Vitamin C and Exercise-Induced Oxidative and Inflammatory Stress in Ultramarathon Athletes

by

Edith Margret Futre (nee Peters)
BA Hons (University of Stellenbosch),
BSc (Med) Hons and M Sc (Med) (University of Cape Town)

Submitted in fulfilment of the requirements for the degree of
Doctor of Philosophy

Department of Immunology, Faculty of Medicine, University of Pretoria

Pretoria

April 2002

Abstract

Literature reveals a paradoxical response of the immune and innate host defence systems to endurance exercise; apparent stimulation following long-term regular training and suppression in response to acute exposure to exhaustive endurance exercise. Two epidemiological studies revealed that clinical manifestation of immunosuppression in the form of increased incidence of upper respiratory tract infection symptoms (URTI) is greater following competitive ultramarathon running than in matched sedentary controls and linearly related to running time. These were followed by three intervention studies in which the efficacy of anti-oxidant supplementation in reducing the incidence of post-race symptoms of infection was assessed following participation in the 90 km Comrades Ultramarathon. Vitamin C alone was found to be more effective than combinations of anti-oxidants in reducing the post-race incidence of URTI symptoms in a study conducted on 178 runners and 162 matched, sedentary controls.

In order to investigate mechanisms by which vitamin C may act in reducing the incidence of URTI infection during the two-week post-race period, intervention studies were conducted at the 1997 and 1999 90 km Comrades Ultramarathons. Runners were required to keep dietary records and ingest prescribed capsules containing 500 mg, 1000 mg, 1500 mg vitamin C or placebo for 10 days prior to the event, complete the 90 km ultramarathon and provide 35 ml blood 14-16 hours before, immediately on completion of, 24 hours and 48 hours post-race. These specimens were subsequently assayed for markers of inflammatory and oxidative stress which included circulating cortisol, adrenaline, vitamin A, vitamin C, vitamin E, C-reactive protein (CRP), serum amyloid A, creatine kinase, lactate dehydrogenase, neutrophils and lymphocytes, neutrophil-derived elastase and myeloperoxidase, the pro-inflammatory-cytokines, interleukin-1ß, interleukin-6, interleukin-8 and tumour necrosis factor-alpha, the anti-inflammatory-cytokine, interleukin-10 (IL-10), and IL-1 receptor antagonist (IL-1Ra), all of which (with the

exception of vitamins A and E and lymphocytes) were significantly elevated on completion of the ultramarathon.

Increased daily intake of vitamin C was accompanied by a dose-related attenuation of the exercise-induced increases in circulating vitamin C, cortisol and neutrophils. Supplementation with the vitamin at doses of ≤ 1000 mg daily appeared to result in augmentation of the exercise-induced inflammatory response as evidenced by significant increases ($p<0.05$) in circulating levels of the acute phase reactant, CRP and in the group receiving 1000 mg daily, an increase in creatine kinase. In the group receiving 1500 mg daily, this apparent pro-inflammatory effect of supplementation with the vitamin was less evident, but circulating cortisol, adrenaline, IL-10 and IL-1Ra concentrations were all significantly reduced ($p<0.05$), suggesting that at this level of supplementation the vitamin may activate a counteracting anti-inflammatory cascade, possibly through inhibition of activation of pro-inflammatory transcription factors.

Although further work involving larger sample sizes is required to confirm these findings, this is the first evidence that vitamin C supplementation attenuates the endogenous, biological anti-inflammatory response to intensive exercise, which may partially explain why the vitamin reduces the incidence of URTI in ultramarathon athletes. On the downside, however, apparent augmentation of exercise-associated inflammatory responses at supplementation levels of 500 and 1000 mg daily, is clearly of concern.

Key Words: **Upper respiratory tract infections, ultramarathon running, vitamin C, oxidative stress, inflammatory stress, cortisol, adrenaline, interleukin-10, interleukin-1Ra, C-reactive protein, amyloid A.**

Abstrak

Die literatuur toon 'n teenstrydige reaksie van die immuun en intrinsieke gasheer weestandsmeganismes ten opsigte van uithouvermoë-oefening; die klaarblyklike stimulasie daarvan as gevolg van langtermyn, gereelde oefening maar ook die demping daarvan wat volg na akute blootstelling aan uitputtende uithouvermoë-oefeninge. Twee epidemiologiese studies het aangedui dat die kliniese manifestasie van immuunonderdrukking in die vorm van 'n verhoging in voorkoms van boonste lugweg infeksie-simptome groter was na mededingende ultramaraton wedlope as in rustende kontroles. Verder, dat hierdie simptome direk verwant was aan die hardlooptyd. Hierdie studie is opgevolg deur drie ander farmakologiese ingrypingstudies waarin die doeltreffendheid van anti-oksidant aanvullings om 'n vermindering in voorkoms van na-wedloopsimptome teweeg te bring, ondersoek is in deelnemers van die 90 km Comrades Ultramaraton. In 'n studiegroep bestaande uit 178 deelnemers en 162 rustende kontroles is gevind dat vitamien C alleen meer effektief was as kombinasies van anti-oksidante, om die na-wedloop voorkoms van boonste lugweg infeksie-simptome te verminder.

Ten einde die meganismes vas te stel waardeur vitamien C die voorkoms van boonste lugweg-infeksie gedurende die twee week- periode na die wedloop verminder, is twee ingrypingstudies onderneem tydens die 1997 en 1999 90 km Comrades Ultramaratons. Deelnemers is versoek om boek te hou van hul dieet, voorgeskrewe kapsules van 500 mg, 1000 mg, 1500 mg vitamien C of plasebo te neem, die 90 km ultramaraton te voltooi en om 35 ml bloed 14-16 uur vooraf, onmiddellik by voltooiing van, 24 uur en 48 uur na die wedloop te skenk. Die bloedmonsters is daarna getoets vir merkers van inflammatoriese en oksidatiewe stres wat insluit: sirkulerende kortisol, adrenalien, vitamien A, vitamien C, vitamien E, C-reaktiewe proteïen (CRP), serum amiloïed A (SAA), kreatien kinase, laktaat dehidrogenase, neutrofiel-afkomstige elastase en miëloperoksidase (MPO), die pro-inflammatoriese sitokiene, IL-1β, IL-6, IL-8, TNF-α, die anti-inflammatoriese sitokien, IL-10 en IL-1 reseptor antagonis (IL-1Ra).

inflammatoriese sitokiene, IL-1β, IL-6, IL-8, TNF-α, die anti-inflammatoriese sitokien, IL-10 en IL-1 reseptor antagonis (IL-1Ra).

Die verhoogde daaglikse inname van vitamien C het gepaard gegaan met 'n dosis-verwante vermindering van oefeningsgeïnduseerde verhogings in sirkulerende vitamien C, kortisol en neutrofiele, sowel as 'n daling in die limfosiete ($p<0.05$). Supplementasie met die vitamien by doserings van ≤1000 mg daagliks het klaarblyklik tot 'n verhoging in die oefeningsgeïnduseerde inflammatoriese respons gelei, soos aangedui deur betekenisvolle verhogings ($p<0.05$) in die sirkulerende vlakke van die akute fase reagens, CRP, en in die groep wat 1000 mg daagliks ontvang het, 'n verhoging in kreatien kinase. In die groep wat daagliks 1500 mg ontvang het, was die klaarblyklike pro-inflammatoriese uitwerking van die vitamienaanvulling minder opsigtelik, maar was konsentrasies van sirkulerende kortisol, adrenalien, IL-10, en IL-1Ra almal betekenisvol minder ($p<0.05$) wat aandui dat by hierdie vlak van supplementasie, die vitamien 'n teenwerkende anti-inflammatoriese kaskade, waarskynlik deur die demping van aktivering van pro-inflammatoriese transkripsie-faktore, aktiveer.

Hoewel verdere studies groter studiegroepe vereis om hierdie bevindings te bevestig, is dit die eerste bewys dat vitamien C aanvullings die endogene, biologiese anti-inflammatoriese respons ten opsigte van intensiewe oefening demp, wat mag verklaar waarom die vitamien die voorkoms van boonste lugweginfeksie in ultra-maraton atlete verminder. In teendeel is die klaarblyklike verhoging van oefeningsverwante inflammatoriese response by aanvullingsvlakke van 500 en 1000 mg daagliks, kommerwekkend.

Kernwoorde: **Boonste lugweg infeksies, ultramaraton wedlope, vitamien C, oksidatiewe en inflammatoriese stres, kortisol, adrenalien, interleukin-10, interleukin-1Ra, C-reaktiewe proteïen, amiloïed A.**

Acknowledgements

I would like to express my sincere gratitude to all who have assisted me in the work which has resulted in the final submission of this thesis. A particular word of thanks is extended to the following persons :

- My supervisor, **Professor R Anderson**, Head of the Department of Immunology, Medical Faculty, Pretoria University, for sharing with me his insight of and specific interest in Vitamin C and oxidative stress, and for all the hours of patient assistance over the last five years. It was indeed a privilege to have been able to work under your guidance.

- **Professor Tim Noakes,** Director of the South African Institute of Sport Science, for introducing me to the broader field of Sport Science and for being a constant source of support and inspiration to me throughout my asssociation with this field of study.

- **Dr David Nieman**, Head of the Department of Health and Exercise Science, Appalachian State University, USA, for the personal support and encouragement to complete this work. I should also like to acknowledge the contribution of his laboratory with the analysis of the cytokine concentrations in the collaborative work of the 1999 study (reported in Chapter Six).

- **Prof Eric Bateman**, Head of the Department of Medicine, Cape Town University, for instigating the original epidemiological study in 1982, which was to introduce me to the field of exercise immunology.

- **Prof Annette Theron,** Associate Professor in the Department of Immunology, University of Pretoria, for expert laboratory assistance, and also the friendship and support over the last five years.

- **Mrs Heidi Fickl**, biomedical scientist in the Department of Immunology, University of Pretoria, for outstanding laboratory assistance, for her thoroughness in teaching me laboratory skills, in particular the development of appropriate ELISA techniques for use in this work and for letting me work in her laboratory. I was indeed privileged to have obtained such training.

- **Mrs Cathy Connolly** from the Biostatistics Unit of the Medical Research Council, Natal for valuable assistance with statistical computations.

- **Dr Vinod Jogessar and Dr Vincent Naiker** from the Department of Haematology of the Nelson Mandela Medical School, Natal University, for providing assistance with the analysis of full blood counts and differential lymphocyte counts in the 1999 study.

- **Dr Elena Nevines,** for always being available to share her expert knowledge of chemistry, her assistance with collation of the original data, patience in teaching me the skills of compiling graphic material, and general moral support during my years in the Department of Physiology.

- **Jeni Goetzsche** and **Lester Joseph** for their assistance with the intervention study conducted at the 1993 Comrades Marathon, **Tim Lucas** and **Desiree Lee** for assistance with the field studies conducted at the 1996 and 1997 Comrades Marathons and to **Andrew McKune** and my research team from the Department of Physiology at the 1999 Comrades Marathon.

- **UNESCO,** the **University of Natal and University of the Witwatersrand Research Funds**, and **Institute of Pathology, University of Pretoria Medical School** for financial contributions to this research. The assistance of the **Roche Pty Ltd** and **Pharma Natura Pty (Ltd)** in formulating and providing the placebo and anti-oxidant supplements used in these studies is also acknowledged.

- **the more than 300 runners** who volunteered to participate in the various studies which I have conducted at the 1993, 1996, 1997 and 1999 Comrades Marathon events. Without your unselfish co-operation, the data reported in this thesis would not have been available.

- **Professor F Güldner,** Head of the School of Medical Sciences, **and my colleagues** in the Department of Physiology of the Nelson Mandela Medical School, Natal University, for granting me the sabbatical leave which provided relief from my teaching responsibilities and the opportunity to attend to the write-up of this thesis. Without this, it would been physically impossible to complete this work.

- **And finally, to my daughter, Fia,** thank you for your encouragement and support and your tolerance of my constant occupation of our home study; **and to my extended family and friends**, thank you, also, for your support and for tolerating my preoccupation with this work over the last few years.

Table of Contents

List of Figures

List of Tables

Table 7.3: Mean (±SEM) plasma interleukin-8 and elastase concentrations.

Recent Publications
which have emanated from this work

Pilot Work (Field Study)

- **Peters E.M.**, Goetzsche J.M., Joseph, L.E., Noakes T.D. Vitamin C as effective as combinations of anti-oxidant nutrients in reducing the incidence of upper respiratory tract infections in ultradistance runners. *S.A. Sports Med.* **4**: 23-27, 1996.

Laboratory Work

- **Peters E.M.**, Anderson, R., Theron, A.J. Attenuation of the increase in circulating cortisol and enhancement of the acute phase response in vitamin C-supplemented ultramarathon runners. *Int J Sports Med* **22**: 120-126, 2001.
- Nieman D.C., **Peters E.M.**, Henson D.A., Nevines E.I., Thompson M.M. Influence of Vitamin C supplementation on cytokine changes following an ultramarathon. *J Interferon & Cytokine Res* **20**: 1029-35, 2000.
- **Peters E.M.**, Anderson, R., Nieman D.C., Fickl H., Joggesar V. Vitamin C supplementation attenuates the increases in circulating cortisol, adrenaline and anti-inflammatory polypeptides following ultramarathon running. *Int J Sports Med* ***22***:537-543, 2001.

Review Articles

- **Peters E.M.** Exercise and Upper Respiratory Tract Infections. *S Afr J Sports Med* **3:** 9-14, 1996.
- **Peters E.M.** Exercise, immunology and upper respiratory tract infections, *Int J Sports Med* **18**, S69-78, 1997.
- **Peters-Futre E.M.** Exercise, Vitamin C and Neutrophil Function. The Missing Link. *Exerc Immunol Rev* **3**: 32-52, 1997.

Chapter in Book

- **Peters E.M.** Vitamins, Immunity and Infection Risk in Athletes In: Nieman D.C., Pederson BK (eds): Nutrition and exercise Immunology, CRC Press, Florida, 2000.

Abbreviations

8-oxodG	8-oxo-2-deoxyguanosine
AA	ascorbic acid
ACTH	adrenocorticotrophic hormone
ANOVA	analysis of variance
AP-1	activator protein-1
APP	acute phase proteins
AVP	arginine vasopressin
CK	creatine kinase
CRF	corticotropin-releasing hormone
CRP	C-reactive protein
CSIF	cytokine synthesis inhibiting factor
Cu	Copper
DHAA	dehydro-ascorbic acid (oxidised form)
DOMS	delayed onset muscle soreness
EBV	Epstein-Barr Virus
EDTA	ethylenediaminetetraacetic acid
FMLP	N-formyl-methionyl-leucyl-phenylalanine
H_2O_2	hydrogen peroxide
HPA	hypothalamic-pituitary-adrenal
HOCL	hypochlorous acid
HPLC	high performance liquid chromatography
IE	infectious episodes
IgA	immunoglobulin A
IL-1β	interleukin-1 beta
IL-1Ra	interleukin-1 receptor antagonist
IL-2	interleukin-2
IL-2R	interleukin-2 receptor
IL-6	interleukin-6
IL-8	interleukin-8
IL-10	interleukin-10

IKK	I-kappa B kinase
L-ascorbate	reduced form of ascorbic acid
LDL	low density lipoprotein
LDH	lactate dehydrogenase
LPS	lipopolysaccharide
MIP	macrophage inhibitory protein
MPO	myeloperoxidase
MSH	alpha-melanocyte stimulating hormone
NF*k*B	nuclear transcription factor, *k*B
NK cells	natural killer cells
OH^-	hydroxyl anion
•OH	hydroxyl radical
$O_2^{\bullet -}$	superoxide anion
PBS	phosphate-buffered saline
PMA	phorbol 12-myristate 13-acetate
PMN	polymorphonuclear leukocyte
PWM	pokeweed mitogen
ROI	reactive oxygen intermediates
ROS	reactive oxygen species
SAA	serum amyloid A
SD	standard deviation
SEM	standard error of the mean
SMI	steroid-mediated immunosuppression
SOD	superoxide dismutase
TNFα	tumour necrosis factor alpha
URT	Upper respiratory tract
URTI	Upper respiratory tract infections
VC	Vitamin C
$\dot{V}O_2$ max	Maximum oxygen consumption

Chapter One

Introduction: early epidemiological work

Physical activity is generally recognised as a therapeutic modality; the beneficial cardiovascular, respiratory and metabolic adaptations resulting from regular prolonged exercise are well recognised. However, the possibility that an improved level of physical conditioning augments immunological function, has only become the subject of serious scientific enquiry in the last three decades and has yielded conflicting results.

A dichotomy of responses has been reported. On the one hand, a high level of physical conditioning, has, in a few carefully controlled recent studies, provided evidence of a lower incidence of infection symptoms and beneficial effects on immune function which may increase resistance to infection (Nieman & Pedersen, 1999). Overtraining, and the combined psycho-physical stress of competitive endurance events and acute bouts of exhaustive endurance exercise, have, on the other hand, been associated with transient suppression of immune and host defences (König *et al.*, 2000).

Nieman (1994) initially proposed a "J" shaped model in explaining the paradoxical relationship between exercise and upper respiratory tract infection (URTI) risk. He postulated that whereas a moderate intensity and quantity of work over a prolonged

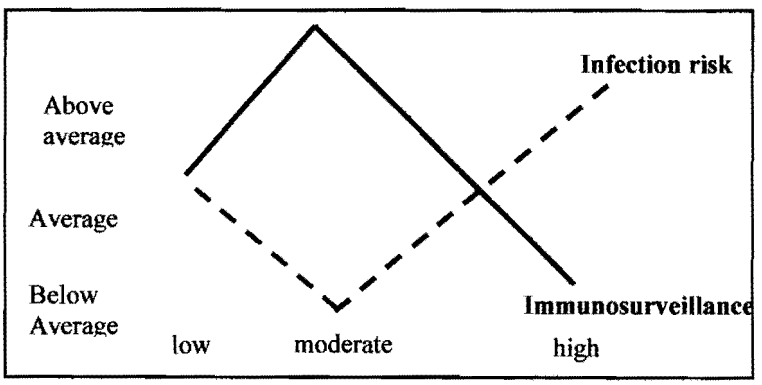

Figure 1.1: The paradoxical relationship between workload, risk of URTI & immunosurveillance athletes (adapted from Nieman, 2000)

period reduces the risk of infection below that of a sedentary individual, once a critical threshold is reached, the more strenuous the exercise, the greater the risk of infection. His recent adaptation of what has become a classic "J" shaped model, incorporates the effect which exercise workload has on immunosurveillance **(Figure 1.1).**

1.1 *Acute effects of competitive prolonged exercise*

Already in 1975, Ryan *et al.* reached the conclusion that "upper respiratory illness causes more disability among athletes than all other diseases combined." This was confirmed by Berglund & Hemmingson (1990) who reported that URTI was the most common reason for absence from training in elite skiers over a 12 month period **(Figure 1.2)**

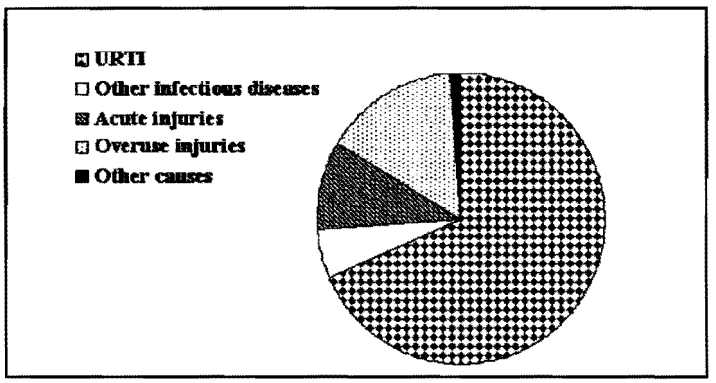

Figure 1.2: URTI, the main medical reason for absence from training in elite Swedish cross country skiers. Data from Berglund & Hemmingson (1990).

Several epidemiological surveys have subsequently confirmed clinical manifestation of immunosuppression in the form of increased incidence of URTI symptoms following participation in competitive marathon and ultramarathon running events (Peters & Bateman, 1983; Peters, 1990; Nieman *et al.*, 1989a; 1990a; Peters *et al.*, 1992; 1993; 1996) and during overtraining (König *et al.*, 2000).

In the first preliminary investigation (Peters & Bateman, 1983), a simple epidemiological survey was conducted on 150 successful finishers in the 1982 Two-Oceans Ultramarathon (56 km) run annually in Cape Town, South Africa, and their age-matched non-running controls who resided in the same households. Thirty-three percent of runners completing the race reported URTI symptoms during the two-week post-race period as opposed to 15.3% in the non-running control group. The

frequency of URTI symptoms was inversely related to the time taken complete the race ($p>0.01$) and almost half of the fastest runners experienced symptoms **(Figure 1.3).**

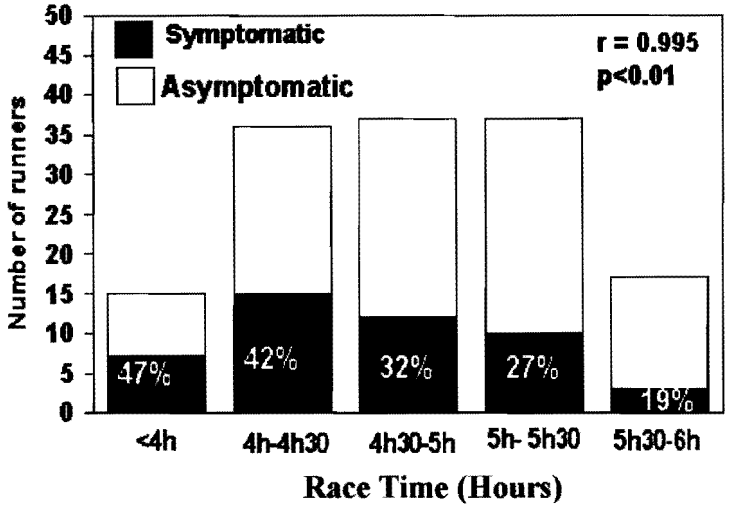

Figure 1.3: Distribution of symptomatic and asymptomatic runners according to time taken to complete the race (n=14; data from Peters & Bateman, 1983)

The finding that the fastest runners experienced the highest incidence of symptoms of infection, was supported by the observation that the runners completing the highest training weekly distance in preparation for the race, experienced the highest incidence of symptoms of infection. This greater incidence of symptoms of infection was attributed to (i) possible drying of the mucosal surfaces resulting from hyperventilation of cold, dry air and/or (ii) immuno-suppression resulting from elevated serum cortisol levels experienced during the race.

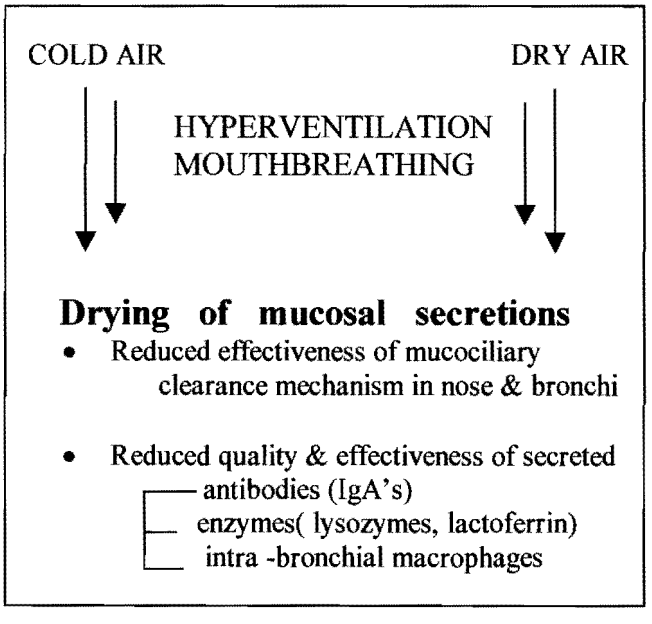

Figure 1.4: Possible mechanism by which distance running predisposes to increased incidence of URTI infection (Peters, 1990)

The finding of a significantly higher incidence of URTI symptoms among runners during the post-race period was confirmed by Peters in 1989 when the study was repeated at the Milo Korkie Marathon, a 56 km race taking place at an altitude of 1800 km above sea-level, between Pretoria and Johannesburg, South Africa. It was hypothesized that local mucosal damage resulting from hyperventilation and mouthbreathing (**Figure 1.4)** would have been greater at this altitude than at sea level due to the lower barometric pressure and concomitant reduction in relative humidity and thus would have resulted in a higher percentage incidence of URTI symptoms among runners competing over the same distance. This was not found in this sample of 108 runners surveyed in this study. A significantly higher incidence of symptoms of infection among the runners during the post-race fortnight than during matched, non-running controls, was, however, confirmed (**Figure 1.5)**.

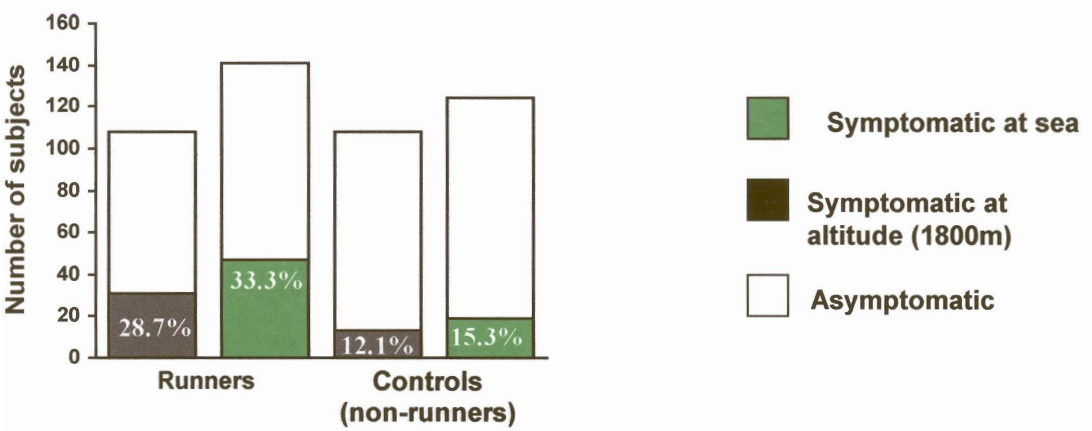

Figure 1.5. The incidence of URTI symptoms during the post- race fortnight in runners completing 56 km ultramarathons at sea level and moderate altitude and matched sedentary controls. Data from Peters & Bateman (1983) and Peters (1990).

In the same year a two-month investigation was conducted into the pre-and post-event incidence of URTI in a group of 273 participants in 5,10 and 20 km events in California (Nieman *et al.,* 1989a). While 25% of the runners training in excess of 25 km.wk^{-1} reported at least one episode of URTI during the two-month period, the incidence was higher (33,3%; p=0.09) in the less serious recreational runners completing less than 25 km.wk^{-1} in training. No increase in URTI incidence was reported in the runners during the 7-day post race period as compared to the incidence in the week prior to the race.

This study was followed by an investigation into the URTI symptom incidence in 2311 participants before and after the Los Angeles Marathon (LAM) [Nieman *et al.*, 1990a] which revealed an increase in odds ratio of infectious episodes (IE) with an increase in pre-race training distance ($p=0.04$). Reported incidence of illness was highest in those runners who completed > 97 $km.wk^{-1}$ while training in preparation for the event. Of the 1828 competitors in the Los Angeles Marathon without infectious episodes (IE) before the race, 12.9% reported IE during the week following the marathon vs. 2.2% in controls (well trained non-participating runners). These researchers concluded that runners may experience increased odds for IE during heavy training or following a marathon race.

Collectively, these independently conducted epidemiological surveys undertaken since 1983 provide consistent evidence of increased infection risk following acute intensive physical exertion.

1.2 *Anti-oxidant intervention studies*

Although the exact mechanisms underlying the increased URTI symptoms following participation in an ultramarathon were not known, I used the predictable increase in infection risk as a model to test the efficacy of anti-oxidant supplements in enhancing resistance to such infections.

I firstly conducted a double-blind, placebo-controlled study on runners and their matched non-running controls participating in the 1990 Comrades Marathon (Peters *et al.*, 1993). Supplementing with 600 mg vitamin C daily for the three-week period prior to the 90 km race resulted in the incidence of post-race URTI symptoms being more than halved in this group of Comrades runners when compared to the incidence amongst the runners receiving placebo. Thirty three percent of the vitamin C supplemented group reported the development of symptoms of URTI as opposed to an incidence of 68% in the runners receiving placebo. This supplementation did not, however, affect the incidence of URTI symptoms in the sedentary controls although previous findings of a significantly ($p<0.01$) shorter duration and a

subjective amelioration of the severity of the symptoms infections (Hemilä, 1992) was confirmed.

Further indirect evidence that the greatest benefit is to be derived from supplementary anti-oxidants was obtained from my findings at the 1991 Comrades Marathon. Supplementing with vitamin A, widely recognised for its anti-infective, rather than anti-oxidant properties (Bloem *et al.*, 1990; Coutsoudis *et al*, 1992), had an insignificant effect ($p<0.05$) on the incidence of URTI in the sample of runners studied (Peters *et al.*, 1992). Measures of blood vitamin A status revealed that neither runners receiving vitamin A supplementation nor runners on placebo were deficient in vitamin A **(Figure 1.6).**

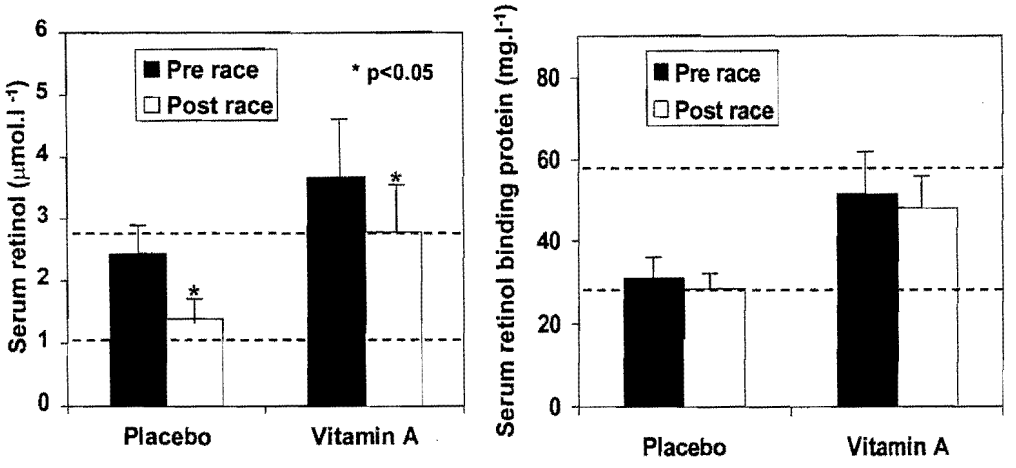

Figure 1.6. Mean (± SD) serum retinol and serum retinol binding protein concentrations in runners on placebo (n=11) and vitamin A supplementation (n=9) before and after the 1992 Comrades Marathon (Peters *et al.*, 1992).

Although both vitamin C and vitamin E have been shown to be of benefit when taken individually, several studies (Packer, 1986; Alescio *et al.*, 1992; Frei, 1994; Herbaczynska-Cedro *et al*, 1994, 1995) have shown that vitamin E in combination with Vitamin C is the most effective in terminating self-propagating free radical chain reactions. I therefore conducted another study in which the efficacy of three different anti-oxidant nutrient combinations were compared with respect to amelioration of URTI symptoms in ultramarathon athletes (Peters *et al.*, 1996).

Runners (n=178) and matched controls who resided with the runner (n=162) were divided into four treatment groups receiving daily supplementation with 500 mg of vitamin C, 400 IU vitamin E and 500 mg vitamin C, and 300 IU vitamin E, 300 mg vitamin C and 18 mg Beta (β) carotene or placebo for a three week period. The incidence of symptoms of URTI was monitored in both runners and controls during the two weeks following the ultramarathon. This was, once again, significantly greater in the runners on placebo than in the sedentary, non-running controls. Of the three groups of runners receiving anti-oxidant supplements, it was the group with the highest mean total (ie. in diet and supplements) daily vitamin C intake (1004 mg) who reported the lowest incidence of symptoms of upper respiratory tract infection **(Figure 1 .7).**

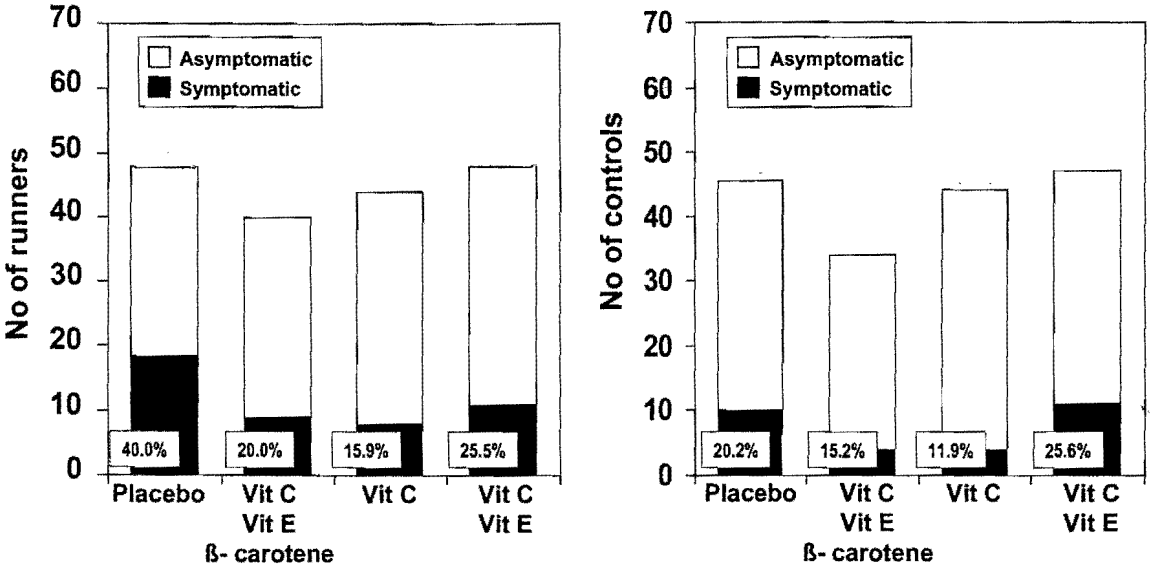

Figure 1.7. Incidence of URTI symptoms in runners (n=178) and controls (n=162) on different anti-oxidant combinations, during the 14 day post-race period. (Peters *et al.*, 1996)

A direct inverse correlation between the combined intake of vitamin C, E and β carotene and incidence of symptoms of URTI did, however, not exist. The relatively lower effect of vitamin E and β carotene supplementation may be attributed to the slow elevation in circulating vitamin E and β carotene levels and the fact that this

supplementation did not extend over long enough a period of time to allow blood levels of vitamin E and β carotene to reach protective levels. Variance in training status and genetic make-up within and between the groups studied, however, also appeared to have an important bearing on the efficacy of anti-oxidant nutrient supplementation in this study.

The major finding of this study, however, supports the notion that a total intake of approximately one gram of vitamin C per day for three weeks prior to the race, does have a protective effect in ultramarathon runners in terms of reducing the URTI risk. This is considerably higher than the daily dosage of 200 mg (for 4 weeks) which has been shown to be associated with accelerated clinical improvement in elderly patients hospitalized with acute respiratory infection (Hunt *et al.*, 1994). The results of this trial also support previous findings of studies examining the effect of vitamin C supplementation on the incidence of URTI in subjects exposed to severe physical stress **(Table 1.1)**.

In 1959 Bessel-Lorck found that the incidence of URTI was 28% lower in 20 vitamin C supplemented children attending a 9-day skiing camp in the Bavarian woods than in 26 non-supplemented children who acted as controls. This was followed by a carefully controlled double blind study conducted on 279 school children in two skiing camps in the Swiss Alps (Ritzel, 1961). Ritzel administered one gram of vitamin C per day to half of these children for a period of a week and reported a decrease in the number of episodes of pharyngitis, laryngitis, tonsillitis and bronchitis in the vitamin C group. In addition to a 45% decrease in the incidence of colds, there was also a 29% decrease in the mean duration of cold episodes and a 61% decrease in the total number of days of illness per person in the group receiving vitamin C. The children in this study were not only exposed to strenuous exercise, but also to the cold, an added environmental stressor (Ritzel, 1961). These findings were supported by those of Sabiston & Radomski (1974) who studied 112 soldiers undergoing military training over a two-week period in the Canadian winter and found less than half the percentage incidence of the common cold in the troops receiving 1g vitamin C per day (11%; n=56) when compared to those on placebo (25%; n=56).

In 1996 Hemilä conducted a meta-analysis of three of the above-mentioned studies of persons exposed to severe physical exertion (Ritzel, 1961; Peters *et al.,* 1993; Sabiston & Radomski, 1974) and reported a pooled ratio of 0.50 of URTI in favour of vitamin C compared to placebo groups during times of heavy stress. Not included in the summed data presented by Hemilä (1996a) were the findings of the study of Pitt and Costrini (1979), which investigated the effect of 2 g/day of vitamin C supplementation vs. placebo during a 2-month military training camp on 674 marine recruits in South Carolina. These findings supported a reduction in the severity of the infections experienced by the military recruits with a substantially lower incidence of pneumonia in the vitamin C group, but did not confirm the findings of a lower incidence of infection observed in previous intervention trials. This study did, however, possess a number of differences in design to the previous studies. As the subjects only received supplementation after two weeks and were followed over a full two-month period, the possibility that an adaptive response, both in terms of physical adaptation and adaptation to the higher intake of vitamin C, may have influenced the effect of the vitamin C supplementation, does need to be taken into consideration (Stanislaw & Klapcinska, 2000). Moreover, as Hemilä correctly observes, the subjects were not subjected to an "acute stress" situation (Hemilä, 1996a).

Another study involving vitamin C supplementation in persons under heavy stress, was conducted by Kimbarowski-Mokrow in the former Soviet Union in 1967. The development of pneumonia was monitored in 226 military recruits who had acquired influenza A infection. In the soldiers who received 300 mg vitamin C supplementation daily (n=114), the incidence of pneumonia was significantly smaller when compared to the incidence in those who received "little vitamin C in food " (n= 112).

Moola (1996) supported the findings of Peters *et al.,* (1992, 1996) that vitamin C supplementation was most effective in reducing the incidence of post-race URTI symptoms in a small group of ultramarathoners, while the reduced effectiveness of a prolonged period of vitamin C supplementation in lowering the incidence of URTI infection symptoms was once again confirmed in the study of Himmelstein *et al.* (1998). In this work, 44 marathon runners and 48 sedentary controls were randomly assigned to a 2-month pre-marathon and 1 month post-marathon regimen of 1000

TABLE 1. 1: VITAMIN C SUPPLEMENTATION STUDIES CONDUCTED ON SUBJECTS SUBJECTED TO HEAVY PHYSICAL STRESS

Authors	Quantity of daily Supplementation	Total daily Vit C intake (mg)	Sample size Active Subjects	Sedentary Controls	Mode of Physical exertion	Duration of Supplementation	% URTI incidence
Bessel-Lorck, 1959	1000mg	not determined	20	-	9-day skiing camp	9 days	17
	non-supplemented		26	-			45
Kimbarowski and	300mg	300mg &	114	-	Soviet military training	not reported	1.8*[a]
Mokrow, 1967	non-supplemented	"little'' in food	112	-			8.9[a]
Ritzel,1961	1000mg	not determined	139	-	7-day skiing camp	1 week	12*
	Placebo		140	-			22
Sabinston & Radomski,	1000mg	not determined	56	-	2 weeks military training	2 weeks-during	11*
1974	Placebo		56	-		training camp	25
Pitt and Costrini, 1979	2000mg	not determined[c]	331	-	8 weeks of military training	8-week study period	90
	Placebo		343	-	in marine recruits		90
Peters *et al*, 1993	Group 1: Vit C: 600mg	1139	43	34	88km run	3 weeks prior to the race	33*[b]
	Group 2: Placebo	494	41	39			68[b]
Peters *et al*., 1996	Group 1: 500mg Vit C& 400IU Vit E	893	40	33	88km run	3 weeks prior to the race	15.9
	Group 2: 600mg Vit C	1004	44	41			20
	Group 3: Vit C: 300mg; Vit E 400IU; Beta Carotene 400IU	665	47	43			25.5
	Group 4: Placebo	585	47	45			40.4
Moola, 1996	Group 1: 600mg Vit C	not determined	11	11	88km run	6 weeks prior to the	30.8
	Group 2: 45mg Beta Carotene		11	11		race	41.7
	Group 3: Placebo		25	19			68
Himmelstein *et al*., 1998	Group1: 1000mg Vit C	1210	41	29	42 km	2 months before	33.3
	Group 2: Placebo	169	30	35		1 month after	43.5

* $p<0.05$ when compared to incidence in unsupplemented groups [a] subjects possessing influenza A who developed pneunonia [b] symtoms lasting ≤ 1 day included in analysis [c] blood vitamin C levels indicated an absence of marginal deficiency in the ontrol group.

mg/ day of vitamin C or placebo. Despite an only small (3.2%), but significant increase in blood vitamin C concentrations in a subsample (n=25) of vitamin C supplemented runners, a 9.9 % difference in the URTI incidence was reported [33.3% vs 42.9 %, among runners receiving vitamin C (n=30) and placebo (n=14) respectively]. Due to the low statistical power of comparisons of these sample sizes, this difference was not statistically significant.

