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

Dr A. Docrat

M.Tech: Chiro., C.C.F.C (T.N.), P.G. Dip-U.T.Med. (UWC)

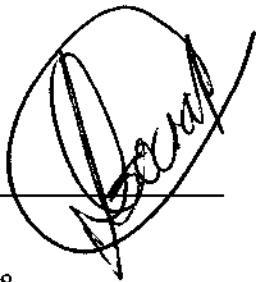**SUPERVISOR:** Prof. E. M. Peters - Futre

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Declaration

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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

Acknowledgements

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, FEV_1, baseline heart rate and blood pressure, RERmax, VO_2 peak, VE_{max}, 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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List of Abbreviations

12PE	12 hours post-exercise
24PE	24 hours post-exercise
48PE	48 hours post-exercise
72PE	72 hours post-exercise
ACTH	Adrenocorticotropic hormone
ANS	Autonomic nervous system
ANOVA	Analysis of variance
BCAA	Branched chain amino acid
BMI	Body mass index
CAM	Cell adhesion molecules
CK	Creatine kinase
CRH	Corticotropin releasing hormone
CRP	C-reactive protein
CSIF	Cytokine synthesis inhibitory factor
CTL	Cytotoxic T Lymphocytes
DOMS	Delayed onset muscle soreness
EDTA	Ethylenediaminetetra acetic acid
ELISA	Enzyme-linked immunosorbent assay
FBC	Full blood count
FVC	Forced Vital Capacity; L/min
F_EV_1	Forced Expiratory Volume in 1 second
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HPA	Hypothalamic pituitary axis
ICAM-1	Intercellular adhesion molecule-1
IFN-γ	Interferon-gamma
IgA	Immunoglobulin A
IL-1α	Interleukin-1 Alpha
IL-1β	Interleukin-1 Beta
IL1-ra	Interleukin-1 receptor antagonist
IL-2	Interleukin-2
IL-3	Interleukin-3
IL-4	Interleukin-4
IL-5	Interleukin-5

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-κB	Nuclear Factor Kappa B
NCF	Neutrophil Chemotactic Factor
NK	Natural killer
OTS	Over training syndrome
PGE2	Prostaglandin E2
PLAC	Placebo
PTRS	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
V_E	Pulmonary Ventilation
VCO_2	Carbon dioxide output
VO_2	Oxygen consumption
VO_2 peak	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-ß), 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 % VO_2 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% VO_{2max}.*

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 (VO_2 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% VO_2 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 Hölmich, 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).

MacIntyre *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^{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 steam (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 hematopoesis (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 hematopoesis (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*

Cytokine Classification	Secreted by	Target cells	Selected Primary Functions
Pro-Inflammatory cytokines			
TNF-α	Macrophages mast cells, NK cells	Macrophages, tumor cells	Induction and expression of adhesion molecules on leucocytes and induction of inflammatory response which activates vascular endothelium increasing its permeability and expression of IL-β; IL-6; IL-8
IL-1α	Macrophages, Monocytes	T helper cells, B cells, NK cells	Co-stimulation of Th1 cells, maturation and proliferation of B cells, activation of NK cells, inflammation, acute phase response, fever
IL-1β	Dendritic cells, B-cells, macrophages	Th cells, B cells, NK cells	Co-stimulation of Th2 helper cells, maturation and proliferation of B cells, activation of NK cells, inflammation, acute phase response, fever
Anti-inflammatory cytokines			
IL-4	Th2-cells, memory CD4+	Activated B cells, T cells macrophages,	Proliferation and differentiation of Th-cells, IgG_1 and IgE synthesis, mediates recruitment and activation of mast cells
IL-10	Th2 cells	B cells, Macrophages	Inhibition of macrophage derived release of TNF-α; IL-2, IL-3, IFN-γ, suppress antigen presentation by macrophages
Immunomodulatory cytokines			
IL-2	Th1-cells	Activated T and B cells, NK cells	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
IL-5	Th2-cells	Activated B cells	Proliferation and differentiation IgA synthesis
IL-12	Macrophages and B-cells	Activated T cells, NK cells	Differentiation into CTL (with IL-2), activation of NK cells
IFN-γ	Th1-cells, Tc-cells, NK cells	Macrophages activated B cells, Th2 cells	Viral replication MHC I expression, Ig class switch to IgG2a, pathogen elimination

Multifunctional Cytokines

IL-6	Monocytes, Macrophages Th2-cells, Stromal cells, Muscle cells	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 hematopoesis, stimulation of cortisol release from adrenal medulla, signal in the synthesis of plasma proteins
TNF-β	Th1 and Tc-cells	Phagocytes, tumor cells	Phagocytosis, cell death
TGF-β	Th3-cells, Monocytes	Monocytes, Macrophages Activated B cells	Increasing IL-1 production by activated macrophages, inducing a class switch to IgA proliferating B cells. Acting as chemoattractant for monocytes and macrophages. Inhibiting the proliferation of cells required for the inflammatory process

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

MHC: Major histocompatibility complex; NK cells: Natural killer cells, CAM: Cell adhesion molecules, IgA: Immunoglobulin A, CTL: Cytotoxic T Lymphocytes, Tc-cells: Effector cytolytic T lymphocytes. *Suzuki *et al.*, 1999; Pedersen *et al.*, 2001; Suzuki *et al.*, 2000, 2002; Smith, 2000; Bagby *et al.*, 1996; Peters, 2004; Pedersen and Hoffman-Goetz, 2000.

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), diapedese 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 blood stream 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, 1993; 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

Organ/Tissue	Effect
Liver	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.
Macrophages	Activates phagocytosis, production of IL-1 and the inflammatory lipid prostaglandin E_2 (PGE_2).
Hypothalamus	Activates the hypothalamic-pituitary-adrenal axis causing the release of corticotropin releasing hormone (CRH), appetite suppression and pyrexia.
Other tissue:	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.

