et al., 2007). Peters et al. (2005) reported increases from 3.7 to 30.2 pg/ml following a downhill ultra-marathon race. It has also been demonstrated that severe exercise for a short duration causes the release of IL-8 into the circulation (Mucci et al., 2000; Suzuki et al., 2002; Smith et al., 2007).

2.2.6. Immunomodulatory Cytokines

2.2.6.1. Interleukin-12

Interleukin-12 (IL-12) is secreted by B-cells and macrophages (Ganong, 2005) and refers to a collection of IL-12 related proteins. The bioactive form of IL-12 is a 75 kDa heterodimer (IL12p70) comprising independently-regulated disulfide-linked 40 kDa (p40) and 35 kDa (p35) subunits. The p40 subunit exists extracellularly as a monomer (IL12p40 1) or dimer (IL-12p40 2) and can antagonize the action of IL12p70 (Bagby et al., 1996; Suzuki et al., 1999; Smith, 2000; Pedersen and Hoffman-Goetz, 2000; Kalinski et al., 2001).

In conjunction with the action of other cytokines (IL-2, in particular), interleukin-12 helps to activate cytotoxic T-cells. It also stimulates the proliferation of NK cells and Th1 cells and is involved in the differentiation of immature T cells into Th1 cells. It is therefore sometimes referred to as a T cell stimulating factor. It also assists in the stimulation and production of interferon-gamma (IFN-γ) and tumor necrosis factor-alpha (TNF-α) which are produced by Th1 cells and NK cells. IL-12 helps to reduce the suppression of IFN-γ which is controlled by IL-4. IL-12 displays anti-angiogenic activity (suppression of new blood vessel production). This is achieved by it increasing the production of IFN-γ, which in turn, stimulates the production of a chemokine called inducible protein-10 (IP-10 or CXCL10). IP-10 mediates the anti-angiogenic effect (Pedersen and Hoffman-Goetz, 2000; Kalinski et al., 2001).

There is conflicting evidence in the literature regarding the effects of exercise on the levels of circulating IL-12 in the blood. For example, some studies reported increased levels following high intensity exercise with heat exposure, while no elevation in the absence of heat exposure has also been reported (Akimoto et al., 2000; Peake et al., 2005a; 2005b).
While inconsistencies and lack of reproducibility have been attributed to the rapid down-regulation of IL-12 levels following exercise and also to different measurement methods and ELIZA kits employed (Akimoto et al., 2000; Suzuki et al., 2002). Suzuki et al. (2003) have more recently differentiated between the response of IL-12p40 and IL-12p70. They reported that following a marathon race in which plasma concentration of IL-6 increased by 80 fold and IL-10 by 100 fold, cytokines promoting cellular immunity including IL-2, IFN-γ, TNF-α and IL-12p70 did not rise. IL-12p40, however, increased markedly. In contrast, an incremental exercise test to exhaustion on a treadmill did not result in an elevation in the IL-12p70 or IL-12p40 content of venous blood immediately after the exercise test when values were adjusted for hemoconcentration and plasma volume changes (Suzuki et al., 2003). One hour post-test IL-12p40 concentrations, however, did increase significantly from 46.1 pg/ml to 72.9 pg/ml (p < 0.01), but IL-12p70 remained unchanged. Suzuki et al. (2003) propose that the increase in the production of IL-12p40 and cytokines mediating inflammation is the result of exercise-induced release of prostaglandin E₂, cAMP, β-adrenergic agents and histamine.

2.2.7. Pro and Anti-inflammatory Cytokine Response to Eccentric Endurance Exercise

Figure 2.1: The Pro-Inflammatory cytokine cascade depicting the negative feedback mechanism

In response to tissue microtrauma which occurs following eccentric exercise, macrophages which are resident in the injured tissue, become activated, releasing TNF-α which has been shown to activate a pro-inflammatory cytokine cascade with the subsequent release of IL-1β and IL-6 (Figure 2.1; Suzuki et al., 1999; Suzuki et al., 2000; Pedersen et al., 2001). Increased circulating concentrations of IL-6, in turn, stimulate the adrenal medulla to increase its release of cortisol into the blood stream which stimulates the release of a number of compensatory anti-inflammatory cytokines, such as IL-10 and IL-1 receptor
antagonists (Figure 2.1; Pedersen and Hoffman-Goetz, 2000). These play a crucial role in containment and isolation of the inflammatory process (Bagby et al., 1996; Peters, 2004).

While the presence of multiple cytokines (TNF-α, IL-1β, IL-6, IL-2 receptors, and IFN-γ) in urine after exercise reflects the expression of a broad spectrum of cytokines in response to exercise (Sprenger et al., 1992), Pedersen et al. (1998) suggest that the type of physical activity as well as the intensity and duration of the exercise may affect the concentrations of pro-inflammatory cytokines measured in the blood. In reviewing the findings in the literature regarding the pro-inflammatory cytokine response to endurance exercise longer than 2 hr (Table 2), Ostrowski et al. (1998), Ostrowski et al. (1999a), Singh et al. (1999), Nieman et al. (2000), Starkie et al. (2001), and Peters et al. (2001) showed that after a marathon race, TNF-α and IL-1β concentrations in the vascular compartment increased by a maximum of approximately 4.3-fold (Starkie et al., 2001), whereas the concentrations of IL-6 increased by as much as 128 fold (Ostrowski et al., 1999). As both concentrations of IL-10 and IL-1ra, however, increase substantially following prolonged eccentric exercise (Nieman et al., 2001; Peters et al., 2001b), it has been suggested that the relatively lesser increment in TNF and IL-1 concentrations in the blood is due to the release of cytokine inhibitors including [IL-1ra and TNF receptors (TNF-R)] and the anti-inflammatory cytokine IL-10 (Ostrowski et al., 1999b; Figure 2.1). These findings suggest that cytokine inhibitors and anti-inflammatory cytokines may restrict the magnitude and duration of the pro-inflammatory response to exercise.

2.3. The Th1 – Th2 Differentiation; Corresponding Cytokine Production And its Effects.

2.3.1. Introduction

Although cytokines are produced by a variety of cells including the vascular endothelium, macrophages and circulating leukocytes, cytokines produced by helper T-cells are special subpopulations that provide help to other cells of the immune system in mounting immune responses.

According to Morel and Oriss (1998), the CD4 T helper (Th) and the CD8 T cytotoxic (Tc) cells can be divided into type 1 (Th1 and Tc1) and type 2 (Th2 and Tc2) cells according to their cytokine profile. Th1 cells drive cellular immunity and produce IL-2, TNF-α and interferon-gamma (INF- IFN-γ), while Th2 cells that activate the humoral response, produce a variety of anti-inflammatory cytokines, including IL-4, IL-5, IL-6, IL-10, and IL-13 (Evans et al., 2000). Type 1 immunity, also referred to as cell mediated immunity, involves macrophage activation and the recruitment and stimulation of cytotoxic cells such as Tc-cells and NK cells. This is stimulated by and effective against intracellular pathogens such as viruses and various intracellular bacteria, including tuberculosis bacilli (Evans et al., 2000; Romagnani, 2000). In contrast, extracellular pathogens, like helminthic (worm) parasites, and most forms of bacteria stimulate
Type 2 immunity which involves the humoral immune response and the production of most types (isotypes) of antibodies.

The balance of both types of T-helper cells can be influenced and regulated by the very cytokines they secrete. For example, IFN-γ, secreted by Th1 cells, can inhibit the proliferation of Th2 cells, while IL10, secreted by Th2 cells, can suppress Th1 functions by inhibiting their cytokine production. The Th2 cytokine IL-4, inhibits the differentiation and/or expansion of Th1 cells. Therefore, the functional subsets of T-helper cells are mutually antagonistic such that the predomination of a subset within an infection, may determine its outcome (Mackay, 2001; Romagnani, 2000; Kelso et al., 1991).

Through the activities of the types of cytokines produced, Th1 and Th2 cells are able to cross regulate each other. This control mechanism helps prevent the inflammatory reactions in response to pathogens, getting out of control. However, it is also possible that imbalances may precipitate inappropriate reactions. It is postulated that some autoimmune disorders may involve overactive Th1 cells, while the exacerbation of other conditions such as allergies, may involve overactive Th2 responses (Romagnani, 2000; Bonecchi et al., 1998 Smith, 2003; 2004).

The molecular mechanisms by which the evolution of these different T-helper cell types occurs from common precursors, are still not completely understood. Thp cells (p for precursor) are believed to be the precursor cells of Th0 cells that, in turn, are thought to be precursor cells that develop into either Th1 or Th2 cells (Romagnani, 2000; Kelso et al., 1991).

While Th1 cells are responsible for stimulating strong cellular immunity, they only activate weak and transient antibody responses. Generally, Th1 responses are stimulated by intracellular pathogens (viruses, some mycobacteria, some yeasts, and some parasitic protozoans) and by IL-12 (Lucey et al., 1996). Th1-type cells secrete high levels of IL-2, TNF-α, and IFN-γ. This activates macrophages and promotes cell-mediated immune responses against invasive intracellular pathogens (Bonecchi et al., 1998; Romagnani, 2000).

On the other hand, chronic stress states have been found to skew the balance against type 1 immunity in favour of type 2 immunity (Elenkov and Chrousos, 1999; Evans et al., 2000; Romagnani, 2000). In particular, IL-6 has been shown to induce Th2 polarization by stimulating the initial production of IL-4.

More recently a third category of Th-cell has been identified. Th3 cells are CD4 (+) regulatory cells associated with immune mechanisms involving oral tolerance towards antigens. These cells are characterised by the secretion of TGF-β and they have suppressive properties for Th1 and Th2 cells (Mosmann and Coffman, 1989; Bonecchi et al., 1998; Romagnani, 2000). The pleiotropic anti-inflammatory growth factor transforming growth factor-β1 (TGF-β1) belongs to a family of growth factors.
that has diverse effects on cellular differentiation, activation, and proliferation. It has also been shown to act as a chemoattractant for monocytes and macrophages and to inhibit the cells required for the inflammatory process. It is therefore regarded as an anti-inflammatory cytokine that aids in wound healing by limiting the inflammatory response (Bonecchi et al., 1998; Elenkov and Chrousos, 1999).

2.3.2 Regulation of the Th1 – Th2 Differentiation

Little is currently known about the physiological regulation of type 1 and type 2 T cell balances. It has been suggested that exercise is an appropriate model to study how the type 1 and type 2 cell balances are regulated. Both cortisol and epinephrine have been found to inhibit the production of type 1 T cell cytokines (Franchimont et al., 2000). Regulation of T-cell activation by the anti-inflammatory cytokines is also a crucial early control element in this process (Romagnani, 2000).

Interleukin-4 (IL-4) is a highly pleiotropic cytokine that is able to influence Th cell differentiation. It is a 20-kd glycoprotein produced by mature Th2 cells and cells from the mast cell or basophil lineage. It drives Th2 responses, mediates the recruitment and activation of mast cells, and stimulates the production of IgE antibodies via the differentiation of B cells into IgE-secreting cells (Mosmann et al., 1986; Kelso, 1995). Early secretion of IL-4 leads to polarization of Th cell differentiation toward Th2-like cells (Mosmann et al., 1986). Th2-type cells secrete their own IL-4, and subsequent autocrine production of IL-4 supports Th2 cell proliferation. The Th2- cell secretion of IL-4 and IL-10 also leads to the suppression of Th1 responses by down-regulating the production of macrophage-derived IL-12 and inhibiting the differentiation of Th1-type cells (Mosmann et al., 1986; Kelso, 1995).

IL-10 is also a potent inhibitor of Th1 cytokines, including both IL-2 and IFN-γ. This activity accounts for its initial designation as cytokine synthesis inhibition factor (Opal et al., 1998; Lalani et al., 1997; Howard and O’Garra, 1992). In addition to its activity as a Th2 lymphocyte cytokine, IL-10 is also a potent deactivator of monocyte/macrophage proinflammatory cytokine synthesis (Clarke et al., 1998).