To date, over 60 studies have examined the effect of vitamin C on the common cold. From the data presently available, it would appear that vitamin C supplementation has the greatest effect in children (Hemilä, 1999) and males with low dietary vitamin C intake (Hemilä, 1997) with the greatest benefit from a dosage of ≥ 2g/day (Mink *et al.*, 1998; Hemilä, 1999). While supplementary vitamin C has not been shown to have preventative effects in most normally nourished subjects in Western countries, substantial treatment benefits have been reported in pneumonia and bronchitis patients (Hemilä & Douglas, 1999). There is also strong cumulative evidence which appears to support its possible beneficial effect in reducing the incidence of infection in those exposed to high levels of acute physical stress (Ritzel, 1961; Peters *et al.*, 1993; 1996; Sabiston & Radomski, 1974; Himmelstein *et al.*, 1998). Variables which may affect the efficacy of supplementation in heavily stressed individuals include duration of supplementation period (Stanislaw & Klapcinska, 2000) and intensity and duration of the physical stress (Hemilä, 1996a; Himmelstein *et al.*, 1998). Further work is required to strengthen this evidence and the inclusion of laboratory measurements of circulating vitamin C concentrations, to establish compliance with treatment, is imperative in all further studies.

Chapter Two

Literature Review

2.1 Exercise-induced modulation of the immune system

Exercise has been described as a form of physical stress which is analogous to trauma, tissue damage, burns, spaceflight and sepsis (Pyne *et al.*, 1998) and results in similar changes in the concentration of neuroendocrine hormones to these forms of stress (Brenner, 1998). There is growing evidence that for several hours subsequent to heavy exertion, various components of both innate (natural or non-adaptive) and acquired (adaptive) immunity exhibit significant perturbations. A brief synopsis of these components and their primary functions is given in Table 2.1:

Table 2.1: Primary components of the immune system.

Component	Prevalence in human peripheral blood	Functions
Innate Immunity		
Physical barriers Skin, epithelial cell barriers mucosal secretions	-	First line of defence Contain IgA antibodies
Proteins/polypeptides Incl lysozyme, complement, acute phase proteins, adhesion molecules (e.g. selectins, integrins)	-	-Recruitment of inflammatory cells to site of infection or inflammation, directing leukocyte trafficking -Antibody like activity (binding to cell surface proteins) -Opsonization of pathogens -Stimulation of phagocytosis
Cellular components		
Polymorphonuclear granulocytes	**60-70 % leukocytes**	Phagocytosis, degradation of damaged tissue, cytokine production, presentation of foreign protein to CD4+ cells
Neutrophils	90% granulocytes	
Eosinophils	2.5% granulocytes	
Basophils	2% granulocytes	
Monocytes(CD14),macrophages	**10-15% leukocytes**	Phagocytosis, cytokine production
Natural killer cells (CD16, 56)	5-20% lymphocytes	Cytotoxicity, cytokine production

Acquired Immunity		
Humoral factors Immunoglobulins Soluble messenger molecules (incl. Cytokines, chemokines)		Antigen binding Activation of immune cells & mediation of leukocyte trafficking; acting as chemical messengers between different immune cells Neutralisation/killing pathogens or tumour cells
Immune Cells: Lymphocytes Helper T cells (CD 4+) Cytotoxic /suppressor T cells (CD 8+) B lymphocytes (CD 19- 23)	**20-25% leukocytes** 60-70% T cells 30-40% T cells 5-15% lymphocytes	 Antigen recognition, cytokine production, B cell activation Cytotoxicity, lymphocyte regulation Antibody production, memory of previous infection

Compiled from Janeway & Travers, 1996; McKinnon, 1999; Guyton, 2000.

In support of the apparently consistent findings in epidemiological surveys following long and ultradistance events, laboratory studies have revealed that the following components of the immune system have been shown to exhibit consistent and reproducible change during and following heavy physical exertion:

- **The distribution of leukocyte subsets**: An exercise-induced leukocytosis has been shown to result from increases in the concentrations of circulating adrenal stress hormones (Brenner *et al.,* 1998) and human growth hormone (Kappel *et al.,* 1993). This leukocytosis results from both an elevation of the total lymphocyte count (due to recruitment of natural killer (NK) cells, B cells and T cells into the blood), as well as increased release of neutrophils into the circulation. During the post-exercise recovery, the leukocyte response is, however, biphasic (Hansen *et al.,* 1991; Nieman *et al.,* 1991; 1994). Correlating strongly with the catecholamine curve (Crary *et al.,* 1983), the exercise-induced increment in lymphocyte number has been shown to be reversed after 30 minutes of recovery, dropping down to below pre-exercise levels (post-exercise lymphocytopenia), and together with eosinophil number, remaining low for 3-6 hours (MacArthy & Dale, 1988; Nieman *et al.,* 1989b; 1991; 1994). In contrast, post-exercise neutrophilia (MacArthy & Dale, 1988; Smith *et al.*, 1990; Nieman *et al.,* 1991; Müns, 1993; Pyne *et al.,* 1995) has, in strong correlation with the increment in cortisol levels, been shown to peak at 1.5 hours into recovery and

slowly return to baseline levels between 6 and 20 hours post-exercise (Nieman, 1995; Pedersen *et al.,* 1997). A marked decrease in circulating NK cells (which have been shown to rise 150-300 % immediately following exhaustive exercise and drop to below pre-exercise levels 1-2 hours into recovery), is associated with decreased cytotoxic capacity of the NK cells (MacKinnon, 1989; Berk *et al*, 1990; Pedersen *et al*, 1989; 1990; 1997; Pedersen and Ullum, 1994; Shephard *et al.,* 1994).

- **Systemic markers of inflammation:**

These include **increases** in

- blood granulocytes (primarily neutrophils and eosinophils) and monocyte phagocytic activity (Smith *et al.,* 1990; Müns *et al.,* 1996; Nieman *et al.*, 1996; Suzuki *et al.,* 1996); concentrations of elastase (Gleeson *et al.,* 1998; Robson *et al.,* 1999) and myeloperoxidase (MPO) [Camus *et al.,* 1992; Bury *et al.,* 1995]
- acute phase reactants {including C-reactive protein (CPR), haptoglobin and fibrinogen} [Strachan *et al.*, 1984; Weight *et al.*, 1991; Castell *et al.*,1997; Pizza *et al.,* 1997]
- complement protein levels (Nieman *et al.*, 1989c; Camus *et al.,* 1994; Castell *et al.*, 1997)
- pro-inflammatory cytokines {including tumour necrosis factor-alpha (TNF α), interleukin –1beta (IL-1β)} [Cannon *et al.,* 1997; Ostrowski *et al.,* 1998a; 1999], interleukin-6 (IL-6) [Bury *et al.,* 1996; Brunsgaard *et al.,* 1997; Weinstock *et al.,* 1997; Ostrowski *et al.,* 1998b] and their inhibitors, TNF- receptor and interleukin -1 receptor antagonist (IL-1Ra) [Ostrowski *et al.,* 1999]
- anti-inflammatory cytokines {including interleukin-10 (IL-10)} [Weinstock *et al.*,1997; Nieman *et al.,* 2000; Suzuki *et al.,* 1999; 2000]
- chemokines such as interleukin-8 (IL-8), macrophage/monocyte inhibitory proteins (MIP) -1α and 1 β (Ostrowski *et al.,* 2001)
- mobilisable anti-oxidative vitamins (particularly vitamin C) [Gleeson *et al.,* 1987; Robertson *et al.*, 1991; Maxwell *et al.,* 1993, Liu *et al.,* 1999]

and **decreases** in

- granulocyte oxidative burst following prolonged exercise (Gabriel *et al.*, 1994; 1995; Robson *et al.*, 1999)

Additional components of the immune system which are also consistently suppressed during the post-exercise open window period include

- T and B lymphocyte numbers and function (Liesen *et al.*, 1988; Mars *et al.* 1998; Pedersen *et al.*, 1998a, Nieman *et al.*, 1999)
- salivary IgA output (Mackinnon et al., 1989, 1993a; Mackinnon & Hooper, 1994; Blannin *et al.*, 1998; Fahlman *et al.*, 2001)
- plasma glutamine concentrations (Keast *et al.*, 1995; Castell *et al.*, 1997)

Despite the increasing knowledge of exercise-induced changes in immune function, the clinical significance of these changes is presently not known and the pathophysiological basis of actual increased post-exercise vulnerability/susceptibility to infection among athletes, is currently poorly understood.

2.2 Aetiology of transient post –exercise immunosuppression

It is well accepted that symptoms of infection can be triggered by infective, inflammatory or allergic factors. In 1994 Pederson and Ullum first identified the existence of an "open window" period during the first 6-20 hours following strenuous exertion showing a temporary drop in lymphocyte numbers, NK cell activity, complement and mucosal IgA levels during this time (**Figure 2.1**).

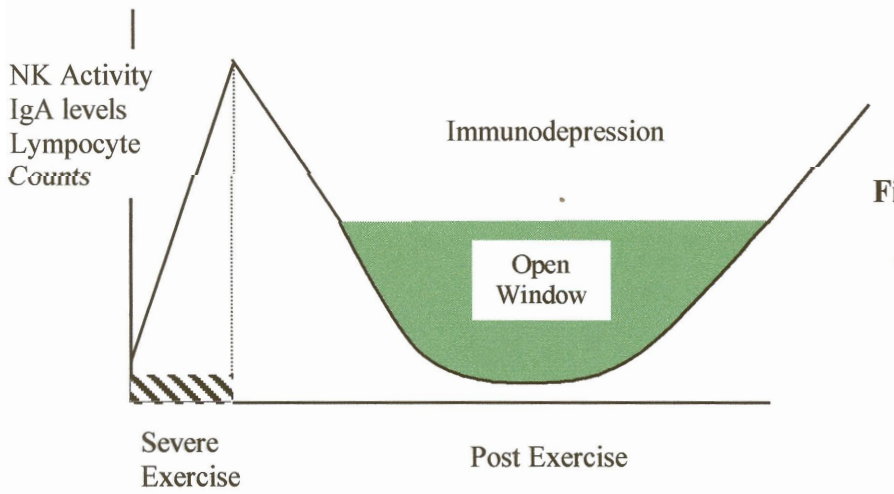

Figure 2.1: The transient post-exercise "open window" period.

This Danish group contend that it is during this transient "open window" period that microbial and viral pathogens can subvert host defenses and the athlete is most vulnerable to developing infections. They subsequently proposed that (i) decreased proliferative response of the lymphocytes (ii) depression of the immune system by corticosteroids produced under physical stress or (iii) harm done to the immune system by oxygen radicals generated during heavy exercise, are possible causes of the transient immunosuppression (Pedersen *et al.,* 1998a).

At present the closest association between markers of reduced immunocompetence and actual infection incidence appears to be between salivary IgA output and the incidence of URTI (Mackinnon & Jenkins, 1993; Pyne & Gleeson, 1998; Gleeson, 2000a, 2000 b). Several studies have shown that salivary IgA concentrations (Tomasi *et al.*, 1982; Mackinnon *et al.,* 1989) and secretion rates (Mackinnon *et al.*, 1993a; Mackinnon & Hooper (1994); Blannin *et al.,* 1998; Fahlman *et al.*, 2001) are depressed following intensive exercise in elite well-trained athletes and have linked these to an increased risk of infection and incidence of URTI in elite athletes (Mackinnon *et al.,* 1993b). Mackinnon & Hooper (1994), examining the secretory IgA response to various exercise conditions, found evidence of a cumulative effect of intense daily exercise on this major effector of resistance against microbial and viral pathogens; well-trained swimmers presented with significantly higher secretory IgA levels than "stale", overtrained swimmers over a 6-month season.

Exercise-induced URTI symptoms cannot, however, be solely attributed to infective origins. A number of excellent reviews have outlined that acute prolonged exercise bouts result in an immunological response which appears to mimic the body's response to inflammation and wound repair (Weight *et al.,* 1991). These include a rise in core body temperature (Cannon & Kluger, 1983), plasma levels of acute phase proteins (Cannon *et al.,* 1991) and cytokines (Pedersen *et al.*, 1998b), accompanied by leukocytosis (Hansen *et al.,* 1991), lymphopenia (Nieman *et al.,* 1995) monocytosis (Nielsen *et al.,* 1996a) and suppressed neutrophil activity (Pyne, 1994).

During prolonged endurance exercise increased ventilatory rates and volumes with actual damage to sensitive mucous membranes in the respiratory tract, and an inflammatory response at the sites of muscle cell damage have been linked to the development of an acute phase reaction (Weight *et al.,* 1991). Shephard & Shek (1996) refer to the "active enmeshment" of the immune system in the muscle tissue repair and inflammation process, and speculate that in this process, protection from respiratory pathogens is compromised.

It has been suggested that the physiologic consequences/metabolic sequelae of sustained exercise are similar, but not analogous, to the acute phase response (Gabriel & Kinderman, 1997). Catecholamines are known to induce a demargination of leukocytes (the increases in leukocyte numbers related mainly to plasma epinephrine concentrations during intense exercise), and as exercise continues, plasma cortisol levels rise inducing an efflux of neutrophils from bone-marrow, and retention of cells in lymphoid tissue. (Brenner *et al.,* 1998). The acute phase response thus manifests with a marked, but transient, neutrophil leukocytosis with concomitant increases in plasma cortisol, creatine kinase (CK), CRP and total protein level. Consistent delayed increases in serum haptoglobin (48 hours post-race) with no change in serum iron level, total iron-binding capacity and serum ferritin concentration have also been reported (Weight *et al.*, 1991). More recent work linking the release of pro-inflammatory cytokines (Ostrowski *et al.,* 1998a, 1989b; 1999) to an acute phase response, have led to the description of a "trauma- like" response to prolonged treadmill running.

Gabriel and Kinderman (1997) have, however, recently emphasized an important distinction between the apparent exercise-induced acute phase protein response and that induced by a bacterial infection. They showed that the leukocytosis following strenuous exercise is associated with impaired granulocyte oxidative burst activity and suppressed defence mechanisms, whereas the leukocytosis present in a bacterial infection is accompanied by priming (sensitising) of neutrophils for increased function and subsequent activation of these cells.

It is well known that indomethacin decreases *in vitro* release of immunosuppressive prostaglandin E_2 from mononuclear cells and restores suppressed post-exercise neutrophil chemiluminescence and NK cell activity (Pedersen *et al.,* 1990). In a work on URTI incidence following participation in the 1996 Two Oceans 56 km Ultramarathon, Schwellnus *et al.* (1997) showed that administration of a topical anti-inflammatory, anti-bacterial spray, Fusafungine, resulted in a lowering of the incidence of URTI in 48 participants when compared to an equal number of runners receiving a placebo during the 9 days following the event.

Gleeson (2000b, 2000c) has recently presented an additional alternative hypothesis to explain the occurrence of URTI when a bacterial or viral cause cannot be identified in the apparently healthy athlete. She proposes that in the case of prior exposure to Epstein-Barr Virus (EBV), exercise-induced suppression of cytotoxic T-lymphocytes may reduce the ability of EBV-specific memory T-cells to maintain control over virus expression and allow EBV shedding into the saliva of asymptomatic healthy carriers. She hypothesises that following exercise-induced disturbance of the cytokine balance and suppression of mucosal antibodies (low levels of salivary IgA), viral reinfection may thus occur in the oral mucosa. These could present with URTI symptoms which would induce a transient inflammatory response and activate the relevant control systems to restore immune functions in previously asymptomatic healthy, elite athletes (Gleeson, 2000b, 2000c).

The most recent state of knowledge thus appears to support the contention that increased infection risk may indeed be caused by the interaction of a combination of current and perhaps prior, pro-infective and pro-inflammatory responses which are modulated by the presence of physical, psychological and environmental stresses placed on the athlete engaging in elite endurance sport. Pyne and Gleeson (1998) further point out that the “transient and modest” nature of the observed changes may be indicative of a "self-modulating immune cell network capable of homeostatic regulation." This perhaps accounts for the rapid post-exercise recovery of most markers of immune response.

2.3 Vitamin C and reduced infection incidence in athletes

In spite of the apparent beneficial effects of vitamin C supplementation on reducing the incidence of post-race URTI, the mechanism by which this is achieved has not been elucidated. The possible involvement of reactive oxidants as mediators of the futile inflammatory response and transient decrease in immune function which accompanies intensive and prolonged exercise was supported by the findings of my three double-blind, placebo-controlled intervention studies (Peters *et al.*, 1992; 1993; 1996) in which statistically significant reductions in the incidence of self-reported symptoms of URTI in the groups supplemented with the anti-oxidative vitamins were observed, with vitamin C being the most effective.

2.3.1 *Properties of vitamin C*

Vitamin C is an organic compound which cannot be synthesized in primates (including humans) and guinea pigs (Burns, 1959). It is thus an essential nutrient in the human diet. The highest concentration of this vitamin is found in the human adrenals, ovaries, brain, pituitary gland, liver, spleen and blood cells (Moser & Bendich, 1991). It has diverse functions in the body which include an essential role in hydroxylation reactions necessary for collagen formation and carnitine synthesis, competitive substrate binding which inhibits the formation of carcinogenic nitrosamines, as well as the facilitation of iron absorption (Burns, 1959; Tolbert, 1985; Glatthaar *et al.*, 1986; Levine, 1986).

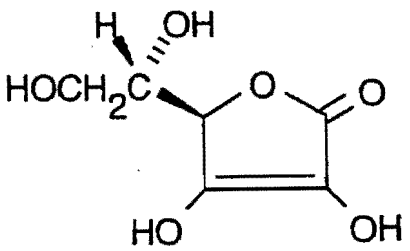

Figure 2.2: Structure of ascorbic acid (AA)

In most animals, the six-carbon ketolactone, L-ascorbate (C_6,H_6,O_8), is synthesised from glucuronic acid or galatonic acid which is derived from D-glucose. Its five membered ring **(Figure 2.2)** includes an endiol substructure with an acidic proton

(pK_a= 4.17) and the compound functions as a reducing agent and co-enzyme in several metabolic pathways.

Perhaps the most widely acknowledged role of ascorbate is that of its function in acting as co-factor for prolyl and lysyl hydroxylases in the biosynthesis of collagen. The hydroxylation steps of collagen biosynthesis are dependent on the enzymes, proline hydroxylase, procollagen-proline 2-oxoglutarate 3-dioxygenase and lysine hydroxylase which are in turn, dependent on AA for maximum activity (Chojkier *et al.,* 1989).

Additional enzymes which are dependent on ascorbate for optimal function include gamma-butyrobetaine and 2-oxoglutararate 4-dioxygenase which are responsible for hydroxylation of carnitine precursors (Otsuka *et al.,* 1999), hydroxypenylpyruvate dioxygenase, which catalyses the hydroxylation and decarboxylation of a tyrosine metabolite (Levine, 1986; Moser, 1992) dopamine β-monooxygenase, an enzyme active in norepinephrine biosynthesis (Dhariwal *et al.,* 1989; Moser, 1992) as well as several enzymes involved in hydroxylation reactions of the cortisol synthesis pathways (detailed on page 30).

2.3.2 *Anti-oxidant actions of vitamin C:*

In addition to its importance in many enzymatic reactions, an important explanation of the protective action of vitamin C during exercise may relate to its ability to directly deactivate/ neutralise the free radicals and other reactive oxidants which are produced during prolonged exercise and oxidative metabolism (Zembron-Lacvny & Szyszka, 2000). AA has been demonstrated to be the most efficient water-soluble anti-oxidant of blood and tissue fluids (Frei *et al.*, 1989). It can act directly with aqueous oxygen- derived reactive species by donating one or two electrons to metal ions, or within redox systems (e.g. hydroxylation reactions). The oxidised form, dehydro-ascorbic acid (DHAA), is immediately converted back to the reduced form. Its anti-oxidant function can also be indirect, by restoring the anti-oxidant potential of the fat-soluble vitamin E when donating an ascorbate electron to the tocopherol radical (Packer, 1986; Moser, 1992) **[Figure 2.3].**

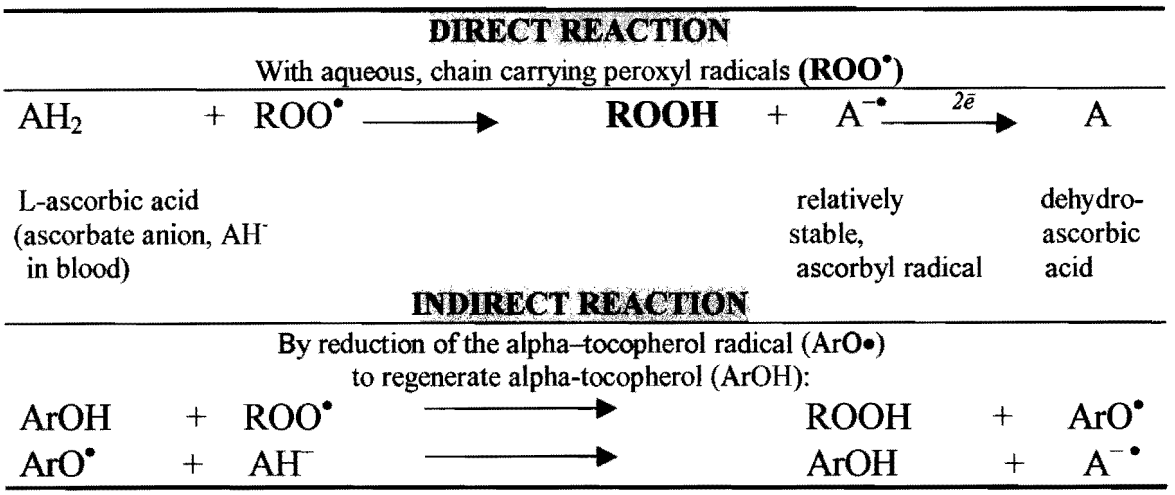

Figure 2.3: The anti-oxidant effect of vitamin C: direct and indirect processes.

It has also been proposed that high AA levels in neutrophils (Moser & Weber, 1984) protect against self-destructive autooxidation (Peters-Futre, 1997). AA is known to scavenge and neutralize effectively $O_2\bullet^-$, •OH, singlet oxygen and HOCl generated during the process of neutrophil activation (Anderson, 1981; 1991, 1995; Bendich *et al,* 1986; Smith *et al.,* 1990b; Peters-Futre, 1997; Ohno *et al.*, 1998; König *et al.,* 2001) **[Figure 2.4].** Release of AA into the extracellular fluid by neutrophils is therefore thought to protect these and other cells from free radical damage (Moser & Weber, 1984; Bendich *et al.,* 1986; Wasko *et al.*, 1989).

Chronic activation of phagocytes resulting from autoimmune disease and infection has been linked to a depletion in total and spleen ascorbate stores (Washko, 1989), as well as to an increase in the oxidised fraction of vitamin C, DHAA (Padh, 1990). Despite evidence of a rise in plasma ascorbate concentrations during prolonged exercise (Fishbaine & Butterfield, 1984; Gleeson *et al.,* 1987; Duthie, 1990; Koz *et al.*, 1992) and an adaptive up-regulation of the anti-oxidant defence system following sustained periods of endurance training (Duthie, 1997; Himmelstein *et al.*, 1998; Brites *et al.*, 1999), prolonged exercise has been associated with elevations in non-mitochondrial generation of reactive oxygen species (ROS) [Davies *et al.*, 1987; König *et al.*, 2001] and a rise in the ratio of oxidised to reduced vitamin C (Siegel & Leibovitz, 1982). Phagocytes and lymphocytes contain greater than 10-fold the

concentration of L-ascorbic acid in blood plasma (Wasko *et al.,* 1989). This, together with the high concentrations of Vitamin C stored in the adrenal and pituitary glands (which can reach about 150 times the concentrations in plasma), suggest the possibility of functional roles of this vitamin in the cells of the immune system during exercise (Moser, 1992; Redman *et al.*, 1995). Animal studies show that AA is involved in regulating a number of neutrophil functions, including chemotactic responses, phagocytosis, hexose monophosphate shunt activity and MPO function (Anderson, 1995). Suppression of phagocytic activity been shown to occur only when AA has been depleted at sites of vigorous phagocyte activity (Van Antwerpen *et al.,* 1991). This suppression has, in turn, been reversed following administration of the vitamin (Anderson *et al.,* 1989; Frei *et al.,* 1989; Wasko *et al.,* 1989).

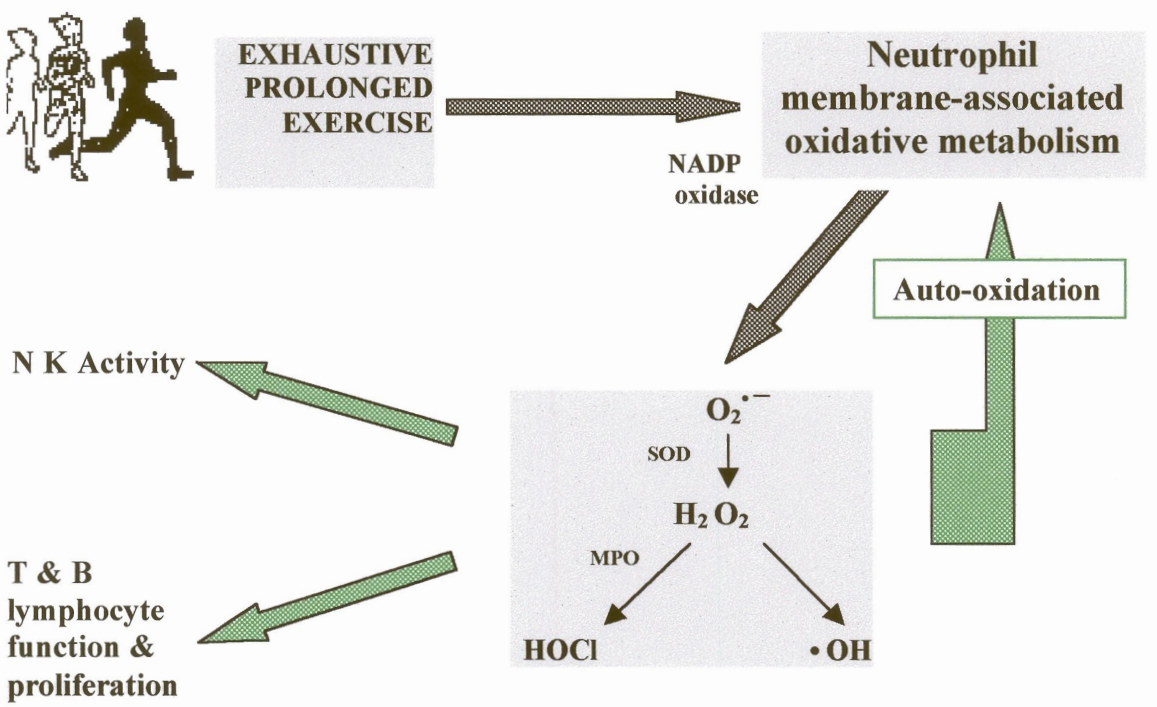

Figure 2.4: Exhaustive, prolonged exercise enhances neutrophil membrane-associated oxidative metabolism. The reactive oxidants produced, in turn, damage the neutrophils, suppressing further oxidative activity in these cells, and cause oxidative inhibition of T and B lymphocyte function and proliferation and NK activity. Adapted from Peters, 1997. ⟹ positive ⟹ negative.

Findings of *in vivo* and *in vitro* measurements in normal and immunocompromised human volunteers and in experimental animals appear to indicate that the concentration of vitamin C is a significant factor in neutralizing the escaped oxidants which accumulate when pro-oxidative events overwhelm available anti-oxidant protection. Anderson & Theron (1979) reported that *in vitro* incubation of human neutrophils with calcium or sodium ascorbate causes stimulation of neutrophil motility and migration to chemoattractants. Neutrophil motility was further investigated *in vivo* following the ingestion of increasing weekly doses (1,2,3 g daily) of sodium ascorbate by normal adult volunteers. Significant stimulation of neutrophil motility was observed following ingestion of 2 g or 3 g daily and a mechanism involving inhibition of MPO-mediated iodination was proposed.

The stimulatory effects of ascorbate on human neutrophil motility *in vivo* and *in vitro* have been associated with inhibition of the autooxidative effect of the MPO/ H_2O_2/haldide system (Anderson, 1981). In a study in which the effect of a single intravenous injection of 1g of ascorbate was monitored, the immunostimulatory and peroxidase inhibitory activity was further related to serum ascorbate level. Anderson *et al.* (1989) identified that a primary biological function of ascorbate in host defence was to neutralize granulocyte-derived HOCl, sustaining the functions of phagocytes by protecting these cells from auto-oxidation by products of their own oxidative metabolism (Anderson & Lukey, 1987).

Evidence is thus accumulating in favour of local vitamin C concentrations at sites of vigorous phagocyte activity being a primary determinant of the magnitude and/or duration of protective immune responses. Supporting the reactive oxidant scavenging properties of vitamin C *in vivo*, an inhibitory effect of Vitamins C and E on the production of phagocyte-derived extracellular ROS has been reported by Herbacynska-Cedro *et al.* (1994). The effect of oral supplementation of vitamins C and E (600 mg of AA and alpha-tocopherol acetate per day for 14 days) to 13 healthy volunteers on leukocyte production of ROS was estimated by lucigenin amplified chemiluminescence in isolated leukocytes that had been stimulated with arachidonic acid. These findings of an inhibition of phagocytic ROS production

following vitamin C supplementation were further confirmed in patients with myocardial infarction (Herbacynska-Cedro *et al.,* 1995) and coronary heart disease in the setting of significant decreases of serum levels of lipid peroxides (de la Fuente *et al.,* 1998), lending support to the theory that anti-oxidant vitamins may provide vasoprotection.

Recent studies have also provided evidence of the importance of AA in mononuclear cells. Schwager and Schulze (1997a; 1997b; 1998), investigating the effect of AA supplementation on lymphocyte function in young pigs who were unable to synthesise ascorbate endogenously, found that increasing extracellular ascorbate concentrations were not only associated with reduced activation of T and B lymphocyte in response to both pokeweed mitogen (PWM) and LPS, and reduced interleukin-2 (IL-2) production by activated lymphocytes (Schwager & Schulze, 1997a), but also decreased production of reactive oxygen intermediates (ROI) by polymorphonuclear leukocytes after supplementation with 5 and 50 mg AA per kg per day (Schwager & Schulze, 1997b). The findings of subsequent work appear to indicate that ascorbate reduces ROI levels via its effect on IL-2R expression and suggests an inverse relationship between cellular levels of ascorbate and the activity of different genes in lymphoid cells (Schwager & Schulze, 1998).

Cooke *et al.* (1998) examined the effects of supplementation with 500 mg vitamin C per day on *in vivo* levels of oxidative DNA damage. Levels of 8-oxo-2-deoxyguanosine (8-oxodG) in molecular cell DNA, serum and urine of human subjects, were significantly decreased which correlated strongly with increases in plasma vitamin C concentration. This is the first evidence in humans which suggests a positive, anti-oxidant role for vitamin C in the regulation of DNA repair enzymes.

The most recent work of Dietrich *et al.* (2002) also supports the numerous *in vitro* findings that vitamin C ameliorates lipid peroxidation which result from exposure to free radicals (Frei, 1994). In a randomised double blind placebo-controlled trial performed on 126 cigarette smokers, 2 months of daily supplementation with 500 mg of vitamin C decreased plasma F2-isoprostane levels in subjects with a high body

mass index by 28.8 pmol/litre when compared with the placebo group (p=0.001). This *in vitro* work supports a protective function of vitamin C in reducing the oxidative damage caused to lipids in cigarette smokers, but what of the effect of vitamin C on the production of free radicals and oxidative damage induced by participation in prolonged exercise (Davies *et al.*, 1987)?

Although two recent works conducted on small sample sizes (Nieman *et al.*,1997; Krause *et al.*, 2001), did fail to show that vitamin C supplementation significantly affected post-exercise neutrophil function (including on neutrophil phagocytosis, bacteriocidal capacity and oxidative burst), several recent studies (Duthie *et al.*,1996; Alessio *et al.*, 1997; Sanchez-Quesada *et al.*, 1998; Vasankari *et al.*, 1998; Ashton *et al.*, 1999; Schröder *et al.*, 2000) have provided evidence of reduced post-exercise oxidative stress following an acute period of vitamin C supplementation.

The effect of Vitamin C supplementation (1g/day) for 7 days and 2 weeks on biomarkers of pro-oxidative plasma thiobarbituric acid reacting substances (TBARS) and anti-oxidative activity oxygen radical absorbance capacity (ORAC) was determined using the TBARS: ORAC ratio to represent oxidative stress (Alessio *et al.*, 1997). This ratio was highest (32%) following 30 min of running exercise when a placebo was given and only rose by 5.8% after one day of vitamin C supplementation as opposed to 25.8% after 2 weeks of supplementation. As the increases in oxidative stress ratios, however, did not reach statistical significance, this study appeared to only support a mild tendency of biomarkers of oxidative stress to tilt the oxidative stress balance towards antioxidant activity after vitamin C supplementation. Of interest, is the apparently more marked effect after an acute period of supplementation than the more prolonged two-weeks of supplementation which appear to support previously reported findings of Duthie *et al.* (1996) and Schröder *et al.*(2000), but require further confirmation.

Ashton *et al.* (1999) have found that acute supplementation with 1000 mg of L-ascorbate in 10 subjects 2 hr before an incremental exercise test to exhaustion, resulted in a significantly lower post-exercise lipid hydroperoxide, malondialdehyde and

electron spin resonance signal intensity. Vasankari *et al.* (1998), studying the effects of supplementation of 2 g vitamin C per day versus placebo on 9 athletes on oxidative stress following a longer 10.5 km maximal run, showed that serum diene conjugation concentration decreased by 11% following the vitamin C trial, but not in the placebo ($p=0.03$), while Sanchez-Quesada *et al.* (1998) confirmed evidence of reduced oxidative stress following a 4-hour athletic race in subjects receiving oral supplementation of 1g of AA per day. In this study, exercise-induced increases in susceptibility of low density lipoprotein (LDL) to oxidation and proportion of LDL(-) were inhibited in the group receiving AA. Schröder *et al.*(2000) provided further evidence of the anti-oxidant actions of vitamin C in exercising individuals with their finding of a 15.3% reduction in the lipid peroxide/total anti-oxidant status ratio (measured by chromogenic method) in professional basketball players following 32 days of supplementation with a three-compound anti-oxidant supplement containing 600 mg α tocopherol, 1000 mg vitamin C and 32 mg β-carotene.

Taken together, the current evidence in favour of protective anti-oxidant effect following Vitamin C supplementation in acutely stressed individuals, is convincing.

2.3.2 *Pro-oxidative potential of vitamin C*

The potentially harmful effects on health of high intakes of AA have been the subject of much debate. In addressing the question of ascorbate toxicity which may be associated with large doses of supplemental intake of vitamin C, most studies have focused primarily on potential increases in oxalate formation, decreased uric acid excretion, impairment of vitamin B status and iron overload. Since kinetic studies using isotopes have proved that the metabolic turnover of vitamin C is limited (with the maximum reaching about 40 mg/day), large doses of vitamin C should not result in increased oxalate and subsequent kidney stone formation (Schmidt *et al.,* 1981). This was shown by Hagler and Herman (1973), who found that daily intakes of 10 g only increased urinary oxalate excretion by 15 to 37 mg per day, which is no more than that resulting from "normal" dietary intake of food, and has subsequently been confirmed by Schmidt *et al.* (1981), Gerster (1997) and Curhan *et al.* (1999).

Physiologists have reasoned that an increased load of AA in the proximal tubule, may, due to competitive inhibition, decrease uric acid reabsorption and lead to ascorbic-acid induced uricosuria (Guyton, 2000). This has not been confirmed by the literature. Relatively small increases in urate excretion at high, non-physiological plasma ascorbate levels, have led to the suggestion that urate may possess a preferential affinity for this transport mechanism or an additional secretory transport system not shared with ascorbate (Rivers, 1987).

While research evidence has consistently demonstrated that vitamin B_{12} in food or the body is not destroyed by AA, reports of possible enhancement of pro-oxidative activity following supplementation with vitamin C do, however, exist (Shilotri & Bhat, 1977; Herbert, 1993).

The theory that megadoses of vitamin C have a pro-oxidant effect was first related to its reaction with iron; that in the presence of iron, AA converts iron stores to catalytic iron which possesses strong pro-oxidant effects (Salonen *et al.*, 1992). An almost optimal iron absorption is obtained with 25-50 mg AA per meal, refuting the possibility of a linear enhancement of iron absorption by the vitamin in healthy, iron-replete individuals (Hallberg, 1985; Bendich & Cohen, 1990). The concern was, however, that in the case of persons born with a gene for increased iron-absorption, high vitamin C intake, which is known to increase absorption of dietary iron (Hallberg, 1985), can cause iron overload and the release of large amounts of catalytic iron from their body stores (Gerster, 1999). This is, however, only applicable if serum ferritin levels are in excess of 120µg/l (Salonen *et al.*, 1992). As these concentrations have rarely been described in elite athletes and are not considered physiological, the possibility of an iron-associated pro-oxidant effect of vitamin C in athletes is unlikely.

Shilotri & Bhat (1977) reported that supplementing adult human volunteers with 200 mg as well as 2000 mg vitamin C per day stimulated hexose monophosphate shunt activity of resting neutrophils. Although bactericidal killing activity was not affected by the moderate dose of vitamin C supplementation, administration of a megadose resulted in a decrement of bacterial killing of the leukocytes. As the megadose of vitamin C

administered was not accompanied by an increase in plasma cortisol, and circulating levels of cyclic nucleotides were not measured, these authors were unable to clarify possible mechanisms with certainty.

A recent debate revolved around the findings of Podmore *et al.*(1998) that administration of 500 mg of Vitamin C to 30 healthy volunteers for 6 weeks resulted in a decrease in 8-oxo-7,8 dihydroguanosine, and an increase in 8-oxo-7,8 - dihydroadenosine in lymphocyte DNA. A major criticism of this study is that intracellular vitamin C concentrations were not measured in the lymphocytes and that increasing the extracellular plasma concentration of ascorbate above 50 µM should not have affected the already saturated intracellular concentration of lymphocytes further (Levine *et al.*, 1998). As the study was not placebo-controlled or double blinded, further well-designed trials are needed to confirm this finding.

Nevertheless, caution is merited and careful consideration needs to be given to possible mechanisms by which vitamin C may exert pro-oxidative effects *in vivo*. On the one hand, Anderson has, based on *in vitro* observations, proposed that vitamin C possesses three properties which might contribute to pro-oxidative activity *in vivo*. Firstly, the vitamin does not scavenge H_2O_2, a cell-permeable, reactive oxidant (Anderson & Lukey, 1987). Secondly, and somewhat paradoxically, vitamin C, by acting as a scavenger of HOCl, prevents auto-oxidative inactivation of NADPH-oxidase, resulting in increased production of H_2O_2 by activated phagocytes (Anderson & Lukey, 1987). Thirdly, vitamin C, probably by complexing with the critical heme group of catalase, inhibits the H_2O_2, neutralizing activity of this enzyme (Orr, 1967; Poulsen *et al.*, 1998). If operative *in vivo*, these pro-oxidative activities of vitamin C may predispose to H_2O_2 - mediated tissue damage and genotoxicity as a result of both increased production and reactivity of this reactive oxidant.