*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,

1995), 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

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

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

Sstimulation 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-∝ 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

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

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, IL2 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, TH_2 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 form 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 (Toft *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; Toft *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; Kaliński *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; Kaliński *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 E_2, cAMP, ß-adrenergic agents and histamine.

2.2.7. Pro and Anti-inflammatory Cytokine Response to Eccentric Endurance Exercise
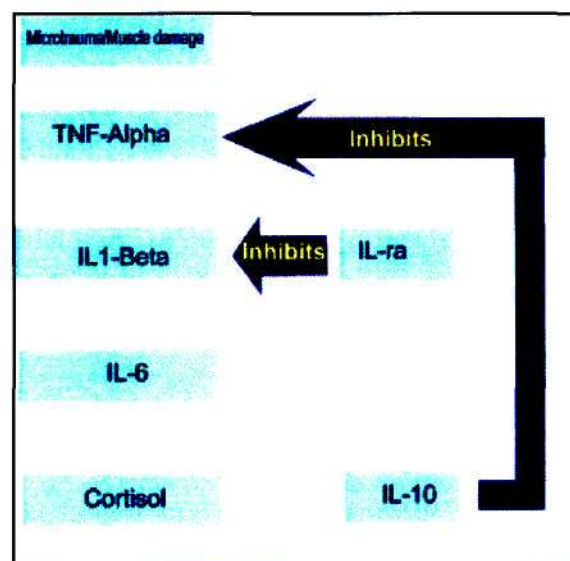
Figure 2.1: The Pro-Inflammatory cytokine cascade depicting the negative feedback mechanism

In response to tissue microtrauma which occurs following eccentric exercise, macrophages which are resident in the injured tissue, become activated, releasing TNF-∝ 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, IL1-β, IL-6, IL-2 receptors, and IFN-g) 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 department 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-b1 (TGF-b1) 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.
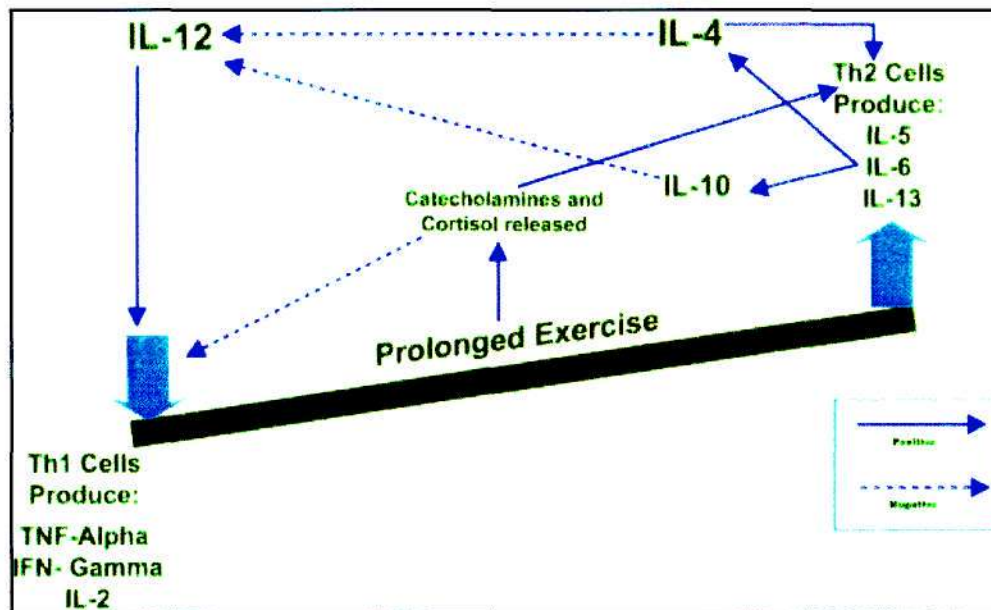
Figure 2.2: The effect of prolonged exercise on the Th1-Th2 cytokine balance following exhaustive exercise

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 β_2-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 a_2-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% VO_2max (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 muscularly 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 antiphlogistic 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:

- o fell within the age category of 20 to 50 years inclusive
- o were asymptomatic
- o 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
- o 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)
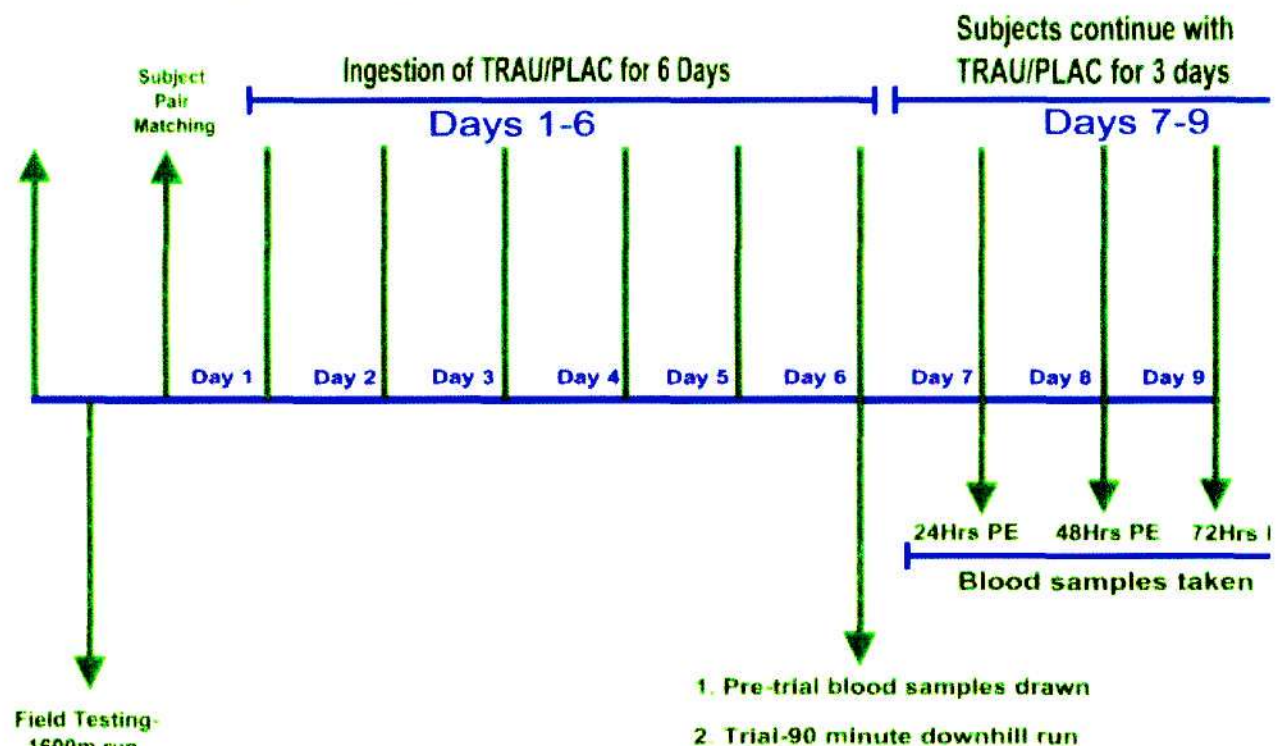
Figure 3.1. Study protocol depicting the time-points.