2.3.3. The Th1 – Th2 Shift Effect During Exercise

To further understand how the type 1 and type 2 T cell balances are regulated, it is of interest to investigate the effect of exercise. As viruses often cause URTI and type 1 T cells are crucial in the defense against intracellular pathogens (Steensberg et al., 2001), the observation of an increased risk of URTI after an acute bout of heavy exercise, may be a consequence of an impaired type 1 T cell response (Smith, 2000).

It has been shown that the immune response to strenuous exercise has some similarities to that of other physical stress conditions such as surgery, trauma, sepsis, and burn (Pedersen and Hoffman-Goetz, 2000; Smith, 2003; 2004). Interestingly, it has recently been demonstrated that major surgery suppresses
maximal production of Th1 cytokines without influencing the Th2 cytokines (Berguer et al., 1999), inferring that surgery and strenuous exercise influence the Th1 and Th2 responses in a similar manner.

Figure 2.2: The effect of prolonged exercise on the Th1-Th2 cytokine balance following exhaustive exercise

During intense and prolonged exercise, lymphocytes are recruited to the blood (Pedersen and Hoffman-Goetz, 2000). However, in the post-exercise period, the number of circulating lymphocytes declines below pre-exercise values. Concomitantly, the plasma levels of proinflammatory cytokines are elevated. Thus the level of plasma IL-6 may be enhanced more than 100-fold (Ostrowski et al., 1999), but increases in plasma IL-12 have been reported to be inconsistent and small (Akimoto et al., 2000).

The greatest elevations in inflammation-mediated cytokine levels have been reported after eccentric exercise. Bruunsgaard et al. (1997) compared concentric and eccentric exercise at the same relative oxygen uptake. They found that although the catecholamine levels did not differ between the two experiments, the creatine kinase (CK) increased almost 40-fold 4 days after eccentric exercise. No changes were observed in the CK concentration in relation to concentric exercise. The IL-6 concentration increased fivefold in relation to eccentric exercise and was significantly correlated with the CK concentration in the subsequent days; no changes were found, however, in relation to eccentric exercise. This study indicates that there was an association between increased IL-6 concentration and muscle damage, but it remains to be shown whether the relationship is causal or not.

Steensberg et al. (2001) reported that the percentage of circulating type 1 T cells decreased after prolonged exercise, whereas the percentage of type 2 T cells did not change, and concomitantly the total number of
circulating T cells declined. Moreover, the post-exercise decrease in circulating T lymphocytes is accompanied by a more pronounced decrease in the type 1 T cell subpopulation.

Plasma IL-2 and IFN-γ have not been shown to rise following intensive short term or prolonged endurance exercise. In vitro studies have shown that acute exercise impairs the ability of stimulated lymphocytes to produce IL-2 (Northoft et al., 1998; Rhind et al., 1996; Steensberg et al., 2001, 2003) a response which is reversed with administration of indomethacin suggesting the involvement of prostaglandins. Suzuki et al. (2003), however, suggest that exercise-induced release of soluble IL-2 receptor which interferes with the measurement may also be a factor contributing to the failure to detect IL-2 in the blood of exercising athletes.

Both animal and human studies, in vivo and in vitro, have revealed that T cells producing IFN-γ and IL-2 are suppressed by cortisol and epinephrine (Elenkov and Chrousos, 1999; Franchimont et al., 2000). These hormones increase during and in response to exercise. Cortisol is thought to operate by inhibiting the production of IL-12 by antigen-presenting cells (Elenkov and Chrousos, 1999) and also by decreasing the ability of T cells to respond to IL-12 (Franchimont et al., 2000). Epinephrine also suppresses the type 1 T cells both at the level of antigen-presenting cells and directly on T cell receptors. Steensberg et al. (2000) found a negative correlation between mean plasma epinephrine and percentage of IL-2-producing CD8 Type 1 cells. These cells express more β2-adreno-receptors on the surface compared with Th1 cells (Pedersen and Hoffman-Goetz, 2000) which may explain that only the percentage of type 1 T cells correlated with epinephrine during exercise (Steensberg et al., 2000) study. No evidence of cortisol correlation with type 1 T cells was found.

Rincon et al. (1997) demonstrated that IL-6 stimulates the production of IL-4 by naive CD4 Type-1 T-cells as plasma IL-6 increases enormously in response to exercise (Ostrowski et al., 1999) and that contracting skeletal muscles release IL-6 (Steensberg et al., 2000). However, exercise induced increases in circulating IL-4 have not been reported. The correlation between plasma IL-6 and percentage of IL-4-producing CD8 Type-1 T-cells may however indicate that the increase in plasma IL-6 during exercise contributes to maintaining an unaltered percentage of Type 2 T-cells and, thereby, influences the balance between the Type-1 and Type-2 T-cells. During exercise, muscle glycogen is reduced. This contributes to enhancing the production of IL-6 and, thereby, the effect of IL-6 on cortisol secretion (Peters, 2004). Furthermore, the level of catecholamines in plasma increases more when subjects exercise under fasting conditions. Thus exercising in a carbohydrate-loaded state might have induced a less pronounced effect on the type 1 or type 2 T cell balance (Steensberg et al., 2000).

Steensberg et al. (2000) postulated that IL-6 probably functions through the HPA axis thereby increasing secretion of cortisol. In accordance, their study demonstrated a correlation between peak plasma IL-6 and
plasma cortisol 1 h post-running (Steensberg et al., 2000). The relatively more pronounced decrease in type 1 compared with type 2 T cells in the recovery period may explain the increased sensitivity to URTI following strenuous exercise, as these infections are often caused by viruses.

2.3.4. The Role of Cortisol

The influence of the stress hormone, cortisol, often incorrectly referred to as an immunosuppressant hormone, on the function of the immune system is complex. However, under normal circumstances and in physiological amounts, cortisol tends to shift the immunological balance away from Th1 mediated and towards Th2 mediated immunity. This has been demonstrated in numerous studies (Agarwal and Marshall, 1998; Visser et al., 1998; Smith, 2003; 2004).

In psychological stress there is a shift in the balance of the immune system away from Th1 activity, which is usually associated with increased cortisol levels and can be reversed with cortisol receptor antagonists (Evans et al., 2000; Glaser et al., 1994). The catecholamine stress hormones are also thought to be important in this respect with similar evidence implicating α2-adrenergic receptors on immune cells (Elenkov and Chrousos, 1999).

In parallel, in addition to the previously described rise in circulating cortisol concentrations occurring during and immediately following acute exercise exposure, long-term endurance training results in immunological changes associated with increased blood cortisol levels and increase in the cortisol/androgen ratio (both the adrenal androgen - DHEA and gonadal androgen / testosterone) (Bernton et al., 1995, Suzuki et al., 2000; Smith, 2003; 2004). Similarly, these immunological changes are most easily described in terms of a shift in the balance of the immune system away from type 1 and towards type 2 immunity. This stress hormone-induced shift away from type-1 towards type-2 immunity has been shown to increase susceptibility to infection as well as duration of an infection once established (Elenkov and Chrousos, 1999). Athletic injury, the progression of which is itself subject to the consequences of reduced type 1 immunity (increasing the possibility of infection) also contributes to the immunological shift in immunity towards type 2 via production of Th2 cytokines triggered by activation of the sympathetic branch of the ANS (Elenkov and Chrousos, 1999; Smith, 2003; 2004).

Fairly consistently, cortisol-induced down-regulation of salivary IgA defence of mucosal surfaces and increased susceptibility to mucosal infections such as upper respiratory tract infection has also been demonstrated (Gleeson et al., 1999; Pedersen and Hoffman-Goetz, 2000; Pedersen et al., 2001). Thus the similarity between the effects of the physical and psychological stress on the immune system indicate that the summative effects of both types of stress are cumulative and synergistic and point to competitive athletes as a particularly vulnerable population with which very effort must be made to reduce the negative impact of both types of stressors. Indeed there is convincing evidence that elite athletes are more
vulnerable to opportunistic infectious illness (Peters and Bateman, 1983; Gleeson, 2000; Mackinnon, 1997; Pedersen et al., 2001; Smith, 2003; 2004).

The rise in serum cortisol concentration following prolonged exercise is well documented (Peters, 2001a; 2001b). Post-exercise concentrations have been shown to rise by more than 50% following 60 minutes of downhill treadmill running (gradient -13.5%) at 75% \( \text{VO}_{2\text{max}} \) (Smith et al., personal correspondence), but ± 200% increases to pm values of 1040 ± 113 (Peters et al., 2001a) and 1179 ± 93.2 mmol/L (Peters et al., 2001b) have been reported following a 90km downhill ultra marathon run despite considerably lowered diurnal cortisol variations in the late afternoon post-race blood samples.

The exercise-induced rise in systemic cortisol concentration is a sequel to the stimulation of the HPA axis and adrenal cortex by IL-6 and increased concentration of circulating cortisol have been reported to and on bone marrow, causing a further release of neutrophils into the blood stream (Starsky et al., 2003; Pedersen and Hoffman-Goetz, 2000; Suzuki et al., 1999). Due to the anti-inflammatory properties of cortisol, which activates the production of anti-inflammatory polypeptides including IL-10 and IL-1ra (Peters, 2001b), inflammation has been theorized to be restrained once cortisol concentrations reach a critical level and the body is given the opportunity to repair the tissue (Van Brandt, 2002).

2.4. The Repeated Bout Effect

The repeated bout effect is described as the adaptation of muscle where a single bout of eccentric exercise protects against muscle damage from subsequent eccentric bouts (McHugh et al., 1999; McHugh, 2003). The mechanisms responsible for this adaptation are poorly understood and are a subject of active study in the literature. Despite numerous studies that have clearly demonstrated the repeated bout effect, little consensus exist as to the actual mechanism. Recently, significant advances in the understanding of the repeated bout effect phenomenon have been reported in the literature (Hortobagyi et al., 1997, 1998, Lavendera and Nosaka, 2008).

The mechanisms are attributed to neural, connective tissue or cellular adaptations (McHugh et al., 1999). Other possible mechanisms include the adaptation in the excitation-contraction coupling mechanism of muscle or adaptation in the inflammatory response following the initial bout of exercise (McHugh et al., 1999; McHugh, 2003, Lavendera and Nosaka, 2008). There has been evidence to suggest that the repeated bout effect is associated with a shift toward greater recruitment of slow twitch motor units (McHugh et al., 1999; McHugh, 2003, Lavendera and Nosaka, 2008).

The neural theory refers to the pattern of recruitment of muscle fibres. It suggests that the change in the pattern of motor unit recruitment intra musculearly results in a reduction in the muscle damage during subsequent sessions of eccentric exercise (Golden and Dudley, 1992). In a similar study carried out by
Hortobagyi et al. (1998) an increase in muscular strength following repeated bouts of exercise was reported. Hortobagyi et al. (1998) concluded that an improved distribution of workload among muscle fibres in the subsequent bouts of exercise occurred and this could be explained by an altered pattern of motor–unit recruitment.

The connective tissue theory predicts that after the first bout of eccentric exercise, the muscles are protected by an overall increase in intramuscular connective tissue (Frieden and Lieber, 1992; McHugh et al., 1999; Frieden and Lieber, 2001; McHugh, 2003). The increase in connective tissue content is thought to be provided by the intermediate filaments within the muscle fibre, which consist of proteins, desmin, vimentin and synemin. These filaments play a role in maintaining the structural integrity of the sarcomere through preserving the sarcomere stretch and overlap during eccentric exercise (Frieden & Lieber, 1992). The non-contractile proteins are thought to be directly involved in the remodelling process of myofilaments disrupted during eccentric contractions. During this process, the muscle–tendon complex is thought to adapt its viscoelastic properties to enable it to absorb and transmit the forces produced during sudden and forceful contractions (Yu et al., 2003a; 2003b).