At this stage there is, however, not enough concrete evidence to support the possibility of a dualistic, differential response to vitamin C supplementation. To quote Poulsen *et al.* (1998) "it is too soon to say whether supplemental doses of vitamin C exert pro-oxidant or mutagenic effects." The rare incidences of conflicting evidence do,

however, justify the need for further research in order to confirm the correctness of the present assumption that megadoses of vitamin C have beneficial anti-oxidant effects to exercising individuals and clarify the reasons for the occasional discrepant findings.

2.3.4 *Suppressed production of cortisol by adrenocortical cells from the adrenal gland as an alternative mechanism of Vitamin C mediated immunostimulation*

In the 1940s vitamin C was first labelled as an "anti-stress" vitamin. This claim was subsequently refuted due to the absence of adequate research data to substantiate it. It has, however, recently been shown that vitamin C may antagonize the immunosuppressive effects of corticosteroids by interfering not only with their synthesis and/or release (Pardue & Thaxton, 1984a), but also with their interactions with target cells (Schwager & Schulze, 1998; Bowie & O'Neill, 2000; Horton *et al.*, 2001).

Numerous reports have indicated that the synthesis and/or release of the major, endogenous immunosuppressive glucocorticoid, cortisol, is regulated by vitamin C (Pardue & Thaxton, 1984a; Goralczyk *et al.*, 1992; Moser, 1992). While vitamin C infusion has been found to enhance adrenal corticoid production in the laboratory of Kodama *et al.* (1994; 1996), the evidence is accumulating in favour of lowered serum cortisol concentrations with and without increases in ACTH when vitamin C status is enhanced. Laboratory work has showed that increasing ascorbate levels in adrenal cortices results in an inhibition of steroidogenesis (Kitabchi, 1967; Sulimovici & Boyd, 1968; Siegel, 1971) and an association between increased dietary intake of vitamin C and a reduction in stress-related increases in circulating corticosteroids has also been described in poultry (Pardue et al. 1985a; Satterlee *et al.*, 1989, 1994; Jones *et al.*, 1999), guinea pigs (Odumosu, 1982; Enwonuwo *et al.*, 1995), rats (Campbell, pers commun), adult patients undergoing surgery (Nathan *et al,* 1991) and elderly women (de la Fuente *et al.*,1998]. Liakakos *et al.*(1975) have shown that AA administration to children reduced mean plasma cortisol values following administration of ACTH. Sumbaev & Iasinskaia (1997) confirmed these

findings by showing that AA not only regulates corticosteroid production, but also activates the hydroxylation of adenyl purines and uric acid in both animals and humans. While Farr *et al.* (1997) provided evidence of reduced weight-loss and less dehydration in AA supplemented broiler chicks exposed to slaughter stress, McKee *et al.*(1997) reported increased weight gain with increased plasma triglyceride concentrations following heat-induced stress in AA supplemented broiler chicks and Campbell (pers commun) evidenced less weight loss together with reductions in thymus involution and adrenal hypertrophy in stressed rats fed 200 mg (the equivalent of a few thousand milligrams in humans) vitamin C per day. It is of interest that each of these sets of findings following AA supplementation appears to be compatible with lowered stress-induced corticosteroid production.

One possible cause of AA- induced reduction in circulating corticosteroid levels may be inhibition of the functional capacity of several enzymes involved in adrenal corticosteroid biosynthesis by vitamin C (Pardue & Thaxton, 1984a; Satterlee *et al.*, 1989). The specific cortical enzymes involved in steroid synthesis are depicted in **Figure 2.5.**

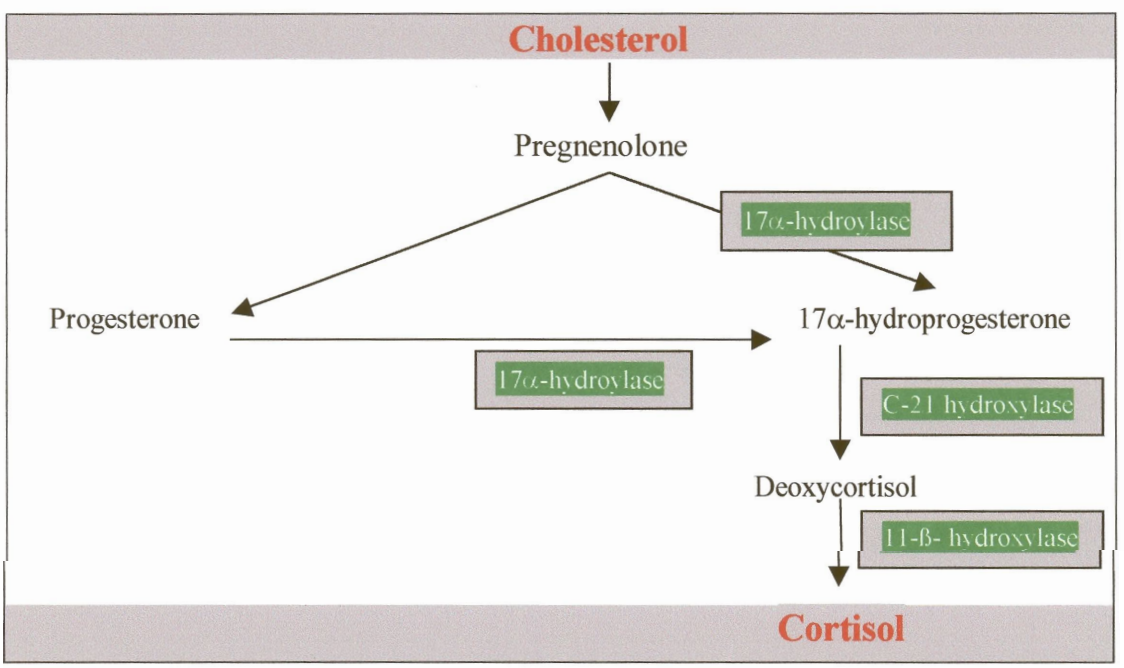

Figure 2.5 Cortical enzymes which regulate adrenal biosynthesis of cortisol (Lindzey & Korach , 1997).

Evidence is available that high adrenal AA levels reduce plasma corticosteroid concentrations due to the ability of the vitamin to (i) inhibit the enzymatic side chain cleavage system that converts cholesterol to pregnenolone (Sulimovici & Boyd, 1968; Shimizu, 1967) and (ii) inhibit C-21 hydroxylase and 11-ß- hydroxylase in the steroidgenesis pathways (Hayono *et al.,* 1956; Cooper and Rosenthal, 1962; Kitabchi, 1967) [**Figure 2. 5**]. Administration of ACTH has, in turn, been reported to inhibit AA transport into the adrenal cortex of the rat and this inhibition has been correlated with a concomitant increase in steroidgenesis (De Nicola *et al.,* 1968).

The immunosuppressive effects of corticosteroids, so-called steroid mediated immunosuppression (SMI), have been reported in numerous species. Following prolonged exercise elevated circulating cortisol levels have been related to decreases in peripheral blood lymphocyte number and proliferation, decreased NK cell cytotoxicity and suppression of neutrophil function (Farrell *et al.*, 1983). A reduction in post-exercise circulating cortisol levels resulting from enhancement of circulating ascorbate levels, would thus have important implications in terms of reducing transient post-exercise SMI. This is also a potential mechanism by which vitamin C increases the proliferative responses of T-lymphocytes *in vitro* (Siegel & Morton, 1977; Anderson *et al.,* 1989; Campbell *et al.*, 1999) and in human subjects (Anderson *et al.*, 1980; de la Fuente, 1998).

2.3.5 *Protection from steroid insult*

Not only has vitamin C supplementation been linked to decreased synthesis of cortisol, but in experiments conducted on broiler chickens, it was found that supplemental AA provided in the diet significantly protected cells from the cytotoxic effects of adrenal steroids and reduced the immunosuppressive effects of elevated circulating cortisol concentrations. These included reduced heterophil/lymphocte ratios (Satterlee *et al.,* 1989), increased agglutinin production (Pardue & Thaxton, 1984b) as well as ameliorated heat-mediated immunosuppression in chicks challenged with sheep erythrocytes (Pardue *et al.*, 1985a) and heat-associated growth inhibition and mortality (Pardue *et al.*, 1985b). Although the previously described

anti-oxidant properties of AA may protect cell membranes from steroid-induced injury, Moffat *et al.*(1972) suggest a possible protective mechanism related to cyclic AMP (cAMP) production within lymphoidal tissue which may account for the findings of Pardue *et al.* (1984b; 1985a; 1985 b). This possibility also requires further verification.

2.3.6 *Reduced exercise-induced mobilisation of vitamin C stores*:

Mobilization of vitamin C from the adrenals has been shown to be a component of an adaptive response to regular and repeated exposure to oxidative stress. This has been confirmed in "fit" runners (Bergholm *et al.*, 1999), runners in a high-training group (Robertson *et al.*, 1991), trained marathon (Gleeson *et al.*, 1987) and ultramarathon runners (Peters *et al.*, 2001a, 2001b - which form a component of this thesis) and in a diverse range of 44 athletes (Rokitzki *et al.*, 1994a), all of whom were found to present with elevated circulating ascorbate levels when at rest viz. well above the 42.4 µmol/l which Brubacher *et al.* (2000) report as the 50th percentile of the plasma concentration for a daily vitamin C intake of 60 mg/day. In addition, Himmelstein *et al.*(1998) found mean plasma vitamin C concentrations were 29.2 % higher in 44 registered participants in the 1994 Duke City Marathon as opposed to those in 48 sedentary controls (80.3 ± 2.99 vs 56.8 ± 4.86 µmol/l), while Brites *et al.*(1999) found that the total plasma anti-oxidant capacity was 25% higher in a group of soccer players engaged in regular training than in matched, sedentary controls. Higher plasma AA, as well as uric acid, α tocopherol and superoxide dismutase were concluded to reflect a compensatory adaptation to high levels of oxidative stress in sportsmen by these researchers. The work of Schröder *et al.* (2000) did, however, not confirm this in 16 professional basketball players ($\bar{X}$ vitamin C, 50.8 ± 22.6µmol/l), but this is most likely due to the relatively small component of aerobic (oxygen-dependent) training completed by these sportsmen.

In 1960 Lipscomb & Nelson found that AA concentration was increased in venous blood from the adrenals preceding onset of glucocorticoid release. Subsequent laboratory studies have shown that the stimulation of cultured porcine adrenocortical

cells by adrenocorticotrophic hormone (ACTH) in the presence of AA results in the production of cortisol with concomitant release of AA (Moser, 1992) which is dose-dependent (Goralczyk *et al.*, 1992). It is therefore possible that mobilisation of vitamin C from the adrenals in response to acute exercise bouts, may be coupled to increased production of cortisol during prolonged exercise. It is well accepted that adrenal cortisol release is a necessity to counter inflammation-mediated tissue damage which is known to result from exercise in athletes (Clarkson *et al.*, 1992) and the majority of studies which have assessed plasma, serum and lymphocyte AA concentrations following acute exercise bouts, have reported an exercise-induced rise in both circulating cortisol and AA concentration (Gleeson & Maughan, 1986; Gleeson *et al.*, 1987; Duthie *et al.*, 1990; Liu *et al.*, 1999, Viguie *et al.*, 1993). However, in recent work on vitamin C supplemented ultramarathoners (Peters *et al,* 2001a, 2001b, which forms a component of the work presented in this thesis), it was found that additional intake of this vitamin (≥1500mg/d) results in an attenuation of both exercise-induced mobilisation of vitamin C and cortisol, adding support to the possibility that exercise-induced adrenal release of cortisol and AA is coupled.

2.3.7 *Immune neuro-endocrine interactions*

Interactions between the immune and endocrine systems are known to play an important role in maintenance of immune homeostasis, particularly in view of the immunosuppressive properties of the glucocorticosteroids. Glucocorticoid release is stimulated by polypeptides known as cytokines, many of which are synthesised and released in the adrenal gland and modulate its secretory activities, as well as help control and mediate interactions among cells involved in immune responses.

2.3.7.1 Cytokine-mediated activation of glucocorticoid release

The onset of inflammation following muscle cell damage is brought about by the release of the "early" or "alarm" pro-inflammatory cytokines, tumour necrosis factor alpha (TNFα) and interleukin-1β (IL-1β), from tissue macrophages, smooth muscle cells and fibroblasts. These act on fibroblasts and endothelial cells and many other

cell types to induce an inflammatory cascade resulting in the production of interleukin-6 (IL-6) and interleukin-8 (IL-8), which act synergistically with TNFα and IL-1β to influence the interaction between the hypothalamic–pituitary-adrenocortical (HPA) axis and the immune system (Marx *et al.*, 1998). These pro-inflammatory cytokines are released sequentially and have been shown to stimulate the release of glucocorticoid hormones, which may represent a homeostatic, counter inflammatory mechanism to dampen excessive immune responses. A number of recent investigations suggest that adrenocortical and adrenomedullary cells have specific receptors for TNFα, IL1β, IL-2 and IL-6 and that these cytokines are also synthesised in adrenocortical and chromaffin cells. *Escherichia coli* LPS is the most potent stimulator of cytokine biosynthesis, not only in leukocytes and macrophages, but also in adrenal cells (Marx *et al.*, 1998). TNFα, IL-2 and IL-6 directly stimulate glucocorticoid production by the cells of the *zona reticularis*, whereas IL-1β has an analogous effect, stimulating catecholamine release by chromaffin cells or activation of the corticotropin-releasing hormone (CRH)/ACTH system (Nussdorfer & Mazzocchi, 1998).

Buckingham *et al* (1994) have reported that oral or peripheral administration of IL-1ß, IL-1α, IL-6 and IL8 to adult male rats, produce increases in serum cortisol concentration and the release of CRF-41 and arginine vasopressin (AVP) from the hypothalamus, while none of these cytokines directly influenced the release of ACTH from pituitary tissue *in vitro.* Päth *et al.*(1997) confirmed these findings showing that IL-6 receptors predominate in the *zona reticularis* and inner zone and that adrenal steroidgenesis could be stimulated by IL-6 in the absence of ACTH, confirming the importance of this pleiotropic cytokine in initiating a suppression of inflammatory activity.

Presently, it can be concluded that IL-6, which is produced in larger amounts than any other cytokine following exercise, may act on the HPA at three levels (**Figure 2.4**):

1. the hypothalamus by stimulating the secretion of CRH
2. pituitary corticotropes by eliciting adrenocorticotropin hormone (ACTH) release

3. the adrenal gland by enhancing steroid-hormone secretion by adrenocortical cells through direct or indirect paracrine mechanisms.

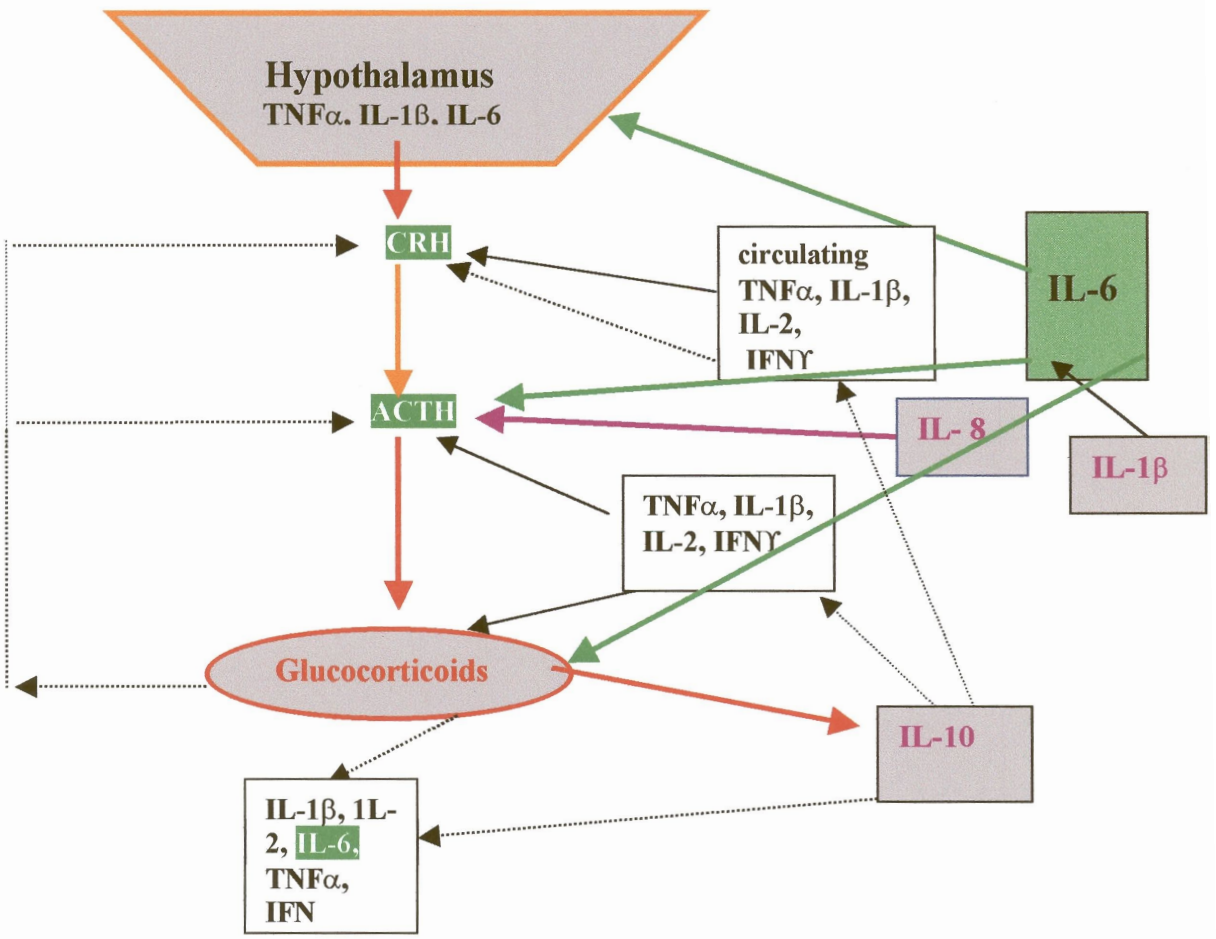

Figure 2.4: The influence of selected cytokines on the hypothalamic- pituitary – adrenal axis ⟶ negative feedback; ⟶ positive feedback (De Waal *et al.*, 1991; Mandrup-Poulsen *et al.*, 1995; Skinkai *et al.*, 1996)

The immunosuppressive activities of corticosteroids affect a wide range of immune and inflammatory cells and their mediators, and are achieved by activation of synthesis of various immunomodulatory polypeptides through the interaction of glucorticoid/ glucocorticoid receptor complexes with glucocorticoid response elements on the promoter regions of steroid responsive genes, as well as by inhibition of the activation of the cytosolic nuclear transcription factors, kappaB (NF*k*B) and activator protein –1 (AP-1), which induce the production of a series of

pro-inflammatory polypeptides (Barnes & Adcock, 1993; Barnes & Karin, 1997; Rahman & MacNee, 1998).

Activation of transcription factor, NF*k*B, a mediator of altered gene expression during inflammation, and implicated in viral infection, has been reported to be inhibited by AA (Schwager & Schulze, 1998; Bowie & O'Neill, 2000; Horton *et al.*, 2001). Schwager & Schulze (1998a) suggest that ascorbate affects immune homeostasis via reactive oxygen intermediate (ROI)–dependent expression of interleukin genes due to sensitivity of NF*k*B to ROIs. Bowie & O'Neill (2000), however, showed that inhibition of NF*k*B by AA was not simply an anti-oxidant effect as redox-insensitive pathways to NF*k*B were also "blocked." Inhibition of TNF-driven I-kappa B kinase (IKK), which inhibits degradation and phosphorylation of I-kappa alpha, an inhibitory protein that dissociates from NF-kappaB, was mediated by the mitogen-activated protein-kinase, p38. The results of this vitro work identify p38 as an intracellular target for high dose vitamin C. This is supported by the findings of Horton *et al.* (2001) who found that administration of 38 mg/kg vitamin C in conjunction with 27 U/kg of vitamin E and 41U/kg vitamin A, inhibited NF*k*B translocation from the cytosol to the nucleus and reduced inflammatory cytokine (TNFα, IL-1β, IL-6) secretion by cardiomyocytes following burn-trauma.

A perspective on the possible association between vitamin C and the pituitary gland, the adrenal glands and the immune system has been described by Kolb (1990). Cu-containing peptidydyl-glycine-alpha-amidiizating–monooxygenase, which is necessary for the formation of alpha-melanocyte stimulating hormone (αMSH) and dependent on ascorbate for its activity, is stored in the pituitary gland. In the event of an ascorbate deficiency, α-MSH formation is inhibited and a stressful situation will result in the increased binding of ACTH to the cells of the *zona fascicularis* and inner *zona reticularis* of the adrenal cortex. This stimulates the release of about 40-60% of the quantity of ascorbate from the adrenal cortex, activating adenylate cyclase and C-21-hydroxylase, and increasing the synthesis and secretion of glucocorticosteroids (Kolb, 1990).

The low molecular weight chemotactic cytokine, interleukin-8 (IL-8), produced by macrophages and stimulated by TNF and IL-1, is another important secondary mediator of the inflammatory response associated with tissue damage and glucocorticosteroid release. It has the ability to both attract neutrophils to damaged tissue and to activate them in response to elevated TNFα and IL-1 (Baggiolini, 1993) which are, in turn, known to stimulate steroid-hormone secretion by adrenocortical cells (Nussdorfer & Mazzocchi, 1998). *In vitro* neutrophil-mediated acute inflammation studies have reported elevated humoral IL-8 concentrations and shown that the administration of a neutralising antibody to IL-8, reduces neutrophil infiltration and neutrophil-mediated tissue injury (Mukaida, 1998). It is possible that this cytokine, which has been shown to be released into the bloodstream after prolonged and intensive, but not moderate exercise (Suzuki *et al.*, 2000), therefore has an important function in activating the hypothalamic- pituitary –adrenal axis and the accumulation of neutrophils in damaged skeletal muscle cells during prolonged eccentric, weight-bearing exercise such as downhill running. Inhibition of its release by vitamin C, would therefore reduce an exercise-induced inflammatory response as well as attenuate glucocorticoid release. This hypothesis requires investigation.

In a randomised, double blind, placebo-controlled study (Nieman *et al.*, 1997) supplementation with 1000 mg of vitamin C for 8 days to 2 groups of 6 distance runners, did not have significant effect on stress hormone concentrations, leukocyte subsets, IL-6, NK cell activity or lymphocyte proliferation following 2.5 hours of intensive running at 75-80% $\dot{V}O_2$ max (N=6). It is, however, a possibility that the duration and intensity of the exercise protocol was not adequate to elicit the oxidative stress and muscle cell damage which results from more prolonged eccentric exercise. Furthermore, dietary CHO intake during exercise may have been an extraneous variable modulating cortisol release into the blood stream via increments in blood glucose concentration in this work. High blood glucose would reduce ATCH activity and cortisol secretion (Tabata *et al*, 1991; Nehlsen-Cannarella, 1997; Nieman *et al.*, 1997), resulting in less release of AA from the adrenal gland into the circulation (Goralczyk, 1992). This may also have applied to the most recent work reported by Pederson *et al.* (2001) in which male recreational runners received either anti-

oxidants (500 mg vitamin C and 400mg vitamin E daily) or placebo 14 days before and 7 days after a 5% downhill 90-min treadmill run at 75 % VO_2 max. and exercise-induced changes in IL-1Ra, IL-6, creatine kinase and lymphocyte numbers did not differ significantly between the supplemented and non-supplemented groups. Further work into the efficacy of vitamin C supplementation in reducing markers of an inflammatory response to prolonged eccentric exercise in which the CHO status of the athletes is carefully standardised, is required.

2.3.7.2 Respondents to glucocorticoid release

Interestingly, cortisol appears to mediate at least some of its immunosuppressive effects through induction of the potent anti-inflammatory cytokine, IL-10 (Skinkai *et al.*, 1996). This soluble protein produced by helper T cells, macrophages/monocytes and B cells, which was originally referred to as cytokine synthesis inhibiting factor (CSIF), has both immunosuppressive and immunostimulatory properties. While increased circulating concentrations of this cytokine inhibit the release of TNFα and IL-1β and induce the production of IL-1Ra (de Waal *et al.*, 1991), inhibition of its bioactivity results in enhanced bacterial clearance, increased expression of proinflammatory cytokines and prolonged survival of mice challenged with *Klebsiella pneumoniae* (Greenberger *et al.*, 1995). Exposure of human monocytes to major group rhinoviruses (the main cause of the common cold) has also recently been reported to result in increased IL-10 production by these cells and down-regulation of their accessory functions (stimulation of T cell and NK cell function). These events (increased IL-10 production) have been proposed to be involved in the pathogenesis of upper respiratory tract infections with these viral pathogens (Stökl *et al.*, 1999). To my knowledge, however, no study has previously been undertaken in which the relationship between intensive exercise, vitamin C status, cortisol and IL-10 production is addressed. It was thus an objective of the studies presented later in this thesis to investigate this relationship.

A further respondent to glucocorticoid release is IL-1Ra, a specific inhibitor of IL-1 activity that acts by blocking the binding of IL-1 to its cell surface receptors. The

antagonist is secreted by several different cell types, including monocytes, neutrophils, macrophages, and fibroblasts. IL-1Ra is thought to be part of a naturally occurring mechanism that limits the extent of the potentially deleterious effects of IL-1. Several cytokines upregulate IL-1Ra production, including IL-6. This cytokine inhibitor has been shown to be elevated following prolonged exercise (Ostrowski *et al.,* 1999), but no study has to date investigated the effect of vitamin C supplementation on its production.

A "grey area' which thus presently exists in the state of knowledge regarding the role of vitamin C in attenuating infection risk following physical exertion, revolves around the possible effect of elevated circulating vitamin C concentrations on adrenal stress hormone production and consequent modulation of the production of IL-10 and IL-1Ra. This question has been addressed in the current study (Chapters 4, 5 and 6).

2.3.8 Exercise, vitamin C status and circulating adrenaline concentrations.

Adrenaline is known to possess anti-inflammatory and immunosuppressive properties (Galbo, 1983) and may accordingly also impact on the magnitude of the post-exercise "open-window" period (Pedersen and Ullum, 1994). During exercise adrenaline is released from the chromaffin granules in the adrenal medulla and plasma concentrations increase almost linearly with the duration of exercise and exponentially with intensity. The expression of beta-2 adrenoreceptors on T, B, and NK cells, macrophages and neutrophils provides the molecular basis for these cells to be targets for catecholamine signaling (Moore and Willoughby, 1995; Van der Poll *et al.*, 1996; Weiss *et al.*, 1996). Beta-2 adrenoreceptors on lymphocytes are linked intracellularly to the adenylate cyclase system for generation of cAMP as a second messenger (Hadden *et al.,* 1970). It has been shown that in most mammals, chromaffin granules of the adrenal medulla have the second highest concentration of AA (the adrenal cortex has the highest) [Dhariwal *et al.,* 1989]. In terms of a possible relationship between blood vitamin C concentration and catecholamine release, an enzyme in the chromaffin granules, dopamine beta-monooxygenase, requires AA *in vitro* to convert dopamine to noradrenaline in the catecholamine biosynthesis

pathway (Levine *et al.*, 1986). No known relationship between blood vitamin C concentration and adrenaline release, has, however, been described.

In conclusion, this review of the relevant literature reveals that the evidence in support of an anti-oxidant function of vitamin C during prolonged, aerobic exercise is strong, but that the effect of vitamin C supplementation on the mobilisation of vitamin C stores, pro-inflammatory cytokines and their natural antagonists and adrenal stress hormone release, requires further investigation. The effect of vitamin C supplementation on neuro-endocrine interactions and local inflammatory response are also open areas for future research some of which have been investigated in the studies described in the following chapters of this thesis.

Chapter Three

Study aims and hypotheses

3.1 *Aims*

To my knowledge no study has previously been undertaken in which the immuno-neuroendocrine relationship following intensive physical stress has been examined following vitamin C supplementation. It was thus the primary purpose of the present series of studies to investigate the relationship between vitamin C intake and adrenal stress hormone and cytokine response to ultramarathon running.

In addition, other relevant aspects addressed in this work include the effect of pre-race supplementation of moderate (500mg/d) and high (>1500mg/day) daily intakes of Vitamin C on mobilization of vitamins A, C and E, differential blood leukocyte counts, platelet counts, neutrophil primary granule enzymes, acute phase reactants and cytokine concentrations. The inflammatory response to ultramarathon running following Vitamin C supplementation compared to the response of runners on placebo to completing the ultramarathon, is also investigated.

More specifically, the proposed work set out to investigate the effects of oral administration of 1000 mg vitamin C (2x 500 mg) daily vs. matched placebo or 500 mg (1 x 500mg daily), 1500 mg (3 x 500 mg) vitamin C vs. matched placebo on the aforementioned parameters of systemic inflammation athletes participating in two 90 km Comrades Marathons events which were run in tropical winter climatic conditions, and downhill, from Pietermaritzburg to Durban, South Africa. These included circulating levels of

- cortisol
- adrenaline
- vitamins A, vitamin C, vitamin E
- C-reactive protein, amyloid A
- creatine kinase, lactate dehydrogenase

- leukocytes (neutrophils, lymphocytes, monocytes, eosinophils, basophils) and platelets
- neutrophil-derived elastase and myeloperoxidase
- pro-inflammatory-cytokines, IL-1ß, IL-6, IL-8, TNFα
- anti-inflammatory-cytokines, IL-10
- IL-1 receptor antagonist

The results of these investigations are presented in chapters 4, 5, 6 and 7, each with a separate introduction, methods, results and discussion section. An integrated discussion of the data is presented in chapter 8.

3.2 *Hypotheses*

The hypotheses to be tested included the following:

1. that downhill ultramarathon running results in elevation of all markers of inflammatory and oxidative stress measured in this study
2. that vitamin C supplementation protects against exercise-associated transient immune dysfunction by ameliorating oxidative and inflammatory stress and attenuating the related increases in circulating cortisol and adrenaline as well as those of immunosuppressive polypeptides.
3. that the response to vitamin C supplementation is dose-dependent.

Chapter Four

Vitamin-C supplementation, oxidative and inflammatory stress in ultramarathoners

4.1 *Introduction*

Acute, prolonged exercise results in transient alterations in systemic inflammatory parameters, which appear to mimic the body's response to infection (Weight *et al.*, 1991; Gabriel & Kinderman, 1997; Nieman, 1995). Characteristics of this response include a rise in core body temperature (Cannon & Kluger, 1974), increased plasma levels of cytokines and acute phase proteins (APPs) [Pedersen et al., 1998a; 2000], neutrophilia, monocytosis and lymphopenia (Keast *et al.*, 1988; MacArthy & Dale, 1988; Pyne *et al.*, 1995; MacKinnon, 1999). Although similarities exist between this apparently futile inflammatory response and that caused by microbial pathogens, there are also some noteworthy differences. For example, the leukocytosis due to microbial infection is associated with priming (sensitization) of the pro-oxidative, pro-inflammatory activities of circulating neutrophils, while that associated with prolonged exercise is accompanied by impairment of these, particularly membrane-associated oxidative metabolism, and diminished host defences (Gabriel & Kinderman, 1997).

Acute and intensive bouts of prolonged physical exertion, such as that experienced by athletes participating in ultramarathon events, have also been reported to result in an increased susceptibility to respiratory infections post-race (Peters & Bateman, 1983). This is preceded by a transient "open-window" period that persists for 6-20 hours immediately post-race during which the numbers of circulating lymphocytes, natural killer cell activity and serum concentrations of IgA and complement components decline to sub-normal values (Pederson & Ullum, 1994). Administration of vitamin C to participants in ultramarathon events has been shown in some studies to decrease the incidence of post-race respiratory infections (Peters *et al.*, 1993; 1996). However, the mechanism by which vitamin C attenuates the increased susceptibility to infection which accompanies prolonged and intensive

exercise has not been established. Potential mechanisms include prevention of oxidant-mediated immunosuppression (Anderson *et al.*, 1989), and/or antagonism of the production of immunoregulatory corticosteroids (Pardue & Thaxton, 1984a).

In an attempt to resolve this issue, I have measured the effects of supplementary vitamin C on systemic parameters of inflammation and oxidative stress, including measurement of cortisol and mobilisable anti-oxidative vitamins, in a group of athletes prior to, and on completion of a 90 kilometer ultramarathon.

4.2 Materials and Methods

4.2.1 *Subjects*

The protocol was approved by the Committee for Research on Human Subjects of the University of the Witwatersrand, Johannesburg, South Africa. Twenty four entrants in the 1997, 90 kilometer Comrades Marathon, which was run from Pietermaritzburg to Durban, volunteered to participate in the study in response to a request for subjects addressed to a large, local running club. They signed informed consent forms which detailed the requirements of the research protocol. Using a placebo-controlled, double-blind design, subjects were divided into experimental and control groups of equal size (n=12). The experimental subjects were given 1000 mg (2 x 500 mg) vitamin C per day for 7 days prior to, on the day of the race and for 2 days following the race, while control subjects were given placebo capsules of similar appearance and taste during these 10 days. Supplements were taken with meals in two equal doses, once in the morning and once in the evening.

4.2.2 *Dietary records*

Each subject was asked to keep a record of his/her dietary intake on the 3 days prior to the race. Total daily vitamin A, C and E intakes during the 3 days preceding the race, including those derived from any additional vitamin and mineral supplements used by the athletes, were determined using the Dietary Manager computer program (Program Management, Randburg, South Africa). Vitamin A, C and E intake of each subject was calculated from the sum of the dietary intake, additional supplements used and the vitamin C supplements given to the experimental group.

4.2.3 *Blood sampling*

Sixteen hours preceding the start of the 90 kilometer race, a 40 ml blood sample was drawn from an ante-cubital vein. Within 30-45 minutes after completion of the race, each subject was required to provide a second 40 ml blood sample. Further blood sampling was done 24 hours and 48 hours following the race. To avoid the effect of diurnal rhythms on cortisol levels, all blood sampling was completed in the late afternoon. Blood was dispensed immediately into glass Vacutainers® with or without the anti-coagulant EDTA (ethylenediaminetetraacetic acid) for plasma and serum samples respectively. In the case of plasma, the blood was fractionated immediately, while for serum, the blood was allowed to clot at room temperature, then fractionated. The resultant plasma or serum was then immediately aliquoted and quick-frozen in liquid nitrogen and stored at –70°C until used in the various assays described below. Serum was used for analysis of cortisol, C-reactive protein (CRP), creatine kinase and vitamin C, while plasma was used for assays of vitamins A and E, interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α) and myeloperoxidase (MPO).

4.2.4 *Vitamins C, A and E*

Vitamin C was extracted from serum using 20% trichloracetic acid and assayed using the 2,4-dinitrophenylhydrazine (Sigma Chemical Co., St Louis, MO, USA) colorimetric method (Attwood *et al,* 1974). Plasma concentrations of vitamins A and E were determined by standard high pressure liquid chromatography (HPLC) procedures following repeated (x3) extraction with hexane and using vitamin A-acetate as the internal standard (Bieri *et al.,* 1983). Quality control was maintained by inclusion of a standard consisting of pooled serum from several healthy adult human donors. With the HPLC procedures the same pool was run with all assays and the standard was extracted and assayed concurrently with all test samples.

4.2.5 *C-reactive protein, cortisol and creatine kinase*

Serum concentrations of the acute phase reactant, CRP (normal range 0-5 μg/ml), were measured by a nephelometric procedure (Behring Nephelometer II) using

reagents purchased from Behringwerke AG, Marburg, Germany. Serum cortisol was assayed using the Gamma Coat radioimmunoassay procedure (Diagnostic Products Corporation, Los Angeles, CA, USA), while creatine kinase was determined using the creatine kinase reagent supplied for use on a SYNCCHRON CX Clinical System (Beckman Instruments Inc, USA)

4.2.6 *Cytokines and myeloperoxidase*

Plasma concentrations of IL-6 and TNF-α were assayed using capture ELISA procedures (Milenia Diagnostic Product Corporation, Los Angeles, CA, USA), with a similar procedure being used for the detection of MPO (MPO-EIA, R & D Systems Inc., Minneapolis, MN, USA).

4.2.7 *Circulating leukocytes and platelets*

Differential leukocyte and platelet counts were performed on EDTA-treated blood using standard, automated, hematological procedures. Plasma volume changes were determined from pre- and post-race hemoglobin and hematocrit values using the method of Dill and Costill (1974).

4.2.8 *Statistical analysis*

Results are expressed as means ± SEM. Baseline results and the change from pre- to post-race values were compared between the placebo (P) and Vitamin C (VCS) groups. Because of the small group sizes and wide ranges in test result values, non-parametric analyses were used. The Wilcoxon Sum Rank Test was used to test for the significance of the differences between the groups and Spearman's correlation coefficient was used as a measure of association. As pre-set *a priori* hypotheses were tested, one-tailed p-values are reported. Statistical analysis was executed using SAS statistical software.

4.3 *Results*

4.3.1 *Compliance*

Of the 24 entrants of the 1997 Comrades Marathon who agreed to participate in the study, 16 complied with all the requirements of the study protocol. These included taking the prescribed capsules, completing the 3-day dietary recalls, completing at least 75 kilometers of the race, and reporting for blood sampling at the 18 hour pre-race, 0.5-1 hour post-, 24 hour post- and 48 hour post-race stations. Ten of the subjects in the experimental group and six of the subjects receiving placebo treatment complied with all of the protocol requirements. The subject characteristics are described in Table 4.1.

Table 4.1: Characteristics of the subjects in the vitamin C-supplemented (VCS) and placebo (P) groups. Data presented as mean (±SEM).

	VCS Group (n=10)	**P Group (n=6)**
Age (yr)	36.6 ± 3.4	44.7 ± 3.7
Mass (kg)	71.5 ± 2.7	64.9 ± 3.7
Body Mass Index (kg/m^2)	23.7 ± 0.9	23.7 ± 0.7
Training distance (km/hr)	72.3 ± 7.8	71.7 ± 3.3
Race finishing time (hr)	9.46 ± 0.2	9.09 ± 0.5

4.3.2 *Blood data*

The 90 km run resulted in a mean decrease of 9.97 (±4.55) and 7.55 (±5.15) % of pre-race plasma volumes in the VCS and P groups, respectively. At each of the three post-race blood sampling time–points, plasma volume changes were insignificantly different between the two groups (data not shown). All blood data reported in this chapter were corrected for plasma volume changes.

4.3.3 *Dietary records*

Analysis of 3 day pre-race records of diet (including additional intake of nutritional supplements) are shown in Table 2 and reveal significantly higher ($p<0.05$) total dietary vitamin C intakes in the supplemented group with non-significant differences in the intakes of vitamins A and E.

Table 4.2: Mean total dietary of vitamins A, C, and E intake of runners in the VCS and P groups. Data presented as mean (±SEM).

	Food Sources	Supplements	Total
VCS Group			
Vitamin A (RE)	348 ± 62.0	0	348 ± 62.0
Vitamin C (mg)	110 ± 29.3	1230 ±132	1339 ± 128 #
Vitamin E (mg)	10.8 ± 1.65	0	10.8 ± 1.65
P Group			
Vitamin A (RE)	515 ± 62.9	0	515 ± 62.9
Vitamin C (mg)	42.2 ± 5.26	41.7 ± 41.6	83.8 ± 42.5
Vitamin E (mg)	6.0 ± 1.14	0	6.0 ± 1.14

#p<0.01 vs P group

The weekly training distances and race finishing times of the athletes indicate that they were non-elite and that there were no significant differences between those who fell into the supplemented and control groups ($p>0.05$) in terms of training status and running time/or intensity.

4.3.4 *Blood vitamins A, C and E*

These results are shown in Table 4.3. Concentrations of vitamin C were significantly higher at the outset in the supplemented group ($p=0.001$), while the values for vitamins A and E did not differ significantly between the groups. Relative to the pre-race values, vitamin C concentrations were increased in the placebo group immediately after the race to a level similar to that of the supplemented group and subsided to pre-race values at 24 and 48 hours thereafter. In contrast, no increase above the pre-race value was observed immediately post-race in the supplemented group.

On completion of the race, plasma concentrations of vitamins A and E increased slightly and to a similar extent in both the control and vitamin C-supplemented groups, returning to pre-race levels 24 hours after the race (Table 4.3).