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 VO_2max 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 VO_2 over a 30 second period was defined as the $VO_{2\,peak}$.

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% CO_2 and 16.7 % O_2 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 tubine sensor which was connected to a tightly fitting facemask. Oxygen and CO_2 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 VO_2 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
- $VO_{2\ max}$
- 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% VO_2 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% VO_2 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 48nm laser capable of detecting and distinguishing fluorescence emissions at 576 and 670nm (eg. 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 0.2ml 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 75mm 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. 900µl 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. 100µl of 10x bulk standard was transferred to the Top Standard tube and was mixed thoroughly

6. A serial dilution was performed by transferring 300µl from the Top Standard to the 1:2 dilution tube and was mixed thoroughly. Several such serial dilutions were conducted by transferring 300µl 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.
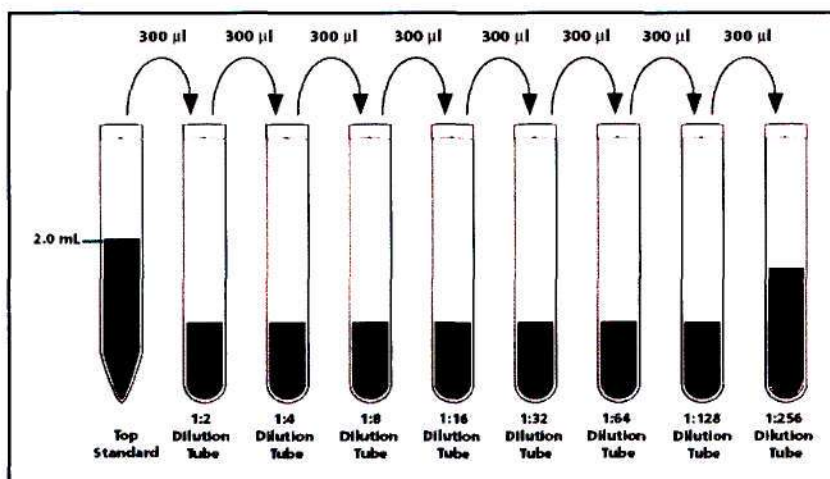
Figure 3.2. Preparation of Human Inflammation Standard Dilutions

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.

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

* 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 pelletted 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).

- 1.5 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).

- 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.

	General post-trial pain seated and relaxed			Post-trial pain during daily living while walking		
DAY after the trial	**Quadriceps**	**Hamstrings**	**Calf**	**Quadriceps**	**Hamstrings**	**Calf**
Day 1: immediately after the run						
Day 2:						
Day 3:						
Day 4:						
Day 5:						

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 $\pm$ 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 5. 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, FEV_1, 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

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

FVC = Forced Vital Capacity; L/min; F_EV_1 = 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 Reponses to the Maximal Incremental Exercise Test in the PLAC and TRAU groups. Data presented as mean ± SEM

Variable	PLAC ($n=10$)	TRAU ($n=10$)	p-value*
HR max (b.min^{-1})	180.27 ± 3.41	175.45 ± 3.11	0.209
RPE max	7.27 ± 0.35	6.91 ± 0.38	0.441
VEmax (L. min^{-1})	98.21 ± 2.79	108.99 ± 4.06	0.370
VO$_2$ Peak (ml.kg.min^{-1})	50.64 ± 1.23	54.42 ± 1.50	0.155
RER Max	1.11 ± 0.02	1.13 ± 0.02	0.626

*Paired t tests, TRAU vs. PLAC; RER = Respiratory Exchange Ratio maximum; VO$_2$ 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$_2$ 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 4.3: Training status of the subjects in the TRAU group and the PLAC group. Data presented as mean ± SEM