McHugh et al. (1999) explain that cellular theory proposes that muscle damage occurs as a result of irreversible sarcomere strain during eccentric contractions. Due to the nature of eccentric exercise during which muscles are lengthened while contracting, sarcomere lengths are highly non-uniform during these types of contractions (Frieden and Lieber, 1992; Hortobagyi et al., 1998; Frieden and Lieber, 2001). Some sarcomeres are stretched beyond the limit of myofilament overlap. The loss of contractile integrity results in sarcomere strain and is seen as the initial stage of damage (McHugh et al., 1999, Yu et al., 2003a, Yu et al., 2003b). Some data suggest that an increase in the number of sarcomeres connected in series, following an initial bout, reduces sarcomere strain during a repeated bout and limits the subsequent damage (Hortobagyi et al., 1998, 1999).

Multiple mechanisms therefore appear to be responsible for the repeated bout effect. Although significant advances have been made in understanding the adaptations seen in muscle tissue after the initial bout of eccentric exercise, a comprehensive unified theory describing all the components involved, remains elusive (Hortobagyi et al., 1998; McHugh et al., 1999).

2.5. The Effect of Traumeel On Inflammatory Markers in the Blood

According to Schroder et al.(1990) the blossoms of Arnica montana have been used for therapeutical purposes because of their anti-phlogistic and analgesic effects. More than 150 chemical substances have been found in Arnica blossoms and, among these, helenalin and its derivatives and flavonoids are the most important. In vitro and in vivo studies have shown the anti-inflammatory and anti-microbial activity of these substances (Kubo et al., 1994; Lyss et al., 1997). Homeopathic preparations of Arnica montana,
often used in combination with other drugs, were reported to have therapeutic effects in conditions like traumas, childbirth, haematomas, dental pain (Gibson et al., 1991; Ventoskovskij and Popov, 1990). A preparation containing active principles from Arnica montana together with other plant extracts and minerals, that could potentially act in synergistic way, has been developed by Heel GmbH and called is called Traumeel®S.

Traumeel®S (Traumeel), a mixture of highly diluted \(10^{-1-10^{-9}}\) extracts from medicinal plants and minerals is widely used in humans to relieve trauma, inflammation and degenerative processes. However, little is known about its possible effects on the behaviour of immune cells (Porozov et al., 2004). Lussignoli et al., 1999 report that the anti-inflammatory effect of Traumeel®S is presumably the result of the activity of single components on the different phases of inflammation. They cite the Handbook Materia Medica, Heel GmbH (1992) which suggests that Aconitum, Chamomilla, Hamamelis and Hypericum may reduce the pain associated with inflammation; Aconitum, Arnica, Hamamelis, Hypericum, Millefolium may have anti-hemorrhagic effects; Arnica, Calendula, Echinacea, Symphytum may accelerate the wound healing; Mercurius solubilis may be a anti-inflammatory and anti-viral agent, Hamamelis may prevent the venous stasis; Hepar sulfuris may improve the cellular breathing. The main indications of Traumeel®S are referred to different types of lesions and inflammatory processes involving muscles and joints, sprains and bruises. Its efficacy has been demonstrated in experimental and clinical studies, even under placebo-controlled trials (Zell et al., 1989; Bohmer and Ambrus, 1992). However, the mechanism by which this drug exerts its therapeutic effects remains to be clarified (Lussignoli et al., 1999).

Conforti et al. (1997) tested Traumeel®S in vivo on acute and chronic experimental inflammatory conditions on rats caused by intra-paw injection of carrageenan (carrageenan oedema) or Freund's complete adjuvant (adjuvant arthritis). The results of their study suggested that the local administration of Traumeel®S reduced oedema development and this inhibition was similar to the effect exerted by aspirin at dose of 30 mg/Kg in the same experimental model. In an adjuvant arthritis model, the intra-paw administration of Traumeel®S every two days led to a significant reduction in acute local inflammation, without affecting the chronic arthritic process (Conforti et al., 1997).

Lussignoli et al. (1999) reported a series of experiments aimed to improve the knowledge of the therapeutic action of Traumeel®S by using an injection intra-paw of a small amount of homologous blood that mimics a traumatic blood extravasation, a condition usually treated with Traumeel®S. They also tested the activity of individual components of Traumeel®S on blood-oedema and of a combination containing only the components with known anti-inflammatory activity. Their preliminary data also indicated a possible role of IL-6 in the Traumeel®S regulation of the inflammatory process, but due to the complex nature of the cytokine network, further work is necessary to clarify this point. Lussignoli et al. (1999) concluded that it is possible that Traumeel®S accelerates the tissue changes involved both in the formation and in the elimination of oedema, with a net beneficial effect on the overall reaction.
Porozov et al. (2004) conducted a study in which effects of Traumeel®S were examined in vitro on the ability of resting and PHA-, PMA- or TNF-α-activated human T cells, monocytes, and gut epithelial cells to secrete the IL-1β, TNF-α and IL-8 over a period of 24-72 hours. While they found that Traumeel®S did not affect T cell and monocyte proliferation, Traumeel®S, however, inhibited the secretion of all three cytokines in resting, as well as activated immune cells. IL-β secretion was reduced by up to 70% in both resting and activated cells, TNF-α secretion was reduced by up to 65 and 54%, respectively, and IL-8 secretion was reduced by 50% in both resting and activated cells (p < 0.01 for all cells). Interestingly, the effect also appeared to be inversely dose-related; maximal inhibition (usually 30-60% inhibition; P<0.01) was seen with dilutions of 10⁻³ - 10⁻⁶ of the Traumeel®S stock material. This suggests that Traumeel®S does not inhibit immune cells functions by exerting a toxic effect.

Heine and Schmotz (1998) have also shown that in whole blood cultures, Traumeel®S had the effect of elevating levels of Transforming Growth Factor (TGF-β), a Th3 cytokine which suppresses Th1 and Th2 cell activity and has also been described as an anti-inflammatory cytokine (Mosmann and Coffman, 1989; Bonecchi et al., 1998; Romagnani, 2000, Table 2.1.).

Although additional studies are needed to clarify the mode of action of Traumeel®S and to demonstrate causative relationship between the inhibition of pro-inflammatory cytokine/chemokine secretion in cell culture and the reported clinical effects of the preparation, this in vitro work of Porozov et al. (2004) and Heine and Schmotz (1998) does shed greater light on a possible mechanism for the anti-inflammatory effects of Traumeel®S observed in clinical use.

While non-steroidal anti-inflammatory drugs act by inhibiting the arachidonic acid pathway and therefore the production of prostaglandins by means of inhibiting the cyclooxygenase and lipoxygenase enzymes (Lussignoli et al., 1999; Porozov et al., 2004, Ganong, 2005), Traumeel®S has, however, been shown to control the generation of reactive oxygen species (ROS) by activated neutrophils. Therefore with the ingestion of Traumeel, increased neutrophilic oxidative burst activity and lymphocyte function is possible as the suppressive action of high levels of ROS on the leucocytes would be neutralised by Traumeel®S (Conforti et al., 1997, Peters, 1997b). It is hence proposed that the inflammatory response is restrained despite stimulation of the innate and secondary adaptive responses to the tissue damage, a finding that is supported by the increased plasma myeloperoxidase concentrations most recently reported in another dimension of this, as yet, unpublished, experimental work (Smith, 2008).

2.6. Traumeel® S and Exercise Induced Muscle Inflammation

Although Traumeel®S is a widely used product, its effects on the immune system following endurance exercise have been published in very few reputable scientific journals. Zell et al. (1989) conducted a double-blinded, placebo-controlled trial in order to test the effect of Traumeel®S ointment on joint mobility and subjective ratings of pain after sports related ankle injury. In their study, Traumeel S
ointment was found to be significantly more effective than placebo in the reduction of pain and the improvement of joint range of motion 10 days following the injury. These findings were consistent with those of Bohmer and Ambrus (1992) and Wright-Carpenter et al. (2004) who reported improved recovery times for the injuries studied in their respective investigations when compared to placebo.

In the first in vivo study conducted on athletes at the Sports Research Institute of Pretoria University (Jordaan, 2006), subjects taking Traumeel® S 7 days prior to a 45 minute downhill run at -10 %, displayed a significantly higher blood cortisol response compared to the placebo group at 48hr, 72hrs and 96hr post exercise. The only markers relevant to a systemic inflammatory response were blood cortisol, leukocyte counts and serum creatine kinase levels. Although subjective perceptions of delayed onset muscle soreness were also quantitated, post-exercise neutrophilia and statistically significant elevations in serum cortisol and CK concentrations were not reported (Jordaan, 2006). It is therefore uncertain whether the exercise was intense enough to elicit an inflammatory response in this study.

27. Conclusion
In conclusion, exercise, especially that of a strenuous and/or eccentric nature, has been shown to have an inflammatory effect that generates a response analogous to the acute phase response. The pro-inflammatory response to muscle damage and consequent soreness has been clearly demonstrated. Although Traumeel® S has been advocated to be effective in accelerating the recovery process and has been used by sportsmen for this purpose for a long time, most of the biochemical studies examining the possible mechanism of action, have been in vitro. The need for a comprehensive in vivo study on endurance athletes which includes a comprehensive analysis of systemic markers of inflammation, with specific reference to the cytokine milieu, therefore still exists and is an open area for future research.
Chapter Three

Materials and Methods

3.1. Study design, ethical approval and insurance

This study was designed as a double-blinded, placebo-controlled clinical trial in which subjects were matched and randomised to Traumeel (TRAU) and Placebo (PLAC) pairs which ingested either Traumeel (TRAU) or Placebo (PLAC) for 9 days; 5 days before, the day of and 3 days after a 90-minute downhill running intervention.

Ethical clearance was obtained from the University of KwaZulu-Natal (UKZN), Nelson R. Mandela School of Medicine, Biomedical Research Ethics Committee (Clearance Number: E017 05). Insurance was obtained from Heel (Pty) Ltd. in Germany, to cover the UKZN in the event of possible research-related injury to the participant. The subjects were recruited by word of mouth from various running clubs in the greater Durban area. Each subject signed an informed consent form which had been approved by the Biomedical Research Ethics Committee of the University of KwaZulu-Natal (Appendix A, B).

3.2. Sample

3.2.1. Size: The study subjects included 18 distance runners and 2 team sport players, matched according to gender, selected anthropometrical characteristics, training age and status, footstrike pattern and performance level and then randomised into TRAU \(n = 10\) and PLAC \(n = 10\) groups.

3.2.2. Inclusion criteria: These included volunteers who:
  - fell within the age category of 20 to 50 years inclusive
  - were asymptomatic
  - were healthy runners who covered a minimum of 40 km per week in training or who were training a minimum of three times per week if participating in team sports, during the six weeks prior to participation in the study
  - were not on any anti-inflammatory or analgesic medication, antioxidant supplements, free of systemic illness, not pregnant and were all non-smokers

3.2.3. Exclusion criteria: Subjects who did not fulfil the inclusion criteria were automatically excluded. In addition, those subjects who were non-compliant with respect to the methodology of the study were also excluded. These included subjects from whom blood samples were unobtainable, who misplaced the tablets, who became ill immediately prior to or post trial and those who did unaccustomed training before and/or after the trial.
3.3. Study Protocol

Prior to the onset of the study, the subjects were required to undergo baseline laboratory and field testing. (Figure 3.1). The laboratory testing took place in the exercise laboratory in the Department of Physiology at the Nelson Mandela School of Medicine and included assessment of the anthropometrical characteristics, lung function and VO$_2$ peak of the subject, while the field test included a 1609 m time-trial on an athletics track.

Thereafter each of the subjects was matched according to gender, physical and performance characteristics. A total of 10 matched pairs of subjects took part in this phase of the study which focused on cytokines. They were randomised to treatment sequences (A and B) using random number tables.

The actual exercise intervention was a 90-minute -6% downhill run on a treadmill at 75% of VO$_2$ peak which was completed 5 days after the subjects started taking either Treatment A or Treatment B. Blood samples were obtained from each subject before (PRE), immediately after (IPE), 24 hours (24PE), 48 hours (48PE) and 72 hours (72PE) after each 90-minute downhill run.

Treatment interventions were coded by Heel (Pty) Ltd. in Baden-Baden, Germany prior to being dispatched to South Africa. Neither the subjects nor the researchers were aware of which treatment was active and which treatment was placebo. The coding was only revealed to the researchers once the study was complete and a preliminary report of the results was made available to Heel (Pty).