Table 4.3: Mean blood concentrations of vitamins A, C and E before and during recovery from the 90 km ultramarathon in the VCS (n=10) and P groups (n=6). Data presented as mean (±SEM).

	Pre-race (-18 hrs)	Post-race (0.5-1 hr)	Post-race (24 hrs)	Post-race (48 hrs)
Vitamin A (µmol/l)				
VCS Group	2.16 ± 0.20	2.72 ± 0.21	2.51 ± 0.01	2.34 ± 0.09
P Group	2.37 ± 0.20	2.48 ± 0.17	2.48 ± 0.23	2.37 ± 0.97
Vitamin E (µmol/l)				
VCS Group	14.7 ± 0.95	16.2 ± 1.20	16.0 ±0.98	14.5 ± 0.95
P Group	14.2 ± 0.78	15.8 ± 1.55	14.6 ±0.04	14.1 ± 0.90
Vitamin C (µmol/l)				
VCS Group	118 ± 5.03#	116 ± 5.03*	101 ± 3.96	97.7 ± 3.96*
P Group	85.8 ± 4.86	107 ± 4.86	84.1 ± 6.69	77.8 ± 4.65

# $p<0.001$ vs P Group * $p<0.05$ vs P group

4.3.5 *CRP, creatine kinase and cortisol*

Serum CRP concentrations, as well as those of creatine kinase and cortisol measured 18 hours before and at various times after completion of the race are shown in **Figures 4.1** and **4.2** and in Table 4.4, respectively.

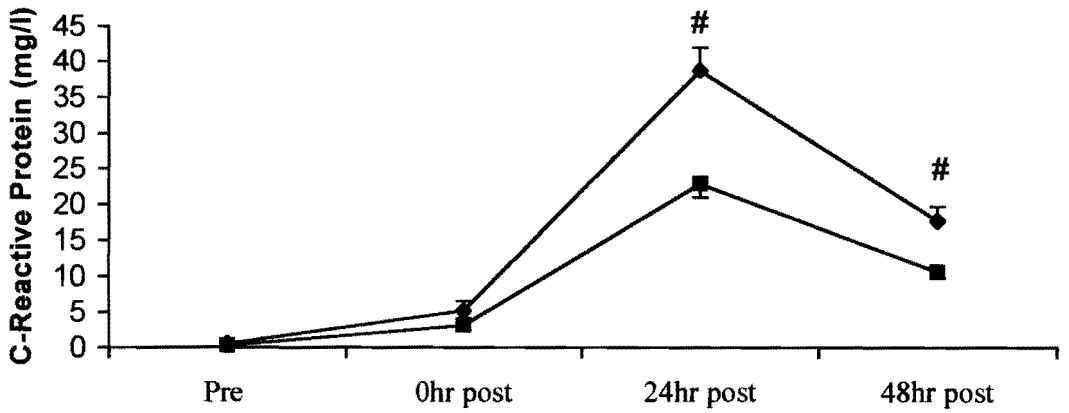

Figure 4.1: Mean (±SEM) serum concentrations of CRP prior to and at varying times (0, 24 and 48 hours) after completion of the ultramarathon race for the ■ P and ◆vitamin C-supplemented groups. #$p<0.05$ vs P.

Creatine kinase was also increased in both groups after the race, reaching a maximum at 0.5-24 hours and declining thereafter to levels, which remained considerably higher than pre-race values **(Figure 4.2)**. Concentrations were significantly higher only in the 24 hour post-race samples of the vitamin C supplemented group.

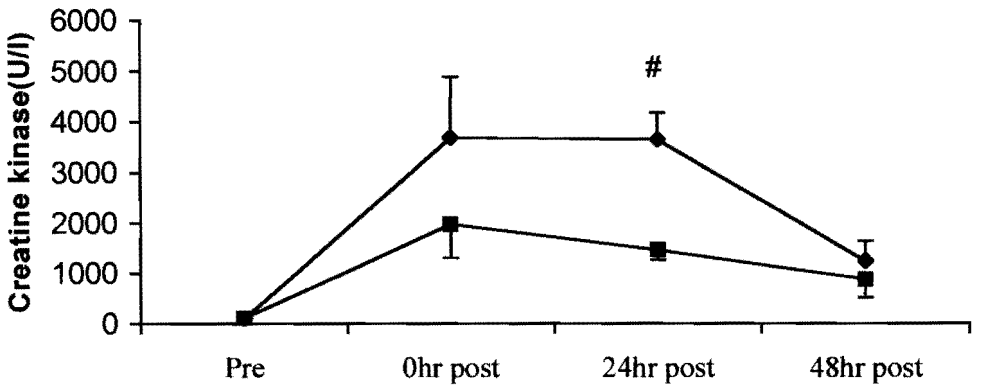

Figure 4.2: Mean (±SEM) serum concentrations of creatine kinase prior to and at varying times (0, 24 and 48 hours) after completion of the ultramarathon race for the ■ P and ◆vitamin C-supplemented groups. #p<0.05 vs P.

Table 4.4: Mean (±SEM) serum cortisol concentrations before and during recovery from the 90 km ultramarathon in the VCS (n=10) and P groups (n=6)

Pre-race	Post-race (-18 hrs)	Post-race (0.5-1 hr)	Post-race (24 hrs)	(48 hrs)
Cortisol (nmol/L)				
VCS Group	227.9 ± 14.1	776.3 ± 62.1#	259.6 ± 64.0	168.2 ± 17.6
P Group	238.9 ± 18.2	1040.3 ± 113	320.9 ± 32.6	184.2 ± 27.1

# p<0.05 vs P group

Mean serum cortisol levels were elevated in both groups immediately after the race, subsiding close to baseline values 24 hours after completion (Table 4.4). The immediate post-race concentrations (adjusted for base-line values) were significantly lower in the vitamin C-supplemented group (p=0.03). Individual immediate post-race serum cortisol concentrations are presented in **Figure 4.3**. An analysis of the association between the pre-race serum vitamin C and post-race cortisol (adjusted for base-line values), is shown in **Figure 4 .4** and revealed a negative correlation of -0.48 (p=0.06).

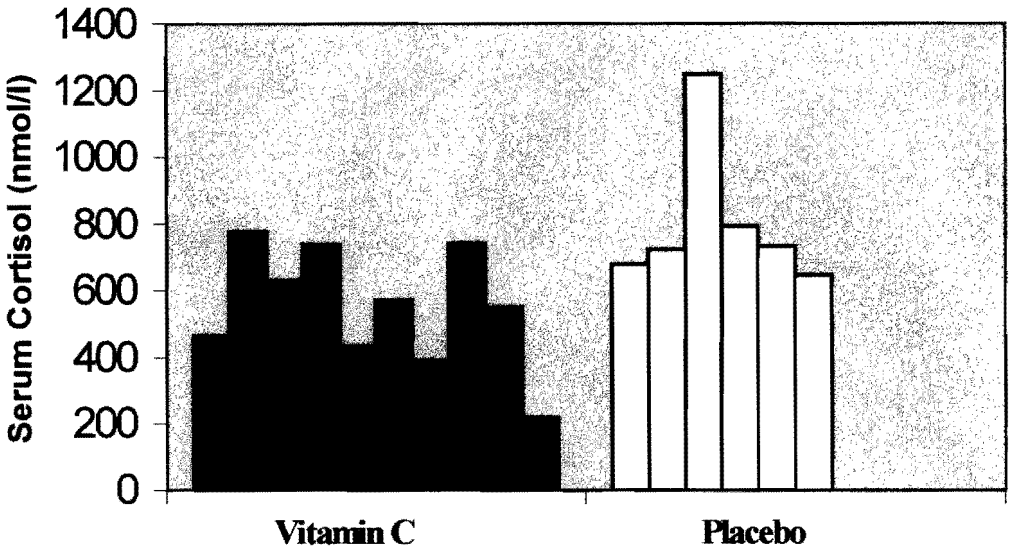

Figure 4.3: Individual 0-hr post-race serum cortisol concentrations (adjusted for base-line concentrations) of the runners in vitamin C and placebo groups.

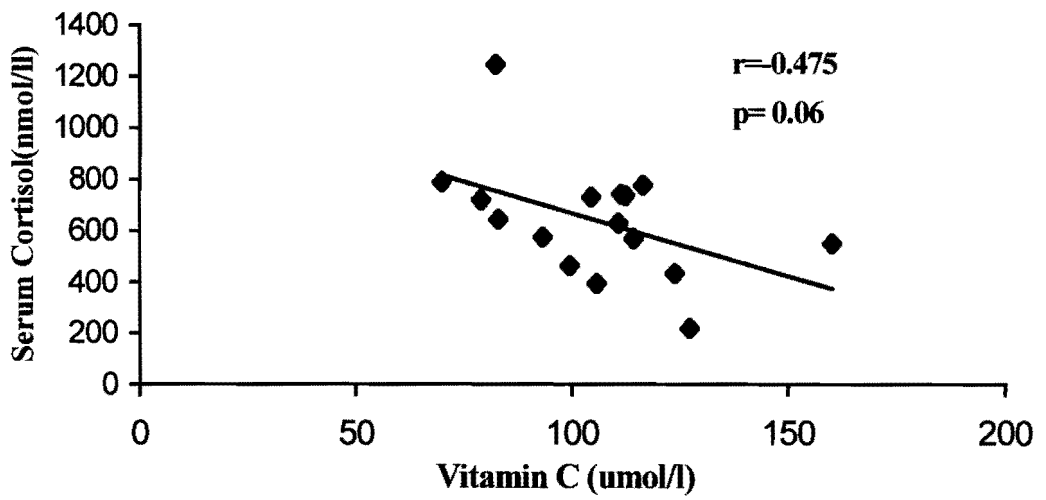

Figure 4.4: The association between pre-race serum vitamin C and immediate post-race serum cortisol (corrected for base-line values) concentrations.

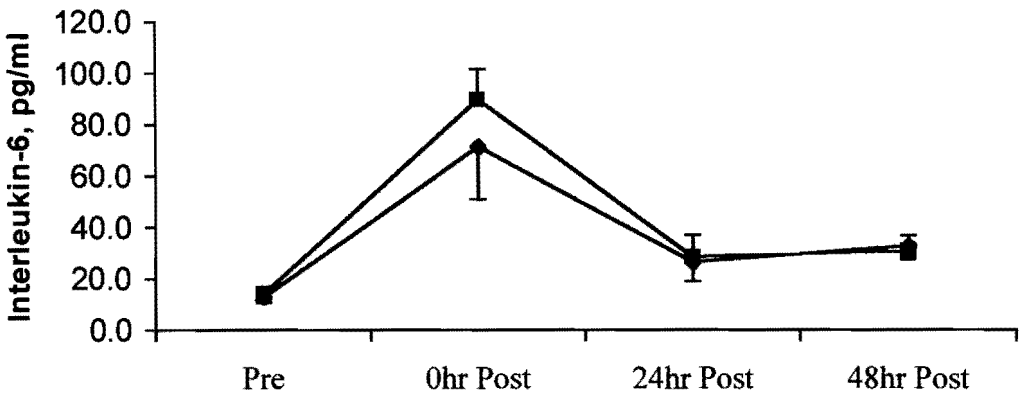

Figure 4.5: Mean (±SEM) plasma concentrations of interleukin-6 prior to and at varying times (0, 24 and 48 hours) after completion of the ultramarathon race for the ■ control and ◆ vitamin C-supplemented groups.

4.3.6 *Cytokines and myeloperoxidase*

The effects of participation in the ultramarathon event on plasma concentrations of IL-6 are shown in **Figure 4.5**. These were dramatically elevated immediately after the race and declined to almost pre-race levels at 24 hours after completion of the event. There were no statistically significant differences, however, between the placebo and vitamin C-supplemented groups. Plasma TNF-α and MPO concentrations were not elevated at any time after the ultramarathon event in either the placebo or vitamin C-supplemented groups (data not shown).

4.3.7 *Circulating leukocytes and platelets*

These are shown in Table 4.5. Circulating concentrations of total leukocytes, neutrophils and monocytes were significantly increased in both groups immediately after the race, returning to pre-race values 24 hours later. The concentrations of total leukocytes, neutrophils and monocytes were higher in the vitamin C-supplemented group immediately after the race, but only the latter achieved statistical significance. The numbers of circulating lymphocytes were decreased to a more-or-less equal extent in both groups immediately after the race, returning to pre-race values 24 hours later.

The neutrophil:lymphocyte ratio increased significantly and to the same extent in both the supplemented and placebo groups immediately after the race (not shown). Circulating platelet counts were unchanged in the placebo group immediately after the race and declined slightly thereafter, while a modest, but significant increase immediately after the race and a decline thereafter was noted in the supplemented group.

Table 4.5: Mean (±SEM) leukocyte subsets and platelet counts in VCS (n=10) and P groups (n=6) before and during recovery from the 90 km ultramarathon.

	Pre-race (18 hrs)	Post-race (0.5-1 hr)	Post-race (24 hrs)	Post-race (48 hrs)
Total leukocytes (10^9/l)				
VCS Group	6.60 ± 0.37	15.7 ± 0.95	7.12 ± 0.57	6.53 ± 0.44
P Group	6.10 ± 0.69	13.7 ± 1.71	6.08 ± 0.65	5.70 ± 0.57
Neutrophils (10^9/l)				
VCS Group	3.49 ± 0.19	13.8 ± 0.14	3.65 ± 0.17	3.11 ± 0.15
P Group	3.18 ± 0.24	11.0 ± 1.08	3.50 ± 0.18	2.86 ± 0.11
Lymphocytes (10^9/l)				
VCS Group	2.47 ± 0.18	1.33 ± 0.13	2.69 ± 0.13	2.52 ± 0.13
P Group	2.19 ± 0.24	1.09 ± 0.14	2.19 ± 0.15	2.25 ± 0.17
Monocytes (10^9/l)				
VCS Group	0.40 ± 0.02	1.11 ± 0.05#	0.57 ± 0.03	0.61 ± 0.04
P Group	0.34 ± 0.04	0.95 ± 0.09	0.44 ± 0.05	0.36 ± 0.04
Platelets (10^9/l)				
VCS Group	261 ± 20.3	279 ± 21.8	244 ± 16.2	232 ±12.0
P Group	245 ± 14.3	241 ± 14.7	210 ± 9.91	197 ±11.7

* $p<0.05$ vs P Group # $p<0.05$ vs P group when corrected for pre-race values

4.5 *Discussion*

Regular physical training, as well as bouts of intensive physical exercise, result in oxidative stress and a subsequent adaptive anti-oxidative response characterized by increased plasma concentrations of superoxide dismutase, vitamin C and vitamin E, the latter two as a result of mobilization from body stores (Garry & Appenzeiler., 1983; Gleeson *et al.*, 1987; Rokitzki *et al.*, 1994b; Kawai *et al.*, 1996; Alescio *et al.*,1997; Brites *et al.*,1999). This adaptive response to oxidative stress is not unique to intensive exercise and has also been described following exposure to tobacco smoke (Chow *et al.*, 1989; Van Antwerpen *et al.*, 1993) or other toxic, prooxidative,

chemicals (Katayama *et al.*, 1991; Kato *et al.*, 1989).

In the present study, the pre-race serum concentrations of vitamin C in individuals in the placebo group were higher than those previously reported from our laboratory for healthy, non-smoking, adult humans (Van Antwerpen *et al.*, 1993) despite their low dietary intake of vitamin C and may reflect the adaptive response to oxidative stress associated with regular training (Gleeson *et al.*, 1987; Brites *et al.*,1999; Himmelstein *et al.*, 1998). On completion of the marathon race, concentrations of circulating vitamin C (adjusted for changes in plasma volume) were even higher than pre-race values in this group, possibly as a result of further enhancement of the adaptive, anti-oxidative response due to intense, physical exertion. Twenty four hours after completion of the race these had subsided to pre-race values. As expected, the pre-race serum vitamin C concentrations were higher in the supplemented group than in the placebo group, but these did not increase above pre-race values on completion of the race, supporting the contention that mobilization of the vitamin from the adrenals in response to oxidative stress is possibly attenuated in these individuals. Plasma concentrations of vitamins A and E increased, albeit moderately, to the same extent in both the placebo and vitamin C-supplemented groups immediately after the race, returning to pre-race values 24-48 hours later.

Intensive exercise has also been reported to cause transient, often substantial, increases in serum cortisol (Skinkai *et al.*, 1996). This was confirmed in the current study. Interestingly however, I observed that the mean, peak increase in serum cortisol, which was detected immediately after the race and had subsided by 24 hours, was less in the vitamin C-supplemented group. The average reduction in serum cortisol in this group was 30% (following correction for the baseline values). This difference was supported by the demonstration of a negative correlation (r=-0.48) between pre-race serum vitamin C and post-race serum cortisol concentrations (adjusted for baseline concentrations). Taken together, these observations appear to support a relationship between vitamin C and attenuation of the exercise-induced cortisol response in ultramarathon athletes.

An association between increased intake of vitamin C and a reduction in stress-related increases in circulating adrenal corticosteroids has previously been described in poultry and has been attributed to the inhibitory effects of the vitamin on several enzymes involved in steroidogenesis (Pardue & Thaxton, 1984a; Satterlee *et al.*, 1992). Moreover, the production of cortisol by adrenocortical cells in response to adrenocorticotrophic hormone is linked to the release of vitamin C (Moser, 1992??). Mobilization of vitamin C from the adrenals as a component of an adaptive response to oxidative stress may therefore be coupled to increased production of cortisol, presumably to counter inflammation-mediated tissue damage. These events may be attenuated by supplementation with the vitamin.

Exercise-associated release of cortisol may, at least, in part, explain the high frequency of respiratory infections in ultramarathon athletes (Peters & Bateman, 1983), as well as the protective effects of supplementary vitamin C (Peters *et al.*, 1993; 1996). Release of the vitamin from the adrenals during oxidative stress may favor synthesis of cortisol with resultant, albeit transient, immunosuppression. The immunosuppressive activities of corticosteroids affect a wide range of immune and inflammatory cells and their mediators, and are achieved by activation of synthesis of various immunomodulatory polypeptides through the interaction of glucorticoid/glucocorticoid receptor complexes with glucocorticoid response elements (GREs) on the promoter regions of steroid responsive genes, as well as by inhibition of activation of the cytosolic nuclear transcription factors, NF*k*B and AP-1, which induce the production of a series of pro-inflammatory polypeptides (Barnes & Karin, 1997).

Recently, vitamin C, at concentrations close to the pre-race values of the supplemented group, has been reported to prevent corticosteroid-induced apoptosis in murine T-lymphocytes, and to antagonize both spontaneous and growth factor withdrawal-related programmed cell death (Campbell *et al.*, 1999). Vitamin C may therefore antagonize the immunosuppressive effects of corticosteroids by interfering not only with their synthesis and/or release, but also with their interactions with target cells. On the other hand, interference with the synthesis and/or actions of

cortisol may have potentially harmful effects as a consequence of enhancement of inappropriate inflammatory responses.

This proposed relationship between increased intake of vitamin C, reduced synthesis of cortisol and prevention of transient acquired immune dysfunction during intensive exercise appears to be supported by the observation of an enhanced acute phase protein response in the supplemented group. Mean serum CRP was elevated in both groups, peaking at 24 hours after completion of the race, but was significantly higher at 24 and 48 hours post-race in the supplemented group. Although the mechanism of the enhanced acute phase response in vitamin C supplemented runners has not been established, it is possible that it is secondary to the attenuation of the cortisol response following intensive exercise.

Circulating IL-6 was elevated to a similar extent immediately post-race in both groups, returning to pre-race values thereafter, while TNF-α remained low throughout. The apparent lack of effect of supplementation on the circulating levels of these cytokines suggests that other mechanisms are involved in vitamin C-associated enhancement of the acute phase response, possibly through increased production of IL-1. Alternatively, peak production of IL-6 may have occurred prior to the completion of the race.

The effects of intensive physical exercise on circulating leukocyte and platelet counts are similar to those which have been described in many previous studies. Immediately post-race there was a transient lymphopenia, which was of a similar magnitude in both groups, in the setting of an increase in circulating neutrophil and monocyte counts. These may occur as a consequence of the release of endogenous catecholamines and cortisol, resulting in altered lymphocyte trafficking (cortisol) and inhibition of the adhesion of phagocytes to vascular endothelium (adrenaline), probably by $ß_2$-adrenoreceptor/ cyclic AMP-dependent mechanisms (Bazzoni *et al.*, 1991; Skinkai *et al.*, 1996). The slight increase in the numbers of circulating neutrophils and monocytes in the vitamin C-supplemented group may reflect the inhibitory effects of the vitamin on oxidant-mediated adhesion of these cells to

vascular endothelium (Pardue & Thaxton, 1984a; Hurst *et al.*, 2001). Failure to detect increased concentrations of circulating MPO in either the placebo or vitamin C-supplemented groups suggests that increased numbers of circulating phagocytes does not necessarily imply systemic activation of these cells. Alternatively, measurement of MPO may be a relatively insensitive marker of systemic activation of phagocytes.

Serum creatine kinase concentrations, as reported previously (Rokitzki *et al.*,1994), were elevated at all times tested after completion of the race, peaking at between 30 min and 24 hours after the race. These were higher in the vitamin C-supplemented group. The reason for this is not entirely clear, others having reported opposite effects in long-distance runners supplemented with a combination of vitamins C and E (Rokitzki *et al.*, 1994), but may reflect an exaggerated inflammatory response to exercise-induced muscle damage.

In conclusion, the possibility that supplementation with vitamin C may abrogate the requirement for mobilization of the vitamin from the adrenals during the adaptive response to exercise-mediated oxidative stress, resulting in decreased synthesis of cortisol and prevention of transient immunosuppression, has potential clinical applications, but requires confirmation in larger scale studies.

Due to the difficulties associated with obtaining full compliance from the runners for work of this nature, this work was restricted to 10 vitamin-C supplemented and 6 runners taking placebos. It was therefore thought important to repeat this work, supplementing with different quantities of vitamin C, in an independent study conducted at the same 90 km ultramarathon, run on exactly the same downhill route, two years later, in order to establish whether the findings of this study were repeatable and to determine whether the trends shown in this study would reach statistical significance in a larger study with greater statistical power.

Chapter Five

Vitamin C supplementation attenuates the increases in circulating cortisol, adrenaline and anti-inflammatory polypeptides following ultramarathon running

5.1 Introduction

My colleagues and I have previously reported that vitamin C supplementation reduces the incidence of post-race upper respiratory tract infections amongst ultramarathon runners (Peters *et al.*, 1993; 1996). In a more recent study (described in chapter 4), I observed that supplementation with 1000 mg of the vitamin over an 8 day period resulted in an average 30% reduction in post-race serum cortisol levels in these athletes. I proposed that the vitamin C-associated decrease in serum cortisol might result from inhibition of enzymes involved in steroidogenesis (Pardue & Thaxton, 1984a; Ehrhart-Bornstein *et al.*, 1998; Satterlee, 1992). Alternatively, because cortisol release from the adrenals may be coupled to concomitant release of vitamin C during oxidative stress (Moser, 1992), it is possible that supplementation with the vitamin may negate the requirement for its mobilization from body stores, with a consequent, albeit secondary, attenuation of the cortisol response (Nussdorfer *et al.*, 1998). Irrespective of the biochemical mechanisms involved, the apparent vitamin C-associated attenuation of the cortisol response to strenuous exercise has potentially important implications for the prevention of transient immune dysfunction in athletes.

In the current study I have again assessed the effects of oral administration of vitamin C, albeit at different doses to those used in my previous study (chapter 4), on the increase in circulating cortisol which accompanies ultramarathon running. Moreover, I have extended my previous study to include measurements of circulating adrenaline, interleukin-10 (IL-10) and the interleukin-1 receptor antagonist (IL-1Ra).

5.2 Materials and Methods

5.2.1 Study design

Approval to conduct the study was obtained from the Human Ethics Committee of the University of Natal Medical School. Forty-five registered entrants for the 1999 Comrades Marathon signed informed consent forms. They were divided into three groups which were matched for age, gender, training status and expected race finishing time:

Group 1 (P; n =15):	Three placebo tablets per day
Group 2 (VC-500; n =15):	One 500 mg vitamin C tablet and two placebo tablets per day
Group 3 (VC-1500; n =15):	Three 500 mg vitamin C tablets per day

Subjects were blinded to their group assignment and were required to ingest one tablet with breakfast, lunch and supper over a 10 day period for 7 days preceding the race, the day of the race and for two days following the race. The vitamin C and placebo tablets were identical in appearance, taste and weight.

On the day prior to the race, subjects were required to complete 24 hour dietary records of their intake and to report for basic anthropometric measurements and blood sampling (35 ml) in the afternoon at a time which coincided with their estimated finishing time (in order to avoid the effect of diurnal rhythms on hormone concentrations). Within 30-45 minutes after completing the race, the subjects again gave 35 ml blood samples and were asked to detail their dietary and liquid intakes on the morning of the race and during the race. The blood sampling was repeated 24 hrs and 48 hrs after the race and subjects were asked to record their post-race dietary intakes for a further 36 hrs.

5.2.2 *Analysis of Dietary Records*

Intake of both food and nutritional supplements was analyzed using the Dietary Manager computer program (Program Management, Randburg, South Africa). Total

daily carbohydrate (CHO) and Vitamin C intakes during the 24 hours before, as well as on the day of the race, and after the race, including those derived from any additional carbohydrate supplements used by the athletes, were determined.

5.2.3 *Treatment of Blood*

Venous blood samples (20ml) were collected in glass Vacutainer tubes containing the anti-coagulant, tripotassium ethylenediaminetetraacetic acid (K_3-EDTA). Full blood counts were conducted on 3ml thereof. The remainder was centrifuged and the fractionated plasma quick-frozen and stored at –70°C for later analysis of Vitamins A and E, glucose, adrenaline, IL-10 and IL-1Ra. An additional 15 ml aliquot was allowed to clot at room temperature, centrifuged for 10 minutes and the serum was quick-frozen and stored at –70°C for later analysis of vitamin C and cortisol.

5.2.4 *Serum Vitamin C, Plasma Glucose, Vitamins A and E*

Vitamin C was extracted from serum using 20% trichloracetic acid and assayed using the 2,4-dinitrophenylhydrazine (Sigma Chemical Co., St Louis, MO, USA) colorimetric method (Attwood *et al.*, 1974). Plasma glucose concentrations were determined spectrophotometrically in pre-race, immediate, 24hr and 48 hr post-race samples. Plasma concentrations of vitamins A and E were determined by standard high performance liquid chromatography (HPLC) procedures following repeated (x3) extraction with hexane and using vitamin A-acetate as the internal standard (Bieri *et al.*, 1983). Quality control was maintained by inclusion of a standard consisting of pooled serum from several healthy adult human donors. With the HPLC procedures the same pool was run with all assays and the standard was extracted and assayed concurrently with all test samples.

5.2.5 *Serum Cortisol, Plasma Adrenaline, IL-10 and IL-1Ra*

Serum cortisol was assayed using the Gamma Coat radioimmunoassay procedure (Diagnostic Products Corporation, Los Angeles, CA, USA) and adrenaline using a radioimmunoassay procedure (DLD Geselschaft fur Diagnostika und medizinishe Geraete mbh, Hamburg, Germany). The plasma IL-10 and IL-1Ra analyses were part of a more comprehensive study on the cytokine profile of ultramarathon runners

which is to be described in chapter 6. These were assayed using quantitative sandwich ELISA kits provided by R&D Systems, Inc. (Minneapolis, MN, USA). A standard curve was constructed using standards provided in the kits. The assays were two step "sandwich" enzyme immunoassay procedures in which samples or standards were incubated in 96-well microtiter plates coated with polyclonal antibodies to the test cytokine as the capture antibody. Following the appropriate incubation time, the wells were washed and a second detection antibody conjugated to either alkaline phosphatase (IL-10) or horseradish peroxidase (IL-1Ra) was added. The plates were incubated and washed, and the amount of bound enzyme-labelled detection antibody was measured by adding a chromogenic substrate. The plates were then read at the appropriate wavelength (490 minus 650 nm for IL-10 and 450 minus 570 nm for IL-1Ra). The minimum detectable concentration of IL-10 was < 0.5 pg/ml and that of IL-1Ra was <22 pg/ml.

5.2.6 *Hematological analyses and adjustments*

Full blood counts were performed on K_3-EDTA treated specimens using standard hematological procedures on an automated STKS model (Coulter Electronics Inc., Hialeah, Florida, USA). Plasma volume changes were determined from pre- and post-race hemoglobin and hematocrit values using the method of Dill and Costill (1974) and subsequent post-race values (0, 24 and 48 hr) were adjusted for these plasma volume changes.

5.2.7 *Statistical Analyses*

Results are expressed as means ± SEM. An initial three-by-four repeated measures ANOVA was used to establish whether the differences between the three groups were significant throughout the 48 hr post-race period and showed that the P and VC-500 groups did not differ significantly in any of the post-race measures. These two sets of data were subsequently pooled and a further two (≤ 500mg per day vs. >1500 mg per day) -by -four repeated measures ANOVA was used to assess the group-time interaction. Wilks' Lambda trace statistic was used as the test statistic with a post-hoc correction to determine the time point of the significant differences. Statistical differences between post-race adrenaline values were determined between

≤ 500mg and >1500mg groups using Students' *t*-tests. Correlation analyses were performed using Pearson's Product Moment Correlation Coefficient. Statistical analysis was done using SAS statistical software.

5.3 Results

5.3.1 *Subjects*

Of the 45 runners recruited to the study only 29 fully complied with the protocol requirements. The characteristics of the individuals in the P, VC-500 and VC-1500 groups are shown in Table 5.1. There were no significant differences between the three groups with respect to age, height, mass, body mass index, training status, and time taken to complete the ultramarathon.

Table 5.1: Mean (±SEM) subject characteristics (n=29).

	Age (years)	**Stature (m)**	**Mass (kg)**	**BMI (kg/m^2)**	**Weekly training distance (km/wk)**	**Race time (hr)**
Placebo (n=7)	39.6 (± 2.7)	1.77 (± 0.04)	70.8 (± 4.4)	22.5 (± 1.1)	77.9(± 9.9)	9.85 (± 0.44)
VC-500 (n=10)	40.9 (± 2.9)	1.72 (± 0.02)	69.3 (± 3.4)	23.4 (± 0.9)	92.0 (± 9.8)	9.65 (± 0.36)
VC-1500 (n=12)	38.7 (± 1.5)	1.74 (± 0.02)	71.1 (± 3.4)	23.4 (± 0.7)	85.0 (± 6.7)	9.60 (± 0.22)

Table 5.2: Mean (±SEM) dietary carbohydrate (CHO) intakes and plasma concentrations of glucose and vitamins A and E on the day preceding the race and day of the race.

	CHO (g)	Plasma glucose (mmol/l)	Plasma vitamin E (μmol/l)	Plasma Vitamin A (μmol/l)
Day preceding the race				
Placebo (n=7)	399(± 29.1)	4.69 (± 0.30)	17.0 (± 1.4)	2.40 (± 0.16)
VC-500 (n=10)	499(± 51.3)	4.95 (± 0.33)	21.1 (± 1.6)	2.57 (± 0.15)
VC-1500 (n=12)	482(± 42.2)	4.74 (± 0.20)	20.7 (± 1.6)	2.29 (± 0.14)
Day of the race				
Placebo (n=7)	315 (±54.8)	6.14 (± 0.57)	16.9 (± 1.6)	2.21 (± 0.19)
VC-500 (n=10)	353(±35.2)	6.47 (± 0.51)	20.7 (± 1.5)	2.51(± 0.17)
VC-1500 (n=12)	488(±65.7)	5.95 (±0.34)	21.2 (±1.8)	2.27(±0.17)

Carbohydrate intake just prior to and during the race averaged 401(±188) g and did not differ significantly between the groups ($p>0.05$; Table 5.2). Likewise, pre- and post-race plasma glucose, vitamin A and vitamin E concentrations were not different between the 3 groups ($p>0.05$; Table 5.2). Total mean vitamin C intake on the day preceding the race (contained in supplements, beverages and foodstuffs ingested) amounted to 94.4 (±60.4), 650 (± 102) and 1603 (±90) mg in P, VC-500 and VC-1500 groups, respectively (data not shown.)

5.3.1 *Serum vitamin C*

Pre-race serum vitamin C was significantly higher in the supplemented groups by comparison with the P group (128 ± 31 and 153 ± 34 µmol/l vs 83 ± 39 µmol/l, **Figure 5.1**). Serum vitamin C concentrations were also significantly lower in placebo compared to VC-500 and VC-1500 groups at the 24 h post-race and 48 h post-race time points (**Figure 5.1**). There was a significant increase ($\bar{X}$ = 42.6 µmol/l) in serum vitamin C in the P group immediately post-race ($p<0.05$).

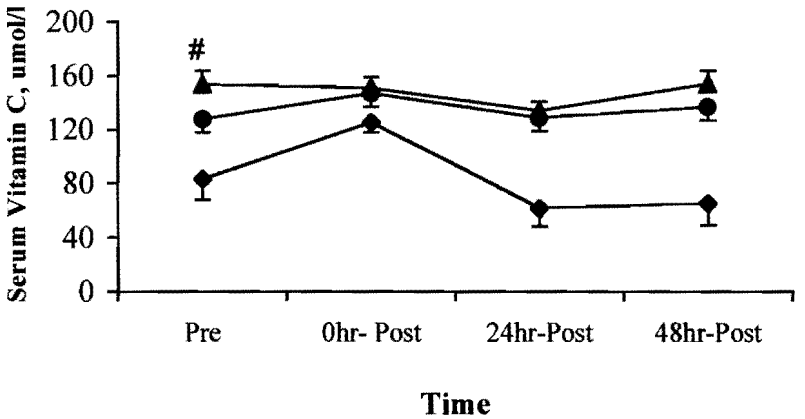

Figure 5.1: Pattern of change in mean serum vitamin C concentrations before and after the 1999 Comrades 90 km ultramarathon in ♦ placebo, ● VC-500 and ▲ VC-1500 groups. Data presented as means ±SEM. Time effect: $p<0.001$; group vs time interaction effect: $p=0.27$, group effect: $p=0.006$ # $p<0.05$ Bonferroni multiple comparison test between ≤500 and >1500mg groups at time point.

This increase in the mean serum vitamin C was attenuated in both of the vitamin supplemented groups (19.3 and -2.84 µmol/l in VC-500 and VC-1500 groups, respectively). At 24 and 48 hrs after completion of the race the serum vitamin C concentrations returned to values which were not significantly different ($p>0.05$) from pre-race values.

Table 5. 3: Hematological profile. Values as mean (±SEM).

Variable	Pre-race	Post-race (0.5-1hr)	Post-race (24 hours)	Post race (48 hours)	Time effect; Interaction effect; Group effect**
Packed cell volume (%)					
Placebo	41.5 (± 2.7)	44.0 (±2.3)	41.6 (± 1.1)	39.4 (± 1.0)	P<0.001
VC-500	42.6 (± 0.7)	43.0 (±0.8)	40.2 (± 0.9)	39.6 (± 0.8)	P=1.10
VC-1500	42.0 (± 0.7)	44.2 (± 1.2)	40.4 (± 1.0)	38.3 (±0.8)	P=0.62
Hemoglobin (g/l)					
Placebo	141 (± 4.5)	146 (± 4.9)	144 (± 3.8)	134 (± 4.5)	P<0.001
VC-500	143 (± 2.5)	145 (± 2.9)	139 (± 3.5)	135 (± 2.9)	P=1.12
VC-1500	142 (± 2.6)	146 (± 3.8)	137 (± 3.2)	130 (± 2.3)	P=0.63
% PV change *					
Placebo		-7.1 (± 3.0)	-2.2 (± 3.6)	9.5 (± 2.5)	P<0.001
VC-500		-1.7 (± 1.8)	7.7 (± 2.3)	12.3 (±2.3)	P=0.11
VC-1500		-6.7 (± 2.2)	5.4 (± 2.6)	15.6 (±2.3)	P=0.63
Total leukocytes(10^9/l)					
Placebo	7.6 (± 1.1)	18.1 (± 2.5)	8.7 (± 1.0)	8.1 (± 1.0)	P<0.001
VC-500	8.0 (± 1.1)	16.6 (± 1.2)	9.4 (± 0.7)	7.8 (± 0.3)	P=0.20
VC-1500	6.5 (± 0.5)	14.2 (± 1.1)	8.1 (± 0.7)	7.2 (± 0.5)	P=0.07
Neutrophils (10^9/l)					
P Group	4.4 (± 0.9)	15.2 (± 2.2)	5.1 (± 0.1)	4.0 (± 0.8)	P<0.001
VC- 500	4.8 (± 1.1)	13,8 (± 0.9)	5.6 (± 0.6)	3.6 (± 0.3)	P=0.10
VC-1500	3.5 (± 0.4)#	11.0(± 1.0)#	4.4 (± 0.1)	3.1 (± 0.2)	P=0.03
Lymphocytes (10^9/l)					
P Group	2.1 (± 0.1)	1.6 (± 0.3)	2.4 (± 0.3)	2.3 (± 0.3)	P<0.001
VC- 500	2.2 (± 0.1)	1.3 (± 0.1)	2.6 (± 0.2)	2.2 (± 0.1)	P=0.95
VC-1500	2.3 (± 0.2)	2.0 (± 0.3#	2.7 (± 0.2)	2.4 (± 0.2)	P=0.22
Neutro:Lymph ratio					
P Group	2.1 (± 0.4)	11.9 (± 2.3)	2.5 (± 1.5)	2.2 (± 1.1)	P<0.001
VC- 500	2.3 (± 0.6)	10.7 (± 1.3)	2.4 (± 0.9)	1.7 (± 0.5)	P=0.08
VC-1500	1.5 (± 0.3)	7.0 (± 1.9)#	2.0 (± 1.5)	1.5 (± 0.8)	P=0.02

*relative to pre-race plasma volume; PV= plasma volume; neutro:lymph ratio=neutrophil :lymphocyte ratio ** Repeated measures ANOVA # p<0.05 Bonferroni multiple comparison test between groups at time point when compared to ≤ 500mg group.

5.3.2 *Blood counts*

Results of the full blood counts are shown in Table 5.3. Packed cell volume and hemoglobin values indicated a varied hydration status with 27.5% presenting with an increase in plasma volume immediately following participation in the ultramarathon. The difference in plasma volume did not differ significantly between the groups.

Significant immediate post-race lymphopenia and neutrophilia was present in all 3 groups with recovery to normal values at 24 and 48 hrs after completion of the race. The smaller relative magnitude of the lymphopenia and neutrophilia, as expressed in the neutrophil:lymphocyte ratio, in the VC-1500 group (n=12) in comparison to the ≤ VC-500 group (n=17) also reached statistical significance ($p<0.05$) over the 48 hour post-race period.

5.3.2 *Circulating cortisol, adrenaline, IL-10 and IL-1Ra concentrations*

Circulating cortisol and adrenaline increased significantly in all 3 groups immediately post-race, subsiding to close to pre-race values (in the case of cortisol) at 24 and 48 hrs after completion of the race (Table 5.4). The increase in both cortisol and adrenaline observed immediately post-race was attenuated in the VC-1500 group relative to the ≤ 500mg groups ($p<0.001$ and $p<0.05$, respectively). Pre-race adrenaline levels were also less in the VC-1500 group ($p<0.05$). The immediate post-race values for IL-10 and IL-1Ra were significantly higher in relation to the pre-race values and subsided to close to pre-race values at 24 and 48 hrs after completion of the race (Table 5.4). However, the increase in the circulating concentrations of these anti-inflammatory polypeptides observed immediately post-race was significantly attentuated in the VC-1500 group ($p=0.05$) when compared to the ≤ 500mg groups.

Correlation analyses between collective data pooled for all subjects (n=124) revealed a significant positive correlation between serum cortisol and IL-10 ($r=0.79$) and inverse correlation between pre-race vitamin C values and post-race serum cortisol ($r=-0.30$; $p<0.05$) Significant ($p<0.05$) correlations were found between

post-race serum cortisol and both IL-10 (r=0.61) and IL-1Ra (r=0.50) as well as adrenaline and IL-1Ra (r=0.71).

