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.

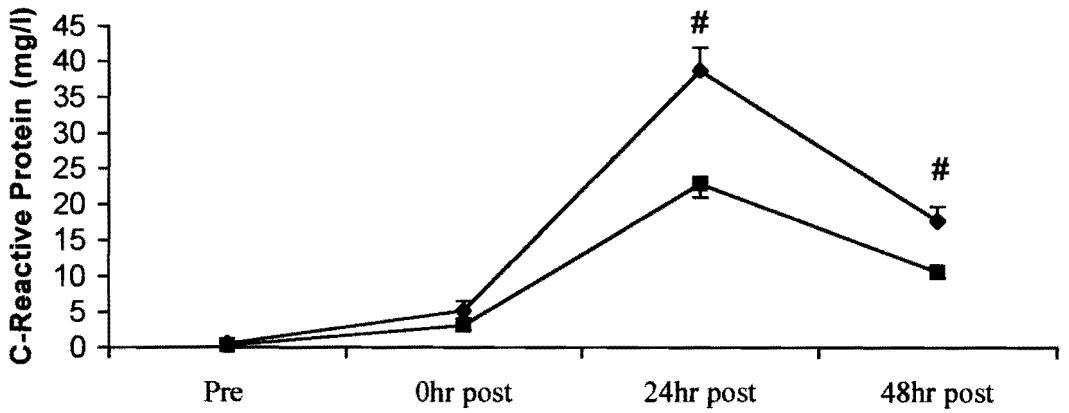

Figure 4.1: Mean (±SEM) serum concentrations of CRP prior to and at varying times (0, 24 and 48 hours) after completion of the ultramarathon race for the ■ P and ◆vitamin C-supplemented groups. #$p<0.05$ vs P.

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.

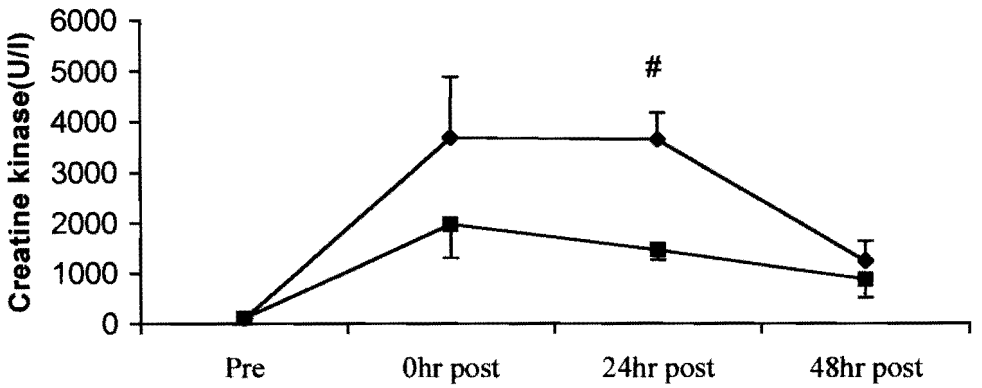

Figure 4.2: Mean (±SEM) serum concentrations of creatine kinase prior to and at varying times (0, 24 and 48 hours) after completion of the ultramarathon race for the ■ P and ◆vitamin C-supplemented groups. #p<0.05 vs P.

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).