Figure 3.1. Study protocol depicting the time-points.
3.3.1. Baseline Laboratory Testing

At the first test, each subject underwent a pre-study evaluation in order to obtain baseline measurements. These included details of exercise training status and anthropometric measures such as height, weight, lung-function, resting blood pressure, resting heart rate and percentage body fat. The latter was evaluated by the sum of the biceps, triceps, suprailliac and subscapular skin folds according to the technique published by Dumin and Womersly (1974). Lung-function was tested using a flow rate spirometer (Jaeger, Wuerzburg, Germany). Each subject was required to obtain indemnity from a physician confirming that they were free from clinical symptoms of upper respiratory tract infections or other diseases.

Before the VO₂max of each subject was determined, they were familiarized with the treadmill to be used in the study (A Powerjog GX10, Sport Engineering Limited, Birmingham, England). The subject was then allowed to warm up for 5 minutes on the treadmill at a speed of 12 km/hr. After the warm-up period, the speed was increased incrementally by 1 km/hr at each minute until fatigue. Fatigue was defined either as the subject reporting a Rate of Perceived Exertion (RPE) of between 8-10, an estimated maximal age-predicted heart rate or a Respiratory Quotient (RQ) of more than 1.15. The highest VO₂ over a 30 second period was defined as the VO₂peak.

During the treadmill tests, respiratory and metabolic responses were assessed using a MetaMax 3B Portable CPX metabolic testing system (Cortex Biophysik, GmbH, Leipzig, Germany). Prior to the assessments, the gas analyzers were calibrated using ambient air and a gas cylinder containing 5.1% CO₂ and 16.7 % O₂ in nitrogen (Air Products, Durban). A two-litre calibration syringe (Erich Jaeger, GmbH, Hoechberg, Germany) was used to calibrate the flow volume sensors. Expiratory flow volume was measured using a triple V transducer containing a 660nm optical tube sensor which was connected to a tightly fitting facemask. Oxygen and CO₂ content of the expired air was measured by oxygen and carbon dioxide analysers, using an electro-chemical cell and infrared system, respectively. Expired gases were analyzed on a breath-by-breath basis by Metasoft® software and the rolling 30 s average was used in order to record VO₂ throughout the incremental exercise test.

3.3.2. Track testing

Each subject was required to undergo an athletic track test which was conducted at the University of KwaZulu-Natal’s sports grounds at the Westville campus. This field test was supervised by a national athletics coach and consultant (Norrie Williamson). After a short warm up session, each subject was timed over approximately 4 laps (1609m) on the athletics track. In addition, the subject’s running style (footstrike pattern, shoe-wear patterns, inner soles and general gait analysis) was assessed while running on the track and downhill (gradient > 5%) The subjects past and current training practices were also analysed. Each subject received feedback from the coach including advice of pre-intervention training and eating procedures.
3.3.3. Matching of subjects

Subjects were then matched according to baseline measurements taken at the first laboratory visit, their anthropometric parameters (mass, height, body-mass indices, percentage body fat), running style, training status and performance time in the 1609m field test. This included:

(i) Gender
(ii) BMI
(iii) Current training profile
(iv) Training age

as well as at least two of the following:

- Foot strike
- $\text{VO}_2\text{ max}$
- 1609m running time and/or best performance over longer endurance running events (5km, 10km, half marathon or marathon), within six months prior to the study trials.

3.3.4. The Intervention and Trial Procedure

Five days prior to the trial each subject was required to orally take 3 Traumeel or Placebo tablets per day (Treatment A or Treatment B) which was provided to them. The subjects were requested to dissolve the tablet under the tongue at least 30 minutes before mealtime (or on an empty stomach). On the day before the trial the subjects were instructed to avoid any heavy physical exercise. For the morning of the trial, the subjects were instructed to eat the following breakfast, three hours beforehand: 30g cornflakes, 150ml of low fat milk and 12-15 g refined sugar.

Each subject was then required to report to the exercise laboratory in the morning. Particular care was taken to ensure that trials took place at the same time of day to avoid possible confounding of the circulating cortisol concentrations. Following a half hour rest period, pre-exercise venous blood samples were obtained from the antecubital vein of each subject. The subjects were allowed to do basic stretching exercises immediately prior to the trial.

Thereafter, each subject underwent a downhill run on a motorised treadmill set at a -6% downhill gradient. Their running speed was set at a workload at which they achieved 75% of the individual maximal oxygen consumption ($75\% \text{ VO}_2\text{ peak}$). During each trial, the subjects’ heart rate was continually monitored. The oxygen consumption was monitored at 20min intervals using a Metamax metabolic analyser (Cortex Biophysik, GMBH, Leipzig, Germany). At 30 minute intervals, the workload was reduced to 50% VO2 peak in order to allow subjects to rehydrate. Each subject was given 500ml of water over the 90-minute period.
3.3.5. Post-Trial Recovery
On completion of the exercise trial a 30ml blood sample was drawn from an antecubital vein immediately after the trial and at 24hrs, 48hrs and 72hrs following the trial. Subjects were requested to return to the exercise laboratory at the same time of the morning on post-trial days to attain further blood samples and to record subjective ratings of delayed onset muscle soreness. All post-trial blood samples were obtained at the same time of day to avoid the influence of diurnal rhythms on cortisol concentrations. The participants were asked to refrain from any strenuous exercise and to avoid the intake of anti-inflammatory medication /painkillers for 3 days after the trial.

3.4 Treatment of the blood samples
Aliquots of 3ml of fresh venous blood were drawn into K$_3$-ethylene diaminetetra-acetic acid (EDTA) tubes for the testing of a full blood count (FBC). Five millilitres of the remainder were allowed to clot at ambient temperature, the serum separated and 0.5ml aliquots thereof stored at -70°C for later analysis of creatine kinase and cortisol. The remaining blood was drawn into vacutainer tubes containing EDTA and the plasma separated. This was stored at -70°C for later measurement.

3.5. Analysis of Blood Samples
3.5.1. Determination of Serum CK and Cortisol Concentrations
Blood samples and serum was sent to a local Chemical Pathology Laboratory (Global Clinical and Viral Laboratories, Jacobs, Durban). Full blood counts including differential leukocyte counts were performed on EDTA treated specimens using standard haematological procedures on an automated STKS model (Coulter Electronics Inc., Hialeah, Florida, USA). Serum was analysed for CK concentration using a Synchron CX, X5Analyser (Beckman Coulter, SA (Pty) Ltd., Halfway House, South Africa).

Serum cortisol was assayed using the Gamma Coat radioimmunoassay procedure (AB Advia Centaur CPAdviacentaur, Siemens Medical Solutions Diagnostics, Deerfield, USA).

3.5.2. Determination of Plasma IL-6 concentration by sandwich ELISA
Plasma IL-6 concentration was determined by sandwich ELISA using a commercial kit (eBioScience, San Diego, CA, USA).

3.5.3. Flow Cytometric Analysis of Cytokine Concentrations
3.5.3.1. Introduction
The BD™ CBA Human Inflammation Kit (Becton, Dickinson and Company) was used to quantitatively measure Interleukin-8 (IL-8), Interleukin-6 (IL-6), Interleukin-10 (IL-10), Tumor Necrosis Factor (TNF),
and Interleukin-12p70 (IL-12p70) protein levels in a single sample. This was done using a flow cytometer equipped with a 48 nm laser capable of detecting and distinguishing fluorescence emissions at 576 and 670 nm (e.g., the BD FACScan™ or BD FACSCalibur™ instruments) and BD Cellquest™ Software.

3.5.3.2. Principle of the test

Six bead populations with distinct fluorescence intensities were coated with capture antibodies specific for IL-8, IL-6, IL-8, IL10, TNF and IL1-12p70 proteins. The six bead populations are mixed together to form the BD™ CBA which is resolved in the FL3 channel of a flow cytometer such as the BD FACScan™ or BD FACSCalibur™ flow cytometer.

The capture beads, PE-conjugated detection antibodies, and recombinant standards or test samples were incubated together to form sandwich complexes. Following acquisition of sample data using the flow cytometer, the sample results were generated in graphical and tabular format using the BD™ CBA Analysis Software. The kit provided sufficient reagents for the quantitative analysis of 50 test samples and the generation of two standard curve sets.

3.5.3.3. Preparation of Human Inflammation Standards

The Human Inflammation Standards were lyophilised and were reconstituted and serially diluted before they were mixed with the capture beads and the PE detection reagent.

This was carried out as follows:

1. One vial of lyophilised Human Inflammation Standards was reconstituted with 0.2 ml of assay diluent in order to prepare a 10x bulk standard. The reconstituted standard was allowed to equilibrate for 15 minutes before the dilutions were made. The vials were agitated vigorously in order to allow mixing, but not vortexed.

2. Twelve 75 mm tubes (BD Flacon™, Cat. No. 352008) were labelled and arranged in the following order: Top standard, 1:2, 1:4, 1:8, 1:16, 1:32, 1:64, 1:128 and 1:256.

3. 900 µl of assay diluent was added to the Top Standard tube.

4. 300 µl of assay diluent was added to each of the remaining tubes.

5. 100 µl of 10x bulk standard was transferred to the Top Standard tube and was mixed thoroughly.

6. A serial dilution was performed by transferring 300 µl from the Top Standard to the 1:2 dilution tube and was mixed thoroughly. Several such serial dilutions were conducted by transferring 300 µl from the 1:2 tube to the 1:4 tube and so on up to the 1:256 tube. All were mixed thoroughly (see figure 3.2 below). The assay diluent serves as the negative control.
The appropriate concentration (pg/ml) of recombinant protein in each dilution tube is displayed in Table 3.1.

Table 3.1: Human Inflammation Standard concentrations after dilution.

<table>
<thead>
<tr>
<th>Protein (pg/ml)</th>
<th>STD</th>
<th>1:2</th>
<th>1:4</th>
<th>1:8</th>
<th>1:16</th>
<th>1:32</th>
<th>1:64</th>
<th>1:128</th>
<th>1:256</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human IL-8</td>
<td>5000</td>
<td>2500</td>
<td>1250</td>
<td>625</td>
<td>312.5</td>
<td>156</td>
<td>80</td>
<td>40</td>
<td>20</td>
</tr>
<tr>
<td>Human IL-10</td>
<td>5000</td>
<td>2500</td>
<td>1250</td>
<td>625</td>
<td>312.5</td>
<td>156</td>
<td>80</td>
<td>40</td>
<td>20</td>
</tr>
<tr>
<td>Human TNF</td>
<td>5000</td>
<td>2500</td>
<td>1250</td>
<td>625</td>
<td>312.5</td>
<td>156</td>
<td>80</td>
<td>40</td>
<td>20</td>
</tr>
<tr>
<td>Human IL-12p70</td>
<td>5000</td>
<td>2500</td>
<td>1250</td>
<td>625</td>
<td>312.5</td>
<td>156</td>
<td>80</td>
<td>40</td>
<td>20</td>
</tr>
</tbody>
</table>

* STD. Standard

3.5.3.4. Plasma Assay Procedure

1. 10μl/test of each Human Inflammation Capture Bead suspension was mixed and vortexed briefly.
2. Mixed beads were pelleted by centrifugation and the supernatant was aspirated.
3. The bead pellets were re-suspended in Serum Enhancement Buffer (equal in volume to the amount removed in step 2) and was vortexed briefly.
   - 30 minute incubation at room temperature was allowed (protected from light).
4. 50μl of mixed beads were transferred to the assay tube.
5. The Human Inflammation Standard was reconstituted in the Assay Diluent (15 min).
6. The Standards were diluted by serial dilutions using the Assay Diluent.
7. The Standard Dilutions and test samples were added to the appropriate sample tubes (50μl/tube).
   - 1.5 hour incubation at room temperature was allowed (protected from light).
8. The samples were washed with 1 ml Wash buffer and centrifuged (100 μl residual volume was left after aspiration).
9. PE Detection Reagent was added (50 μl/test).
1. 1.5 hour incubation at room temperature was allowed (protected from light).