Table 5.4: Mean (±SEM) stress hormone and anti-inflammatory polypeptide concentrations

	Pre-race	Post-race** (0.5-1hr)	Post-race** (24 hours)	Post race** (48 hours)	Time effect; Interaction effect; Group effect***
Serum Cortisol (nmol/l)					
P Group	347 (± 41.5)	1179 (± 93.2)	323 (± 59.6)	329 (± 70.9)	P<0.001
VC- 500	260 (± 33.5)	1205 (± 97.5)	300 (± 26.3)	284 (± 27.1)	P= 0.003
VC-1500	248 (± 32.9)	770 (± 64.7)#	262 (± 21.8)	329 (± 46.5)	P= 0.02
Plasma Adrenaline (pg/ml)					
P Group	93.4 (± 16.7)	204 (± 48.7)			
VC- 500	140 (± 49.1)	257 (± 59.4)	ND	ND	
VC-1500	56.0(± 8.83)*	120 (± 21.5)*			
Plasma IL-10 (pg/ml)					
P Group	0.46 (± 0.26)	83.1 (± 22.9)	0.61 (± 0.23)	0.62 (± 0.30)	P<0.001
VC- 500	0.56 (± 0.24)	69.6 (± 18.5)	0.91 (± 0.34)	0.80 (± 0.27)	P=0.001
VC-1500	0.35 (± 0.15)	31.5 (± 8.81)#	0.39 (± 0.14)	0.30 (± 0.07)	P=0.01
Plasma IL-1Ra (pg/ml)					
P Group	176 (± 21.8)	2850(± 1084)	320(± 29.4)	249 (± 23.8)	P<0.001
VC-500	184 (± 24.8)	4241(± 1051)	439 (± 63.6)	327 (± 56.3)	P=0. 07
VC-1500	193 (± 15.5)	1519(± 434)#	282 (± 24.7)	288 (± 25.1)	P=0.04

# p<0.05 Bonferroni multiple comparison test between groups at time point; * p<0.05 vs ≤500 group; Students t-test, adjusted for base-line values; **adjusted for plasma volume changes from pre-race ;*** Repeated measures ANOVA; ND = not done

5.4 Discussion

The increase in circulating concentrations of the adrenal immunosuppressive, anti-inflammatory hormones cortisol, adrenaline and noradrenaline, which accompanies intensive physical exercise is well-documented (Keast *et al.*, 1988; Skinkai *et al.*, 1996, Suzuki *et al.*, 1999). An earlier laboratory study (Nieman *et al.*, 1997) on 6 pairs of runners failed to report an effect of vitamin C supplementation on immune

response to 2.5 hours of treadmill running. I have, however, reported that 1000mg vitamin C supplementation (total mean intake: 1339 mg /day) in ultramarathoners is associated with attenuation of the increase in serum cortisol observed immediately post-race following an ultramarathon lasting 9 -11 hours (Chapter 4). In the current study I have investigated the effects of vitamin C supplementation, at different doses (500 mg and 1500 mg/daily) to those used in my previous study (Chapter 4), on the cortisol response which accompanies participation in the same 90 km ultramarathon and included measurements of circulating adrenaline and those of the anti-inflammatory polypeptides, IL-10 and the IL-1Ra in an extension of this study.

As previously reported by myself (chapter 4) and others (Gleeson *et al.*, 1987; Brites *et al.*, 1999), vitamin C levels were increased in the placebo group on completion of the ultramarathon and subsided at 24 and 48 hrs thereafter. This apparent mobilization of vitamin C appears to represent an adaptive response to exercise-induced oxidative stress (Brites *et al.*, 1999). Pre-race serum vitamin C values and those measured 24 and 48 hrs after completion of the race were also significantly higher in the vitamin-supplemented group than those of the placebo groups. Interestingly, the difference in serum vitamin C between the placebo (51.4 % higher than the pre-race value) and vitamin-supplemented groups was considerably less and statistically insignificant immediately post-race. The corresponding average changes in circulating vitamin C concentrations in the immediate post-race VC-500 and VC-1500 groups were 13.0 % and -0.02% respectively. These observations confirm my previous findings (described in chapter 4) that supplementation with vitamin C appears to negate the requirement for mobilization of the vitamin from the adrenal gland and other body storage sites during intensive physical stress (Borish, 1998).

Somewhat surprisingly the pre-race serum vitamin C levels observed following supplementation with 500 mg daily of the vitamin in the current study (Chapter 5, Figure 5.1) were similar to those observed following supplementation with 1000 mg daily of the vitamin described in the previous chapter (Table 4.3). However, the differences observed between these two supplementation regimens with respect to

mobilization of the vitamin following intensive exercise, as well as the effects on circulating cortisol, suggest that pre-race serum concentrations of the vitamin at these doses may not necessarily reflect tissue concentrations.

In agreement with my previous study (chapter 4), administration of vitamin C at 1500 mg/daily, but not at 500 mg/daily, significantly attenuated (average decrease of 34.7% relative to P group) the immediate post-race increase in serum cortisol. Pre-race concentrations of serum cortisol, as well as those measured at 24 and 48 hrs after completion of the ultramarathon event, were somewhat lower, although not significantly so, in both vitamin-supplemented groups relative to the P group. These observations are also in agreement with a recent report in which administration of vitamin C (1000 mg/daily) in combination with vitamin E to healthy, elderly humans was accompanied by a significant decrease in serum cortisol and improved immune function (De la Fuente *et al.,* 1998) and confirm previous findings on animals (Pardue & Thaxton, 1985a; Satterlee, 1989; 1994; Enwonuwo *et al.,* 1995; Jones *et al.,* 1999).

Although blood sampling for adrenaline concentrations should ideally have been performed immediately on completion of the race, this was not logistically possible in a competitive event of this nature. It is, however, noteworthy, that circulatory adrenaline concentrations were reduced significantly following a week of supplementation with vitamin C both prior to and following the stressful competitive event when compared to those of the unsupplemented runners. The average decreases relative to the P group were of 40% and 41% respectively in the group of athletes supplemented with 1500 mg vitamin C daily, but were not significantly lower ($p<0.05$) in those supplemented with $\leq$ 500 mg/daily vitamin C.

It is possible that the observed vitamin C-related attenuation of the exercise-induced increase in circulating cortisol and adrenaline may, in part, explain the reported decrease in the incidence of upper respiratory infections in vitamin C-supplemented ultramarathon athletes. Both of these adrenal hormones possess potent anti-inflammatory, immunosuppressive properties and may impact on the magnitude of

the post-exercise "open-window" period (Pedersen & Ullum, 1994) with a delayed manifestation of actual symptoms of infection following varying incubation periods. Corticosteroids have been shown to mediate these immunomodulatory actions by interaction with cytosolic glucocorticoid receptors (Barnes & Adcock, 1993; Barnes & Karin, 1997; Rahman & MacNee, 1998), while adrenaline operates via cyclic AMP-coupled $ß_2$-adrenoreceptors on immune and inflammatory cells (Moore & Willoughby, 1995; Van der Poll *et al.*, 1996; Weiss *et al.*, 1996).

The proposed relationship between vitamin C-associated suppression of cortisol release from the adrenals and possible potentiation of immune function, is further strengthened by the observation that the dramatic increase in the circulating concentration of the broad-spectrum anti-inflammatory cytokine, IL-10 (Borish, 1998), observed immediately after completion of the ultramarathon event, was significantly attenuated in the group of athletes supplemented with 1500 mg/daily of the vitamin. This is also supported by the coefficient of correlation of 0.79 between the circulating concentrations of cortisol and IL-10 obtained from the findings of this study. Production of IL-10 by immune and inflammatory cells is potentiated by corticosteroids (Suzuki *et al.,* 1999) and adrenaline (Van der Poll *et al.*, 1996). Interleukin-10, in turn, acts on monocytes/macrophages to stimulate release of IL1-Ra (Borish, 1998), an endogenous antagonist of the pro-inflammatory cytokine, IL-1. Interestingly it has recently been reported that rhinoviruses, the predominant cause of the common cold, increase the production of IL-10 by monocytes, suggesting that increased levels of this cytokine may contribute to the pathogenesis of infection with these viral pathogens (Stöckl *et al.,* 1999). It is therefore possible, but not proven, that vitamin C supplementation, through attenuation of the cortisol, adrenaline and IL-10 responses which accompany intensive exercise, may contribute towards the prevention of the resultant transient immunosuppression which predisposes to upper respiratory tract infections (Peters *et al.,* 1993; 1996).

The biochemical mechanisms by which vitamin C supplementation attenuates the adrenal hormone response to exercise-induced oxidative stress remains to be established. However, my observation that the release of both cortisol and

adrenaline is attenuated by supplementation with the vitamin appears to favor a mechanism by which the release of these anti-inflammatory hormones is coupled to mobilization of vitamin C from the adrenals (Moser, 1992), as opposed to inhibitory effects of the vitamin on the synthesis of these hormones (Pardue & Thaxton, 1984a; Satterlee, 1992). Oxidative stress is presumably the trigger for the combined release of vitamin C, cortisol and adrenaline from the adrenals, with all three cooperating to protect against inflammation-mediated tissue damage.

In conclusion, oral supplementation with vitamin C at 1500 mg daily attenuated the increases in the production of the immunosuppressive adrenal hormones, cortisol and adrenaline, which accompanies intensive exercise, as well as the production of the anti-inflammatory polypeptides IL-10 and IL-1Ra. The findings of this study did not, however, reveal a linear dose-dependent response. Instead, the combined results of this work and my previous studies (Peters *et al.*, 1993; 1996; chapter 4) in which total vitamin C ingestion varied from 1139 and 1004 mg/day, respectively in the early studies (Peters *et al.*, 1993, 1996) and 1339 mg /day in my most recent work (chapter 4) appear to point towards a threshold value existing at approximately 1000 mg per day. As it is possible, however, that inhibitory effects of a vitamin C intake of 650 mg daily do indeed occur, but are only evident at earlier time-points during the race, as opposed to on its completion, the relationship, if any, between these immunomodulatory effects of a daily dosage ranging from 650-1603 mg vitamin C and the possible protective effects of this vitamin against post-exercise upper respiratory tract infection, do require further investigation.

This chapter limited itself to a selected set of related findings which confirm the initial finding of an attenuation of the exercise-induced elevation of cortisol described in the previous study in chapter 4. A more comprehensive description of the cytokine profile of this group of runners is presented in chapter 6.

Chapter Six

Influence of vitamin C supplementation on cytokine changes following an ultramarathon

6.1 Introduction

Vitamin C (ascorbate) is a water-soluble vitamin present in the cytosolic compartment of the cell and the extracellular fluid. Of all essential nutrients, vitamin C has generated the greatest interest for its potential influence on host defense mechanisms and the immune system (Hughes, 1999). The concentration of vitamin C is unusually high in activated neutrophils and macrophages (Washko *et al.*, 1991; Wolf, 1993). Supplemental amounts of vitamin C have been shown to alter many different indices of human immune responses (Anderson *et al.*, 1980; Jacob *et al.*, 1991; Campbell *et al.*, 1999). Vitamin C also provides *in vivo* antioxidant protection primarily as an aqueous-phase peroxyl and oxygen radical scavenger, and is concentrated in those tissues and fluids which have a high potential for radical generation (Jacob & Burri, 1996). Vitamin C exerts a protective effect on neutrophil-mediated cell injury by scavenging reactive oxygen metabolites following physical trauma (Dwenger *et al.*, 1992; Jonas *et al.*, 1993). Free radical-mediated processes appear to be an important component of exercise-induced muscle and lymphoid tissue damage and inflammation (Azenabor & Hoffman-Goetz, 1999; Goldfarb, 1999). Numerous recent studies have indicated that vitamin C supplementation attenuates exercise-induced oxidative stress (Sanchez-Quesada *et al.*, 1998; Vasankari *et al.*, 1998; Ashton *et al.*, 1999: Schröder *et al.*, 2000).

The concentration of vitamin C in the adrenal cortex is higher than in any other organ (Redmann et al., 1995). Although poorly defined in humans, vitamin C depletion or supplementation appears to alter serum cortisol levels in some animal models (Kodama *et al.*, 1994; Satterlee *et al.*, 1994; Enwonwu *et al.*, 1995; Redmann *et al.*, 1995; Jones *et al.*, 1999). This may be important in view of the well-defined role of cortisol in leukocyte trafficking and function following heavy

exertion as well as the well described immuno-suppressive actions of cortisol (Cupps & Fauci, 1982)

Given the importance of vitamin C to the immune system, the effect of supplemental amounts of this nutrient in altering immune function and quenching the reactivity of exercise-induced free radicals, and its potential role in altering serum cortisol levels, a randomized, double-blind, placebo-controlled study was designed to investigate the influence of supplemental vitamin C on the immune response to 2.5 hours of treadmill running by 12 marathoners (Nieman *et al*, 1997). Vitamin C compared to placebo supplementation (1000 mg/day for 8 days) had no significant effect on the pattern of change in cortisol, IL-6, or other immune measures following the exercise bout. This study, however, had a small number of subjects (six in each group), induced a relatively low degree of physiologic and oxidative stress, and the carbohydrate intake of the subjects, which has been shown to significantly affect the parameters measured (Nehlsen-Cannarella *et al.*, 1997; Nieman *et al*, 1998), was not controlled. A subsequent field study performed on ultramarathoners showed an attenuation of the post-exercise cortisol response in runners supplemented with vitamin C (Chapter 4). As a follow-up and extension of these studies, I investigated the influence of vitamin C supplementation at two levels (500 and 1,500 mg/day) on the pattern of change in concentration of serum cortisol and plasma cytokines in runners following a competitive ultramarathon.

6.2 Materials and Methods

The Comrades Marathon is a 90 km race event held each year during the winter in South Africa. Twenty-nine entrants to the 1999 Comrades Marathon (same as in chapter 5) volunteered to be subjects in this study, and complied with all aspects of the research design. Subject selection criteria included: 1) non-smoking; 2) no clinical signs of infection; 3) no intake of analgesic or anti-inflammatory medication prior to and during the race; 4) no regular use of vitamin C supplements; and 5) willingness to adhere to all aspects of the study design. The protocol was approved

by the Human Ethics Committee of the University of Natal Medical School and informed consent was obtained from subject.

6.2.1 *Research Design*

The study was based on a 3 (three groups) by 4 (four blood samples) repeated measures design. Subjects were divided into three groups:

Group 1 (Placebo): Placebo supplement (three placebo tablets per day).
Group 2 (VC-500): One 500 mg vitamin C tablet and two placebo tablets per day.
Group 3 (VC-1500): Three 500 mg vitamin C tablets per day.

Each subject in each group received three tablets per day, with one tablet ingested with breakfast, lunch, and supper. The vitamin C and placebo tablets were identical in appearance, taste, and weight, and subjects were blinded to their group assignment. Subjects ingested the supplements for seven days prior to the race, on race day, and for two days after (10 days total). Diet and fluid intake was recorded on the morning of and during the 90 km event, with carbohydrate intake determined through use of the Dietary Manager software program (Program Management, Randburg, South Africa).

On the afternoon preceding the race (about 14-16 h pre-race), height, body mass, age, and training history were recorded.

6.2.2 *Blood Samples and Assays*

A 30 ml venous blood sample was also collected on the afternoon preceding the race (14-16 h prior to the race). Post-race venous blood samples were collected within 30-45 min after completion of the race event, and then again 24 h and 48 h post-race. Plasma and serum aliquots were stored at -70°C, and analyzed for cortisol, vitamin C, and cytokine concentrations. The serum vitamin C, cortisol, IL-10 and IL-Ra data are part of a study examining the effects of vitamin C supplementation

on indices of immune function following ultramarathon running. This paper, however, focuses on the plasma cytokine data from this larger study.

Serum cortisol was assayed using a competitive solid-phase ^{125}I radioimmunoassay (RIA) technique (Diagnostic Products Corporation, Los Angeles CA). Serum vitamin C was extracted from the serum using 20% trichloracetic acid and assayed using the 2,4-dinitrophenylhydrazine colorimetric method (Sigma Chemical Co., St Louis, MO, USA).

Total plasma concentrations of interleukin-1β (IL-1β), interleukin-6 (IL-6), interleukin-8 (IL-8), interleukin-10 (IL-10), interleukin-1 receptor antagonist (IL-1Ra), and tumor necrosis factor-α (TNFα) were determined using quantitative sandwich ELISA kits provided by R&D Systems, Inc. (Minneapolis, MN). A standard curve was constructed using standards provided in the kits and the cytokine concentrations were determined from the standard curves using linear regression analysis. The assays were two step "sandwich" enzyme immunoassays in which samples and standards were incubated in a 96-well microtiter plate coated with polyclonal antibodies for the test cytokine as the capture antibody. Following the appropriate incubation time, the wells were washed and a second detection antibody conjugated to either alkaline phosphatase (IL-1β, IL-6, IL-10, TNFα) or horseradish peroxidase (IL-8, IL-1Ra) was added. The plates were incubated and washed, and the amount of bound enzyme-labeled detection antibody was measured by adding a chromogenic substrate. The plates were then read at the appropriate wavelength (490 minus 650 nm for IL-1β, IL-6, IL-10, and TNFα; 450 minus 570 nm for IL-8 and IL-1ra). The minimum detectable concentration of IL-1β was <0.1 pg/ml, of IL-6 was <0.094 pg/ml, of IL-8 was <10 pg/ml, of IL-10 was < 0.5 pg/ml, of IL-1Ra was <22 pg/ml, and of TNFα was 0.18 pg/ml.

All post-race blood data reported in this chapter were adjusted for plasma volume changes. These were calculated from pre- and post-race hematocrit and hemoglobin differences (given in chapter 5)

6.2.3 *Data Analysis*

Results are expressed as mean ± SEM. A three by four repeated measures ANOVA with two between-subjects factors (placebo, VC- 500, VC-1500) and one within-subjects factor (time of measurement) was used to analyze the data. When Box's M suggested that the assumptions necessary for the univariate approach were not tenable, the multivariate approach to repeated measures was used. In the latter case, Pillais trace statistic was used as the test statistic. When the group x time interaction p-value was ≤ 0.05, the Tukey multiple comparison test was used to compare groups at a particular time point. When it was determined that the placebo and VC- 500 groups did not differ in any of the post-race measures, the data were reanalyzed using a two (placebo and VC- 500 combined compared to VC-1500) by four repeated measures ANOVA. Pearson correlations were used to test the association between serum cortisol and plasma cytokine concentrations post-race.

6.3 *Results*

Twenty-nine subjects ranging in age from 27 to 54 years fully complied with all protocol requirements (Table 6.1). This included 7 in the placebo group, 10 in the VC-500 group, and 12 in the VC-1500 group. Age, body mass, and stature did not differ significantly between groups. Serum vitamin C concentrations were significantly lower in the placebo compared to VC-500 and VC-1500 groups at the pre-race, 24 h post-race, and 48 h post-race time points (Table 6.2).

Table 6.1 Subject Characteristics (n=29)

Characteristic	Mean (±SEM)	Range
Age (yr)	39.7(±1.30)	27.5-54.0
Body Mass (kg)	70.4(±2.04)	53.2-97.0
Stature (m)	1.74 (±0.02)	1.57-1.89
Body mass index (kg/m^2)	23.2 (±0.50)	18.7 –28.7
Race Time (hrs)	9.73 (±0.18)	7.38-11.08
Weekly Training distance (km)	87.9 (± 4.92)	70.0-120

Serum cortisol increased in all groups immediately following the race, but significantly less so in the VC-1500 group (**Figure 6.1** and Table 6.2).

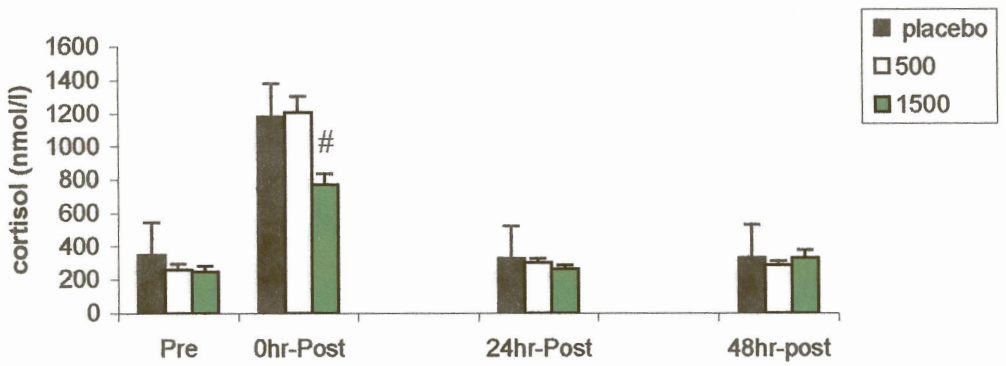

Figure 6.1 Serum cortisol concentrations before and after the 1999 Comrades 90 km ultramarathon in placebo, VC-500, and VC-1500 groups. Data presented as mean ±SEM. # $p<0.01$, Tukey multiple comparison test between groups (VC-1500 and ≤500mg) at time point.

All measured plasma cytokine concentrations were significantly elevated immediately post-race, with the magnitude of increase for TNF-α and IL-1β much smaller than for IL-6, IL-8, IL-10 and IL-1ra (**Figures 6.2-6.5**, Table 6.3). IL-10, IL-8, TNF-α, and IL-1β returned to pre-race levels within 24 hours, whereas IL-6 and IL1Ra dropped back to resting levels with 48 hours.

Group x time interaction statistics between the three groups were not significant for any of the plasma cytokines. However, when the placebo and VC-500 groups were combined (N=17) and compared to VC-1500 (N=12), immediate post-race plasma concentrations were significantly lower in the VC-1500 group for IL-1Ra (-57%) and IL-10 (-57%), with a trend measured for IL-6 (-27%, P=0.11) and IL-8 (-26%, P=0.14) (**Figures 6.2-6.6).** Table 6.2 presents the differences between the VC-1500 *and ≤500mg Vit C in immediate post-race data.*

Table 6.2: Mean ±SEM immediate post-race cortisol and cytokine concentrations in ≤500mg Vit C and ≥ 1500 mg Vit C groups

	≤500 (n=17)	≥1500 (n=12)	Significance*
Serum Cortisol (nmol/l)	1194 ± 95	770± 64.7#	p<0.01
Plasma IL-Ra	3668 ± 1064	1519± 434#	P=0.04
Plasma IL-10	75.2 ± 20.3	31.5± 8.81#	p= 0.01
Plasma IL-6	108± 20.2	78.4 ± 5.8	p=0.11
Plasma IL-8	27.1 ± 5.4	20.9 ± 2.3	p=0.14

* Tukey multiple comparison test between two groups at time point # p<0.05

Table 6.3 : Serum vitamin C, and plasma IL-1β and TNF-α concentrations before and after the 1999 Comrades 90K ultramarathon. Placebo group, n=7; 500 mg/d vitamin C supplement group (VC-500), n=10; 1500 mg/d vitamin C supplement group (VC-1500), n=12. Data presented as mean ±SE.M

Parameter	Pre-race	Post-race (0.5-1 hr)	Post-race (24-hrs)	Post-race (48-hrs)	Time effect; Interaction effect
Vitamin C (µmol/l)					**p<0.001**
Placebo	82.9 ±10.8	125.5 ±6.5	61.3 ±6.8	65.2 ±11.9	**p=0.065**
VC-500	127.8 ±10.2*	146.9 ±12.3	128.9 ±7.9*	136.9 ±9.7*	
VC-1500	153.4 ±10.2*	150.9 ±7.8	134.6 ±7.4*	153.4 ±8.5*	
TNF-α (pg/ml)					**p<0.001**
Placebo	5.86 ±0.77	6.46 ±1.10	4.70 ±0.62	4.90 ±0.84	**p=0.419**
VC-500	7.45 ±1.47	10.24 ±2.17	7.20 ±1.14	6.83 ±1.30	
VC-1500	4.63 ±0.66	6.63 ±0.70	5.05 ±0.43	4.37 ±0.48	
IL-1β(pg/ml)					**p<0.001**
Placebo	0.22 ±0.10	0.44 ±0.11	0.14 ±0.07	0.14 ±0.07	**p=0.124**
VC-500	0.13 ±0.05	0.55 ±0.15	0.11 ±0.04	0.20 ±0.09	
VC-1500	0.04 ±0.01	0.29 ±0.06	0.06 ±0.01	0.02 ±0.01	

* p<0.05, Tukey multiple comparison test between two groups (≤500mg Vit C and ≥ 1500 mg Vit C) at time point

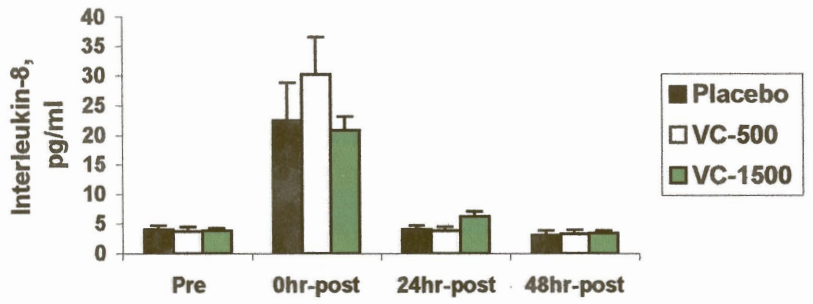

Figure 6.2: Plasma IL-8 concentrations before and after the ultramarathon in placebo, VC-500, and VC-1500 groups. Data presented as mean ±SEM.

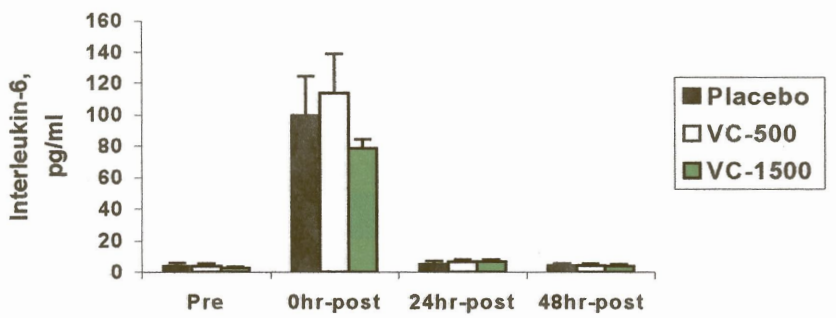

Figure 6.3 Plasma IL-6 concentrations before and after the ultramarathon in placebo, VC-500, and VC-1500 groups. Data presented as mean ±SEM

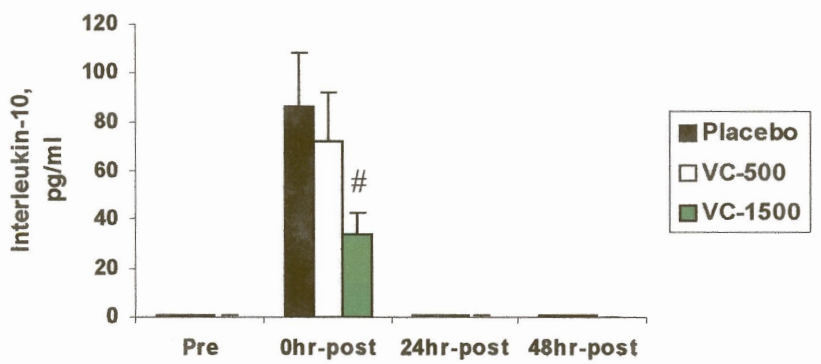

Figure 6.4: Plasma IL-10 concentrations before and after the ultramarathon in placebo, VC-500, and VC-1500 groups. Data presented as mean ±SEM; #$p<0.05$, Tukey multiple comparison test between groups at time point.

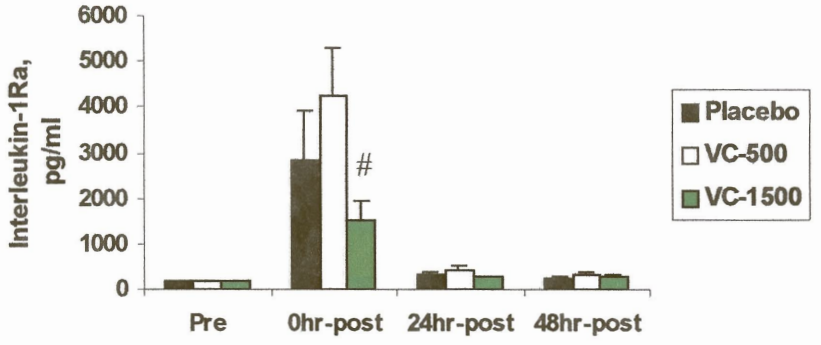

Figure 6.5: Plasma IL-1Ra concentrations before and after the ultramarathon in placebo, VC-500, and VC-1500 groups. Data presented as mean ±SEM; #p<0.05, Tukey multiple comparison test between groups at time point.

For all subjects combined, pre-race serum vitamin C concentrations were negatively correlated with post-race cortisol concentrations (r=-0.33, p=0.08). Post-race cortisol concentrations were significantly correlated with post-race IL-10 (r=0.65, p<0.01), IL-6 (r=0.47, p=0.01), IL-1β (r=0.43, p=0.02), and IL-1ra (r=0.42, p=0.03). When pre- and post-race data were pooled (N=124), serum cortisol concentrations were significantly correlated with IL-6 (r-0.83), IL-10 (r=0.79), IL-8 (r=0.78), IL-1Ra (r=0.69), IL-1B(r=0.57). These data are presented in **Figures 6.6-6.11**.

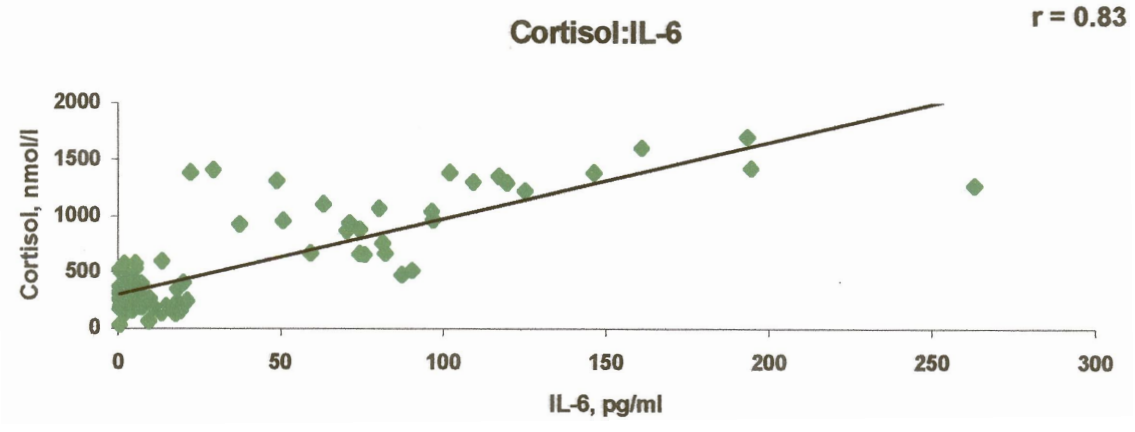

Figure 6.6: The coefficient of correlation between serum cortisol and Il-6

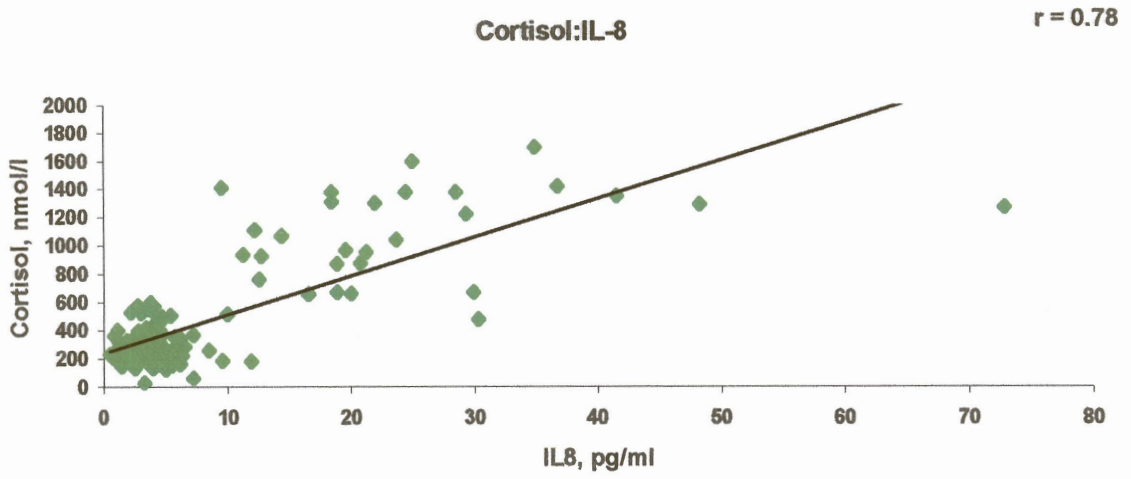

Figure 6.7: The coefficient of correlation between serum cortisol and Il-8

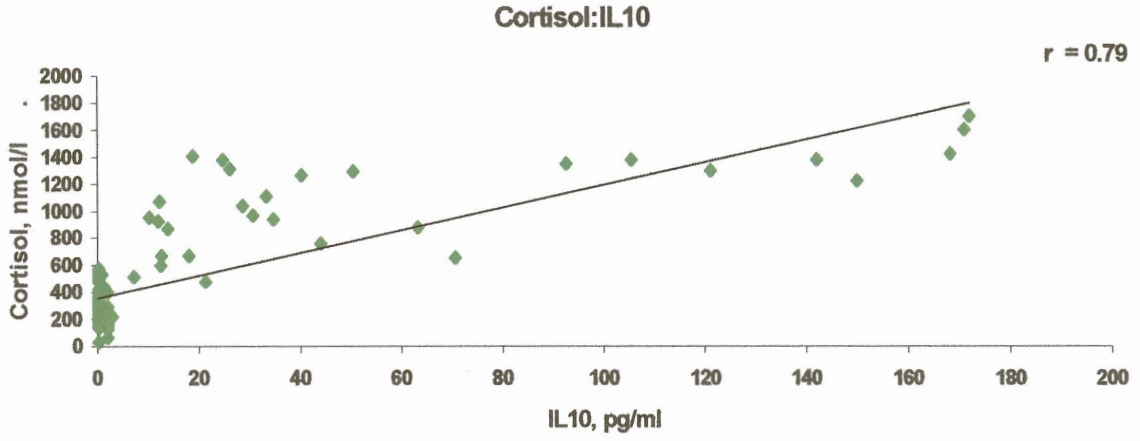

Figure 6.8: The coefficient of correlation between serum cortisol and Il-10

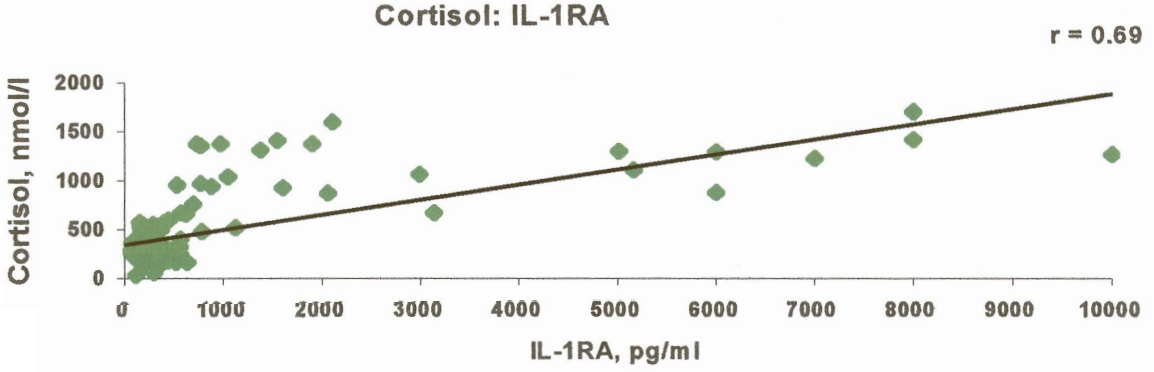

Figure 6.9: The coefficient of correlation between serum cortisol and Il-1RA

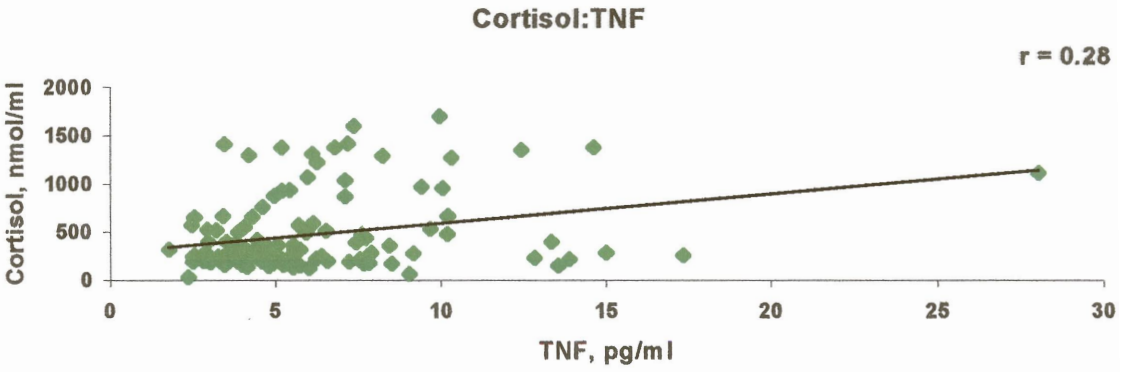

Figure 6.10: The coefficient of correlation between serum cortisol and TNF

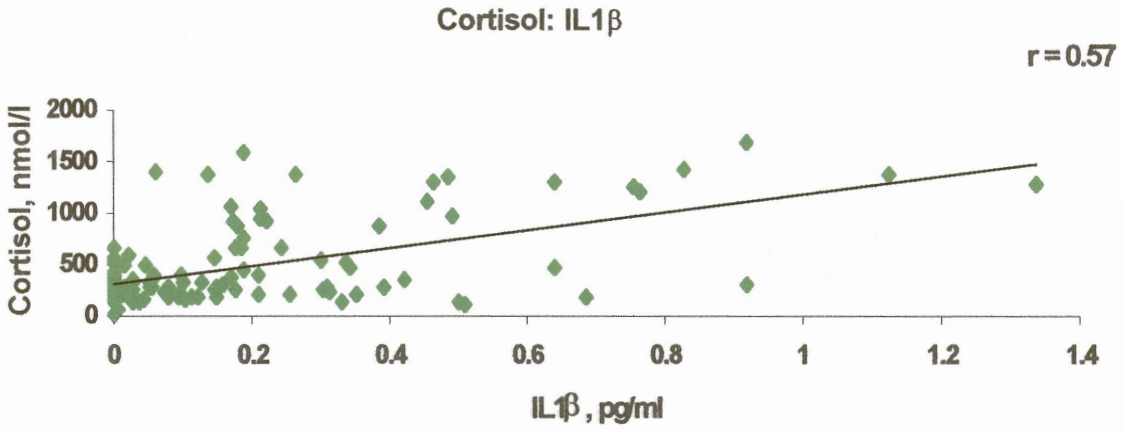

Figure 6.11: The coefficient of correlation between serum cortisol and IL1β

6.4 Discussion

The data from this study and others confirm that plasma concentrations of IL-6, IL-8, IL-10, and IL-1Ra, but not those of TNF-α or IL-1β, rise strongly following prolonged and intensive exertion, falling to near pre-race levels within 24 hours (Drenth *et al.*, 1995; Nehlsen-Cannarella *et al.*, 1997; Ostrowski *et al.*, 1998; Nieman *et al.*, 1998; Suzuki *et al.*, 2000). Strenuous physical exercise of limb muscles typically results in muscle soreness and injury, especially when the exercise is intense and prolonged such as in ultramarathon running. An inflammatory response

to the muscle injury is initiated, characterized by movement of fluid, plasma proteins, and leukocytes into the injured area and metabolically active tissues (Suzuki *et al.*, 2000). Cytokines help regulate the inflammatory cascade, with TNF-α, IL-1β, IL-6, and interferons working synergistically. An exaggerated response is prevented via several pathways, including the production of the anti-inflammatory cytokine, IL-10 and cytokine inhibitor, IL-1Ra, as well as the anti-inflammatory action of cortisol.