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

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

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.
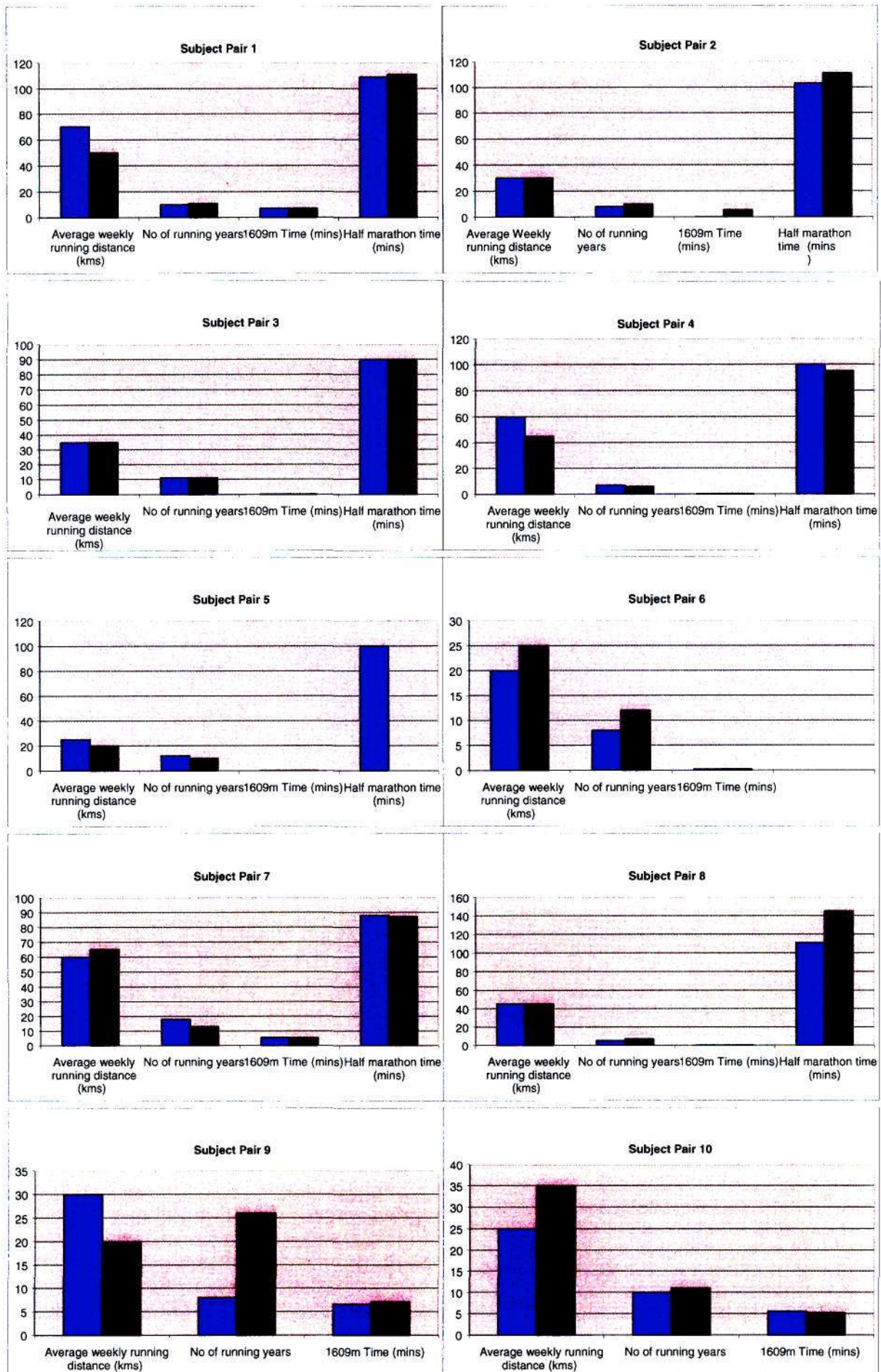
Figure 4.1. The matching of the subject pairs with respect to their training status and performance.

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

Parameter	TRAU ($n = 10$)	PLAC ($n = 10$)	p-value*
Average Speed (Km/hr)	13.29 ± 0.21	12.84 ± 0.25	0.020
Distance (km)	20.13 ± 0.29	19.41 ± 0.38	0.030
Mean RPE	4.82 ± 0.26	4.91 ± 0.25	0.780
Mean Heart Rate	159.5 ± 1.87	160.7 ± 2.09	0.630
Maximum Heart Rate	175.5 ± 3.11	180.3 ± 3.41	0.210
% of Heart Rate Maximum	83.9 ± 1.32	85.4 ± 0.66	0.390
Maximum RPE	6.91 ± 0.38	7.27 ± 0.35	0.440

*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 ($p = 0.398$; 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

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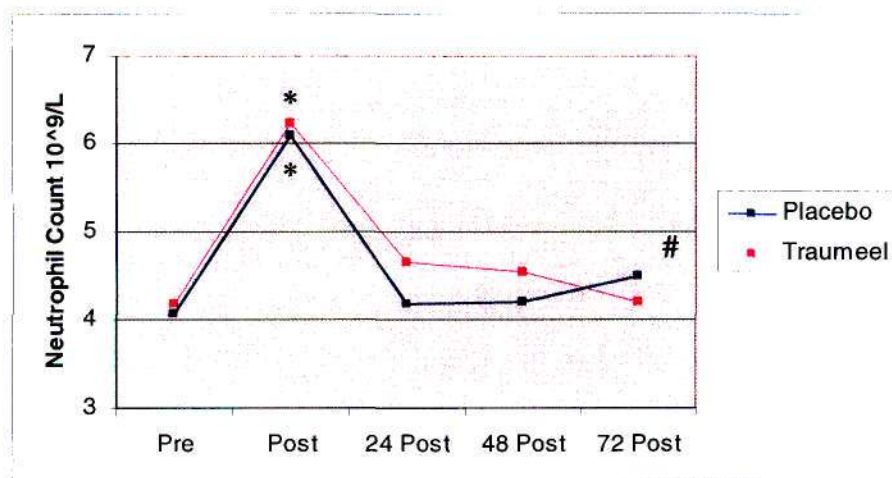
Figure 4.3 : Mean ± SEM neutrophil concentrations in the TR AU ($n = 10$) and PLAC groups ($n = 10$) before and after the 90-minute treadmill trial. # $p < 0.05$ Time-effect, repeated measures ANOVA, Wilks Lambda Test Statistic, *$p < 0.05$ Simple contrast *post hoc* comparison vs PRE