10. The samples were washed with 1 ml Wash buffer and centrifuged.

11. 300μl of Wash Buffer was added to each assay tube and the samples were analysed.

3.5.3.5. Cytometer Setup Bead Procedure

1. The Cytometer setup beads (vortexed before adding) were added to setup tubes A, B, and C (50μl/tube).

2. 30 min. incubation period at room temperature was allowed (protected from light).

2. 50μl of FITC Positive Control was added to tube B and 50μl of PE Positive Control was added to tube C.

3. 400μl of Wash Buffer was added to tubes B and C.

4. 450μl of Wash Buffer was added to tube A.

5. Tubes A, B, and C were then used for the cytometer setup.

3.6. Subjective Rating of Perceived Muscle Soreness

Subjective ratings of muscular soreness were determined at the beginning of each day following the trial with a numerical 0-10 scale where 0 = no pain at all, 5 = moderate, 10 = severe/maximal pain. The quadriceps, hamstrings and calf muscles were assessed under four categories: General pain (subject seated and relaxed), daily living (walking/moving), Pressure pain (palpation of the muscle area) and Stretch pain (stretching the muscle) using the tabulation shown below.

<table>
<thead>
<tr>
<th>DAY after the trial</th>
<th>General post-trial pain seated and relaxed</th>
<th>Post-trial pain during daily living while walking</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Quadriceps</td>
<td>Hamstrings</td>
</tr>
<tr>
<td>Day 1: immediately after the run</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 2:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 3:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 4:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 5:</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

0 = no pain at all; 5 = moderate 10 = severe/maximal
3.7. Statistical Analyses

Data were analysed in SPSS version 15.0 (SPSS Inc., Chicago, Illinois, USA). A p-value < 0.05 was considered as statistically significant and data are expressed as mean ± SEM. Baseline and field trial outcomes were compared between the two groups using paired t-tests. Repeated measures analysis of variance (ANOVA) testing was used to compare quantitative normally distributed outcomes over time between the experimental (TRAU) and placebo-control (PLAC) groups. Wilks' Lambda was used as test statistic with a Simple Contrasts post hoc correction to determine the time-point of the significant differences between treatments. Variables that were not normally distributed were log transformed. Significant time-group interactions indicated significant treatment effects. Profile plots were generated to examine trends. There was very little missing data, but for some outcomes, the final time point result was missing in a few (up to four) cases. In this case, repeated measures ANOVA was conducted on four time points as well as five and the results were compared. If there was no difference in the conclusion then the five time-point comparison was reported. For the DOMS analysis, the average of the three muscle types and modes were computed for each day. Repeated measures ANOVA was performed on these averaged measurements over time, which were normally distributed.
Chapter 4

Results

4.1. Subject Characteristics

Twenty subjects (12 men, 8 women), aged between 20 and 50 years fully complied with all inclusion criteria set for the study. The subject characteristics are provided in Table 4.1. Paired student t-tests indicate that the mean ± SEM of the two groups were not significantly different (p < 0.05) in terms of age, stature, mass, percentage body fat, body mass index, FVC, FEV1, heart rate and blood pressure at rest (Table 4.1).

Table 4.1: Physical characteristics of the participants in the PLAC and TRAU groups. Data presented as mean ± SEM

<table>
<thead>
<tr>
<th>Variable</th>
<th>PLAC (n=10)</th>
<th>TRAU (n=10)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>32.60 ± 2.21</td>
<td>30.00 ± 1.80</td>
<td>0.255</td>
</tr>
<tr>
<td>Stature (cm)</td>
<td>170.33 ± 2.99</td>
<td>171.48 ± 2.81</td>
<td>0.346</td>
</tr>
<tr>
<td>Mass (kg)</td>
<td>66.29 ± 2.74</td>
<td>68.53 ± 2.49</td>
<td>0.288</td>
</tr>
<tr>
<td>% Body Fat</td>
<td>16.25 ± 1.23</td>
<td>15.86 ± 0.86</td>
<td>0.807</td>
</tr>
<tr>
<td>Body Mass Index (kg.m⁻²)</td>
<td>22.67 ± 0.41</td>
<td>23.24 ± 0.50</td>
<td>0.323</td>
</tr>
<tr>
<td>FVC (L)</td>
<td>4.71 ± 0.33</td>
<td>4.65 ± 0.20</td>
<td>0.832</td>
</tr>
<tr>
<td>FEV1 (L)</td>
<td>3.67 ± 0.21</td>
<td>4.00 ± 0.20</td>
<td>0.295</td>
</tr>
<tr>
<td>HR Rest (b.min⁻¹)</td>
<td>63.89 ± 2.08</td>
<td>63.40 ± 1.42</td>
<td>0.976</td>
</tr>
<tr>
<td>Systolic Blood Pressure (mm Hg)</td>
<td>121.2 ± 1.35</td>
<td>123.3 ± 1.54</td>
<td>0.416</td>
</tr>
<tr>
<td>Diastolic Blood Pressure(mm Hg)</td>
<td>75.80 ± 1.22</td>
<td>78.60 ± 1.17</td>
<td>0.213</td>
</tr>
</tbody>
</table>

FVC = Forced Vital Capacity; L/min; FEV1, Forced Expiratory Volume in 1 second; HR Rest = Resting Heart Rate

Physiological response to the incremental maximal exercise test is presented in Table 4.2

Table 4.2: Physiological Responses to the Maximal Incremental Exercise Test in the PLAC and TRAU groups. Data presented as mean ± SEM

<table>
<thead>
<tr>
<th>Variable</th>
<th>PLAC (n=10)</th>
<th>TRAU (n=10)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>HR max (b.min⁻¹)</td>
<td>180.27 ± 3.41</td>
<td>175.45 ± 3.11</td>
<td>0.209</td>
</tr>
<tr>
<td>RPE max</td>
<td>7.27 ± 0.35</td>
<td>6.91 ± 0.38</td>
<td>0.441</td>
</tr>
<tr>
<td>VEmax (L.min⁻¹)</td>
<td>98.21 ± 2.79</td>
<td>108.99 ± 4.06</td>
<td>0.370</td>
</tr>
<tr>
<td>VO₂ Peak (ml.kg.min⁻¹)</td>
<td>50.64 ± 1.23</td>
<td>54.42 ± 1.50</td>
<td>0.155</td>
</tr>
<tr>
<td>RER Max</td>
<td>1.11 ± 0.02</td>
<td>1.13 ± 0.02</td>
<td>0.626</td>
</tr>
</tbody>
</table>

*Paired t tests, TRAU vs. PLAC; RER = Respiratory Exchange Ratio maximum; VO₂ peak = relative peak oxygen consumption; HR max = Maximal Heart Rate
The TRAU and PLAC groups did not differ significantly in terms of mean HR max, RPE max, VE max or VO\textsubscript{2} peak (p > 0.05).

### 4.1.2 Training Status and Performance during Field Testing

The mean ± SEM of data relating to the training status and performance in competitive field events of the two groups is given in Table 4.3. These indicate that the gender-matched pairs were also well matched in terms of average weekly running distance, number of running years, 1609 run time, performance in 5km, 10 km, half marathon and/or marathon times. Where possible, subjects who were training partners and hence trained in similar conditions were matched.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>TRAU (n = 10)</th>
<th>PLAC (n = 10)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Average weekly running distance (km)*</td>
<td>39.00 ± 0.70</td>
<td>38.00 ± 4.02</td>
<td>0.760</td>
</tr>
<tr>
<td>Number of running years*</td>
<td>10.30 ± 0.17</td>
<td>11.10 ± 1.27</td>
<td>0.700</td>
</tr>
<tr>
<td>1609m time (min)*</td>
<td>5.88 ± 0.04</td>
<td>6.16 ± 0.24</td>
<td>0.160</td>
</tr>
<tr>
<td>5km time (min)**</td>
<td>18.67 ± 0.06</td>
<td>19.50 ± 0.19</td>
<td>0.300</td>
</tr>
<tr>
<td>10km time (min)**</td>
<td>42.75 ± 0.51</td>
<td>38.50 ± 0.47</td>
<td>0.200</td>
</tr>
<tr>
<td>Half marathon time (min)**</td>
<td>101.83 ± 2.42</td>
<td>102.38 ± 4.19</td>
<td>0.650</td>
</tr>
<tr>
<td>Marathon time (min)****</td>
<td>233.00 ± 6.60</td>
<td>228.50 ± 5.15</td>
<td>0.670</td>
</tr>
</tbody>
</table>

\*n = 8; \**n = 6; \***n = 5; \****n = 3

A series of graphs depicting the matching of the subject pairs with respect to their training status and running performance is presented in Figure 4.1.
Figure 4.1. The matching of the subject pairs with respect to their training status and performance.
The mean ± SEM performance values during the 90-minute downhill trial are given in Table 4.4. While average speed and distance covered was slightly greater \((p < 0.05)\) in the TRAU group during the 90-minute downhill running trial, relative degree of exertion as reflected by the mean RPE, heart rate and maximum heart rate and RPE was not significantly different \((p > 0.05)\).

**Table 4.4: Performance of the subjects TRAU and PLAC groups in the 90-minute downhill running trial. Data presented as mean ± SEM**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>TRAU ((n = 10))</th>
<th>PLAC ((n = 10))</th>
<th>(p)-value(^{\circ})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Average Speed (Km/hr)</td>
<td>13.29 ± 0.21</td>
<td>12.84 ± 0.25</td>
<td>0.020</td>
</tr>
<tr>
<td>Distance (km)</td>
<td>20.13 ± 0.29</td>
<td>19.41 ± 0.38</td>
<td>0.030</td>
</tr>
<tr>
<td>Mean RPE</td>
<td>4.82 ± 0.26</td>
<td>4.91 ± 0.25</td>
<td>0.780</td>
</tr>
<tr>
<td>Mean Heart Rate</td>
<td>159.5 ± 1.87</td>
<td>160.7 ± 2.09</td>
<td>0.630</td>
</tr>
<tr>
<td>Maximum Heart Rate</td>
<td>175.5 ± 3.11</td>
<td>180.3 ± 3.41</td>
<td>0.210</td>
</tr>
<tr>
<td>% of Heart Rate Maximum</td>
<td>83.9 ± 1.32</td>
<td>85.4 ± 0.66</td>
<td>0.390</td>
</tr>
<tr>
<td>Maximum RPE</td>
<td>6.91 ± 0.38</td>
<td>7.27 ± 0.35</td>
<td>0.440</td>
</tr>
</tbody>
</table>

\(^{\circ}\)Paired \(t\)-tests TRAU vs. PLAC

### 4.2 Serum Creatine Kinase

The mean ± SEM serum CK concentrations in the TRAU and PLAC groups is depicted in Figure 4.2. The CK concentration rose significantly in response to 90-minutes of downhill running in both groups \((p < 0.001)\), peaking at 24 hours post exercise and remained significantly elevated in the 48 PE and 72 PE serum samples \((p < 0.05)\) despite relatively large variances within the groups. The time effect between the TRAU and PLAC groups was highly significant \((p < 0.001)\), but the treatment-time interaction did not reach statistical significance \((p = 0.398; \text{repeated measures ANOVA, Wilks' Lambda test statistic})\). Simple contrasts post-hoc analyses revealed that the absolute CK concentration differences were only statistically significant at the IPE time point \((p = 0.04)\).

![Figure 4.2: Mean ± SEM serum creatine kinase concentrations of the TRAU \((n = 10)\) and PLAC \((n = 10)\) groups before and after 90-minutes of downhill running on a treadmill, \# \(p < 0.05\) Time-effect, repeated measures ANOVA, Wilks Lambda Test Statistic \ **) \(p < 0.05\) Simple contrast post hoc comparison between groups at time point](image-url)
4.3. Neutrophil Count

Figure 4.3: Mean ± SEM neutrophil concentrations in the TR AU (n = 10) and PLAC groups (n = 10) before and after the 90-minute treadmill trial. # p < 0.05 Time-effect, repeated measures ANOVA, Wilks Lambda Test Statistic, *p < 0.05 Simple contrast post hoc comparison vs PRE

Figure 4.3 reflects that the absolute neutrophil counts (10^9 L^-1) were significantly increased at the IPE time point (p < 0.001) and returned to near pre-trial values within 24 hours in both the TRAU and PLAC groups. The time effect between the TRAU and PLAC groups was highly significant (p < 0.003), but the treatment-time interaction did not reach statistical significance (p = 0.940; repeated measures ANOVA, Wilks’ Lambda test statistic).