The significant reduction in serum cortisol concentrations in the VC-1500 group following the ultramarathon, confirms the results of my earlier study, described in chapter 4. Ostrowski *et al.* (1998b) have provided data supporting the production of IL-6 by neutrophils and macrophages in the area of damaged muscle cells followed by IL-1β and IL-1Ra production by mononuclear cells in the blood compartment. The findings of this work also appear to support this with substantial rises in IL-6 (> 25 fold) and IL-1Ra (>20 fold) being evident following participation in the ultramarathon **(Figures 6.3 and 6.5).** The >5-fold rise in IL-8 concentrations **(Figure 6.2)** supports the work of Suzuki *et al.*, (2000) which showed that this neutrophil chemotactic chemokine is released into circulation after prolonged and intensive, but not moderate exercise.

In the current study the circulating concentrations of IL-1β and TNF-α were only marginally increased post-race, unlike those of IL-6, IL-8 and IL-10. The trivial increases in these cytokines may on the one hand, reflect their relative instability in the circulation as they have been shown to be removed from the circulation rapidly and to later be detected in the urine (Sprenger *et al.*, 1992). It is also possible that peak responses had subsided at the time of sampling (30-45 minutes post-race). Alternatively, complexing to circulating receptors and/or proteolytic degradation may also account for my failure to detect meaningful increases in the circulating concentrations of these two cytokines. The latter contention is supported by reports that both IL-1β and TNF-α are degraded by human neutrophil elastase (Lee & Downey, 2001), a protease, which as previously reported (Gleeson *et al*,1998; Robson *et al.*, 1999) and confirmed in the present study (chapter 7), is elevated in

the circulation during prolonged and intensive exercise, presumably as a consequence of intravascular activation of neutrophils.

Carbohydrate ingestion has been reported to attenuate post-exercise increases in cortisol, IL-6, IL-1Ra, and IL-10 by maintaining blood glucose levels (Nieman *et al.*, 1998). In the present study, daily supplementation with 1500 mg, but not 500 mg of vitamin C during the week prior to the race was also associated with lower post-exercise concentrations of cortisol and the anti-inflammatory cytokines, IL-1Ra and IL-10. This effect was not confounded by race day intake of carbohydrate, with all groups ingesting large, but comparable, quantities due to the extreme conditions of the Comrades Ultramarathon race. Pre-race serum vitamin C correlated negatively, but weakly, with post-race serum cortisol levels, while post-race serum cortisol correlated positively with post-race plasma Il-10, IL-6, IL-1β and IL-1Ra levels. When pre- and post-race data were pooled (N=124), correlation co-efficients between serum cortisol and the polypeptides measured, exceeded 0.68 for all but TNF and IL-1β **(Figures 6.6-6.11)**.

These high correlations lend support to the previously described interactions between pro-inflammatory cytokines, cortisol and the anti-inflammatory polypeptide cascade (Mandrup-Poulsen *et al.*, 1995). While glucocorticoid release has been shown to be activated by the pro-inflammatory cytokines, IL-6 and IL-8 (Figure 2.4), the anti-inflammatory cytokine, IL-10, which induces the production of cytokine inhibitor, IL-1Ra, is well known to respond to increased circulating cortisol concentrations. The apparent attenuation of IL-6, IL-8, IL-10 and IL-1Ra in the post-race samples of the VC-1500 group therefore confirms a possible link between these cytokines and the significantly lower serum cortisol concentrations following prolonged and intensive exertion. In this study, the association is particularly significant in terms of IL-10, IL-1Ra and cortisol. Possible reasons for this, which may well relate to inhibition of the activation of the pro-inflammatory transcription factors, have been described in chapter 5.

Another mechanism may explain the association between vitamin C supplementation and the reduced post-race cytokine levels observed in the VC-1500 group. During exercise, the increase in oxygen uptake by active muscles causes an increase in the generation of reactive oxygen species (ROS). Strenuous exercise also causes an influx of neutrophils into muscle tissues which are considered to be one of the main sources of extracellular ROS (Peters, 1997). ROS cause a wide spectrum of cellular damage and may mediate leukocyte apoptosis (Azenabor & Hoffman-Goetz, 1999; Campbell *et al.*, 1999). As discussed in chapter 2, the majority of studies have indicated that vitamin C supplementation does not completely prevent, but attenuates exercise-induced oxidative stress. This has been confirmed by increases in the levels of serum diene conjugation (Vasankari *et al.*, 1998), thiobarituric acid reactive substances (Rokitzki *et al.*, 1994b), malondialdehyde and exhaled pentane (Kanter et al., 1993; Ashton *et al.* ,1999; Thompson *et al.*, 2001b), the oxidation of low-density lipoproteins (Sanchez-Quesada *et al.*, 1998; Ashton *et al.*, 1999) and electron spin resonance (Ashton *et al.* ,1999). There is growing evidence that by protecting cells from oxidative damage, inflammation and cytokine production may be reduced by vitamin C supplementation (Grimble, 1997; Chen et al., 1998; Schwager & Schulze, 1998b; Bijur, 1999). It has also been reported that vitamin C prevents glucocorticoid-induced apotosis in murine lympocytes (Campbell *et al.*, 1999).

As neutrophil ROS have damaging effects on the neutrophils themselves (**Figure 2.3**), these cells acquire a high level of ascorbic acid for protective purposes (Wolf, 1993). As it has been shown that Vitamin C supplementation can attenuate neutrophil oxygen radical production (Dwenger *et al.*, 1992), it is possible that a mechanism by which vitamin C supplementation prior to prolonged and intensive exercise may lower post-race cytokine levels, is by reducing neutrophil oxidative stress on muscle cells (Goldfarb, 1999).

In summary, runners completing the 90 km Comrades Marathon experienced substantial increases in concentrations of serum cortisol and plasma IL-6, IL-8, IL-10 and IL-1Ra. Although these increases were significantly attenuated in runners

ingesting ≥1500 mg, but not ≤500 mg vitamin C supplements for one week prior to the race and on race day, and these attenuations reached statistical significance only in the cases of IL-10 and IL-1Ra, a larger scale study with greater sample sizes is required to establish whether the trends towards possible attenuation of post-race IL-6 and IL-8 ($p = 0.11$; 0.14) were meaningful and could reach statistical significance.

An interesting novel finding in this paper was the trend towards the higher circulating concentrations of the inflammatory cytokine, IL-6, and the chemotactic cytokine, IL-8, in the group ingesting 500 mg vitamin C. In the next chapter, the question of muscle inflammation is investigated in more depth and the cytokine response in the three groups is integrated with that of acute phase proteins and elastase, a marker of neutrophil degranulation.

Chapter Seven

Vitamin C supplementation and inflammatory response to downhill ultramarathon running

7.1 Introduction

It is well established that prolonged exercise results in delayed muscle soreness (DOMS) which peaks after 24-48 hours and subsides after 5-7 days (Lambert & Dennis, 1994). This has been attributed to actual tissue damage which occurs during repetitive contraction of muscle fibres. The damage has been shown to be further exacerbated when the eccentric component of contraction is increased as occurs during downhill long distance running when muscles are used in a "breaking" motion (Schane *et al.*, 1983; Sorichter *et al.*, 1999).

The Comrades marathon, a gruelling 90 km downhill foot-race from Pietermaritzburg to Durban, South Africa, provides the ideal stimulus for the development of an inflammatory response. As the evidence regarding the effect of vitamin C supplementation on systemic markers of inflammation following eccentric exerise is presently conflicting showing either no effect (Nieman *et al*, 1997; Pedersen *et al.*, 2001) an attenuation (Schmidt *et al.*, 1988; Hurst *et al.*, 2000) or evidence of an increased pro-inflammatory response (Childs *et al.*, 2001) and in view of the apparent evidence of enhanced acute phase response in runners who supplemented with 1000 mg vitamin C per day for 10 days before this 90 km ultramarathon (reported in chapter 4), it was the purpose of this study to reinvestigate and extend the previous study on the effect of oral vitamin C supplementation on markers of acute phase response and muscle damage following ultramarathon running.

In the study presented in this chapter, I thus examined the effects of higher and lower dosages of Vitamin C supplementation on systemic markers of an inflammatory response in participants in the same event two years later.

7.2 Materials and methods

7.2.1 *Study design*

Approval to conduct the study was obtained from the Human Ethics Committee of the University of Natal Medical School. Forty-five registered entrants for the 1999 Comrades Marathon (same as described in chapters 5 & 6) signed informed consent forms and agreed to participate in the study. They were divided into three groups which were matched for age, gender, training status and expected race finishing time.

Each subject was required to take three tablets per day over a 10 day period; one tablet with breakfast, lunch and supper on the 7 days preceding the race, day of the race and two days following the race. In this double-blind study, the first group (VC-500; n=15) were required to take one 500mg Vitamin C tablet in the morning, and a placebo tablet of equal taste and appearance with lunch and supper, while the second group (VC-1500; n=15) were required to take one 500mg Vitamin C tablet with each meal and the third group (P; n=15) were required to take a placebo tablet with each meal.

On the day prior to the race, subjects were required to complete 24 hour dietary records of the intake both in terms of food and supplements and to report for basic anthropometric measurements and blood sampling on the afternoon prior to the race at a time which co-incided with their estimated finishing time (in order to avoid the effect of diurnal rhythms on hormone concentrations). Within 30-45 minutes after completing the race, the subjects again gave 35 ml blood samples and were asked to detail their dietary and liquid intakes on the morning of the race and during the race. The blood sampling was repeated 24 hours and 48 hours after the race and subject were asked to record their post-race dietary intakes for a further 36 hours after the race.

7.2.2 *Treatment of blood*

Venous blood samples collected in glass Vacutainer tubes containing ethylenediaminetetra-acetic acid (K_3-EDTA) were used for determination of full blood counts. A 15ml aliquot was allowed to clot at room temperature and centrifuged for 10 minutes; portions of serum were quick-frozen and stored at -70°C for later analysis of vitamin C, cortisol, C-reactive protein, amyloid A, creatine kinase and lactate dehydrogenase. The remainder was drawn into vacutainer tubes containing K_3-EDTA and the plasma stored at -70 °C for later analysis of vitamin E and A, glucose, elastase and interleukin-8 concentrations.

7.2.3 *Hematological analyses and adjustments*

Full blood counts were performed on K_3-EDTA treated specimens using hematological procedures on an automated STKS model (Coulter Electronics Inc., Hialeah, Florida, USA). Plasma volume changes were determined from pre- and post-race hemoglobin and hematocrit values using the method of Dill and Costill (1974) and all subsequent post-race concentrations (0, 24 and 48 hr) were adjusted for these plasma volume changes.

7.2.4 *Serum acute phase reactants, creatine kinase, lactate dehydrogenase, cortisol, plasma IL-8 and elastase*

Serum concentrations of the acute phase reactants, CRP (normal range 0-5 µg/ml) and amyloid A (normal range 6-8 µg/ml), were measured by a nephelometric procedure (Behring Nephelometer II) using reagents purchased from Behringwerke AG, Marburg, Germany, while creatine kinase and lactate dehydrogenase were determined using the creatine kinase and lactate dehydrogenase reagent supplied for use on a SYNCCHRON CX Clinical System (Beckman Instruments Inc, USA). Serum cortisol was assayed using Gamma Coat radioimmunoassay procedure (Diagnostic Products Corporation, Los Angeles, CA, USA) and plasma elastase (µg/l) using a PMN elastase ELISA kit provided by MERCK (Darmstadt, Germany).

The plasma IL-8 analyses were part of a more comprehensive study on the cytokine profile of ultramarathon runners which is detailed in chapter six, but have been adjusted for plasma volume changes in this report. These were assayed using quantitative sandwich ELISA kits provided by R&D Systems, Inc. (Minneapolis, MN, USA). A standard curve was constructed using standards provided in the kits. The assays were two step "sandwich" enzyme immunoassay procedures in which samples or standards were incubated in 96-well microtiter plates coated with polyclonal antibodies for the test cytokine as the capture antibody. Following the appropriate incubation time, the wells were washed and a second detection antibody conjugated to horseradish peroxidase was added. The plates were incubated and washed, and the amount of bound enzyme-labelled detection antibody was measured by adding a chromogenic substrate. The plates were then read at the appropriate wavelength (450 minus 570 nm). The minimum detectable concentration of IL-8 was < 10 pg/ml.

7.2.5 *Statistical Analyses*

Results are expressed as means (± SEM). As the mean levels between the placebo and VC-500 groups were significantly different for some of the laboratory measures reported in this chapter, it was not possible to pool the data from these two groups, as was done in chapters 5 and 6, but necessary to analyse the data from the three groups separately. Due to the small sizes of the groups and the large variability of the test result values within the groups, conservative non-parametric statistics were used. A Kruskal- Wallis test was used to compare the means of the three groups at each of the time points. If this revealed significance, a two- tailed Wilcoxon two-sample test was used to establish the whether the difference between placebo group and VC-500 or VC-1500 was significant ($P<0.05$). Spearman's correlation coefficient was used as a measure of association. Statistical analysis was executed using SAS statistical software.

7.3 *Results:*

7.3.1 *Subjects*

Of the initial 45 athletes registered to participate in the study, only 29 fully complied with the protocol requirements of the study. The characteristics of the subjects are provided in Table 1. There were no significant differences between the three groups with respect to age, height, mass, body mass index, training status, and time taken to complete the ultramarathon.

Table 7.1: Subject Characteristics (n=29)

Characteristic	Mean (±SEM)	Range
Age (yr)	39.7 (±1.30)	27.5-54.0
Body Mass (kg)	70.4 (± 2.04)	53.2-97.0
Stature(m)	1.74 (± 0.02)	1.57-1.89
Body mass index (kg/m^2)	23.2 (± 0.50)	18.7 –28.7
Race Time (hrs)	9.73 (± 0.18)	7.38-11.08
Weekly Training distance (km)	87.9 (± 4.92)	70.0-120

7.2.2 *Serum vitamin C*

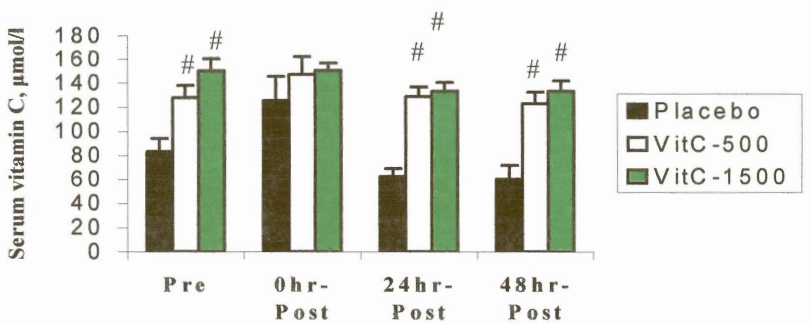

Figure 7.1: Mean (± SEM) serum Vitamin C concentrations before and after participation in a 90 km ultramarathon. # p<0.01, two- tailed Wilcoxon test vs placebo group at this time point.

Pre-race serum vitamin C was significantly higher in the supplemented groups by comparison with the P group (plasma volume adjusted data presented in chapter 5). The significant increase (X= 42.6 µmol/l) in mean serum vitamin C in the P group immediately post-race was less in both of the vitamin supplemented groups (19.1

and –2.84 µmol/l in VC-500 and VC-1500 groups, respectively). At 24 and 48 hrs after completion of the race the serum vitamin C concentrations returned to close to pre-race values.

7.2.3 *Carbohydrate intake and plasma glucose*

These results are presented in chapter 5. Carbohydrate intake just prior to and during the race averaged 401(188) g and did not differ significantly between the groups. Likewise, pre- and post-race plasma glucose concentrations were not different between the 3 groups ($p>0.05$).

7.2.4 *Blood counts*

Selected results of the full blood counts are shown in Table 7.2. Packed cell volume and haemoglobin concentrations indicated a varied hydration status ranging from a mean percent plasma volume drop of 1.7 in the VC-500 group to 7.1 in the placebo group in the immediate post-race samples (**Figure 7.2**). Although 72.5 % of the sample (n=29) presented with a decrease in plasma volume immediately following completion of the ultramarathon, the greatest majority (62.1 and 100 %,), presented with increases in plasma volume in the 24 hour and 48hr post-race samples, respectively. The difference in plasma volume between the groups was insignificant ($p>0.05$).

Significant immediate post-race lymphopenia and neutrophilia was present in all 3 groups with recovery to normal values at 24 and 48 hrs after completion of the race. Circulating neutrophil count was significantly lower in the VC-1500 group (n=12) in immediate post-race samples. samples. Post-race lymphopenia was also not as pronounced in the VC-1500 group, resulting in the immediate post-race neutrophil:lymphocyte ratio being lowest in this group($p<0.05$). Mean monocyte levels rose significantly in all three groups as a result of the prolonged exertion and remained elevated for the 48 hour post race period with no significant difference between the response in the 3 groups.

Table 7.2: Hematological profile. Values as mean (±SEM)

Variable	Pre-race	Post-race (0.5-1hr)	Post-race (24 hours)	Post race (48 hours)
% PV change *				
P Group		7.11(±2.98)	-2.23 (±3.55)	9.45(±2.54)
VC-500		1.73(±1.80)	7.74 (±2.26)	12.3(±2.25)
VC-1500		6,73(±2.29)	5.43(±2.84)	15.6(±2.23)
Total leukocytes(10^9/l)				
P Group	7.62(±1.07)	18.1 (±2.5)	8.66(±1.03)	8.09(±1.04)
VC-500	7.96(±1.09)	16.6(±1.2)	9.37(±0.68)	7.75(±0.31)
VC-1500	6.52(0.48)	14.2(±1.1)	8.14(±0.66)	7.24(±0.46)
Neutrophils(10^9/l)				
P Group	4.43 (±0.91)	15.2(±2.2)	5.14(±0.13)	.04(±0.79)
VC- 500	4.82(±1.08)	13,8(±0.9)	5.64(±0.57)	.59(±0.26)
VC-1500	3.45(±0.35)#	11.0(±1.0)#	4.43(±0.08)	3.10(±1.1)
Lymphocytes (10^9/l)				
P Group	2.11(±0.12)	1.6(±0.3)	2.4(±0.3)	2.3(±0.3)
VC- 500	2.21(±0.12)	1.3(±0.1)	2.6(±0.2)	2.2(±0.1)
VC-1500	2.25(±0.21)	2.0(±0.3)#	2.7(±0.2)	2.4(±0.2)
Neutro:Lymph ratio				
P Group	2.10 (±0.37)	11.9(±2.3)	2.5(±1.5)	2.2(±1.1)
VC- 500	2.31 (±0.62)	10.7(±1.3)	2.4(±0.9)	1.7(±0.5)
VC-1500	1.53(±0.25)	7.0(±1.9)#	2.0(±1.5)	1.5(±0.8)
Monocytes (10^9/l)				
P Group	0.46 (± 0.05)	1.36(±0.15)	0.76 (±0.10)	0.73(±0.10)
VC-500	0.61 (±0.07)	1.00(±0.25)	1.00 (±0.10)	0.76(±0.06)
VC-1500	0.61(±0.05)	1.05(±0.14)	0.79 (±0.07)	0.79(±0.05)

*calculated from packed cell volumes and Hb concentrations; expressed as percentages relative to pre-race plasma volume; PV= plasma volume; neutro:lymph ratio = neutrophil:lymphocyte ratio # $p<0.05$ Wilcoxon test, vs placebo at this time-point.

7.2.1 Circulating cortisol

Circulating cortisol increased significantly in all 3 groups immediately post-race, with serum cortisol subsiding to close to pre-race values at 24 and 48 hrs after completion of the race (**Figure 7.2**). The increase in cortisol observed immediately post-race was significantly attenuated in the VC-1500 group relative to the VC-500 and placebo groups ($p<0.01$).

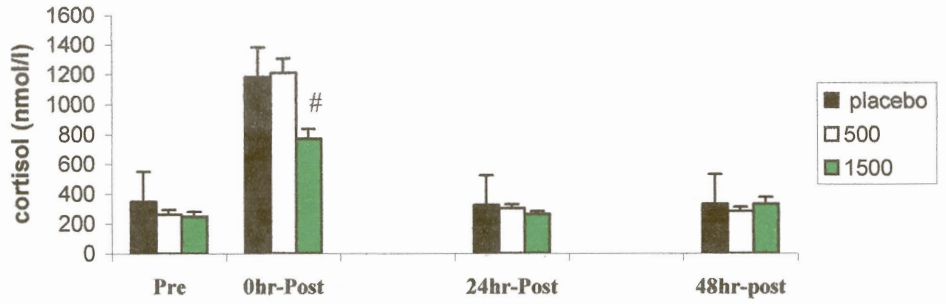

Figure 7.2: Mean (± SEM) serum cortisol concentrations before and after participation in a 90 km ultramarathon. # p<0.01, Wilcoxon test between groups at time point

7.2.6 Circulating concentrations of plasma IL-8 and elastase

The mean immediate post-race concentrations of the chemotactic cytokine, IL-8, were more than five- fold higher than mean pre-race concentrations and subsided to close to pre-race concentrations at 24 and 48 hrs after completion of the race (Table 7.3). These plasma IL-8 concentrations also correlated strongly (r= 0.67) with absolute neutrophil numbers in the circulation (Table 7.2). The increase in the mean circulating concentrations of IL-8, observed immediately post-race, was greater in numbers in the circulation (Table 7.2). The increase in the mean circulating concentrations of IL-8, observed immediately post-race, was greater in the 500 mg group than in the placebo group, but the difference was not statistically different (p=0.14).

Table 7.3: Mean (±SEM) plasma interleukin-8 and elastase concentrations

Group	**Pre-race**	**0hr-post race**	**24hr-post-race**	**48hr post-race**	**Time Effect; Interaction Effect**
Plasma Interleukin-8 (pg/ml)					
Placebo	4.0 (±0.5)	22.5 (±3.9)	4.0 (±0.5)	2.8.0 (±0.5)	P<0.01
VC-500	3.7 (±0.7)	30.2 (±6.4)	3.8 (±0.7)	3.2 (±0.7)	P=0.14
VC-1500	3.8 (±0.4)	20.9 (±2.3)	6.1 (±1.0)	3.3 (±0.5)	
Plasma Elastase (µg/l)					
Placebo	51.0 (±3.45)	206.7 (±71.0)	61.2 (±28.5)	40.3 (±6.91)	p<0.01
VC-500	43.6(±12.9)	159.3(±40.1)	57.0(±14.1)	46.4(±16.8)	p=0.11
VC-1500	44.4(±14.1)	114.5(±39.4)	54.5 (±16.3)	39.2(±8.1)	

The prolonged running event resulted in a 2.6-4 fold increase in mean elastase concentrations which did not differ significantly between the 3 groups at any of the time points ($p>0.05$).

7.2.7 *Serum CRP and amyloid A concentrations*

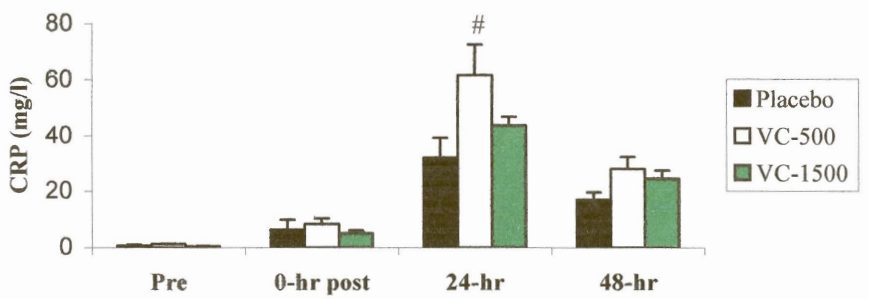

Figure 7.3: Mean (±SEM) serum CRP concentrations before and after participation in a 90 km ultramarathon # $p<0.01$, Wilcoxon test, vs placebo at time point.

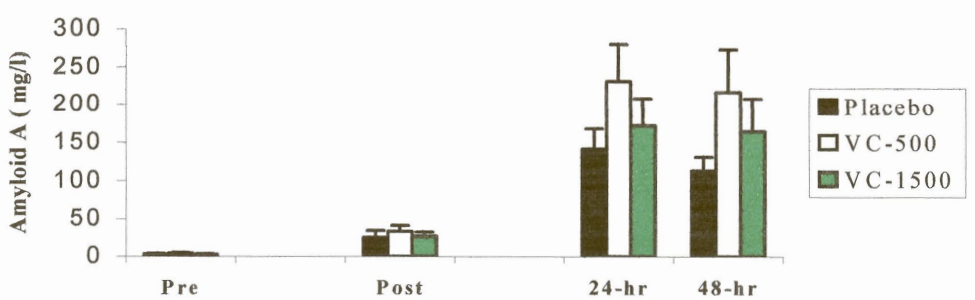

Figure 7.4: Mean (±SEM) serum amyloid A concentrations before and after participation in a 90 km ultramarathon.

Both acute phase proteins measured in this study revealed a similar trend (**Figures 7.3 and 7.4**). When concentrations in the three groups at the 24 and 48 hour post-race time-point were compared, a Kruskal-Wallis test revealed a significant

Both acute phase proteins measured in this study revealed a similar trend (**Figures 7.3 and 7.4**). When concentrations in the three groups at the 24 and 48 hour post-race time-point were compared, a Kruskal-Wallis test revealed a significant difference in the circulating CRP concentration ($p<0.05$) at 24 and 48 hours post-race, but due to large intra-group variance, not in the amyloid-A concentrations ($p<0.05$). A subsequent Wilcoxin two-sample test revealed significant differences ($p=0.03$; $p=0.04$) between the VC-500 and P groups for serum CRP at the 24 and 48 hour post-race time-points respectively. The increments in mean CRP concentrations were, however, not significant at any of the post-race time-points in the VC-1500 group. The correlation between pre-race vitamin c concentration and pre-race CRP concentrations at the three time-points, was not significant ($r= 0.2, 0.01, 0.1$ respectively).

7.2.4 Serum Creatine kinase and Lactate Dehydrogenase

Creatine kinase rose between 15 and 22-fold following the 90km race, reaching peak concentrations 24 hours post-race. Due to large intra-group variation, the difference between the groups was not significant ($p>0.05$) at any of the four time-points measured (Figure 7.5). Lactate dehydrogenase rose more than two-fold following completion of the 90 km race and remained elevated for the entire 48 hour period. **(Figure 7.6).**

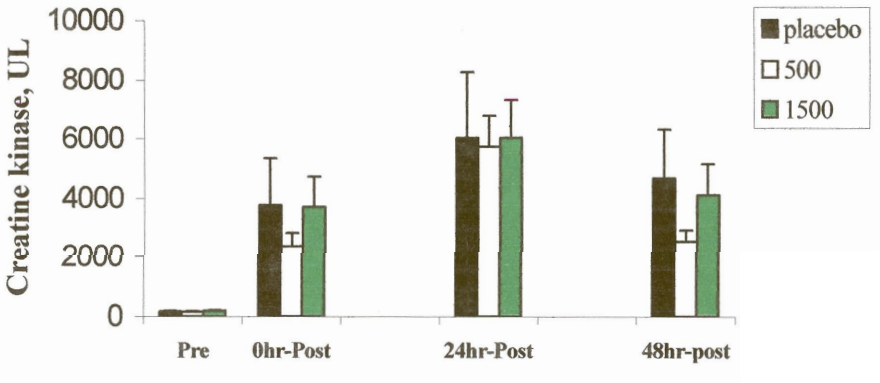

Figure 7.5: Mean (±SEM) serum creatine kinase concentrations before and after participation in a 90 km ultramarathon

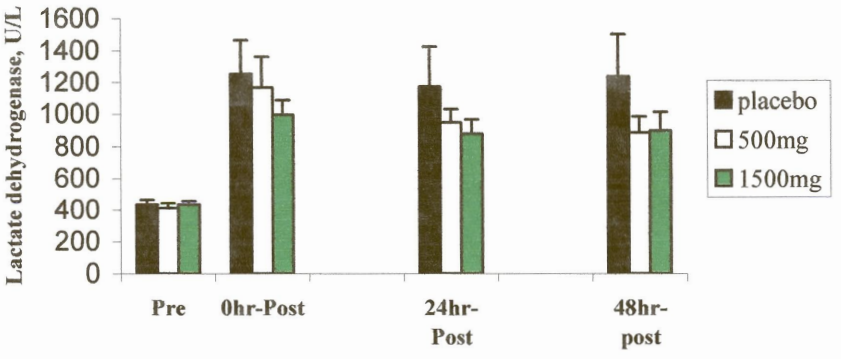

Figure 7.6: Mean ± serum lactate dehydrogenase concentrations before and after participation in a 90 km ultramarathon

7.4 Discussion

When examining the aetiology of the DOMS experienced by distance runners, previously described histologically identifiable disruptions of myofibrils and streaming of Z lines (Friden, 1984), excretion of 3-methylhistidine in urine (Lambert & Dennis, 1994), leakage of creatine kinase (CK) and lactate dehydrogenase (LDH) into the blood stream (Clarkson *et al.*, 1992) and prostaglandin-activated release of CRP into the blood stream (Strachan *et al,* 1984) all confirm local cell damage in the previously active muscles. This is further supported by reports of intra- and extracellular oedema, disruption of T-tubules, and the presence of macrophages, satellite cells and fibroblasts in muscle 1-2 days after prolonged weightbearing exercise (Warhol *et al.,* 1985).

The findings of this study confirm the typical sequence of systemic and metabolic as well as local inflammatory changes associated with the above-described histological damage to muscle fibres. Participation in this 90 km downhill ultramarathon resulted in an elevation of LDH and CK (Evans & Cannon, 1991), confirming the presence of local muscle cell damage. This was accompanied by a pronounced neutrophilia and elevated serum cortisol concentration and a mean increase in plasma elastase

which exceeded 300%. Release of neutrophil elastase, an indicator of neutrophil activation, is known to be related to the amount of exercise and structural damage elicited by mechanical loading of skeletal muscle.

The chemokine, interleukin-8, known to mediate inflammation via its ability to attract and activate neutrophils in the damaged tissues (Baggiolini, 1993; Bazzoni 1991), is a key player in the pro-inflammatory cytokine cascade which triggers the orchestrated metabolic and local inflammatory response to tissue injury and infection. In this study, the mean increase in Il-8 exceeded 500% and was accompanied by substantial increases in IL-Iß and Il-6 (results reported in chapter 6), as well as the acute phase proteins secreted from the liver, C-Reactive protein and amyloid A.

When the results of each of the markers of an inflammatory response measured in this study are considered collectively, a trend towards a pro-inflammatory response is apparent in the VC-500 group when compared to the placebo group. Despite the relatively small sample sizes of the three groups and the conservative non-parametric statistic which was used, serum CRP, an important marker of acute phase response, was significantly elevated in the 24 hour post-race sample of the VC-500 group when compared to the placebo group.

Although the elevation of the mean of the immediate and 48 hour post-race CRP samples, all post-race amyloid A and IL-8 concentrations in the VC-500 group, did not reach statistical significance, they do add support to the possibility of an augmented post-race pro-inflammatory response in the group receiving 500 mg vitamin C daily.

Collectively, these data are also supported by recent findings. The significant increment in the 24 hour post-race acute phase protein concentration in the VC-500 group firstly confirms my previous finding of an increased acute phase response (CRP) and reduced anti-inflammatory response (lower serum cortisol concentrations) reported in the group receiving 1000 mg vitamin C in my previous

study (Chapter 4). The recent findings of Childs *et al.* (2001) who showed that supplementation with the equivalent of 750-1000 mg daily for seven days prior to a brief, intense session of eccentric resistance exercise, resulted in significant increases in indices of oxidative stress which included serum free iron, lipid hydroperoxides and 8-iso prostaglandin F_2 alpha, also lend support to this finding. In addition, these findings are further confirmed by the trend towards higher post-race IL-8 ($p=0.14$; Table 7.3) and IL-6 concentrations (reported in chapter 6), as well as the significantly lower cortisol and anti-inflammatory cytokine response (reported in chapter 5).

In contrast to both my previous study (chapter 4) and the findings of Childs *et al.* (2001), creatine kinase and lactate dehydrogenase concentrations were unaffected by supplementation with Vitamin C. Further work using larger sample sizes is required to elucidate this question.

Notwithstanding factors such as the relatively small sample sizes, the trends of a pro-inflammatory response in the VC-500 group, are too striking to ignore and may well confirm *in vitro* findings of an ascorbic acid–induced pro-inflammatory response. These include inhibition of the H_2O_2, neutralizing activity of this enzyme by complexing with the heme group of catalase (Orr, 1967; Poulsen *et al.*, 1998), the inability of the vitamin to scavenge H_2O_2 (Anderson & Lukey, 1987) and its paradoxical action in preventing the auto-oxidative inactivation of NADPH-oxidase by acting as a scavenger of HOCl, which results in increased production of H_2O_2 by activated phagocytes (Anderson & Lukey, 1987). This hypothesis requires further investigation.

Chapter Eight

Concluding Comments

The primary purpose of the studies described in this thesis was to investigate the relationship between relatively short-term (7-10 days) supplemental oral vitamin C intake of 500-1500 mg/d and adrenal stress hormone and cytokine response to downhill ultramarathon running. Three hypotheses were set prior to undertaking this work (and outlined in chapter 3), namely that :

1. downhill ultramarathon running results in elevation of all markers of inflammatory and oxidative stress measured in this study
2. vitamin C supplementation protects against exercise-associated, transient immune dysfunction by ameliorating oxidative and inflammatory stress and attenuating the related increases in circulating cortisol and adrenaline as well as those of immunosuppressive polypeptides
3. the response to vitamin C supplementation is dose-dependent

The first hypothesis is confirmed by the findings of this work. In each of the ultramarathons, the prolonged eccentric exercise stress was associated with apparent mobilisation of vitamin C from body stores, which was accompanied by substantial increases in circulating levels of leukocytes, cortisol, adrenaline, MPO, elastase, IL-6, IL-8, IL-10, IL-1Ra, CRP, SAA, creatine kinase and lactate dehydrogenase. These findings confirm the presence of considerable oxidative stress and systemic manifestations of an inflammatory response to this exercise load.

The second hypothesis is only partly supported by the findings of this study. Oral administration of vitamin C (≥ 1 gram/day) significantly attenuated the exercise-associated

- mobilisation of vitamin C from body stores
- increase in circulating cortisol and adrenaline

- increase in circulating IL-10, the immuno-suppressive, cortisol-induced cytokine, as well as the IL-1 antagonist with anti-inflammatory action, IL-1 Ra

Although these are novel findings in exercising humans, they do, in part (cortisol data), confirm and extend previous findings of *in vitro* work in animals and selected human populations. Firstly, the finding of an attenuation of the stress response as reflected in serum cortisol concentrations, which was confirmed in two independent studies, may present as corollary to the laboratory work of Goralczyk *et al.* (1992) on porcine adrenocortical cells which identified that ascorbate levels in adrenal cortices regulated steroidgenesis, but also appears to support the hypothesis more recently presented by Halliwell (1996), that ascorbate may inhibit the activity of the important steroidgenic enzyme, 11-ß- hydroxylase, through its pro-oxidant effects. This draws upon the earlier work of Jenkins, who in 1962 described that ascorbate may function as a pro-oxidant by reducing transition metal ions, and in turn, drive the Fenton reaction and enhance oxidative stress (Jenkins, 1962).

Further, a reduction in stress-related increases in circulating corticosteroids following administration of ascorbate has been described in poultry (Pardue *et al.,* 1985a; Satterlee *et al.,* 1989; 1994; Jones *et al.*, 1999), guinea pigs (Odumosu, 1982; Enwonuwo *et al.*,1995), rats (Campbell, pers commun), adult humans undergoing surgery (Nathan *et al,* 1991) and elderly women (De la Fuente *et al.*,1998).

A recent study conducted at the University of Alabama (Campbell, 2000, pers comm.) in which the effect of Vitamin C supplementation on groups of rats which were stressed by complete physical immobilization was investigated, provide strong support to these findings. Administration of 200 mg Vitamin C per day resulted in prevention of the corticosterone response to the stressor. Plasma cortisol levels were not significantly different from those in the non-stressed control rats, but remained significantly lower (mean =300%) than the hormone concentrations in rats which were subjected to the physical immobilisation, but not fed supplemental Vitamin C. Additionally, the stressed rats fed vitamin C exhibited less weight loss, less thymus

involution and less depletion of adrenal ascorbic acid and decreased adrenal hypertrophy compared to those stressed, and not provided vitamin C. These effects of vitamin C were accompanied by enhancement of immune function (increased immunoglobulin levels).

The supplementation dose of vitamin C used in the two studies on ultramarathoners presented in this thesis was considerably smaller [mean = 12-23 mg/kg vs. >300 mg/kg (dependent on mass) used in the work of Campbell *et al.* (2000)]. This may account for the fact that Campbell *et al.* (pers comm.) evidenced a 300% reduction in stress-induced circulating cortisol concentrations as opposed to the 30% and 35% mean attenuations observed in the post-race samples of the ultramarathoners receiving 1000 and 1500 mg vitamin C daily. Despite the use of moderate dosages of vitamin C, the potential physiological impact of what was, nevertheless, a statistically significant attenuation of the widely-described, exercise-associated increase in circulating cortisol, cannot be underestimated. This is supported by the concomitant significant ($p<0.05$) reduction in plasma adrenaline, IL-10 and IL-1Ra concentrations in the vitamin C-supplemented runners (reported in chapters 5 and 6) which point towards a multi-faceted reduction of post-exercise, steroid-mediated immunosuppression (SMI), which may be one of the mechanisms explaining the reduction in incidence of URTI which I have found following vitamin C supplementation in earlier work (1993, 1996).

Of additional interest was the reduction in vitamin C mobilised from body stores in the vitamin C supplemented runners following the ultramarathon run. This is supported by the finding of Campbell *et al.* (pers comm.) of less depletion of adrenal AA following vitamin C supplementation. As vitamin C mobilisation from the adrenal cortex does appear to be coupled to the release of cortisol and adrenaline from the adrenal cortex (Moser, 1992), it is quite possible that oxidative stress was the trigger for their release, with all three working in unison in an attempt to reduce inflammation-mediated tissue damage. As discussed in greater depth in chapters 2, 4 & 5, the reduction in cortisol secretion associated with administration of vitamin C

may be due to decreased synthesis of cortisol or to the protection from steroid insult (Pardue & Thaxton, 1984b).

However, contradictory to the second hypothesis, oral administration of vitamin C doses of 500 and 1000 mg daily did not diminish, but rather potentiated, objective systemic parameters of the exercise-induced inflammatory response (acute phase reactants) and muscle damage (creatine kinase at 1000 mg daily). Since vitamin C at 500 mg daily did not detectably affect cortisol or adrenaline levels, as well as those of the anti-inflammatory polypeptides, IL-10 and IL-1Ra, it appears that potentiation of inflammatory responses at supplementation levels of <1000 mg daily are mediated by alternative mechanisms. It may be that at these doses, vitamin C functions predominantly as a pro-oxidant. This has recently been shown in the results of a study by Childs *et al.* (2001) in which vitamin C supplementation (12.5 mg/kg body mass for 7 days; presumably this would be equivalent to around 500-1000 mg daily depending on body weight) not only resulted in increases in serological indices of exercise-induced muscle damage (creatine kinase, and lactate dehydrogenase) but also inflammation (free serum iron, lipid hydroperoxides and 8-isoprostaglandin F-2 alpha concentrations). Although circulating acute phase reactants, cortisol, adrenaline, IL-10 and IL-1Ra concentrations were not measured in this study, the results are compatible with a predominantly pro-oxidative, pro-inflammatory effect of vitamin C at concentrations of 500-1000mg daily, which is in agreement with my findings.

As previously mentioned in chapter 2, this evidence of a pro-oxidative, pro-inflammatory effect of vitamin C could occur by inhibition of catalase (Orr, 1967) or somewhat counter-intuitively, by anti-oxidative mechanisms, whereby the vitamin protects the free radical generating systems of both phagocytes (respiratory burst) and myocytes (oxidative phosphorylation) resulting in increased production of H_2O_2 which can activate various pro-inflammatory cascades (Anderson & Lukey, 1987). This, however, seems unlikely since there was no clear evidence of increased numbers of phagocytes in the circulation, or of elevations in markers of systemic activation of these cells (MPO, elastase, IL-6, IL-8) in the vitamin supplemented

groups. Alternatively, and perhaps more likely, it is possible that cortisol, adrenaline, IL-10 and IL-1Ra and their combined anti-inflammatory impact are indeed suppressed by administration of the vitamin at doses of 500 mg daily, but that this suppression is only detectable earlier in the time-course of the ultramarathon event, as opposed to on completion of the race, following, in some cases, more than 20 hours of exercise.