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
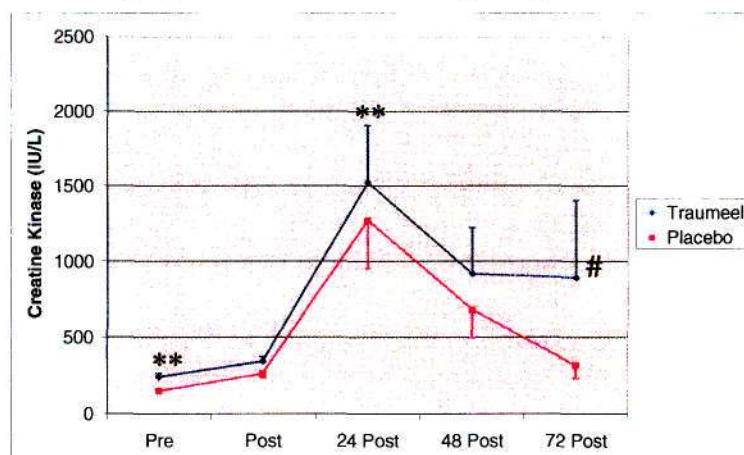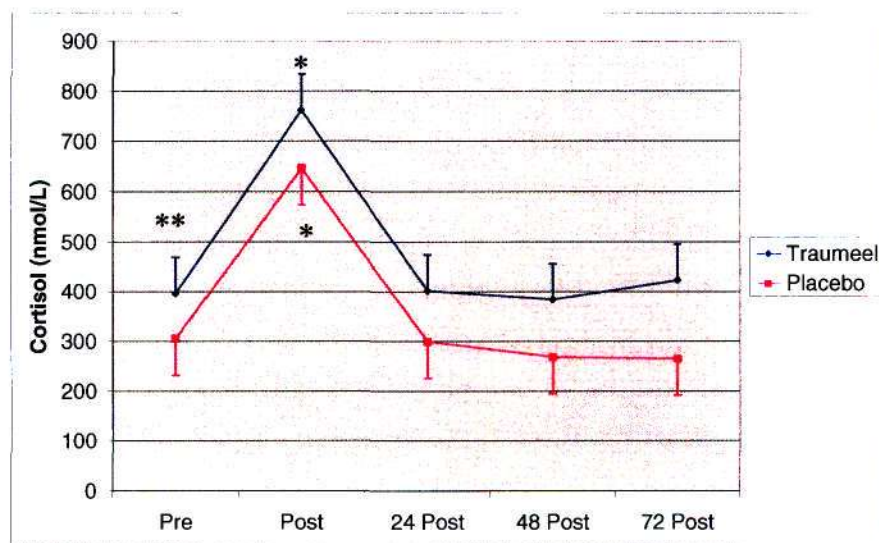

Figure 4.4: Mean ± SEM serum cortisol concentrations of the TRAU ($n = 10$) and PLAC ($n = 10$) groups before and after 90-minutes of downhill running on a treadmill *$p < 0.05$ Paired *t*-tests vs PRE; ** $p < 0.05$ Simple contrast *post hoc* comparison between groups at time point

Figure 4.4 graphically displays changes in serum cortisol concentrations for the 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
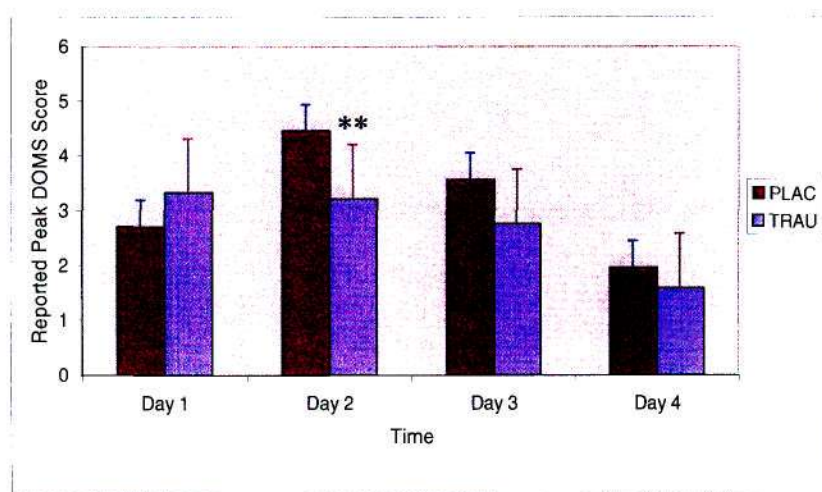
Figure 4.5: Mean ± SEM reported peak DOMS scores of the TRAU ($n = 10$) and PLAC ($n = 10$) groups following participation in a 90-minute downhill run ; ** $p < 0.05$ Simple contrast *post hoc* comparison between groups at time point