4.4. Cortisol

Figure 4.4: Mean ± SEM serum cortisol concentrations of the TRAU (n = 10) and PLAC (n = 10) groups before and after 90-minutes of downhill running on a treadmill *p < 0.05 Paired t-tests vs PRE; ** p < 0.05 Simple contrast post hoc comparison between groups at time point
Figure 4.4 graphically displays changes in serum cortisol concentrations for the TRAU and PLAC groups. The TRAU group increased to a peak of 754.2 ± 340.5 nmol/l while the control group reached a peak of 600.6 ± 148.4 nmol/l \( (p < 0.001) \). In both groups, the decline in serum cortisol concentrations was significant at 24 hours \( (p < 0.001) \) and by 72 hours, both groups had returned to near-pre trial cortisol levels.

Repeated measures ANOVA revealed that the time effect was highly significant \( (p < 0.001) \) in both treatment groups, but the group-time interaction did not reach statistical significance \( (p = 0.767) \). Simple contrast post hoc analyses verified these findings \( (p > 0.05) \) at each of the time points.

4.5. DOMS

Figure 4.5: Mean ± SEM reported peak DOMS scores of the TRAU \( (n = 10) \) and PLAC \( (n = 10) \) groups following participation in a 90-minute downhill run; ** \( p < 0.05 \) Simple contrast post hoc comparison between groups at time point

The mean ± SEM post-trial DOMS scores reported by the TRAU group over the 3-day post-exercise recovery period, were significantly different from those reported by the PLAC group \( (p = 0.04) \). Figure 4.5 depicts that the mean DOMS score peaked at Day 2 post trial and declined steadily thereafter. The majority of subjects reported that pain was elicited during activities and walking as opposed to at rest. The muscle groups most affected were the quadriceps muscles, followed by the calf muscles. The hamstring muscle group was the least affected. Five subjects reported pain in all muscle groups. The overall means of the DOMS scores over the four days following the trial were analysed using repeated measures ANOVA tests not did not reveal a significant group-time interaction over the three days post exercise \( (p = 0.68) \). The difference between the two groups was significant 24 hours post-trial \( (p < 0.03; \) Figure 4.5).
4.6. Plasma TNFα concentrations

The mean ± SEM of TNFα immediately before, after and 24 hours after the 90-minute trial run are presented in Figure 4.6. The 90-minute run did not result in a significant change in TNFα ($p > 0.05$) when compared to the pre-test values. The values remained within the normal range throughout the 24 hour period pre and post trial.

![Figure 4.6: Mean ± SEM plasma TNFα concentrations (pg/ml) of subjects in the TRAU ($n = 10$) and PLAC ($n = 10$) before and after participation in the 90-minute trial at 3 time-points](image)

4.7. Plasma IL-6 concentrations

The mean ± SEM of IL-6 concentrations determined by ELISA immediately before, after, 24, 48 and 72 hours after the 90-minute downhill trial run, are presented in Figure 4.7. The downhill run resulted in a small (5-fold, 5.2 fold), but significant elevation in IL-6 ($p < 0.05$) when compared to the pre-test values. The difference in the exercise-induced change between the two groups was, however, not statistically significant ($p > 0.05$). IL-6 concentrations reached baseline levels within 24 hours following the run and did not fluctuate during the subsequent 48 hour period monitored.

![Figure 4.7: Mean ± SEM plasma IL-6 concentrations (pg/ml) of subjects in the TRAU ($n = 10$) and PLAC ($n = 10$) before and after participation in the 90-minute trial at 5 time-points, # $p < 0.05$ Time-effect, repeated measures ANOVA, Wilks Lambda Test Statistic, * $p < 0.05$, Simple contrast post hoc comparison vs. PRE](image)
4.8. Plasma IL-8 concentrations:

The mean ± SEM of IL-8 concentrations immediately before, after and 24 hours after the 90-minute downhill trial run, are presented in Figure 4.8. In the TRAU and PLAC groups, there was a significant elevation in IL-8 ($p < 0.05$) when compared to the pre-test values. The difference in the exercise-induced change between the two groups was, however, not statistically significant ($p > 0.05$). IL-8 concentrations reached baseline levels in both groups within 24 hours following the run.

4.9. Plasma IL-10 concentrations:

Figure 4.9 reveals that the mean ± SEM plasma IL-10 concentrations rose significantly in both groups following the downhill exercise protocol ($p < 0.001$; $p = 0.03$) with mean concentrations peaking at 9.99
and 10.11 pg/ml respectively. The time effect was statistically significant throughout the 24 hour period, but the time-group interaction ($p > 0.05$) did not reveal a significant difference between the two groups.

4.10. Plasma IL-12p70 concentrations:

The mean ± SEM of IL-12p70 concentrations immediately before, after and 24 hours after the 90-minute downhill trial run, are presented in Figure 4.10 and Table 4.5. Due to the large variance in the results, the exercise trials were not found to result in a statistically significant increases in the plasma IL-12p70 (pg/ml) concentrations and no significant group-time effect was observed in these cytokine concentrations ($p > 0.05$).

Table 4.5: Plasma TNF-α, IL-8, IL-10, IL-12p70 concentration preceding and following the trial run. Data presented as mean ± SEM

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Pre</th>
<th>Post</th>
<th>24 Hrs Post</th>
<th>$p$-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNFα (pg/ml)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TRAU Group</td>
<td>ND</td>
<td>1.12 ± 0.79</td>
<td>ND</td>
<td>-</td>
</tr>
<tr>
<td>PLAC Group</td>
<td>1.49 ± 0.69</td>
<td>2.91 ± 1.21</td>
<td>3.84 ± 2.11</td>
<td>0.547</td>
</tr>
<tr>
<td>IL-8 (pg/ml)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TRAU Group</td>
<td>11.62 ± 0.69</td>
<td>23.94 ± 1.63</td>
<td>5.28 ± 2.30</td>
<td>0.129</td>
</tr>
<tr>
<td>PLAC Group</td>
<td>11.73 ± 2.69</td>
<td>17.60 ± 7.06</td>
<td>13.24 ± 0.94</td>
<td>0.327</td>
</tr>
<tr>
<td>IL-10 (pg/ml)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TRAU Group</td>
<td>1.85 ± 0.42</td>
<td>9.99 ± 1.35</td>
<td>3.0 ± 0.59</td>
<td>0.019</td>
</tr>
<tr>
<td>PLAC Group</td>
<td>±5.28 ± 0.81</td>
<td>±10.11 ± 1.52</td>
<td>±6.0 ± 1.60</td>
<td>0.433</td>
</tr>
<tr>
<td>IL-12p70 (pg/ml)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TRAU Group</td>
<td>3.67 ± 0.71</td>
<td>6.70 ± 1.48</td>
<td>4.73 ± 0.56</td>
<td>0.633</td>
</tr>
<tr>
<td>PLAC Group</td>
<td>8.61 ± 1.04</td>
<td>7.88 ± 2.07</td>
<td>10.38 ± 3.06</td>
<td>0.082</td>
</tr>
</tbody>
</table>

*Time Effect, Repeated Measures ANOVA, Wilks Lambda Test Statistic, ND: Not detected
While Table 4.5 presents the results of flow cytometric bead array analyses at only three time points before and after the 90-minute trial, Table 4.6 presents the results of the Elisa analysis of IL-6 at each of the 5 time points in the study.

Table 4.6: Plasma IL-6 concentration preceding and following the trial run. Data presented as mean ± SEM

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Pre</th>
<th>Post</th>
<th>24 Hrs Post</th>
<th>48 Hrs Post</th>
<th>72 Hrs Post</th>
<th>p-value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-6</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TRAU Group</td>
<td>1.82 ± 0.29</td>
<td>8.89 ± 0.98</td>
<td>2.71 ± 0.65</td>
<td>2.84 ± 0.37</td>
<td>2.00 ± 0.43</td>
<td>0.000</td>
</tr>
<tr>
<td>PLAC Group</td>
<td>2.73 ± 0.43</td>
<td>12.31 ± 3.23</td>
<td>2.52 ± 0.46</td>
<td>3.40 ± 0.63</td>
<td>2.95 ± 0.66</td>
<td>0.637</td>
</tr>
</tbody>
</table>

* Time Effect, Repeated Measures ANOVA, Wilks Lambda Test Statistic
Chapter 5
Discussion

This trial presented with numerous challenges. Although it would have been ideal to use each subject as their own control and conducted a classic cross-over, double-blinded-placebo controlled trial, this was not possible due to the well-described confounding repeated bout effect (McHugh et al., 1999, 2003). As previously described, the training effect of the first 90-minute downhill running trial would have resulted in an attenuation of most markers of the inflammatory response to eccentric exercise (Hortobagyi et al., 1997, 1998; McHugh et al., 1997, 1999; McHugh, 2003; Lavendera and Nosaka, 2008). This would have significantly impacted the results of the second trial run even after the wash-out period and has been shown to persist for up to 3 months post-exercise (Hortobagyi et al., 1997, 1998; McHugh, 2003). Therefore matched pairs were selected to participate in the study and every attempt was made to ensure optimal matching. It was felt that this study design was superior to randomizing subjects when the sample size was so small.

As is evident in Tables 4.1, 4.2 and Figure 4.1, subjects in the two groups were not significantly different ($p > 0.05$) in terms of age, stature, mass, percentage body fat, body mass index, FVC, FEV$_1$, RER Max, VO$_2$ Peak, VE$_{max}$, and heart rate at rest. They were also well matched with respect to their average weekly running distance, number of running years, 1609 run time, performance in 5km, 10km, half marathon and/or marathon times. Where possible, subjects who were training partners and hence trained in similar conditions, were matched.

5.1. Pre-Trial Data

Although every attempt was made to match the subjects in terms of the factors mentioned above, it would appear that the subjects in the TRAU group entered the trial slightly less well-rested than those in the PLAC group. This is reflected by the differences in the mean creatine kinase concentrations between the two groups in the pre-trial samples ($p < 0.05$). We also confirmed the findings of Jordaan, (2006) of a higher pre-trial Cortisol concentration in the TRAU group following the 5 days of Traumeel administration. As the anti-inflammatory effect of Cortisol is well recognized, this may be a beneficial effect of Traumeel and does require further verification in studies with larger sample sizes.

5.2. Trial Performance

Subjects in TRAU group ran slightly faster and covered a slightly greater distance during the 90-minute run than subjects in PLAC group ($p < 0.05$). This difference was statistically significantly ($p = 0.03; p = 0.02$). However, with respect to the relative degree of exertion as reflected by the mean RPE, heart rate and maximum heart rate and RPE, there were no significant differences between the TRAU and the PLAC
groups during the 90-minute downhill trial run ($p > 0.05$). Although this may have indicated a slightly superior training status in the TRAU group, analyses of the training and race profile (Table 4.3 and Fig 4.1.) indicates no difference between the groups.

The possibility of a lesser relative perception of effort and chronotropic adaptation to the higher work rate following 5 days of TRAUMEEL administration in the TRAU group requires further investigation. Although this has not been previously shown in scientific studies, numerous anecdotal reports do exist. It is important that the post-trial recovery rates are evaluated in relation to the increased amount of work done and greater power output in the TRAU group during the 90-minute trial.

5.3. Post-Trial Data

The effect of the 90-minute downhill running trial was as follows:

- A significant increase in leucocytes/neutrophil count in both treatment groups.
- A significant increase in blood cortisol concentration in both treatment groups.
- A significant increase in creatine kinase concentration in both treatment groups.
- No change in the concentrations of plasma:
  - TNF-α
  - IL-12p70
- An increase in the concentrations of plasma:
  - IL-6
  - IL-8
  - IL-10

in both TRAU and PLAC groups.