If this is the case, one would question why there is no clear relation between the dose of the vitamin administered to the athletes and the magnitude of the acute phase response (ie. at 1500 mg daily there was no significant enhancement of CRP and SAA)? This may be due to the initiation of counteracting, anti-inflammatory mechanisms at higher doses of the vitamin. For example, vitamin C, at fairly high concentrations, has been reported to inhibit the activation of NF*k*B by multiple pro-inflammatory stimuli, including TNFα, IL-1β, IL-6, in cultured endothelial cells *in vitro* by a non-oxidative mechanism involving activation of p38 mitogen-activated protein-kinase (Bowie & O'Neill, 2000; Horton *et al.,* 2001). If operative *in vivo,* such a mechanism would explain the unusual dose-response relationship between administration of vitamin C and the magnitude of the exercise-induced inflammatory response.

The third hypothesis set prior to undertaking this work was that the response to vitamin C supplementation is dose-dependent. This hypothesis is rejected by the findings of this series of studies and earlier pilot work. Taken together, the findings of this work do not reveal a simple linear, dose-dependent response in any of the parameters of immune and oxidative stress monitored in this work.

Scope and limitations of the work

Due to difficulties encountered in obtaining full compliance from ultradistance runners who had dedicated many months of hard training to completing the ultramarathon and were highly committed to this goal and the practical problems associated with obtaining immediate post-race samples from participants in one of

the world's largest ultramarathon events (>10,000 participants), the work reported in this thesis was unfortunately limited to relatively small sample sizes and restricted to blood sampling. As a confirmatory measure as well as an extension, the work done at the 1997 Comrades Marathon, was therefore replicated in an independent follow-up study in which groups of different subjects were exposed to the almost identical stressor at the 1999 Comrades Marathon in order to determine whether the findings were repeatable.

The *in vivo* findings of attenuation of adrenal cortisol release and vitamin C mobilisation were duplicated in the follow-up study and additional attenuation of adrenaline and anti-inflammatory polypeptide release further strengthened the findings. However, although the evidence in favour of an inhibition of cortisol synthesis with increases in circulating AA concentrations is strong, further possible mechanisms of action and interaction can, at this stage, only be debated. Future laboratory and *in vivo* work which attempts to elucidate actual mechanisms of this apparent interaction between adrenal vitamin C and stress hormone release, as well as anti-inflammatory polypeptide secretion by immune cells following vitamin C supplementation in humans who are exposed to physical stress, is required to validate these findings and the hypotheses made.

With regard to the possibility of a pro-inflammatory response in the VC-500 group, the strength of the evidence is limited by the smaller sizes of the three groups compared and more restricted statistical power. Further work is therefore required on larger sample sizes to confirm these findings before the focus is shifted to elucidating possible biochemical mechanisms of action.

8.3 Additional directions for future research

The possibility of a differential and paradoxical response following supplementation with 500-1000 mg/ day requires further investigation. It will be noted in the work reported in chapters 6 and 7, that runners in the VC-500 group presented with the highest mean concentrations of CRP, amyloid A, IL-6 and IL-8 which confirms the

finding of significantly higher CRP concentrations in the group receiving 1000 mg Vitamin C reported in Chapter 4. As mentioned above, these preliminary findings which appear to point towards an apparent overriding pro-inflammatory response within an attenuated anti-inflammatory milieu, require further investigation.

A further important direction for future research is the effect of supplementation with quantities of vitamin C exceeding 1500 mg/d. At this level of supplementation the desirable actions of supplementation with vitamin C on reducing serum cortisol concentrations, appeared to be retained in the setting of apparent suppression or containment of the pro-inflammatory actions of the vitamin. If this is really the case, and if the protective effects of the vitamin against post-race development of URTI are due to attenuation of cortisol-mediated suppression of specific immune responses, as opposed to a non-specific, pro-inflammatory, adjuvant-type effect of the vitamin, which may occur at doses of ≤ 1000 mg/day, then future research should focus on supplementation at levels in excess of 1500 mg vitamin C daily.

Related to the above-mentioned question of optimal dosage, is the determination of the most desirable /optimal duration of the supplementation period. Isolated reports which implicate a possible adaptive response following months/years of high dietary vitamin C intake, do exist (Hemilä, 1997; Stanislaw & Klapcinska, 2000). In the series of studies described in this thesis, the supplementation period was purposefully limited to 10 days in order to exclude the possibility of an adaptive response influencing the results. This matter does, however, require investigation and may explain some of the discrepant findings previously reported in the literature.

In conclusion, while highlighting the possibility of a pro-inflammatory response to vitamin C supplementation, the findings of the work described in this thesis have identified possible mechanisms which may account for the reduction of transient post-race SMI in ultramarathoners following 7-10 days of supplementation with 1000-1500 mg vitamin C per day. It is hoped that this will stimulate further research designed to elucidate exact molecular/

biochemical mechanisms of action and optimal dosages and periods of supplementation, bearing in mind the potential of the vitamin to augment potentially harmful pro-oxidative responses.

Addendum, June 2002

Following submission of this thesis, a number of reports which have focused on vitamin C, have appeared in the literature. I should like to comment briefly on these.

In a recent report, Nieman *et al.*(2002) describe a study on ultramarathoners in which a vitamin C supplemented group was compared to runners receiving placebo without any differences in cortisol, cytokine, lipid hydroperoxide, F_2-isoprostane, immune cells counts or mitogen stimulated lymphocyte proliferation in runners consuming a mean of 115g carbohydrate per hour and supplemented with 1500 mg per day for 10 days.

Of significance is the fact that the carbohydrate intake of these runners during the course of the race was more than double of the average consumption of runners in the studies reported in this thesis. In addition, plasma glucose concentrations (which ranged from 7.94 to 7.07 mmol/litre in the Vitamin C supplemented group during and after the race in the work of Nieman *et al.*) were also higher than reported in my work (5.95 ±0.34 mmol/litre after the race in the VC-1500 group).

Glucose has been shown to be a potent inhibitor of ascorbate uptake by a variety of different cell types, including immune and inflammatory cells, as well as endothelial cells and intestinal brush vessels (Malo & Wilson, 2000). L-ascorbate transport is a Na^+ dependant, electrogenic process which is known to be "modulated" by glucose; glucose has been shown to interfere with the ascorbate transporter from the internal side of the membrane (Malo & Wilson, 2000). The antagonistic effects of glucose on ascorbate absorption may therefore well account for the discrepancy in the findings reported by myself and Nieman *et al.*(2002).

High glucose concentrations are also capable of triggering endothelial cell apoptosis (Ho *et al.*, 1999). Exposure to high blood glucose concentrations has been shown to enhance the production of reactive oxygen species and lead to an imbalance of nitric oxide and ROS and the subsequent induction of apoptosis, after 24 hours. Both of these glucose-induced effects have been shown to be reversed by Vitamin C (Ho *et al.*, 1999).

Another important recent finding is the ability of vitamin C to attenuate post-ischaemic oxidative changes, decrease platelet activating factor (PAF) and PAF-like lipids and reduce myeloperoxidase activity (Lloberas *et al.*, 2002). This, together with the recent finding that the gene encoding the ascorbic acid transporter, S1c23a1), is a necessity in the perinatal period (Sotiriou *et al.,* 2002) and that inactivation of this gene results in the death of new born mice, highlights the value of sufficient dietary intake of Vitamin C.

These recent findings therefore also provide further support to possible therapeutic benefits of vitamin C supplementation which surpass the theory that the only proven value of ascorbate is for the prevention of scurvy and do place the divergent findings on marathoners which can possibly be related to the antagonistic effects of glucose on ascorbate uptake, into perspective. Actual verification of the possible antagonism of vitamin C by glucose in ultramarathoners, is, however, an important direction for future research.

References

Alessio HM, Cao G, Cutler RG. Total antioxidant protection following Vit E and C supplementation and submaximal exercise. *Med Sci Sports Exerc* **24**: S16, 1992.

Alessio HM, Goldfarb AH. Cao G. Exercise-Induced oxidative stress before and after Vitamin C Supplementation. *Med Sci Sports Exerc* **25**: 208-214, 1997.

Anderson R, Lukey PT. A biological role for ascorbate in the selective neutralization of extracellular, phagocyte derived reactive oxidants. *Ann NY Acad Sci* **498**: 219-247, 1987.

Anderson R, Oosthuizen R, Maritz R, Van Rensburg AJ. The effects of increasing weekly doses of ascorbate on certain cellular and humoral immune functions in normal volunteers. *Am J Clin Nutr* **33**: 71-6, 1980.

Anderson R, Smit MJ, Joone GK, Van Staden AM. Vitamin C and Cellular Immune Functions: Protection against hypochlorous acid-mediated inactivation of glyceraldehyde-3-phosphate dehydrogenase and ATP generation in human leukocytes as a possible mechanism of ascorbate immunostimulation. *Ann N Y Acad Sci* **587**: 33-48, 1989.

Anderson R, Theron AJ. Effects of Ascorbate on Leukocytes. *S Afr Med J* **56**: 394-9, 1979.

Anderson R, Van Antwerpen VL. Vitamins in the maintenance of optimum immune functions and prevention of phagocyte-mediated tissue damage and carcinogenesis. *Bibliotheca Nutrio et Dieta* **52**: 66-74, 1995.

Anderson R. Mechanisms of Vitamin C-mediated anti-inflammatory and immunomodulatory activity. *Bibliotheca Nutrio et Dieta* **55**: 135-147, 2001.

Anderson R. Phagocyte-derived oxidants as mediators of inflammation-associated damage. *S Afr J Sci* **87**: 594-596, 1991.

Anderson R. Ascorbate-mediated stimulation of neutrophil motility and lymphocyte transformation by inhibition of the peroxidase/H_2O_2/halide system in vitro and in vivo. *Am J Cl Nutr* **34**: 1906-1911, 1981.

Anderson R. Effects of Ascorbate on Normal and Abnormal Leukocyte Functions. In Hanck A. (ed). Vitamin C. New Clinical Applications in Immunology, Lipid Metabolism and Cancer. Bern: Hans Huber Publishers, pp 23-52, 1982.

Anderson R. Phagocyte-derived oxidants as mediators of inflammation-associated damage. *S Afr J Sci* **87**: 594-596, 1991.

Anderson R. The activated neutrophil -formidable forces unleashed. *S Afr Med J* **85**: 1024-1027, 1995.

Ashton T, Young IS, Peters JR, Jones E, Ejackson SK., Davies B, Rowlands CC. Electron spin resonance spectroscopy, exercise, and oxidative stress: an ascorbic acid intervention study. *J Appl Physiol* **87**: 2032-6, 1999.

Attwood EC, Robey ED, Ross H, Bradley F, Kramer JJ. Determination of platelet and leukocyte vitamin C levels found in normal subjects. *Clin Chim Acta* **54**: 95-105, 1974.

Azenabor AA, Hoffman-Goetz L. Intrathymic and intrasplenic oxidative stress mediates thymocyte and splenocyte damage in acutely exercised mice. *J Appl Physiol* **86**: 1823-7, 1999.

Babior BM. Oxidants from Phagocytes: agents of defence and destruction. *Blood* **64**: 959-964, 1984.

Baggiolini M. Novel aspects of inflammation: Interleukin-8 and related chemotactic cytokines. *Clinical Investig* **71**: 812-814, 1993.

Barnes PJ, Adcock I. Anti-inflammatory actions of steroids: molecular mechanisms. *Trends Pharmacol Sci* **14**: 436-441, 1993.

Barnes PJ, Karin M. Nuclear factor kB – a pivotal transcription factor in chronic inflammatory diseases. *New Engl J Med* **336**: 1066-1071, 1997.

Bazzoni G, Dejana E, Del Mashcio A. Adrenergic modulation of human polymorphonuclear leukocyte activation. Potentiating effect of adenosine. *Blood* 77: 2042-2048, 1991.

Bendich A. Vitamins and Immunity. *J Nutr* **122**: 601-3, 1992.

Bendich A, Machlin LJ, Scandura O, Burton GW, Wayner DDM. The anti-oxidant role of Vitamin C. *Adv Free Rad Biol Med* **2**: 419-44, 1986.

Bendich A, Cohen M. Ascorbic acid safety: analysis of factors affecting iron absorption. *Toxiol Lett* **51**: 189-201, 1990.

Benoni G, Bellavite P, Adami A, Chrumbolo S, Lippi G, Brocco G, Giulini GM, Cuzzolin L. Changes in several neutrophil functions in basketball players before, during and after a sports season: *Int J Sports Med* **16**: 34-37, 1995.

Bergholm R, Mäkimattila S, Valkonen M, Liu ML, Lahdenperä S, Taskinen MR, Sovijarvi, A, Malmberg P, Yki-Jarvininen H. Intense physical training decreases circulating antioxidants and endothelium-dependant vasodilation *in vivo*. *Artherosclerosis*, **145**: 341-349, 1999.

Berglund B, Hemmingson P. Infectious disease in elite cross country skiers: a one-year incidence study. *Clin Sports Med* **2**: 19-23, 1990.

Bessel-Lorck C. Erkaeltungsprophylaxe bei Jugendlichen im Skilager (Common cold prophylaxis in young people at a ski camp). *Med Welt* **44**: 2126-2127, 1959.

Bieri JG, Tolliver TJ, Catignani GL. Simultaneous determination of retinol and α-tocopherol in serum and plasma by liquid chromatography. *Clin Chem* **29**: 708-712, 1983.

Bijur GN, Briggs B, Hitchcock CL, Williams MV. Ascorbic acid-dehydroascorbate induces cell cycle arrest at G2/M DNA damage checkpoint during oxidative stress. *Environ Mol Mutagen* **33**: 144-52, 1999.

Blannin AK, Robson PJ, Walsh NP, Clark AM. Glennon L, Gleeson M. The effect of exercising to exhaustion at different intensities on saliva immunoglobulin A, protein and electrolyte secretion. *Int J Sports Med* **19**: 547-52, 1998.

Bloem, MW, Wedel M, Egger RJ, Speek AJ, Schrijer J, Saowakontha S, Scheurs W. Mild Vitamin A deficiency and risk of upper respiratory tract disease and diarrhoea in pre-school and school children in North Eastern Thailand. *Am J Epidemiol* **131**: 20332-339, 1990.

Borish, L. IL-10: Evolving concepts. *J Allergy Clin Immunol* **101**: 293-297, 1998.

Bowie AG, O'Neill LA. Vitamin C inhibits NF-kappa B activation by TNF via the activation of p38 mitogen-activated protein kinase. *J Immunol* **165**: 7180-8, 2000.

Brenner I, Shek, PN, Zamecnik J, Shepard RJ, Stress hormones and the immunological responses to heat and exercise. *Int J Sports Med* **19**: 130-143, 1998.

Child RB, Wilkinson DM, Fallowfield JL, Donnelly AE. Elevated serum antioxidant capacity and plasma malondialdehyde concentration in response to a simulated half marathon run. *Med Sci Sports Exerc* **11**: 1603-1607, 1998.

Childs A, Jacobs C, Kaminski T, Halliwell B, Leeuwwenburgh C. Supplementation with vitamin C and N-acetyl-cysteine increases oxidative stress in humans after an acute muscle injury induced by eccentric exercise. *Free Rad Biol Med* **31**: 745-753, 2001.

Chojkier M, Houglum K, Solis-Herruzo J, Brenner DA. Stimulation of collagen gene expression by ascorbic acid in cultured human fibroblasts. A role for lipid peroxidation? *J Biol Chem* **264**: 16957-16962, 1989.

Chow CK, Arriess, GR, Changchit C. Increased vitamin E content in the lungs of cigarette-smoked rats. *Ann NY Acad Sci* **570**: 425-427, 1989.

Chretien JH, Garagusi VF. Correction of corticosteroid-induced defects of polymorhonuclear neutrophil function by ascorbic acid. *J Reticuloendothel Soc* **14**: 280-286, 1973.

Clarkson PM, Nosaka K, Braun, B. Muscle function after exercise-induced muscle damage and rapid adaptation. *Med Sci Sports Exerc* **24**: 5112-520, 1992.

Clarkson PM, Thompson HS. Anti-oxidants: what role do they play in physical activity and health? *Am J Clin Nutr* **72**: 637S-46S, 2000.

Cooke MS, Evans MD, Podmore ID, Herbert KE, Mistry N, Mistry P, Hickenbotham PT, Hussieni A, Griffiths HR, Lunec J. Novel repair action of vitamin C upon *in vivo* oxidative DNA damage. *FEBS Letters* **439**: 363-7, 1998.

Cooper DY, Rosendahl O. Action of noradrenaline and ascorbic acid on C-21 hydroxylation of steroids by adrenocortical microsomes. *Arch Biochem* **96:** 331-335, 1962

Coutsoudis A, Kiepiela P, Coovadia HM, Broughton M: Vitamin A supplementation enhances specific IGA antibody levels and total lymphocyte numbers while improving morbitity in measels. *Paed Infect Dis J* **11**: 203-9, 1992.

Crary B, Hanser B, Boreseynco M, Kuts I, Hoban C, Ault KA, Weiner L, Benson H. Epinephrine induced change in the distribution of lymphocyte subsets in humans. *J Immuno* **131**: 1178-1181, 1983.

Cupps TR, Fauci AS. Corticosteroid-mediated immunoregulation in man. *Immunological Rev* **65**: 133-55,1982.

Curham GC, Willet WC, Speiser FE. Intake of vitamins B_6 & C and the risk of kidney stones on women. *J Am Ass Nephrol* **10**: 840-5, 1999.

Davies KJA, Quintanilha AT, Brooks GA, Packer L. Free radicals and tissue damage produced by exercise. *Biochem Biophys Res Com* **107**: 1198-1205, 1987.

De la Fuente M, Fernandez MD, Buegos MS, Soler A, Prieto A, Miquel J. Immune function in aged women is improved by ingestion of vitamins C and E. *Can J Physiol* **76**: 373-380, 1998.

De Waal MR, Abrams J, Bennett B, Figdor CG, de Vries JE. Interleukin-10 (IL-10) inhibits cytokine synthesis by human monocytes: an autoregulatory role of IL-10 produced by monocytes. *J Exp Med* **174**: 1209-12120, 1991.

Dekkers, C, van Dooren LJP, Kemper HCG. The Role of Antioxidant Vitamins and Enzymes in the Prevention of Exercise Induced Muscle Damage. *Sports Med* **21**: 213-238, 1996.

Dhariwal K, Wasko P, Hartzell W, Levine M. Ascorbic acid within chromaffin granules. *J Biol Chem* **264**: 15404-15409, 1989.

Dietrich M, Block G, Hudes M, Morrow JD, Norkus EP, Traber MG, Cross CE, Packer L. Antioxidant supplementation decreases lipid peroxidation biomarker F(2)-isoprostanes in plasma of smokers. *Cancer Epidemiol Biomarkers Prev* **11**: 7-13, 2002.

Dill DB, Costill DL. Calculation of percentage changes of blood, plasma and red cells in dehydration. *J App Physiol* **37**: 247-248,1974.

Drenth JPH, Van Uum SHM, Van Deuren M, Pesman GJ, Van der Ven-Jongekrijg J, Van der Meer JWM. Endurance run increases circulating IL-6 and IL-1ra, but downregulates ex vivo TNF-a and IL-1b production. *J Appl Physiol* **79** : 1497-503, 1995.

Dufaux B, Order U. Plasma elastase, α1-antitrypsin, neopterin, tumour necrosis factor α , and soluble interleukin-2 receptor after prolonged exercise. *Int J Sports Med* **10**: 434- 438, 1989.

Duthie GA, Maughan RJ, Morrice PC, Arthur RJ. Anti-oxidant adaptation to exercise. In: Biochemistry of Exercise IX, Maughan RJ. Shirreffs SM(Eds): Champaign Il: Human Kinetics, pp 465-470, 1996.

Duthie GA, Robertson JD, Maughan, RJ, Morrice PC. Blood Anti-oxidant Status and Eryhrocyte Lipid Peroxidation following distance running. *Arch Biochem Biophys* **282**: 78-83, 1990.

Dwenger A, Funck M, Lueken B, Schweitzer G, Lehmann U. Effect of ascorbic acid on neutrophil functions and hypoxanthine/xanthine oxidase-generated, oxygen-derived radicals. *Eur J Clin Chem Clin Biochem* **30**: 187-91, 1992.

Dziedziak W. The effect of incremental cycling on physiological functions of peripheral blood granlocytes. *Biol Spt* **7**: 239-247,1990.

Ehrhart-Bornstein M, Hinson JP, Borstein SR, Scherbaum WA, Vinson GP. Intra-adrenal interactions in the regulation of adrenocortical steroidogenesis. *Endocrine Rev* **19**: 101-143, 1998.

El-Hag A, Clark RA. Down regulation of human natural killer cell activity against tumours by the neutrophil myeloperoxidase system and hydrogen peroxide. *J. Immunol* **133**: 3291-8, 1984.

El-Hag A, Lipsky PE, Bennett M, Clark RA. Immunomodulation by neutrophil myeloperoxidase and hydrogen peroxide; differential susceptibility of human lymphocyte functions. *J Immunol* **136**: 3320-6, 1986.

Enwonwu CO, Sawiris P, Chanaud N. Effect of marginal ascorbic acid deficiency on saliva level of cortisol in the guinea pig. *Arch Oral Biol* **40**: 737-42, 1995.

Eskola J, Ruuskanen O, Soppi E, Viljanen MK, Jarvinen M, Toivonen M, Kouvalainen K Effect of sport stress on lymphocyte transformation and antibody formation. *Clin & Experiml Immunol* **32**: 339-345, 1978.

Evans R, Currie L, Campbell A. The distribution of ascorbic acid between various cellular components of blood in normal individuals, and its relation to the plasma concentration. *Brit J Nutr* **47**: 473, 1982.

Fahlman MM, Engels HJ, Morgan AL, Kolokouri I. Mucosal IgA resoponse to repeated Wingate tests in Females. *Int J Sports Med* **22**: 127-131, 2001.

Farr A, Salman HK, Krautman BA, Gonzales LA, Macdonald A. Effect of high level vitamin C dosage at 32 hours prior to slaughter on processing parameters of broiler chickens. *Poultry Sci* **67** (suppl): 85 (abstr)

Farrell P, Garthwaite T, Gustafson A. Plasma adrenocorticotropin and cortisol responses to submaximal and exhaustive exercise. *J Appl Physiol* **55**: 1441-4, 1983.

Fishbaine B, Butterfield G. Ascorbic Acid Status of running and sedentary men *Int. J Vit Nutr Res* **54**: 273, 1984.

Frei B, Stocker R, Ames BN. Anti-oxidant defences and lipid peroxidation in human blood plasma. *Proc Natl Acad* **85**: 9748-9752, 1988.

Frei B, England L, Ames BN. Ascorbate is an outstanding anti-oxidant in human blood plasma. *Proc Nat. Acad Sci* **85**: 6377-6381, 1989.

Frei B. Reactive oxygen species and anti-oxidant vitamins: mechanisms of action. *Am J Med* **97**: 5S-13S, 1994.

Friden J. Muscle soreness after exercise: implications of morphological changes. *Int J Sports Med* **5**: 57-66, 1984.

Gabriel H, Kindermann W. The acute immune response to exercise; what does it mean? *Int J Sports Med* **18**: S28-41, 1997.

Gabriel H, Muller H-J, Kettler K, Brechttel L, Urhausen A, Kindermann W. Increased phagocytic capacity of the blood, but decreased phagocytic activity per individual circulating neutrophil after an ultradistance run. *Eur J Appl Physio* **71**: 281-284, 1995.

Gabriel H, Muller HJ, Urhausen A, Kindermann W. Suppressed PMA-induced oxidative burst and unimpaired phagocytosis of circulating granulocytes one week after a long endurance run. *Int J Sports Med* **15**: 441-445, 1994.

Galbo H. Hormonal and metabolic adaptation to exercise. George Thieme Verslag, Stuttgart, 1983.

Garry PJ, Appenzeller O. Vitamins A and C and endurance races. *Annal Sports Med* **1**: 82-84, 1983.

Gerster H. No contribution of ascorbic acid to renal calcium oxalate stones. *Annal Nutr Met* **41**: 269-82, 1997.

Gerster H. High dose vitamin C a risk for persons with high iron stores? *Int J Vit Nutr Res* **69**: 67-82, 1999.

Glathaar BE, Hornig DH, Moser U. The role of ascorbic acid in carcinogenesis. *Adv Exper Med & Biol* **206**: 357-77, 1986.

Gleeson M, Maughan R (abstract). Acute effect of exercise on ascorbate content of plasma and lymphocytes. *Proc Nutr Sci* **73**: 501-505, 1987.

Gleeson M, McDonald W, Pyne DB, Cripps AW, Francis JL, Fricker PA, Clancy RL. Salivary IgA levels and infection risk in elite swimmers. *Med. Sci Sports Exerc* **31**: 67-73, 1999.

Gleeson M, McDonald WA, Cripps AW, Pyne DB, Clancy RL, Fricker PA, Exercise, stress, and mucosal immunity of long term intensive training in elite swimmers, *Clin Exp Immunol* **102**: 210-216, 1995.

Gleeson M, Walsh NP, Blannin AK, Robson PJ, Cook L, Donnelly AE, Day SH. The effect of severe eccentric exercise –induced muscle damage on plasma elastase, glutamine and zinc concentrations. *Eur J Appl Physiol* 77: 543- 546, 1998.

Gleeson M. Epstein-Barr Virus in Association with Respiratory Illness in Elite Athletes. In : Rimmer J.S. & Katelaris C.H (eds). Proceedings of the XVII International Congress of Allergology and Clinical Immunology, Hogrefe & Huber Publishers, Seattle, 2000c.

Gleeson M. Mucosal Immune Responses & Risk of Illness in Elite Athletes. *Exerc Immunol Rev* **6**: 5-42, 2000b.

Gleeson M. Mucosal immunity and respiratory illness in elite athletes. *Int J Sports Med* **21**: S33-43, 2000a.

Gleeson, M, Robertson J, Maughan R. Influence of exercise on ascorbic acid status in man. *Clin Sci* **73**: 501-505, 1987.

Goldfarb AG. Antioxidants: role of supplementation to prevent exercise-induced oxidative stress. *Med Sci Sports Exerc* **25**: 232-236, 1992.

Goldfarb AH. Nutritional antioxidants as therapeutic and preventive modalities in exercise-induced muscle damage. *Can J Appl Physiol* **24**: 249-66, 1999.

Goralczyk R, Moser UK, Matter U, Weiser H. Regulation of steroid hormone metabolism requires L-ascorbic acid. *Annal NY Acad Sci* **669**: 349-51, 1992.

Green HN, Medllanby E. Vitamin A as an anti-infective agent. *BJM* **2**: 691-696, 1928

Green RL, Kaplan SS, Rabin BS, Stanitski CL, Zdziarski U, Immune Function in Marathon Runners. *Annal Allerg* **47**: 73-75, 1981.

Greenberger MJ, Strieter RM, Krunkel SL, Danforth JM, Goodman RE, Stadiford TJ. Neutralisation of IL-10 increases survival in a murine model of *Klebsiella pneumoniae. J Immunol* **155**: 722-9, 1995.

Grimble RF. Effect of antioxidative vitamins on immune function with clinical applications. *Int J Vit Nutr Res* **67**: 312-320, 1997.

Guyton AC. Textbook of Medical Physiology, Tenth Edition, WB Saunders Company, 2000.

Hadden JW, Hadden EM, Middelton E. Lymphocytye blast transformation: Demonstration of adrenergic receptors in human peripheral lymphocytes. *Cell Immunol* **1**: 583-595, 1970.

Hagerman FC, Hikida RS, Staron RS, Sherman WM, Costill DL. Muscle damage in Marathon Runners. *Phys Sports Med* **12**: 39-46, 1984.

Hagler l, Herman RH. Oxalate metabolism. *Am J Cl Nutr* **26**: 758-65, 1973.

Halliwell B. Vitamin C: anti-oxidant or pro-oxidant *in vivo*? *Free Rad Res* **25**: 439-454, 1996.

Hallberg L. The role of vitamin C in improving the critical iron balance situation in women. *Int J Vit Nutr Res* **Supp 27**: 177-187, 1985.

Hansen JB, Wilsgaard l, Osterud B. Biphasic changes in leukocytes induced by strenuous exercise. *Eur J Appl Physiol* **62**: 157-161, 1991.

Hanson PG, Flaherty DK. Immunological responses to training in conditioned runners. *Clin Sc* **60**: 225-218, 1981.

Hayono M, Saba RI, Dorfman RI, Hechter O. Some aspects of the biogenesis of adrenal hormones. *Recent Prog Horm Res* **12**: 79-123, 1956.

Heath GW, Ford ES, Craven TE, Macera CA, Jackson KL, Pate RR. Exercise and the incidence of upper respiratory tract infections. *Med Sci Sports Exerc* **23**: 152-157, 1991.

Heath GW, Macera CA, Nieman DC. Exercise and upper respiratory tract infection: Is there a relationship? *Sports Med* **14**: 353-356, 1992.

Heiss F. Unfallverhutung beim Sport. Shorndorf, K Hoffman , pp 17-19, 1971.

Hemilä H. Vitamin C and the common cold. *Br J Nutr* **65**: 3-16, 1992.

Hemilä H. Does Vitamin C alleviate the symptoms of the common cold? A review of the current evidence. *Scand J Infect Dis* **26**: 1-6, 1994.

Hemilä H. Vitamin C intake and susceptibility to the common cold. *J Nutr* 77: 59, 1997.

Hemilä H. Vitamin C and common cold incidence: a review of studies with subjects under heavy stress. *Int J Sports Med* **17**: 379-384, 1996a.

Hemilä H. Vitamin C and Infectious Diseases. In Poaletti R., Sies. H., Bug., J., Grossi E., Poli., (eds) Vitamin C. The state of the art in disease prevention sixty years after the Nobel Prize, Springer-Verslag, Italia, Milano, pp74, 1998.

Hemilä H. Vitamin C Supplementation and Common Cold Symptoms: Problems with Inaccurate Reviews. *Nutr* **12**: 804-806, 1996.

Hemilä H. Vitamin C supplementation and common cold symptoms; factors affecting the magnitude of the benefit. *Med Hypotheses* **52**:171-8, 1999.

Hemilä H. Douglas RM. Vitamin C and acute respiratory infections. *Int J Tubercul Lung Dis* **3:** 756-61, 1999.

Herbaczynska-Cedro K, Wartanowicz M, Panczenko-Kresowska B, Cedro K, Klosiewicz-Wasek B, Wasek W. Inhibitory effect of vitamins C and E on the free radical production in human polymorphonuclear leukocytes. *Europ J Clin Invest* **24**: 316-9, 1994.

Herbaczynska-Cedro K. Supplementation with Vitamin C and E suppresses leucocyte oxygen free radical production in patients with myocardial infarction. *Eur Heart J* 71: 245-9, 1995.

Herbert V. Viewpoint. Does mega- do more good than harm, or more harm than good? *Nutr Today*, 29-32, Jan/Feb 1993.

Herbert V. Pro-oxidant effects of anti-oxidant vitamins. Introduction. *J Nutr* **126**: 1197-2000S, 1996.

Himmelstein SA, Robergs RA, Koehler KM, Lewis SL, Qualls CR. Vitamin C supplementation and upper respiratory tract infections in marathon runners, $JEP_{on\ line}$, **1**(2), 1998.

Hornig D. Requirements of vitamin C in man. *S Afr Med J* **3**: 294-296,1981.

Ho FM, Liu CS, Huang PJ, Shiah SG, Lin-Shiah SY. Nitric oxide prevents apoptosis of human endothelial cells from high glucose exposure during early stage. *J Cell Biochem* **75**: 258-63, 1999.

Horton JW, White DJ, Mass DL, Hybki DP, Hauddek S, Giroir B. Antioxidant vitamin theraiypy alters burn trauma-mediated cardiac NF-kappaB activation and cardiomyocyte cytokine secretion. *J Trauma-Injury, Infect, Crit Care* **50**: 397-406, 2001.

Hughes DA. Effects of dietary antioxidants on the immune function of middle-aged adults. *Proc Nutr Soc* **58**: 79-84, 1999.

Hunt C, Chakaravorty NK, Annan G, Habibzadeh N, Schorah CJ. The clinical effects of vitamin C supplementation in elderly hospitalized with acute respiratory infections. *Int J Vit Nutr Res* **64**: 202-209, 1994.

Klein M. A randomised, controlled trial of Vitamin A in children with severe measles. *New Engl J Med* **323**: 160-164, 1990.

Hurst TL, Bailey DM, Powell JR, Williams C. Immune function changes in downhill running following ascorbic acid supplementation. (Abstract) *Med Sci Sports Exerc*, 2002, *in press.*

Huupponen, MRH, Makinen LH, Sen CK, Rankinen T, Vaisanen S, Rauramao R. The effect of N-Acetylcysteine on exercise induced priming of human neutrophils. A Chemiluminescence Study. *Int J Sports Med* **416**: 399-403, 1995.

Jacob RA, Burri BJ. Oxidative damage and defense. *Am J Clin Nutr* **63**(suppl): 985S-90S, 1996.

Jacob RA, Kelley DS, Pianalto FS. Immunocompetence and oxidant defense during ascorbate depletion of healthy men. *Am J Clin Nutr* **54** (suppl):1302S-9S, 1991.

Janeway CA, Travers P. Immunobiology. The Immune System in Health and Disease (2nd Edition). London. Current Biology Ltd. 1996.

Jariwalla RJ, Harakeh S. Antiviral and immunomodulatory activities of ascorbic acid. *Subcell Biochem* **25**: 215-231, 1996.

Jenkins J. The effect of ascorbic acid on adrenal stroid synthesis *in vitro*. *Endocrinol* **70**: 267-271, 1962.

Jonas E, Dwenger A, Hager A. *In vitro* effect of ascorbic acid on neutrophil-endothelial cell interaction. *J Biolumin Chemilumin* **8**: 15-20, 1993.

Jones RB, Satterlee DG, Cadd GG. Timidity in Japanese quail: Effects of vitamin C and divergent selection for adrenocortical response. *Physiology & Behaviour* **67**: 117-120, 1999.

Kanter M, Nolte L, Holloszky J. Effects of an anti-oxidant vitamin mixture on lipid peroxidation at rest and post-exercise. *J Appl Physiol* **74**: 965-969, 1993.

Kappel M, Hansen MB, Diamant JOL, Jergensen A, Gyhrs A, Pedersen BK. Effects of an acute bolus growth hormone infusion on the human immune system. *Horm Metabol Res* **25**: 579-585, 1993.

Katayama T, Momota Y, Watanaba Y, Kato N. Elevated concentrations of α-tocopherol, ascorbic acid and serum lipids in rats fed polychlorinated biphenyls, chlorobutanol, or phenobarbital. *J Nutr Biochem* **2**: 92-96, 1991.

Kato, N, Momota Y, Kusuhara T. Changes in distribution of α-tocopherol and cholesterol in serum lipoproteins and tissues of rats by dietary PCB and dietary level of protein. *J Nutr Sci Vitaminol* **35**: 655-660, 1989.

Kawai Y, Iwane H, Takanami Y, Shimomitsu T, Katsumura T. Evaluation of anti-oxidant capacity of serum after strenuous endurance exercise. *Med Sci Sports* **28**(5) suppl (abstract): 541, 1996.

Keast D, Arnstein D, Harper W, Fry RW, Morton AR, Depression of plasma glutamine concentration after exercise stress and its possible influence on the immune system. *Med J Aust* **162**: 15-18, 1995.

Keast D, Cameron K, Morton AR. Exercise and the Immune response. *Sports Med* **5**: 248-267, 1988.

Kehrer JP. Free radicals as mediators of tissue injury and disease. *Crit Rev Toxiol* **23**: 20-48, 1993.

Kimbarowski JA, Mokrow NJ. Coloured precipitation reaction of the urine according to Kimbarowski (FARK) as an index of the effect of ascorbic acid during treatment of viral influenza [Fabige Ausfallungsreaktion des Harns nach Kimbarowski (FARK), als Index der Wirkung von Ascorbinsaure bei Behandlung der Virusgrippe] *Deutsche Gesundheitswesen.* **22**(51): 2413-8, 1967.

Kitabchi AE. Ascorbic Acid in steroidgenesis. *Nature* **215**: 1385-1386, 1967.

Kodama M, Kodama T, Murakami M, Kodama M. Vitamin C infusion treatment enhances cortisol production of the adrenal via the pituitary ACTH route. *In Vivo* **8**: 1079-85, 1994.

Kodama M, Inoue F, Kodama T, Kodama M. Intraperitoneal administration of ascorbic acid delays turn-over of 3H-labelled cortisol in the plasma of an ODS rat, but not in the Wistar rat. Evidence in support of the cardinal role of vitamin C in the progression of glucocorticoid synthesis. *In Vivo* **10**: 97-102, 1996.

Kolb E. Some recent discoveries of metabolism and function of ascorbic acid (Review). *Z Gesamte Inn Med* **45**: 205-10, 1990.

König D, Grathwwohl D, Weinstock C, Northoff H, Berg A. Upper respiratory tract infections in athletes: Influence of lifestyle, type of sport, training effects and immunostimulant intake. *Exerc Immunol Rev* **6**: 102-120, 2000.

König D, Wagner C-H, Elmadfa I, Berg A. Exercise and Oxidative Stress: Significance of Antioxidants with reference to inflammatory, muscular and systemic stress. *Exerc Immunol Rev* **7**: 108-133, 2001.

Koz M, Erbas D, Bilgihan A, Aricioglu A. Effects of acute swimming exercise on muscle and erythrocyte malondialdehyde, serum myoglobin and plasma ascorbic acid concentrations. *Can J Physiol Pharmacol* **70**: 1392 - 1395, 1992.

Krause R, Patruta S, Daxbock F, Fladerer P, Biegelmayer C, Wewnisc A. Effect of vitamin C on neutrophil function after high intensity exercise. *EurJ Clin Invest* **31**: 258-63, 2001.

Lambert MI, Dennis SC. Delayed Onset Muscle Soreness: *SA J Sports Med* **1**:18-20, 1994.

Laney PH, Levy JA, Kipp DE. Plasma cortisol and adrenal ascorbic acid levels after ACTH treatment with a high intake of ascorbic acid in the guinea pig. *Ann Nutr Metab* **34**: 85-92, 1990.

Lee WL, Downey GP. Leukocyte elastase: physiological functions and role in acute lung injury. *Am J Resp Crit Care Med* **164**: 896-904, 2001.

Lehr HA, Frei B, Arfors KE. Vitamin C prevents cigarette smoke-induced leukocyte aggregation and adhesion to endothelium *in vivo*. *Proc Natl Acad Sci USA* **91**: 7688-7699, 1994.

Levine M, Duruvala C, Park JP, Rumsey SC, Wang Y. Does vitamin C have a pro-oxidant effect? *Nature* **395**: 231, Sept 1998.

Levine M. New concepts in the biology and biochemistry of ascorbic acid. *N Eng J Med* **314**: 892-901, 1986.

Levine M, Asher A, Pollqrd H, Zinder O. Ascorbic acid and catelcholamine secretion from cultured chromaffin cells. *J Biol Chem* **258**: 1311-5.1983

Lewicki R, Tchorzewski H, Denys A, Kowalska M, Golinska A. Effects of Physical Exercise some parameters of immunity in conditioned sportsmen. *Int J Sports Med* **8**: 309-314, 1987.

Liakakos D, Douglas D, Ikkos C, Anoussakis C, Vlachos P, Jouramani G. Inhibitory effect of ascorbic acid (vitamin C) on cortisol secretion following adrenal stimulation in children. *Clin Chim Acta* **65**: 251-255, 1975.

Liesen H, Kleiter K, Muucke S, Order U, Widenmayer W, Riedel H. Leukozyten und Lymphozyten Subpopulationen bei den Spielen der Feldhockeynationalmanschaft wahrend der Olympiavorbereitung. *Dtsch Z Sportsmed* **40**: 41-52, 1988.

Lindzey J, Korach K. Steroid Hormones. In *Endocrinology: basic and clinical principles*, P Com (ed). Totowa, NY Human Press, pp 49-60, 1997.

Lipscomb HS, Nelson DH. Dynamic changes in ascorbic acid and corticosteroids in adrenal vein blood after ACTH. *Endocrinol* **66**: 1441476, 1960.