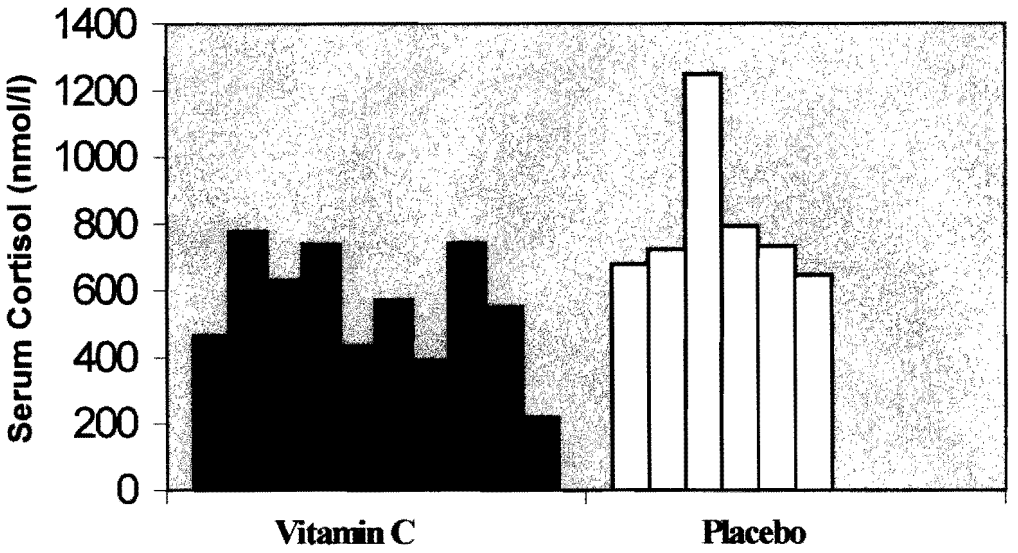

Figure 4.3: Individual 0-hr post-race serum cortisol concentrations (adjusted for base-line concentrations) of the runners in vitamin C and placebo groups.

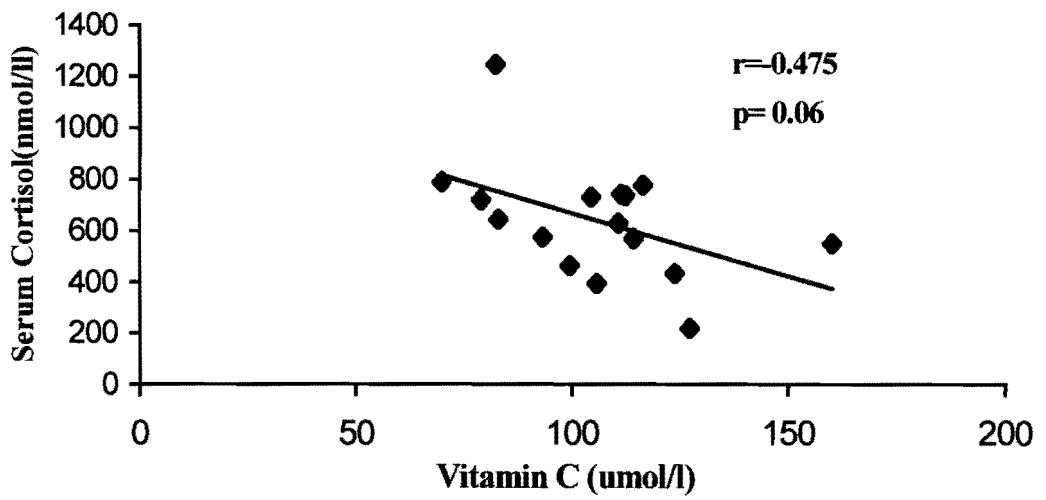

Figure 4.4: The association between pre-race serum vitamin C and immediate post-race serum cortisol (corrected for base-line values) concentrations.

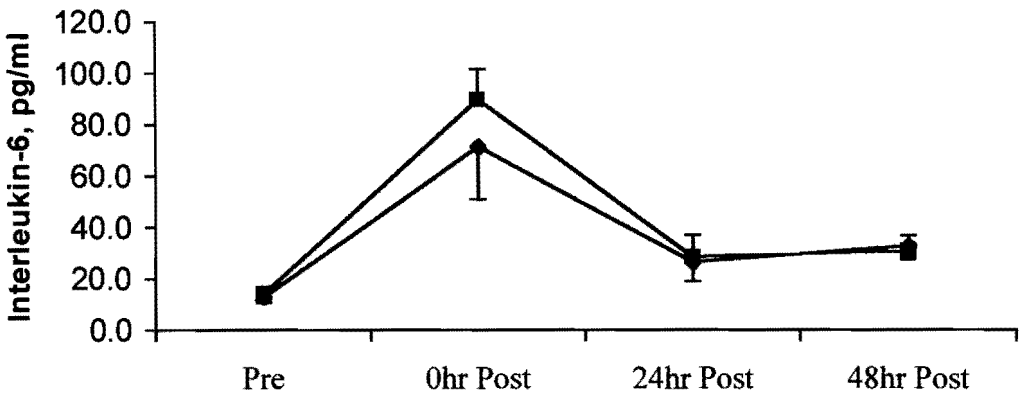

Figure 4.5: Mean (±SEM) plasma concentrations of interleukin-6 prior to and at varying times (0, 24 and 48 hours) after completion of the ultramarathon race for the ■ control and ◆ vitamin C-supplemented groups.

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.