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

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

4.8. Plasma IL-8 concentrations:
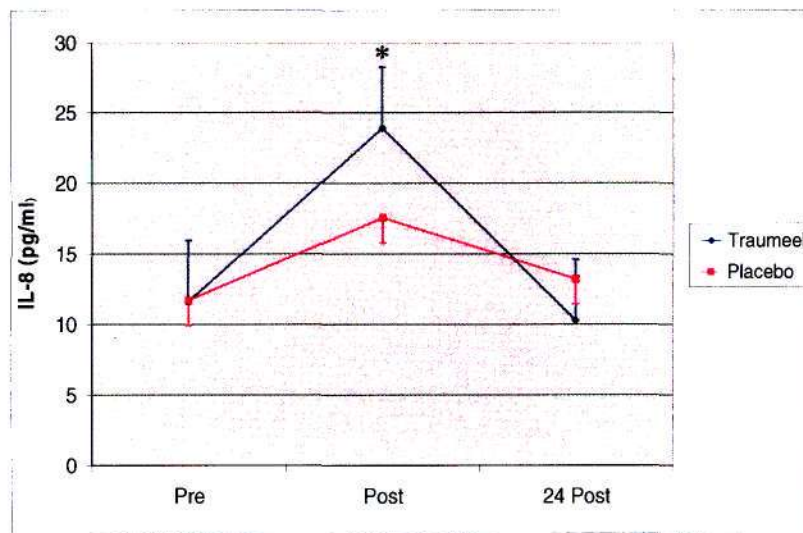
Figure 4.8: Mean ± SEM plasma IL-8 concentrations (pg/ml) of subjects in the TRAU ($n = 10$) and PLAC ($n = 10$) before and after participation in the 90-minute trial at 3 time-points, *$p < 0.05$, Simple contrast *post hoc* comparison vs. PRE

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:
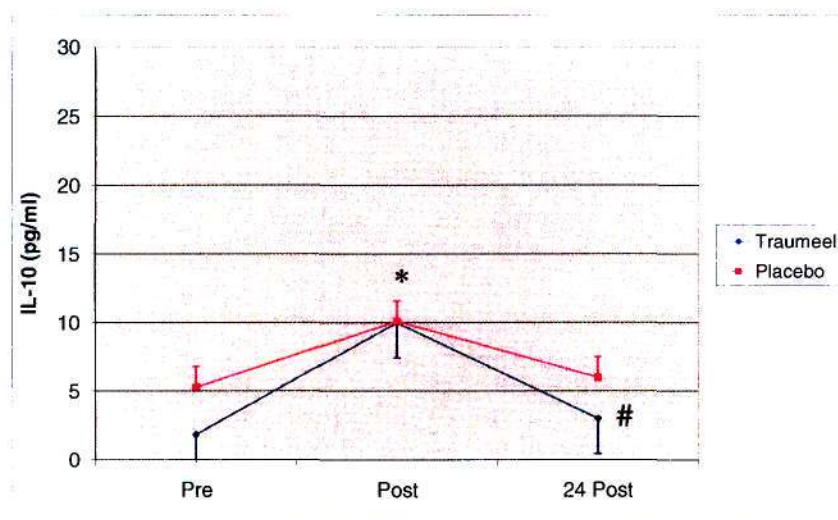
Figure 4.9: Mean ± SEM plasma IL-10 concentrations (pg/ml) of subjects in the TRAU ($n =10$) and PLAC ($n = 10$) before and after participation in the 90-minute trial at 3 time-points, # $p < 0.05$ Time-effect, repeated measures ANOVA, Wilks Lambda Test Statistic, *$p < 0.05$, Simple contrast *post hoc* comparison vs. PRE

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:

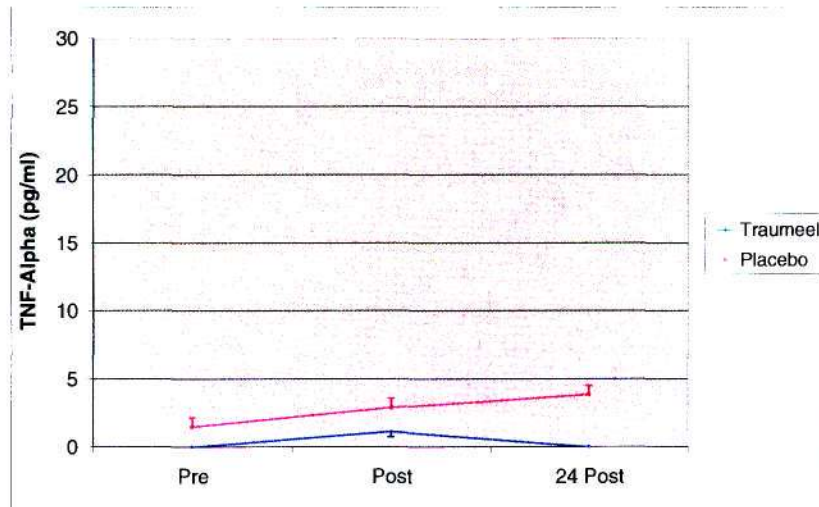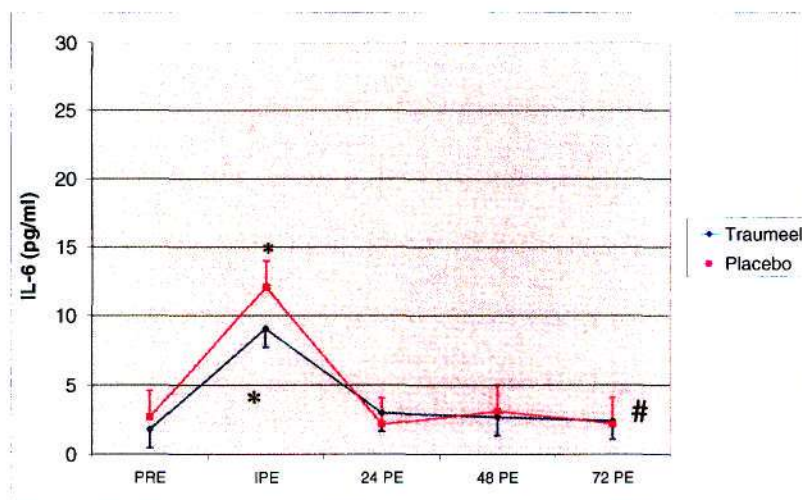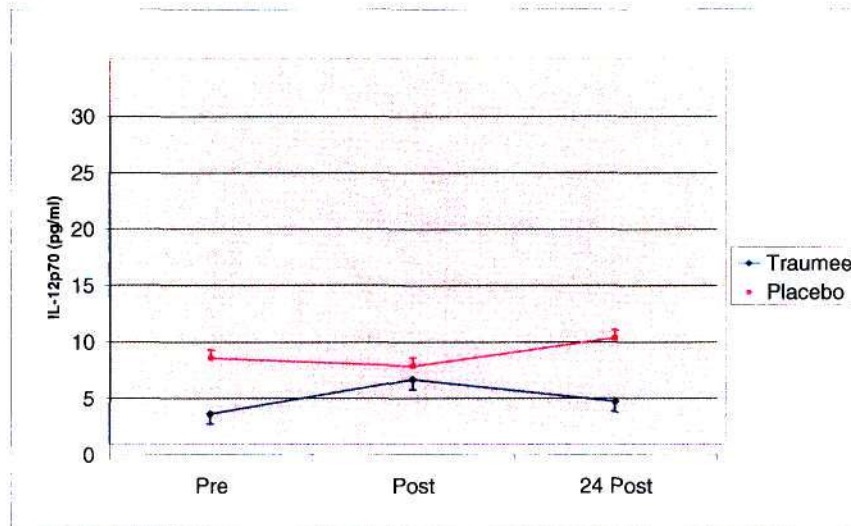Figure 4.10: Mean ± SEM plasma IL-12p70 concentrations (pg/ml) of subjects in the TRAU ($n = 10$) and PLAC ($n = 10$) before and after participation in the 90-minute trial at 3 time-points