Despite the relatively small downhill gradient (-6%), these confirm the findings of Ostrowski et al., 1998b, 1999; Singh et al., 1999; Nieman et al., 2000; Peters et al., 2001b; Starkie et al., 2001; McKune, 2006; Smith et al., 2007 with respect to Cortisol, creatine kinase and IL-6, IL-8, IL-10 cytokine concentrations.

5.4. Serum Creatine Kinase

Our findings following the 90-minute bout of downhill running support those of McKune et al. (2006) who found that a 60 minute bout of downhill running at a gradient of -13% resulted in substantial elevation of CK post-exercise. Interestingly our CK concentrations peaked at 24PE (1394.8 ± 273.96 in the TRAU Group and 915.9 ± 192.46 in the PLAC group).

Of greatest interest is the fact that the faster running speed and greater distance covered on the treadmill by the runners in the TRAU group, elicited a greater evidence of muscle damage in the TRAU group ($n = 10$) in the IPE samples ($p < 0.05$), but not in the 24PE, 48PE, and 72PE samples. This would appear to reflect a
more rapid recovery in the TRAU group. The faster mean recovery rate was, however, not of statistical significance ($p > 0.05$). this was most likely due to large intra-group variance.

5.5. Neutrophil Counts

These results confirm that the downhill running protocol of 90-minutes, at a gradient of -6% and running speed which elicited 75% of VO$_2$ peak, was sufficient to result in a significant elevation ($p < 0.03$) in neutrophil counts in both TRAU and PLAC groups.

They confirm the previously described exercise-induced neutrophilia which has been reported to be related to increasing serum cortisol concentrations (Pedersen, 1997) and to also be indicative of an acute reaction to the microscopic muscle damage inflicted by the downhill running protocol.

The findings of this study revealed that despite the faster running speed, no significant time-treatment interaction was shown between the TRS and PLAC groups in terms of WBC and neutrophil response to the exercise protocol over the three-day period. This appears to indicate that TRAU does not act by inducing the mobilisation of neutrophils in response to microscopic muscle damage.

5.6. Serum Cortisol

As particular care was taken to ensure that post trial blood sampling occurred at the same time of day, the diurnal rhythm of cortisol release from the adrenal gland was accounted for. The pre-exercise mean of 419.8 ± 209.8 and 320.3 ± 88.7 (which falls into the upper bracket of the morning reference range of cortisol) in the TRAU and PLAC groups is attributed to the fact that all trials took place in the morning, most starting before 9am.

The significantly higher pre-trial concentrations in the TRAU group following 5 days of supplementation, confirm the findings of Jordaan (2006) who also reported elevated pre-trial cortisol concentrations in the TRAU group. As large variations in baseline cortisol concentrations do exist between individuals (Peters, 2001 b), the finding, however, requires validation in a larger study before possible mechanisms of action are investigated.

The concentration of cortisol rose significantly following the 90-minute run in both the TRAU and PLAC, but had returned closer to baseline levels 24 hours post the trial-run with a significant ($p = 0.001$) time-effect in both groups. As is reflected by the close to parallel alignment of the line graphs of the two groups, the group-time effect was, however, not statistically significant ($p = 0.767$).

The almost two-fold (200%) rise in serum cortisol concentrations exceeds the 50% rise reported by McKune et al. (personal correspondence, 2006) following 60 minutes of running at a steeper gradient of -
13.5%. The rise would most likely have been caused by the damaging effect of downhill running on muscle fibers in both the TRAU and PLAC groups over the 90 minute period. It is well described in the literature that the debris produced by the muscle tissue damage activates the macrophages that are resident in the muscle tissue, which produce pro-inflammatory cytokines, including TNF-α and IL-1β, which, amongst other functions, increase cell membrane permeability and the influx of lymphocytes, neutrophils, monocytes to the damaged tissue and activate the release of cortisol from the adrenal cortex (Malm et al., 1999; Pederson and Toft, 2000). The resultant release of anti-inflammatory cytokines into the circulation (Figure 2) is important in restraining the inflammatory response and accelerating the healing process ((Van Brandt, 2002).

Interestingly, despite the consistently higher mean cortisol concentrations in the TRAU group over the 5 time points, the difference between the two groups was not statistically significant. As the range of serum cortisol concentration is great between individuals, the large variance in combination with the low power of the study, is the most likely reason for this. A larger study involving greater sample sizes is thus imperative to clarify this potentially important anti-inflammatory mechanism by which Traumeel may act.

5.7. Plasma TNF-α

The 90-minute run did not result in a significant change in TNF-α concentrations when compared to the pre-test values ($p = 0.243$, $p = 0.165$). TNF-α concentrations were, however, undetected in numerous samples and the values remained within the normal range throughout the 24 hour period pre and post trial (Nieman, et al., 2001, although Hirose et al., 2004). No time effect ($p = 0.167$) and no group time-interaction ($p = 0.547$) was noted.

Numerous reports (Ostrowski et al., 1998; Croisier et al, 1999; Ostrowski et al., 1999; Nieman et al. 2000; Toft et al., 2000; Starkie et al., 2001; Hirose et al., 2004; Smith et al., 2007), especially those following eccentric exercise, indicate that there is no significant exercise-induced increase. Firstly, the half-life of circulating TNF-α is short (14-18 minutes) and the kidney is a major organ for TNF clearance. A rapid renal clearance from the circulation into the urine has therefore been suggested as one of the reasons for the failure of TNF-α concentrations to be elevated post exercise (Seckinger et al., 1989 Shek and Shephard, 1998; Suzuki et al., 2002). Exercise has also been shown to stimulate the release of soluble TNF-α receptors which inactivate systemic TNF-α release (Ostrowski et al., 1999). Thirdly, prolonged exercise results in the release of IL-10 which inhibits NF-κB translocation, promoting degradation of mRNA for TNF-α (Opal et al., 1998) and also attenuates surface membrane expression of TNF-α receptors and promotes shedding of the receptors into the circulation (Dickensheets et al., 1997; Joyce et al., 1994).
The failure of plasma TNF-α concentration to rise and the inability to detect a difference between the two treatment groups is thus an expected finding in in vivo work of this nature which involves the response to prolonged exercise.

5.8. Plasma Interleukin - 6

The mean ± SEM of plasma IL-6 concentrations immediately before, after and 24 hours after the 90-minute trial run are presented in Table 4.6. The downhill run resulted in a relatively small (±5.2 fold), but significant elevation in IL-6 (p < 0.05) when compared to the pre-test values. The difference in the exercise-induced change between the two groups was, however, not statistically significant (p > 0.05). There appeared to be a significant time effect (Table 4.6, (p < 0.001). The concentrations of IL-6 reached baseline levels within 24 hours following the run and did not fluctuate during the subsequent 48 hour period monitored.

Circulating IL-6 concentrations have been shown to increase both during and following bouts of severe exercise, especially when muscle mass recruitment is large and exercise is of an eccentric nature. Many studies have demonstrated increased levels of IL-6 following bouts of prolonged exercise which results in the recruitment of large muscle mass which has been attributed to increased metabolic activity in muscle ((Ostrowski et al., 1998b, 1999, Singh et al., 1999, Nieman et al., 2001, Starkie et al. 2001 and Peters et al., 2001a; 2001b; Smith et al., 2007).

The exercise-induced increase in IL-6 was, however, relatively modest when compared to previous reports in the literature. Several factors could account for this.

Firstly, relatively low metabolic activity during downhill running when compared to running uphill or on a level surface, would explain relatively small exercise-induced increases in IL-6 measured immediately post exercise. In this regard our findings confirm those of Ostrowski et al.(1998b); Nieman et al.(2001); Smith et al.(2007).

In terms of an inflammation responsive reaction occurring after completion of the eccentric exercise, the timing of the post-exercise sample may have been an important factor. Previous research (Ostrowski et al.,1998b) has shown that when the increase in IL-6 is the result of muscle damage, it only peaks in the blood stream after 30-minutes. As our samples were taken immediately post-exercise, this may also account for the moderate increase we reported.

It is, however, interesting that despite the greater distance covered, faster running speed and higher CK concentrations in the TRAU group, IL-6 concentrations were not significantly higher in the TRAU group.
pointing towards the possibility of an attenuated inflammatory response in this group. This requires further investigation in a larger study.

5.9. Plasma Interleukin - 8

The downhill run resulted in an approximately 9-fold increase in the serum concentrations of IL-8 in the TRAU group and the PLAC group. This represented a significant elevation ($p < 0.05$) in IL-8 serum concentration between the pre and post test concentrations. However, the difference in the exercise-induced change between the two groups was, not statistically significant ($p > 0.05$). IL-8 concentrations reached baseline levels within 24 hours following the run. The Repeated Measures ANOVA test revealed that neither the increase in concentrations over time nor the time-group interactions were significantly different ($p = 0.129$, $p = 0.327$).

The results of this study concur with the results of other studies which revealed that following periods of intense eccentric exercise or periods of exhaustive exercise, serum IL-8 concentrations have been shown to be significantly elevated in the blood (Nieman et al., 2000; Suzuki et al., 2000; Nieman et al., 2001; Ostrowski et al., 2001; Smith et al., 2007). In both groups, the downhill running protocol therefore not only resulted in increased neutrophil concentrations in the blood, but also increased the concentrations of the chemotactic cytokine, IL-8. As the concentrations of the two groups were, however, not significantly different between the two groups despite the higher workload in the TRAU group, the findings of this study do not provide evidence of increased chemotactic stimulus for phagocytes following ingestion of Traumeel®S.

5.10. Plasma Interleukin - 10

The mean ± SEM plasma IL-10 concentrations rose significantly in both groups following the downhill exercise protocol ($p < 0.001; p = 0.03$) with mean concentrations peaking at 9.99 and 10.11 pg/ml respectively. The time effect was statistically significant throughout the 24 hour period ($p = 0.02$), but the time-group interaction ($p > 0.05$) did not reveal a significant difference between the two groups.

IL-10 is part of the primary anti-inflammatory cytokine group and acts by inhibiting pro-inflammatory cytokine production by monocyte and macrophage activation. The findings of this study support those of Peters et al. (2001b), Suzuki et al. (2002), Smith et al (2007) who reported that high intensity exercise of extended durations may induce the release of IL-10 into the circulation.

As the downhill protocol used in the study, did according to the raised CK levels and degree of post-exercise neutrophilia, induce muscle microtrauma, post-exercise elevation of IL-10 was an expected finding (Peters et al., 2001b; Peters, 2004). The rise in IL-10 was, however, not different between the TRAU and PLAC trials, despite the significantly greater running distance and faster running speed, a
finding which is in alignment with that of the cortisol, IL-6 and IL-8 results. This supports the possibility of a protective effect in the TRAU group.

5.11. Plasma Interleukin – 12p70

Due to the large variance in the results, the exercise trials were not found to result in a statistically significant increase in serum IL-12p70 (pg/ml) concentrations and no significant group-time effect was observed in the cytokine concentrations ($p > 0.05$). This confirms the findings if Akimoto et al. (2000) and Suzuki et al. (2002).

While the inconsistencies and lack of reproducibility have been attributed to the rapid down-regulation of IL-12 levels following exercise and to different measurement methods (Suzuki et al., 2002; Smith et al., 2007), it is also of interest that the p70 subunit and not the p40 subunit was measured. In view of the important function of IL-12 in activating Th1 cells, this failure of IL-12p70 to increase significantly, was an expected finding. On the one hand, it may be interpreted to indicate that the exercise protocol was not intense enough to downregulate the Th1 cells and hence cell-mediated immunity. On the other hand, measurement of the p40 subunit may have provided more valid data and would have been a more advisable choice in view of the recent findings of Suzuki et al. (2003).
Chapter 6

Conclusions and Recommendations

Despite the shortcomings of this study which include a relatively small sample size and limited statistical power, several interesting observations were made.

1. The subjects in the TRAU group presented with higher pre-trial blood CK and cortisol concentrations than the PLAC group after administration of Traumeel® S for 5 days. This requires further investigation.

