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*

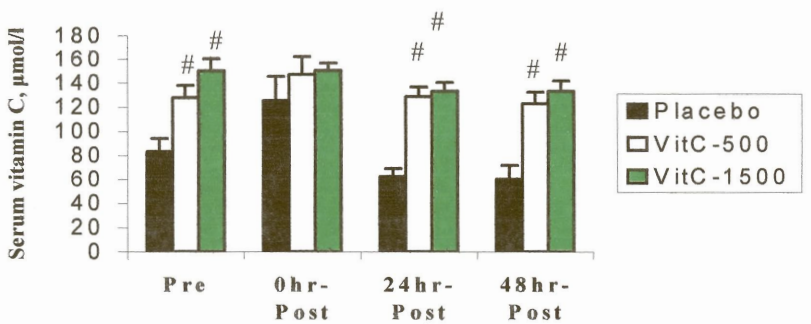

Figure 7.1: Mean (± SEM) serum Vitamin C concentrations before and after participation in a 90 km ultramarathon. # p<0.01, two- tailed Wilcoxon test vs placebo group at this time point.

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 ($p<0.01$).

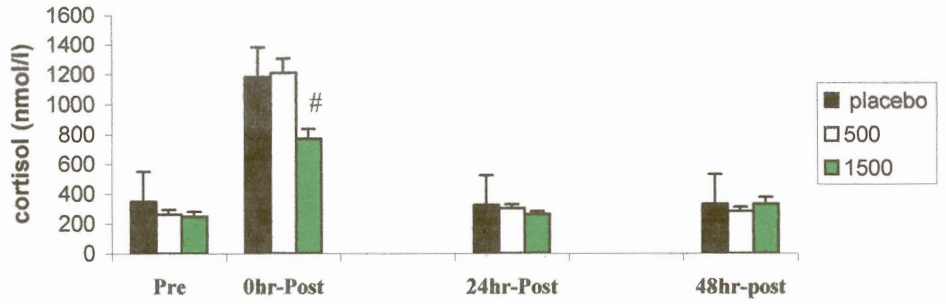

Figure 7.2: Mean (± SEM) serum cortisol concentrations before and after participation in a 90 km ultramarathon. # p<0.01, Wilcoxon test between groups at time point

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*

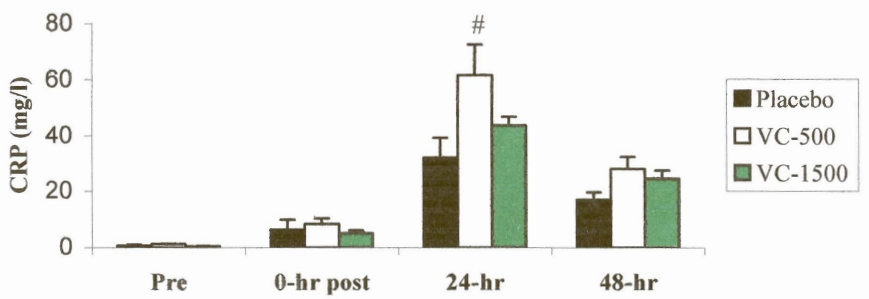

Figure 7.3: Mean (±SEM) serum CRP concentrations before and after participation in a 90 km ultramarathon # $p<0.01$, Wilcoxon test, vs placebo at time point.

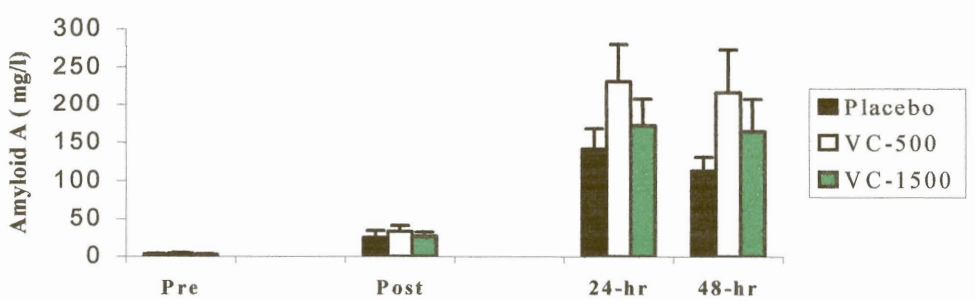

Figure 7.4: Mean (±SEM) serum amyloid A concentrations before and after participation in a 90 km ultramarathon.

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).**

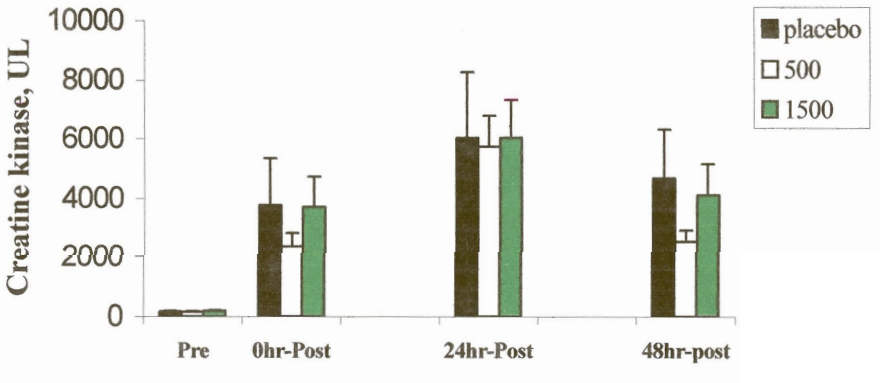

Figure 7.5: Mean (±SEM) serum creatine kinase concentrations before and after participation in a 90 km ultramarathon

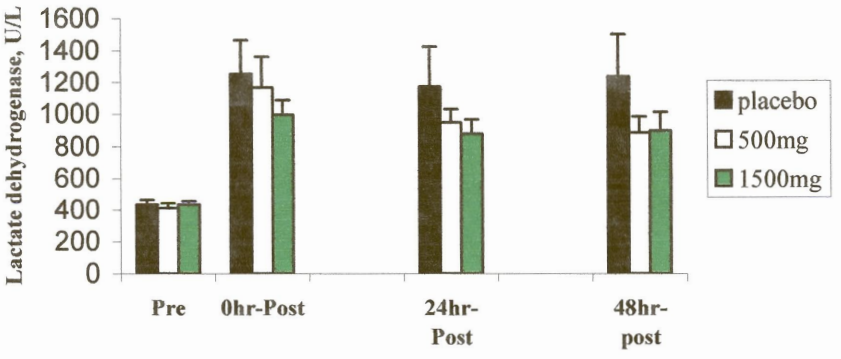

Figure 7.6: Mean ± serum lactate dehydrogenase concentrations before and after participation in a 90 km ultramarathon

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.