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

Parameter	Pre	Post	24 Hrs Post	* p-value
TNFα (pg/ml)				
TRAU Group	ND	1.12 ± 0.79	ND	-
PLAC Group	1.49 ± 0.69	2.91± 1.21	3.84 ± 2.11	0.547
IL-8 (pg/ml)				
TRAU Group	11. 62 ±0.69	23.94 ± 1.63	5. 28 ± 2.30	0.129
PLAC Group	11. 73 ± 2.69	17.60 ± 7.06	13.24 ± 0.94	0.327
IL-10 (pg/ml)				
TRAU Group	1.85 ± 0.42	9.99 ± 1.35	3.0 ± 0.59	0.019
PLAC Group	±5.28 ± 0.81	±10.11 ± 1.52	6.0 ± 1.60	0.433
IL-12p70 (pg/ml)				
TRAU Group	3.67 ± 0.71	6.70 ± 1.48	4.73 ± 0.56	0.633
PLAC Group	8.61 ± 1.04	7.88 ± 2.07	10.38 ± 3.06	0.082

*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

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

* 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.*, 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:

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

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APPENDIX A

DEPARTMENT OF PHYSIOLOGY
FACULTY OF HEALTH SCIENCES
DEPARTMENT OF PHYSIOLOGY
NELSON R. MANDELA MEDICAL SCHOOL

SUBJECT INFORMATION SHEET

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

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 establishing the mechanisms which result in this muscle soreness and whether this can be minimized by the use of homeopathic preparations, we shall be doing a variety of analyses on your blood samples before and after exercise.

Who will qualify to participate in this study? Thirty healthy long-distance athletes
- aged 20–50 years
- non-smokers, non-diabetic, free of cardiovascular impairments (as verified by medical practitioner)
- not showing clinical signs of infection (as verified by medical practitioner)
- taking no medication (e.g. analgesics or anti-inflammatories)
- covering a minimum of 30 km per week in training

What will be expected from volunteers in this study?
You will be asked to:
- not use any analgesics, anti-infective or 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 diet for the duration of the trials
- maintain the same type of training during the trial period
- avoid any heavy physical exercise the day before each exercise trial.

Outline 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
  - Assessment of height, mass and percentage body fat (to be estimated from skinfold thicknesses)
  - 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, expired air will be collected and analysed as running speed will increase by

- **Field visit – University of KZN – Sports grounds Francois Rd Track (approx 1 week before trial)**
  - Warm up at relevant pace – subject will be timed over 4 laps (1609m) on track with heart rate and time recorded.
  - Recording and analysis of past and current training processes
  - Analysis of running style and feedback (as above)
  - Race schedule analysis for past month and following month (as above)
  - Explanation of pre- intervention training and eating procedures.

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

The protocol of each of the intervention trial will be the following:
5 days prior to the trial – 1 x TRAUMEEL tablets 3 X per day (provided) sublingually before meals.
The morning of the trial – the following breakfast is to be eaten 3 hrs before the trial :30g cornflakes, 150ml of low fat milk and 12-15 g refined sugar
The exercise trial – 30ml blood sample to be taken on arrival
  - exercise protocol – wearing a heart rate monitor, six subjects to run on treadmill in the exercise laboratory for 90 minutes in a relaxed atmosphere TV screens/music will be available to entertain the runners on the treadmill and free communication between runners and research assistants will be encouraged. The running speed will be individualized according to the results of the VO₂ max tests and the 1609m time trial.
  - 30ml blood sample will be drawn 1, 24, 48 and 72 hours after the trial.

The order of the trials is to be **randomized**. By this we mean that you will be placed into treatment sequences using random number tables. You may therefore not be receiving the treatment in the same order as the other subject(s). As it is to be **a double blind study,** neither the researchers or you will be informed regarding which treatment you are on. The products are to be coded as placebo or active substance 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 peak 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 at various race distances from 5km to Comrades marathon (as relevant to your goals / Training)
- a list of training paces relevant to your current ability
- information on your running style and relevance of your current running shoes to this style
- an indication, based on the information supplied, as to the relevance of your current training to running goals

A furthering of the knowledge regarding supplementation procedures which will optimize both your performance and the stiffness you feel following exercise, will be obtained from this work. You will be the first to be notified of the results of this study.

Will you be exposed to adverse effects of the exercise or side effects from Traumeel?