2. In both TRAU and PLAC groups, the exercise-induced increase in IL-6, IL-8 and IL-10 concentrations, provided evidence of an inflammatory response to the downhill running protocol and an upregulation of the Th2 cells. The failure of IL-12p70 to increase significantly, however, appears to indicate that the downhill running protocol was not intensive enough to downregulate Th1 cells and suppress cellular immunity.

3. Although the subjects in the TRAU group did not report a superior competitive performance or training status profile before the study ($p > 0.05$), the average running speed and distance covered in the 90-minute trial was greater than the subjects in the PLAC group. This was confirmed by higher 24hr post-trial CK concentrations. Despite this, their mean and maximum RPE ($p = 0.441$) and mean and maximum heart rate ($p = 0.209$) was not greater. In addition, blood cortisol, IL-6, IL-8 and IL-10 concentrations were not significantly higher in the TRAU group. In view of the higher work output of the TRAU group during the 90-minute trial, this is, however, indicative of a possible protective effect of Traumeel® S and is worthy of further investigation.

A number of directions for future research can also be identified from the findings of this study. These include the following:

1. In future studies, the p40 subunit of IL-12 should be measured in order to verify the conclusion that downregulation of Th1 cells does not occur following an exercise protocol of this nature and that Traumeel does not act on the cellular component of the immune system.

2. Every attempt also needs to be made to ensure that subject pairs complete the same amount of work on the treadmill over the specified time-period in order to ensure that the intervention designed to induce muscle damage, is equal for the two groups. While more precise matching of
pairs may, however, be difficult, a larger sample size would ensure that significant differences would not exist in a randomized, double-blind study of this nature.

3. Molecular investigations into the expression of NF-κB would be valuable in confirming the findings of the inflammation responsive cytokine milieu in a further study, while measurement of TGF-β would provide valuable additional insights into the anti-inflammatory properties of Traumeel.
References


160. Ullum H, Haahr PM, Diamant M, Palmo J, Halkjaer Kristensen J, Pedersen BK. Bicycle exercise enhances plasma IL-6 but does not change IL-1 a, IL-1 b, IL-6, or TNF-a pre-mRNA in BMNC. *J Appl Physiol* 77: 93–97, 1994.


APPENDIX A

DEPARTMENT OF PHYSIOLOGY
FACULTY OF HEALTH SCIENCES
Mandela Medical School

Subject Information Sheet

Study Title: The effect of an anti-inflammatory homeopathic product on systemic markers of inflammation following prolonged exercise.

Introduction: This study will investigate the effect of Traumeel, an homeopathic anti-inflammatory product, on the delayed muscle soreness (DOMS) which occurs following prolonged exercise. As we are interested in examining the mechanisms which result in muscle soreness and whether this can be minimized by the use of homeopathic preparations, we shall be doing a series of analyses on your blood samples before and after exercise.

What will qualify you to participate in this study? Only healthy long-distance athletes aged 20-50 years will be considered for this study.

What will be expected from volunteers in this study? You will be asked to:
- complete a medical history questionnaire
- write down your running style and relevance of your current running shoes to this trial
- write down a list of training paces relevant to your current ability
- maintain your usual diet for the duration of the trials.
- maintain your usual training, the same type of training during the trial period
- not use any analgesics, anti-inflammatory or Vitamin C preparations in addition to the prescribed study supplements for 2 weeks before and for the duration of the trials
- maintain your usual exercise routine during the trial period
- not use any medication (e.g. analgesics or anti-inflammatory) during the study

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Outlines of tests and trials:
- First Laboratory Visit - Exercise Laboratory, Physiology Department, Nelson Mandela Medical School: approx two weeks before trial
  - Interview regarding exercise training status, medical status
  - Weight, height
  - Lung function test
  - Resting heart rate and blood pressure measurement
  - VO2 peak test on treadmill: After a 5 minute warm-up at a relevant pace, the subject will be timed over 4 laps (1609m) on track after a 5 minute warm-up at a relevant pace. The expired air will be collected and analysed as running speed will increase in a stepwise manner. The TV screens/music will be available to entertain the runners on the treadmill
  - Recording and analysis of past and current training processes
  - Explanation of pre-intervention training and eating procedures.

Further queries? Should you have any queries or wish to obtain further details regarding this study, please do not hesitate to contact us.

Norrie Williamson - 031-2017687

Field Visit - University of KZN - Sports grounds Transwel Rd Training (approx 4 weeks before trial)
- Warm-up in release pace - subjects will be trained over 4 laps (1609m) on track
- Recording of pre and post exercise heart rate
- Recording of pre and post exercise heart rate
- Analysis of running style and feedback (as above)
- Basic schedule analysis for pre and postrace (as above)
- Explanation of pre-intervention training and eating procedures.

Interpreting Trial to be conducted in the Exercise Laboratory, Nelson Mandela School of Medicine.

The protocol of each of the intervention trial will be as follows:
- 5 days prior to the trial - 1 x TRAUMEEL; 1 x TRAUMEEL; 1 x TRAUMEEL; 1 x TRAUMEEL; 1 x TRAUMEEL
- The day of the trial - the following breakfast is to be eaten 3 hrs before the trial
- VO2 peak test: 350m of running at 45-50, 40-45 and 42-35 g reduced pace
- Muscle soreness - Serial blood samples to be taken on arrival
- resting heart rate and blood pressure measurement until the subject is comfortable according to the results of the VO2 max test and the 1500 time trial
- Muscle soreness will be drawn at 1, 24, 48, and 72 hours after the trial.

The order of the trials is to be randomized. By this means data that will be placed in treatment sequences using random number tables. You may therefore not be receiving the treatment in the same order as the other subject. As in all double-blind studies, neither the researcher nor you will be informed regarding which treatment you are on. The results are to be coded in place of active substances in the chemical laboratory before distribution to the subjects.

How can you benefit from participation in this study? Following completion of all aspects of the trial, you will receive:
- the results of your VO2 max test
- the results of your full blood counts and other biochemical/laboratory tests which were run on your blood samples
- a prediction of your current ability (potential in various race distances from 5km to Comrades marathon) on your running style and relevance of your current running shoes to this study
- a list of running tests relevant to your current ability

Further questions? Should you have any queries or wish to obtain further details regarding this study, please do not hesitate to contact us.

Professor Edith Peters-Ture - 031-201-4232/3/261-3863/30

Study Director
Department of Physiology
Faculty of Health Sciences
University of KwaZulu-Natal

Further questions? Should you have any queries or wish to obtain further details regarding this study, please do not hesitate to contact us.

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Department of Physiology
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APPENDIX B

UNIVERSITY OF KWAZULU-NATAL

Verification from General Practitioner

Dear Dr __________________________

I hereby agree to participate in a research study to be performed by Edith Peters-Futre and co-workers in the Department of Physiology at the University of KwaZulu-Natal Medical School.

I understand that the base procedures involved in this study are to include:
- Assessments of training history, anthropometric measures, lung function, selected baseline clinical measures and an exercise test (VO2 peak) during 1st Laboratory visit (approx 2 weeks before 90-minute trial in the laboratory).
- Field test conducted by Norrie Williamson which will consist of a 4 mile (6.4km) time-trial on the KZN athletics track with heart rate and time recorded (1 week before 90-minute trial in the laboratory).
- Sublingual intake of 1 x TRAUMEEL or placebo tablets 3 x per day 30 minutes before meals each meal (i.e. 3 x per day) for 5 days prior to the exercise trial to be held in the Exercise Laboratory, Nelson R Mandela School of Medicine.
- Having a 30ml blood sample taken on arrival at the 90-minute exercise trial.
- Laboratory Exercise Trial: Assessment of treadmill at an intensity of 75% VO2 max. 20% above age predicted maximum heart rate will be permitted and 30 and 60 minutes into the run.
- Having a 30ml blood sample drawn 1, 24, 48 and 72 hours after both trials.
- Completing a numerical rating of muscle soreness experienced during the 72 hours after both exercise trials.

The details of these procedures have been explained to me in full. I am aware that a certain level of discomfort may occur when the blood is taken and that the exercise trial will involve strenuous prolonged exercise which may be accompanied by certain medical risks and result in muscle soreness in the 48-72 hours post test. I also understand that I do not have recourse against the University in the case of an acute medical event occurring while I am running on the treadmill.

I understand that participation is entirely voluntary and that I may withdraw from the study at any time.

Signed ____________________________

Date ____________________________

Principal investigator's signature

<table>
<thead>
<tr>
<th>Day, Date and Time of Session</th>
<th>Training Mileage</th>
<th>Intensity Sessions</th>
<th>Rating of Perceived Exertion and General Comments</th>
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<tr>
<td></td>
<td>Type:</td>
<td>Duration:</td>
<td>% of max (e.g., 50%):</td>
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<td>Type:</td>
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<tr>
<td></td>
<td>Type:</td>
<td>Duration:</td>
<td>% of max (e.g., 50%):</td>
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<tr>
<td>Rate of perceived exertion</td>
<td>1 = Very easy</td>
<td>2 = Easy</td>
<td>3 = Moderate</td>
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<td></td>
<td>4 = Fairly</td>
<td>5 = Exhausted</td>
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<td></td>
<td>Exhausting</td>
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**APPENDIX C**

**UNIVERSITY OF Kwa-Zulu Natal**  
**DEPARTMENT OF PHYSIOLOGY**  
**NELSON MANDELA MEDICAL SCHOOL**

The effect of Traumed supplementatioa on inflammatory response to prolonged exercise.

### ATHLETE'S QUESTIONNAIRE

Name: ____________________________  
Date of Birth: __/__/____  
Age: ______  
Address: ____________________________  
Phone Numbers:  
Home: ____________________________  
Work: ____________________________  
Cell: ____________________________  
Athletics Club: ____________________________  
Occupation: ____________________________

### TRAINING DETAILS:

Average weekly running distance: _______ km

**Running History**

Serious/Recreational: _______  
Age started running: _______ (____-____)

Level of training during these years:  
Total number of marathons run: _______

Describe normal terrain used for training (to be discussed / confirmed by athletics coach):  
Take into consideration: number of days on flat - number on hills, distance on hills, group training, etc.

Other sports played in last 12 months (squash, rugby weights, etc):  
List peak events for each year of training age (or last 5 years):  
List races / competitions over the last 6 months (all sports):  
List races / competitions over the last 2 months (all sports):  
Any training group partner in study? If so who?:  
Describe your running shoes (make / model and age / distance covered):  
Describe your running foot strike (heel-toe / forefoot / pronation):  

Check the bow to fill in after test:  

Please sign: ____________________________  
Principal investigator’s signature: ____________________________

### MEASUREMENTS

Pregnancy Test (if female only):  
Lung Function Tests  
FVC: _______ FEV1: _______  
FVC/FEV1: _______  
Height (cm): _______ Mass (kg): _______

Skin - fold Measurements  
Biceps: _______ ave: _______  
Triceps: _______ ave: _______  
Suprailiac: _______ ave: _______  
Subscapular: _______ ave: _______  
Body fat percentage: _______

Resting heart rate: _______  
Recovery heart rate: _______  
Running heart rate: _______  
Blood pressure: _______

Results of VO2 max test:

Speed Km/hr  
VO2 ml/kg/min RQ VE VO x HR RPE

Running speed at 75% VO2 max: _______
APPENDIX D

**Trial Record**

<table>
<thead>
<tr>
<th>Time</th>
<th>Workload</th>
<th>Distance</th>
<th>H R</th>
<th>VO2</th>
<th>RPE</th>
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<tr>
<td>30 min</td>
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<td>60 min</td>
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<td>90 min</td>
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**Pre-Trial Mass:**

**Post-Trial Mass:**

**Training Record:**

<table>
<thead>
<tr>
<th>Tablets</th>
<th></th>
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**DOMS Record:**

**Study Title:** The effects of an anti-inflammatory homeopathic product on systemic markers of exercise-induced inflammation

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<tr>
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<th>Quads</th>
<th>Hamstrings</th>
<th>Calf</th>
<th>Quads</th>
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General post-trial pain tested and released
Post-trial pain during daily living while walking

0 = no pain at all
5 = moderate
10 = severe/maximal