Liu ML, Bergholm R, Kimattila S, Lahdenperä S, Valkonen M, Hilden H, Yki-Jarvininen H, Taskinen MR. A marathon run increases the susceptibility of LDL to oxidation in vitro and modifies plasma antioxidants. *Am J Physiol* **276**: E1083-E1091, 1999.

Lloberas N, Torras J, Herrero-Fresneda I, Cruzado JM, Riera M, Hurtado I, Grinyó JM

FASEB J, **10**: 1096, 2002.

MacArthy DA, Dale MM. The leucocytosis of exercise-a review and model. *Sports Med* **6**: 333-363, 1988.

Machlin LJ, Bendich A. Free radical tissue damage: protective role of anti-oxidant nutrients *FASEB J* **1**: 441-445, 1985.

MacKinnon LT, Chick TW, Van As A, Tomasi TB. Decreased secretory immunoblobulins following intense endurance exercise. *Sports Train Med Rehab* **1**: 209-218,1989.

MacKinnon LT, Ginn E, Seymour GJ. Decreased salivary immunoglobin A secretion rate after intense interval exercise in elite kayakers. *Eur J Applied Physiol* **67**: 180-184, 1993a.

MacKinnon LT, Ginn E, Seymour GJ. Temporal relationship between exercise-induced decreases in salivary IgA and subsequent appearance of upper respiratory tract infection in elite athletes. *Aust J Sc Med Sports* **25**: 94-99, 1993b.

MacKinnon LT, Hooper S. Mucosal (Secretory) Immune System responses to exercise of varying intensity and during overtraining. *Int J Sports Med* **15**: S179-183, 1994.

MacKinnon LT, Jenkins DJ. Decreased salivary immunoglobin A after intense interval exercise before and after training. *Med Sci Sports Exerc* **25**: 678-683, 1993.

MacKinnon LT. Advances in Exercise Immunology. Human Kinetics, Champaign, 1999.

MacKinnon LT. Current challenges and future expectations in Exercise Immunology: back to the future. *Med Sci Sports Exerc* **26**: 191- 194, 1994.

MacKinnon LT. Exercise and Natural Killer Cells. What is the relationship? *Sports Med* **7**: 141-149, 1989.

Maderasco EG, Woronick CL, Hickingbotham N, Jacobs L, Bhagavan H. A randomized trial of replacement anti-oxidant vitamin therapy for neutrophil locomotory dysfunction in blunt trauma. *J Trauma* **31**: 1142-1150, 1991.

Mandrup-Paulsen T, Nerup J, Reimers JI, Pociot F, Andersen HU, Karlsen A, Bjerre U, Bergholdt R. Cytokines and the endocrine system. I. The immuneendocrine network. *Eur J Endocrinol* **133**: 660-670, 1995.

Malo C, Wilson JX. Glucose modulates vitamin C transport in adult human small instestinal brush border vesicles. *J Nutr* **130**: 63-9, 2000.

Mars M, Govender S, Weston A, Naidoo V, Chuturgoon A. High intensity exercise: a cause of lymphocyte apoptosis? *Biochem Biophys Res Com* **249**: 366-370, 1998.

Marx C, Ehrhart-Bornstein M, Scherbaum WA, Bornstein SR. Regulation of adrenocortical function by cytokines- relevance for immune-endocrine interaction. *Horm Metab Res* **30**: 41`6-420, 1998.

Maxwell SRJ, Jekeman P, Thomason H. Changes in plasma antioxidant status during eccentric exercise and the effect of vitamin supplementation. *Free Radical Research Commun* **19**: 191-202, 1993.

McKee JS, Harrison PC, Riskowski GL. Effects of supplemental ascorbic acid on the energy conversion of broiler chicks during heat stress and feed withdrawal. *Poulty Sci* **76**: 1278-86, 1997.

Mink KA, Dick EC, Jennings LC, Inhorn SL. Amelioration of rhinovirus colds by vitamin C supplementation. *Proc Int Symp Med Virology*, Anaheim, California, 1987.

Moffat AC, Patterson DA, Currey S, Owen P. Inhibition in vitro of cyclic 3'5'nucleotide phospodiesterase activity by drugs. *Eur J Toxicol* **5**: 160-162, 1972.

Moolla ME. The effect of supplemental anti-oxidants on the incidence and severity of upper respiratory tract infections in ultra-marathon runners. Unpublished M Phil dissertation, University of Cape Town, 1996.

Moore AR, Willoughby DA. The role of cAMP regulation in controlling inflammation. *Clin Exp Immunol* **101**: 387-389, 1995.

Moorthy AV, Zimmerman SW. Human leukocyte responses to an endurance run. *Eur J Appl Physiol* **38**: 271-276, 1978.

Moser U, Bendich A. Vitamin C. In: *Handbook of Vitamins* (2nd edition). Machlin LJ (ed). Marcel Dekker Inc. pp 198-232, 1991.

Moser U, Weber F. Uptake of ascorbic acid by human granulocytes. *Int J Vit Nutr*. Res. **54**: 47-53, 1984.

Moser UK. Physiology and metabolism of ascorbic acid. In: Wenk C, Fenster R, Völker L (eds): Ascorbic Acid in Domestic Animals: Proceedings of the Shecond Symposium, Basel, F. Hoffmann-La Roche, 3-16, 1992.

Moser U. Uptake of ascorbic acid by leukocytes. *Ann N Y Acad Sc* **498:** 200-15, 1987.

Moshage H. Cytokines and the hepatic acute phase response. *J Pathol* **181**: 257-166, 1997.

Mukaida N, Matsumoto T, Yokoi K, Harada A, Matsushima K. Inhibition of neutrophil-mediated acute inflammation injury by an anti-body against interleukin-8 (IL-8). *Inflam Res* **47**: 151-7, 1998.

Müns G. Effect of long distance running on polymorphonuclear neutrophil function of the upper airways. *Int J Sports Med* **15**: 96-99, 1993.

Müns GI, Rubinstein I, Singer P. Neutrophil chemotactic activity is increased in nasal secretions of long distance runners. *Int J Sports Med* **171**: 56-59, 1996.

Nagatomi R, Kaifu T, Okutsu M, Zhang X, Kanemi O, Ohmori H. Modulation of the immune system by the Autonomic Nervous System and its implications in immunonological changes after training. *Exerc Immun Rev* **6**: 54-74, 2000.

Nathan N, Vandoux JC, Feiss P: Role of Vitamin C on adreno-cortical effects of etomidate (French). *Annales Francaises de Anesthesie et de Reanimation* **10**: 329-32, 1991.

Nehlsen-Cannarella SL, Fagoaga OR, Nieman DC, Henson DA, Butterworth DE, Schmidt RL., Bailey EM., Warren BJ., Utter A, Davis JM. Carbohydrate and the cytokine response to 2.5 hours of running. *J Appl Physiol* **82**: 1662-7, 1997.

Nelson-Cannarella SL, Nieman DC, Balk-Lamberton AJ, Markoff PA, Chritton DB, Gusewitch G, Lee JW. The effects of moderate exercise training on immune response, *Med Sci Sports Exerc* **23**: 64, 1991.

Nielsen HB, Secher NH, Christensen NJ, Pedersen BK, Lymphocytes and NK cell activity during repeated bouts of maximal exercise. *Am J Physiol* **271** (Regulatory Integrative comparative Physiology, 40): R 222-R227, 1996a.

Nielsen, HB, Secher NH, Kappel M, Hanel B, Pedersen BK. Lymphocyte, NK and LAK Cell Responses to Maximal Exercise. *Int J Sports Med* **17**: 60-65, 1996b.

Nieman DC, Berk LS, Simpson-Westerberg M, Arabatzis K, Youngberg S, Tan SA, Lee JW, Eby WC. Effects of Long- Endurance Running on Immune System Parameters and Lymphocyte in Experienced Marathoners. *Int J Sports Med* **10**: 317-323, 1989b.

Nieman DC, Henson DA, Butterworth DE, Warren BJ, Davis JM, Fagoaga OR, Nelson-Cannarella SL. Vitamin C supplementation does not alter the immune response to 2.5 hours of running. *Int J Sports Nutr* **7:** 173-84, 1997.

Nieman DC, Henson DA, Gusewitch G, Warren BJ, Dotson RC, Butterworth DE, Nelson-Cannarella SA. Physical activity and immune function in elderly women. *Med Sc Sports Exerc* **25**: 823-831, 1993.

Nieman DC, Henson DA, McAnulty SR, McAnulty L, Swick NS, Utter AC, Vinci DM, Opiela SJ, Morrow JD. Influence of vitamin C supplementation on oxidative and immune changes after an ultramarathon. *J Appl Physiol* **92**: 1970-77, 2002.

Nieman DC, Johansen LM, Lee JW, Arabatzis K. Infectious episodes in runners before and after the Los Angeles Marathon. *J Sports Med Phys Fitness* **30**: 316, 1990a.

Nieman DC, Johansen LM, Lee JW. Infectious episodes in runners before and after a road race. *J Sports Med Phys Fitness* **30**: 289-294, 1989a.

Nieman DC, Miller AR, Henson DA, Warren BJ, Gusewitch G, Johnson RL, Davis JM, Butterworth DE, Herring JL, Nelson Cannarella SL. Effect of high- versus moderate-intensity exercise on lymphocyte subpopulations and proliferative response. *Int J Sports Med* **15**: 199-212, 1994.

Nieman DC, Nehlsen-Cannarella SL, Donohue KM, Chritton DB, Haddock BL, Stout RW, Lee JW. The effects of acute moderate exercise on leukocyte and lymphocyte subpopulations. *Med Sci Sports Exerc* **23**: 578-586, 1991.

Nieman DC, Nehlsen-Cannarella SL, Fagoaga OR, Henson DA, Utter A, Davis JH., Williams F., Butterworth DE. Influence of mode and carbohydrate on the cytokine response to heavy exertion. *Med Sci Sports Exerc* **30**: 671-8, 1998.

Nieman DC, Nehlsen-Cannarella SL, Henson DA, Warren BJ. Granulocyte monocyte phagocytosis, but not oxidative burst is increased after 2.5h of running (Abstract). *Med. Sci. Sports Exerc.*(Suppl) **28**: 191- 194, 1996.

Nieman DC, Nehlsen-Cannarella SL, Markoff PA, Balk-Lamberton H, Yang H, Chritton DBW, Lee JW, Arabatzis K. The effects of moderate exercise training on natural killer cells and upper respiratory tract infections. *Int J Sports Med* **11**: 467-473, 1990b.

Nieman DC, Pedersen BK. Exercise and Immune function. Recent developments. *Sports Med* **27**: 73-80, 1999.

Nieman DC, Peters, EM, Henson DA, Nevines EI, Thompson MM. Influence of Vitamin C supplementation on cytokine changes following an ultramarathon. *J Interferon & Cytokine Res* **20** (11): 1029-35, 2000.

Nieman DC, Simandle S, Henson DA, Warren BJ, Suttles J, Davis JM, Buckley KS, Ahle JC, Butterworth DE, Fagoago OR, Nelson-Cannarella SL. Lymphocyte Proliferate Response to 2.5 hours of Running *Int J Sports Med* **16**: 404-408, 1995.

Nieman DC, Tan SA, Lee JW, Berk LS. Complement and immunoglobin levels in athletes and sedentary controls. *Int J Sports Med* **10**: 124, 1989c.

Nieman DC. Carbohydrates and the Immune Response to prolonged Exertion. In: Nieman D.C., Pederson BK (eds): Nutrition and exercise Immunology, CRC Press, Florida, 2000.

Nieman DC. Exercise, Infection and Immunity. *Int J Sports Med* **15**: S131-9, 1995.

Nieman DC. Exercise, upper respiratory infections and the immune system. *Med Sci Sports Exerc* **26**: 128, 1994.

Nieman DC. Physical Activity, fitness and infection, in Exercise and Health; a concensus of Current Knowledge, Bouchard, C., Ed., Human Kinetics Publishers, Champaign, 1993.

Northhof HC, Wienstock A, Berg A. The cytokine response to strenuous exercise. *Int J Sports Med* **15**: S167-171. 1994.

Nussdorfer GG, Mazzochi G. Immune-endocrine interactions in the mammalian adrenal gland: facts and hypotheses. *Int Rev Cytol* **183**: 143-184, 1998.

Odumosu A. Ascorbic Acid and cortisol metabolism in hypovitaminosis C in guineapigs *Int J Vit Nutr Res* **52**: 176-85, 1982.

Ohno H, Oh-ishi S, Ookawara T, Kizaki T, Miyazaki H, Haga S, Nakao C, Sato Y, Radak, Z: Physical exercise and superoxide dismutases. *Medicina Sportiva* **2**: 219-229, 1998.

Orr CWM. Studies on ascorbic acid. I. Factors influencing the ascorbate-mediated inhibition of catalase. *Biochem* **6**: 2295- 3000, 1967.

Ortega E, Collazos ME, Maynar M, Barriga C, De la Fuente M. Stimulation of the phagocytic function of neutrophils in sedentary men after acute moderate exercise. *Eur J. Appl Physiol* **66**: 60-64, 1993.

Osterback L, Qvarnberg Y. A Prospective Study of Respiratory Infections in 12-year-old children actively engaged in Sports. *Acta Physiol Scand* **76**: 944, 1987.

Ostrowski K, Herman C, Bangash A, Scherling P, Nielsen JN, Pedersen BK. A trauma-like elevation of plasma cytokines in humans in response to treadmill running. *J Physiol* **513**: 889-94, 1998a.

Ostrowski K, Rohde T, Asp S, Schjerling P, Pedersen BK. The cytokine balance and strenuous exercise: TNF-α, IL-1β, IL-6, IL-1ra, sTNF-r2 and IL-10. *J Physiol (London)* **515**: 287-291, 1999.

Ostrowski K, Rohde T, Zacho M, Asp S, Pedersen BK. Evidence that interleukin-6 is produced in human skeletal muscle during prolonged running. *J Physiol (Lond)* **508**: 949-53, 1998b.

Ostrowski K, Rohde T, Asp S, Schjerling P, Pedersen BK. Chemokines are elevated in plasma after strenuous exercise in humans. *Eur J Appl Physiol* **84**: 244-5, 2001.

Otsuka M, Matsuzawa M, Ha TY, Arakawa N. Contribution of a high dose of L-ascorbic acid to carnitine synthesis in guinea pigs fed high fat diets. *J Nutr Sci Vitaminol* **45**: 163-171, 1999.

Packer L. Oxidants, antioxidant nutrients and the athlete. *J Sports Sci* **15**: 353-363, 1997.

Packer L. Oxygen radicals and anti-oxidants in endurance exercise, in Benzi G., Packer L., Silliprandi N. (eds). Biochemical Aspects of physical exercise. Elserivier, Amsterdam, 1986.

Padh H. Cellular functions of ascorbic acid. *Biochem Cell Biol* **68**: 1166-73, 1990.

Pardue SL, Thaxton JP. Ascorbic acid and physiological stress. In Wegger I, Tagwerker FJ, Moustgaard J (eds). Proceedings of Workshop on Ascorbic Acid in Domestic Animals, Copenhagen: The Royal Danish Agriculture Society, 25-31, 1984a.

Pardue SL, Thaxton JP. Evidence for amelioration of steroid-mediated immunosuppression by ascorbic acid. *Poultry Sci* **63**: 1262-1268, 1984b.

Pardue SL, Thaxton JP, Brake J Role of ascorbic acid exposed to high environmental temperature. *J Appl Phys* **58**: 1511-6, 1985a.

Pardue SL, Thaxton JP, Brake J Influence of supplemental ascorbic acid on broiler performance following exposure to high environmental temperature. *Poultry Sci* **64**: 1334-8, 1985b.

Päth G, Bornstein SR, Ehrhart-Bohrnstein M, Scherbaum WA. Interleukin 6 and Interleukin-6 receptor in the Human Adrenal Gland: Expression and effects on Steroidgenesis. *J Cl End Met* **82**: 23443-2349, 1997.

Pauling L. The significance of the evidence about ascorbic acid and the common cold. *Proc Nat Acad Sci* **68**: 2678, 1971.

Pedersen BK, Bruunsgaard H, Klokker M, Kappel M, MacLean DA, Nielsen HB, Rohde T, Ullum H, Zacho M. Exercise induced immunomodulation-possible roles of neuroendocrine and metabolic factors. *Int J Sports Med* **18** (Suppl): S2-7, 1997.

Pedersen BK, Bruunsgaard H, Ostowski K, Krabbe K, Hansen H, Krzywkowski K, Toft A, Sondergaard SR, Petersen EW, Ibfelt T, Schjerling P. Cytokines in Aging and Exercise. *Int. J Sports Med* **21**: S4-S9, 2000.

Pedersen BK, Ostowski K, Rohde T, Bruunsgaard H. The cytokine response to strenuous exercise. *Can J Physiol Pharm* **76**: 505-11, 1998b.

Pedersen BK, Tvede N, Christensen LD. Klarlund K, Kragbag S, Halkaejr-Kristenen J. Natural killer cell activity in peripheral blood of trained and untrained persons. *Int J Sports Med* **10**: 129-131, 1989.

Pedersen BK, Ullum H. N K Response to Physical Activity: possible mechanisms of action. *Med Sci Sports Exerc* **26**: 140, 1994.

Pederson BK, Tvede N, Klarlund K, Christensen LD, Hanse F R, Galbo H, Kharazmi A, Halkjer-Kristenen J. Indomethacin in vitro and in vivo abolishes post-exercise suppression of natural killer cell activity in peripheral blood. *Int J Sports Med* **11**: 127-132, 1990.

Pederson BK. Rohde T. Ostowski K. Recovery of the Immune System after exercise. *Acta Physiologica Scand* **162**: 325-332, 1998a.

Pederson EW, Ostowski K, Ibfedt T, Richelle M, Offord E, Halkjer-Kristenen J, Pederson BK Effect of vitamin supplementation on cytokine response and on muscle damage after strenuous exercise. *Am J Phyiol Cell Physiol* **289**: 1570-5, 2001.

Peters EM, Anderson R, Nieman DC, Fickl H, Jogessar V. Vitamin C supplementation attenuates the increase in circulating cortisol, adrenaline and anti-inflammatory polypeptides following ultramarathon running. *Int J Sports Med* **22**: 537—543, 2001b.

Peters EM, Anderson R, Theron AJ. Attenuation of the increase in circulating cortisol and enhancement of the acute phase response in vitamin C-supplemented ultramarathon runners. *Int J Sports Med* **22**: 120-126, 2001a.

Peters EM, Bateman ED. Ultramarathon running and upper respiratory tract infections. *S Afr Med J* **64**: 582-584, 1983.

Peters EM, Campbell A, Pawley L. Vitamin A fails to increase resistance to upper respiratory infection in distance runners. *S Afr J Sports Med* **7**: 3-7, 1992.

Peters EM, Goetzsche JM, Grobbelaar B, Noakes TD. Vitamin C supplementation reduces the incidence of post-race symptoms of upper respiratory tract infection in ultradistance runners. *Am J Cl Nutr* **57**: 170, 1993.

Peters EM, Goetzsche JM, Joseph LE, Noakes TD. Vitamin C as effective as combinations of anti-oxidant nutrients in reducing the incidence of upper respiratory tract infections in ultradistance runners. *S Afr J Sports Med* **4**: 23-27, 1996.

Peters EM. Exercise, immunology and upper respiratory tract infections. *Int J Sports Med* **18**: S69, 1997.

Peters EM. Altitude fails to increase susceptibility of ultramarathon runners to upper respiratory tract infections. *S Afr J Sports Med* **5**: 4-8, 1990.

Peters EM. Anti-oxidant nutrient supplementation and prolonged exercise. *The Leech* **62**(2), 1993.

Peters EM. Exercise induced toxicity...is anti-oxidant supplementation the answer? *Micronutrients*, May, 1996.

Peters-Futre EM. Exercise, Vitamin C and Phagocyte Function. The Missing Link. *Exerc Immunol Rev* **3**: 32-52, 1997.

Petrova IV, Kuzmin SN, Kurashakova TS, Suzdailnitskii RS, Perskin BB. Neutrophil phagocytic activity and the humeral factors of local immunity under intensive physical loading. Zhurnal Mikrobiologii *Epidermiologii Immunobiologii* **12**: 53-57, 1983.

Pettipher ER, Labasi, JM, Salter ED, Stam EJ, Cheng JB, Griffiths RJ. Regulation of tumour necrosis factor production by adrenal hormones *in vivo*: insights into the anti-inflammatory activity of rolipram. *Brit J Pharmacol* **117**: 1530-1534, 1996.

Pincemail J, Camus G, Roesgen A, Dreezen E, Bertrand Y, Lismonde M, Deby-Dupont, C. Deby C. Exercise induces pentane production and neutrophil activation in humans. Effects of propanolol. *Europ J Appl Physiol* **61**: 319- 322, 1990.

Pitt H A, Costrini A. M. Vitamin C prophylaxis in marine recruits. *JAMA* **241**: 908-911, 1979.

Pizza FX, Cavender D, Stockard A, Baylies H, Beighle A. Anti-inflammatory doses of ibuprofen: effect on neutrophils and exercise induced muscle injury. *Int J Sports Med* **20**: 98-102, 1999.

Pizza FX, Flynn MG, Boone JB, Rodriguez-Zayas JR, Andres FF. Serum haptoglobin and ferritin during a competitive running and swimming season. *Int J Sports Med* **18**: 233-7, 1997.

Pizza FX, Mitchell JB, Davis BH, Starling RD, Holtz RW, Bigelow N. Eexercise-induced muscle damage: effect on circulating leukocyte and lymphocyte subsets. *Med Sci Sports Exerc* **27**: 363-70, 1995.

Playfair JHL. Immunology at a Glance. Blackwell Scientific Publications, Oxford, 1979.

Podmore ID, Grriffiths HR, Herbert KE, Minstry N, Minstry P, Lunec J. Pro-oxidant effect of Vitamin. *Nature* **392**: 559, April 9, 1998.

Poulsen HE, Weiman A, Salonen KN, Loft S, Cadet J, Douki T, Ravanat JL. Does Vitamin C have a pro-oxidant effect? *Nature* **395**: 231, September, 1998.

Pyne DB, Baker MS, PA Fricker, McDonald WA, Telford RD, Weideman MJ. Effects of an intensive 12 wk training program by elite swimmers on neutrophil oxidative activity. *Med Sci Sports Exerc* **27**: 536-542, 1995.

Pyne DB, Baker MS, Telford RD, Weideman MJ. Neutrophil oxidative activity is differentially effected by moderate and intense interval exercise. *Med Sci Sports Exerc* **25**: S102, 1993.

Pyne DB, Gleeson M, Effects of intensive exercise training on immunity in athletes. *Int J Sports Med* **19** (Supp3): S183, 1998.

Pyne DB, McDonald WA, Gleeson M, Flanagan A, Clancy RL, Fricker PA. Mucosal Immunity, respiratory illness, and competitive performance in elite swimmers. *Med Sci Sports Exerc* **33(3)**: 348-53, 2000.

Pyne DB. Regulation of neutrophil function during exercise. *Sports Med* **17**: 245-58, 1994.

Rahman I, MacNee W. Role of transcription factors in inflammatory lung diseases. *Thorax* **53**: 601-612, 1998.

Redmann A, Mobius K, Hiller HH, Oelkers W, Bahr V. Ascorbate depletion prevents aldosterone stimulation by sodium deficiency in the guinea pig. *Eur J Endocrinol* **133**: 499-506, 1995.

Reid MR, Drummond PD, MacKinnon LT. The effect of moderate aerobic exercise and relaxation on secretory Immunoglobulin A. *Int J Sports Med* **22**: 132-137, 2001.

Ritzel G. Critical analysis of the role of Vitamin C in the prophylaxis and treatment of the common cold (In German) *Helvetica Medica Acta* **28**: 63-68, 1961.

Ritzel G. Ascorbic acid and the common cold (letter) *JAMA* **235**:1108, 1976.

Robertson JD, Maughan RJ, Duthie GG, Morrice PC. Increased blood antioxidant systems of runners in response to training load. *Clin Sc* **80**: 611-618, 1991.

Rivers, JM. Safety of high level vitamin C ingestion. *Ann NY Acad Sci* **498**: 445-454, 1987.

Robson PJ, Blannin AK, Walsh NP, Castell LM, Gleeson M. Effects of exercise intensity, duration and recovery on in vitro neutrophil function in male athletes. *Int J Sports Med* **20**: 128-35, 1999.

Rodriquez AB, Barriga C, de LaFuente M. Phagocytic function of blood neutrophils in sedentary young people after physical exercise. *Int J Sports Med* **12**: 276-280, 1991.

Rokitzki L, Hinkel S, Klemp C, Cufi D, Keul J. Dietary, serum and urine ascorbic acid status in male athletes. *Int J Sports Med* **15**: 435-440, 1994a.

Rokitzki L, Logeman E, Sagredos AN, Murphy M, Wetzel-Roth W, Keul J: Lipid peroxidation and antioxidant vitamins under extreme endurance stress. *Acta Physiol Scand* **151**: 149-58, 1994b.

Ryan AJ, Darymple W, Dull BH, Kaden W, Lerman SJ. Round Table, upper respiratory tract infections in sports. *Phys Sports Med* **3**: 29, 1975.

Sabiston BH, Radomski MW. Health problems and vitamin C in Canadian Northern Military Operations. DCIEM Report NO 74-R-1012. Downsvew, Ontario, Defence Research Board, 1974.

Sagone AL, Kamps S, Campbell R. *Photochem Photobiol* **28**: 909-915, 1987.

Salonen JT, Nyyssonen K, Korpela H, Tuomilehto J, Seppanen R, Salonen R. High stored iron levels are associated with excess risk of myocardial infarction in Eastern Finnish men. *Circulation* **2**: 803, 1992.

Sanchez-Quesada JL, Jorba O, Payes A, Otal C, Serra-Grima R, Gonzalez-Sastre F, Ordonez-Llanos J. Ascorbic acid inhibits the increase in low-density lipoprotein (LDL) susceptibility to oxidation and the proportion of electronegative LDL induced by intense aerobic exercise. *Coron Artery Dis* **9**: 249-55, 1998.

Satterlee DG, Aquilera-Quintana I, Munn BJ, Krautmann BA. Vitamin C amelioration of the adrenal stress response in broiler chickens being prepared for slaughter. *Comp Biochem Physiol* **94**: 569-574, 1989.

Satterlee DG, Jones RB, Ryder FH. Effects of ascorbyl-2-polyphosphate on adrenocortical activation and fear-related behaviour in broiler chickens. *Poultry Sci* **73**: 194-201, 1994.

Satterlee DG. Ascorbic acid and physiological stress in poultry. In: Wenk G, Fenster R, Völker R(eds). Ascorbic Acid in Domestic Animals: Proceedings of the Second Symposium. Basel: F.Hoffmann-La Roche, 1992: pp 43-59.

Saywer DW, Donowitz GR, Mandell GL. Polymorphonuclear neutrophils: an effective antimicrobial force. *Rev Infect Dis* **11**: S1532-S1544, 1989.

Schane JA, Johnson SR, Vandenakker CB, Delayed Onset muscle soreness and plasma CK and LDH activities after downhill running. *Med Sci Sports Exerc* **15**: 51-56, 1983.

Schmidt KA, Hagmaeier V, Hornig DH, Vulleumier JP, Rutishauser G. Urinary oxalate excretion after large intakes of ascorbic acid in man. *Am J Cl Nutr* **34**: 305-311, 1981.

Schmidt KA, Steinhilber D, Moser U, Roth HJ. L-ascorbic acid modulates 5-lipogenase activity in human polymorphonuclear leukocytes. *Int Ach Allergy Appl Immunol* **85**(4), 441-5, 1988.

Schouten WJ, Vershuur R, Kemper HCG. Habitual physical activity, strenuous exercise, and salivary immunoglobin A levels in young adults: The Amsterdam Growth and Health Study. *Int J Sports Med* **9**: 289-293, 1988.

Schouten WJ, Vershuur R, Kemper HCG. Physical activity and Upper Respiratory Tract Infections in a normal Population of young men and women: The Amsterdam Growth and Health Study. *Int J Sports Med* **9**: 451-5, 1988.

Schröder H, Navarro E, Tramullas A, Mora J, Galiano D. Nutrition anti-oxidant status and oxidative stress in professional basketball players: effects of a three compound anti-oxidative supplement. *Int J Sports Med* **21**: 146-50, 2000.

Schwager J, Schulze J. Influence of ascorbic acid on the response to mitogens and interleukin production of porcine lymphocytes. *Int J Vit & Nutr Res* **67**: 10-6, 1997a.

Schwager J, Schulze J. Dependence of growth, bone metabolism and functions of polymorphonuclear leukocytes on ascorbic acid in pigs. *Int J Vit & Nutr Res* **68**: 48-58, 1997b.

Schwager J, Schulze J. Modulation of interleukin production by ascorbic acid. *Vet Immunol Immunopathol* **64**: 45-57, 1998.

Schwellnus M, Kiesig M, Derman W, Noakes TD. Fusafungine reduces symptoms of upper respiratory tract in runners after a 56 km race. *Med Sci Sports Exerc* **29**: S296, 1997.

Sen CK. Oxidants and Anti-oxidants in Exercise. *J Appl Physiol* **79**: 675-86, 1995.

Senecsko F. White blood cell count and adherence in sportsmen and non-training subjects. *Acta Physiol. Pol* **34**: 5-6, 1983.

Shek PN, Sabiston BH, Burguet A, Radomski MW. Strenuous Exercise and Immunological Changes. A multiple- Time Point Analysis of Leukocyte Subsets, CD4/CD8 Ratio, Immunoglobulin Production and NK Response. *Int J Sports Med* **16**: 466-474, 1995.

Shephard RJ, Shek PN. Impact of physical activity and sport on the immune system. *Rev Environ Health* **11**: 133, 1996.

Shephard RJ, Shek PN. Heavy Exercise, Nutrition and Immune Function: Is there a connection? *Int J Sports Med* **16**: 491-496, 1995.

Shephard RJ. Rhind S, Shek PN. Exercise and the Immune system. Natural Killer Cells, Interleukins and Related Responses. *Sports Med* **18**(5): 340-369, 1994.

Shilotri PG, Bhat KS. The effect of mega doses of vitamin C on bacteriocidal activity of leukocytes. *Am J Cl Nutr* **30**: 1077, 1977.

Shimuzi K. Effects of ascorbic acid on the cholesterol side chain cleavage system in the rat ovary. *Biochem J* **103**: 16-17, 1967.

Siegel BV, Leibovitz B. The Multifactorial Role of Vitamin C in Health and Disease. In: Hanck A (ed). Vitamin C. New Clinical Application in immunology, lipid metabolism and cancer. Bern, Hans Huber Publishers, pp 9-21, 1982.

Siegel BV, Morton JI. Vitamin C and the immune response. *Experientia* **33**: 393-394, 1971.

Sies H. Oxidative stress: Introduction. In: Sies H(ed). Oxidative stress: Oxidants and Anti-oxidant. New York: Academic Press, 1991.

Siushansian R, Tao L, Dixon SJ, Wilson JX. Cerebral astrocytes transport ascorbic acid and dehydroascorbic acid through distinct mechanisms regulated by cyclic AMP. *J Neurochemistry* **68**(6): 2378-85, 1997.

Skinkai S, Watanabe S, Asai H, Shek PN. Cortisol response to exercise and post-exercise suppression of blood lymphocyte subset counts. *Int J Sports Med* **17**: 597-603, 1996.

Smith JW, Telford RD, Mason IB, Weiderman MJ. Exercise, training, and neutrophil microbicidal activity. *Int J Sports Med* **11**: 179-187, 1990.

Smith JW, Weidermann MJ. The exercise and immunity paradox: A neuroendocrine/cytokine hypothesis. *Med Sci Res* **18**: 749-753, 1990.

Sorichter S, Puschendorf B, Mair J. Skeletal muscle injury induced by eccentric muscle action. Muscle proteins as markers of muscle fibre injury. *Exerc Immunol Rev* **5**: 5-21, 1999.

Sotiriou S, Gispert S, Cheng J, Wang Y, Chen A, Hoogstraten-Miller S, Miller GF, Kwon O, Levine M, Guttentag SH, Nussbaum RL. Ascorbic-acid transporter S1c23a1 is essential for vitamin C transport into the brain and for perinatal survival. *Nat Med* **8**: 445-5, 2002.

Sprenger H, Jacobs C, Nain M, Gressner AM, Prinz H, Weserman W, Gemsa D. Enhanced release of cytokines, interleukin-2 receptors, and neopterin after long-distance running. *Clin Immunol Immunopathol* **63**: 188-195, 1992.

Stanislaw P, Klapcinska B. Effectiveness of supplementation of physically active male subjects with anti-oxidant vitamins. *Medicina Sportiva* **4**: E98, 2000.

Stöckl J, Vetr H, Majdic O, Zlabinger G, Keuchler E, Knapp W. Human major group rhinoviruses downmodulate the accessory function of monocytes by inducing Il-10. *J Clin Invest* **104**: 957-965, 1999.

Strachan AF, Noakes TD, Kotzenberg G, Nel AE, de Beer FC. C-Reactive Protein during long distance running. *Brit Med J* **289**: 1249-1251, 1984.

Sulimovici S, Boyd GS. The effect of ascorbic acid *in vitro* on the rat ovarian cholesterol side chain cleavage enzyme system. *Steroids* **12**: 127-49, 1968.

Sumbaev VV, Iasinskaia IM. Effect of ascorbic acid on uric acid formation in men and animals. *Ukrainskii Biokhimicheskii Zhurnal* **69**: 116-20, 1997.

Suzuki K, Totsuka M, Nakaj S, Yamada M, Kudo S, Liu Q, Sugawara K, Yamaya K, Sato K. Endurance exercise causes interaction among stress hormones, cytokines, neutrophil dynamics, and muscle damage. *J Appl Physiol* **87**: 1360-1367, 1999.

Suzuki K, Yamada M, Kurakake S, Okamura N, Yamada K, Liu Q, Kudoh S, Kowatari K, Sugawara K. Circulating cytokines and hormones with immunosuppressive but neutrophil-priming potentials rise after endurance exercise in humans. *Eur J Appl Physio* **81**: 281-7, 2000.

Suzuki K, Nnaganuma S, Totsuka M, Suzuki K.-J, Mochizuki M, Shiraishi M, Nakaji S, Sugawara K. Effects of Exhaustive Endurance Exercise and its One-week Daily Repetition on Neutrophil Count and Functional Status in Untrained Men. *Int J Sports Med* **17**: 205-212, 1996.

Tabata I, Ogita M, Miyachi M, Shibayama H. Effect of low blood glucose on plasma CFR, ACTH, and cortisol during prolonged physical exercise. *J Appl Physiol* **71**: 1807-1812, 1991.

Taga K, Cherney B, Tosato G. IL-10 inhibits apototic cell death in human T cells starved of IL-2. *Int Immunol* **5**: 1599-608, 1993.

Taylor C, Rodgers G, Godma C, Baynes RD, Bothwell TH, Bezwoda WR, Kramer F, Hattingh J. Hematologic iron-related, and acute phase protein responses to sustained strenous exercise. *J Appl Physiol* **62**: 464-469, 1987.

Thompson D, Williams M, Kingsley M, Nicholas CW, Lakomy F, McArdle F, Jackson MJ. Muscle soreness and damage parameters after prolonged intermittent shuttle-running following acute vitamin C supplementation. *Int J Sports Med* **22**: 68-75, 2001a

Thompson D, Williams C, McGregor SJ, Nicholas CW, McArdle F, Jackson MJ, Powell JR. Prolonged vitamin C supplementation and recovery from demanding exercise. *Int J Sports Nutr Exerc Met* **11**: 466-481, 2001b

Tolbert BM. Metabolism and function of ascorbic acid and its metabolites. *Int J Vit Nutr* Res **27**: 121-132, 1985.

Tomasi TB, Trudeau FB, Czerwinski D, Erredge S: Immune parameters in athletes before and after strenuous exercise. *J Clin Immunol* **2**: 173-178, 1982.

Van Antwerpen L, Theron AJ, Meyer MS, Richards GA, Wolmarans L, Booysen U, Van Der Merwe CA, Sluis –Cremer CK, Anderson R. Cigarette smoke-mediated oxidant stress, phagocytes, vitamin C, vitamin E, and tissue injury. *Ann N Y Acad Sc* **686**: 53-645, 1993.

Van der Poll T, Coyle SM, Barbosa CC, Lowry SF. Epinephrine inhibits tumour necrosis factor -α and potentiates interleukin 10 production during human endoxemia. *J Clin Invest*

97: 713-719, 1996.

Vasankari TJ, Kujala U, Sarna S, Ahotupa M. Effects of ascorbic acid and carbohydrate ingestion on exercise induced oxidative stress. *J Sports Med & Phys Fitness* **38**: 281-5, 1998.

Vasankari TJ, Kujala U, Vasankari TM, Ahotupa M. Reduced oxidised LDL levels after a 10 month exercise program. *Med Sc Sports Exerc* **30**: 1496-1501, 1998.

Vasankari TJ, Kujala U, Vasankari TM, Vuorimaa T, Ahotupa M. Increased serum and low-density-lipoprotein anti-oxidant potential after anti-oxidant supplementation in endurance athletes. *Am J Cl Nutr* **65**: 1052-6, 1997.

Viguie C, Frei B, Shigenega M, Ames B, Packer L, Brooks G. Antioxidant status and indices of oxidative stress during consecutive days of exercise. *J Appl Physiol.* **75**: 566-72, 1993.

Von Weiss M, Fuhrmansky J, Lulay R, Weiker H. Haufigkeit und Ursache von Immunoglobinmangel bei Sportlern. *Dtsch Z Sportsmedizin* **36**: 146-153, 1985.

Wagmer PG, Jorgenson MS, Arden WA, Jackson BA, Stimulus secretion coupling in porcine adrenal chromaffin cells: acute effects of glucocorticoids. *J Neuroscience Res* **57**: 643-50, 1999.

Walsh NP, Blannin AK, Clark AM, Cook L, Robson PJ, Gleeson M. The effects of high-intensity intermittent exercise on saliva IgA, total protein and alpha-amylase. *J Sports Sci* **17**: 129-34, 1999.

Warhol MJ, Siegel AJ, Evans WJ, Silverman LM. Skeletal muscle injury and repair in marathon runners after competition. *Am J Path* **118**: 331-339, 1985.

Washko P, Rotrosen D, Levine M. Ascorbic acid in human neutrophils. *Am J Clin Nutr* **54** (suppl): 1221S-7S, 1991.

Washko P, Rotrosen D, Levine M. Ascorbic acid transport and accumulation in human neutrophils. *J Biol Chem* **264:** 18996-19002, 1989.

Weight LM, Alexander D, Jacobs P. Strenuous Exercise: Analogous to the acute phase response? *Clin Sci* **81**: 677-683, 1991.

Weinstock C, König D, Harnischmacher R, Keul J, Berg A, Northoff H. Effect of exhaustive exercise stress on the cytokine response. *Med Sci Sports Exerc* **29**: 345-54, 1997.

Weiss M, Schneider EM, Tarnow J, Mettler S, Krone M, Teschemacher A., Lemoine H. Is inhibition of oxygen radical production of neutrophils by sympathomimetics mediated via beta-2 adrenoreceptors? *J Pharmacol Exp Therapeut* **278**: 1105-1113, 1996.

Weiss SJ. Tissue Destruction by Neutrophils. *New Eng J Med* **320**:365-376, 1989.

Wolf G. Uptake of ascorbic acid by human neutrophils. *Nutr Rev* **51**: 337-8. 1993.

Wong CW, Smith SE, Thong YH, Opdebeeck JP, Thorton JR. Effects of Exercise stress on various immune functions in horses. *Am J Vet Res* **53**: 1414-1417, 1992.

Yamada M, Suzuki K, Kudo S, Totsuka M, Simoyama T, Nakaji S, Sugawara K. Effect of exhaustive exercise on human neutrophils in athletes. *Luminescence* **15**:15-20, 2000.

Zembron-Lacvny A, Szyszka K. Exercise modification of the pro-oxidant-anti-oxidant equilibrium. *Medicina Sportiva* **4**: 247-255, 2000.

Zoschke DC, Messner RP. Suppression of human lymphocyte mitogenesis mediated by phagocyte-released reactive oxygen-species: comparative activities in normals and in

chronic granulomatous disease. *Clin Immunol Immunopathol* **32**: 29-40, 1984.