As with all exercise exposure, it is possible that an accident may occur (e.g. that you may perhaps fall). In the event of your heart or one of your other organs having a disorder which has to date, not been diagnosed, the increased blood flow to the heart and increased mean blood pressure during exercise may also result in a heart problem or other organ damage occurring while you are exercising. This is the reason why we have been so careful in terms of who we have allowed to participate in the study. Do, however, also be assured that there will be medical personnel present or on call throughout all aspects of the testing and that the laboratory is fully equipped with first aid and emergency equipment.

In terms of possible side-effects from the Traumeel supplementation that you will be using, although some people do sometimes develop mild headaches, diarrhoea or nausea, recent studies have shown that these resolve without discontinuation of the treatment and it has not been proven that these are directly related to ingestion of Traumeel.

As with all prolonged exercise involving downhill running, you should expect a certain amount of muscle soreness 48-72 hours post-exercise. This will be carefully monitored using a 0-10 numerical scale recording your levels of discomfort. It is also worth noting that previous studies and coaches suggest that the downhill training session will assist in protecting you from muscle soreness and muscle inflammation during other downhill sessions over the following 3 months (Nosaka K, Sakamoto K, Newton M, Sacco P. How long does the protective effect on eccentric exercise-induced muscle damage last? Med Sci Sports Exerc., 33⁻ 1490-1495). Should you be participating in a competitive event involving downhill running following participation in this trial, you are therefore assured of a beneficial training effect for at least 3 months thereafter.

Can you withdraw from the study?
As participation is entirely voluntary, you may withdraw from the study at any time without penalty.

Will your individual results remain confidential?
Yes. The records identifying the participant will be kept confidential and, to the extent permitted by the applicable laws and/or regulations, will not be made publicly available. If the results of the study are published, the participant's identity will remain confidential.

Sponsorship of the study/Financial compensation
The Traumeel and placebo products to be used during the study will be manufactured in Germany, and packaged and distributed to you by Heel Pty Ltd, South Africa in Johannesburg.

Further queries?

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

Professor Edith Peters-Futre – (031) 260-4237(w); 261-3869(h).

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

Athletics Coach and Consultant: Norrie Williamson – 031-2017687
www.coachnorrie.co.za

Post-graduate research assistants:
Dr Aadil Docrat, Department of Physiology
Ms Megan Smith, Department of Physiology
Ms Smith and Dr Docrat will be assisting with the implementation of this study and writing up sections of the results as part of their M (Med) Sc Degree by coursework requirements.

APPENDIX B

Day, Date and Time of Session	Training Mileage	Intensity Sessions	Rating of Perceived Exertion and General Comments
		Type: Duration: % of max (eg. 50%):	
		Type: Duration: % of max (eg. 50%):	
		Type: Duration: % of max (eg. 50%):	
		Type: Duration: % of max (eg. 50%):	
Rate of perceived exertion	1= Very easy 4= Fairly Exhausting	2= Easy 5=Exhausted	3= Moderate

APPENDIX C

UNIVERSITY OF KWA-ZULU NATAL
DEPARTMENT OF PHYSIOLOGY
NELSON MANDELA MEDICAL SCHOOL

The effect of Traumeel supplementation on inflammatory response to prolonged exercise.

ATHLETE'S QUESTIONNAIRE

Name:
_____

Date of Birth:
_____ / _____ / _____ (dd/mm/yyyy)

Age: _____

Address:
_____
_____
_____
_____

Phone Numbers
Home: _____

Work: _____

Cell: _____

Athletics Club:
_____

Occupation:
_____

TRAINING DETAILS:

Average weekly running distance: _____ km

Running History

Serious\Social : _____

Age started running: _____ (....yrs)

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 event(s) for each year of training age (or last 5 years)
....
....
....
....
....

List races / competitions over the last 6 months (all sports)
....
....

List races planned for next 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)
....

Do you use orthotics?

....

Describe the wear pattern on your shoes?

....

Personal Best times in last 12 months:
5 km time trial .... 8km time trial .... 10km ....

15 km .... 21.1km .... 32km ....

42.2km .... 56 km .... Comrades ....

1609m time trial time (will be tested as part of study) ....

Pace to be used for test (will be filled in after test) ....

Please sign: _____ **Date:** _____

Principal investigator's signature: _____

MEASUREMENTS

Pregnancy Test(if female only)..

Lung Function Tests

FVC: _____ FEV$_1$: _____

FVC/FEV$_1$: _____

Height (cm): _____ Mass (kg): _____

Skin – fold Measurements

Biceps: _____ _____ _____ ave: _____

Triceps: _____ _____ _____ ave: _____

Suprailliac: _____ _____ _____ ave: _____

Subscapular: _____ _____ _____ ave: _____

Body fat percentage: _____

Resting heart rate: _____

Resting blood pressure: _____

Results of VO$_2$ max test:

Speed Km/hr	VO$_2$ ml/kg/min	RQ	VE	VO$_2$	HR	RPE

Running Speed at 75% VO2 peak": ....

APPENDIX D

		Trial Record			
Trial No:					
Name:				**Date:**	
Breakfast:				**Time:**	
Resting HR:				**Resting BP:**	
Ambient Temperature:					
Dry Bulb:		**Wet Bulb:**		**Black Bulb:**	
Pre-Trial Mass:		**Fan: On / Off**		**Stretches:**	

Time	Workload	Distance	H R	VO2	RPE
30 min					
60 min					
90 min					

Post-Trial Mass:			**Post BP:**	
Training Record:			**Tablets:**	
DOMS Record:				

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

Name.............................   Date of Trial: ....

DAY	General post-trial pain seated and relaxed			Post-trial pain during daily living while walking		
	Quads	Hamstrings	Calf	Quads	Hamstrings	Calf
Day 1: immediately after the run						
Day 2: after the run						
Day 3: after the trial						
Day 4: after the trial						
Day 5: after the trial						

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