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ORIGINAL ARTICLE

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The effect of 2 weeks vitamin C supplementation on immunoendocrine responses to 2.5 h cycling exercise in man

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Abstract An increased systemic concentration of stress hormones (of the hypothalamic-pituitary adrenal axis) and some cytokines may contribute to the depression of immune cell function typically observed after prolonged exercise. The aim of the present study was to determine the effect of 2 weeks of supplementation with vitamin C (VC) on cortisol, adrenocorticotrophic hormone, interleukin-6, oxidative stress and neutrophil responses to a single bout of endurance exercise. Nine healthy endurance-trained males exercised for 2.5 h at 60% $\dot{V}O_{2max}$ after 2 weeks of placebo (PLA) or VC (1,000 mg day^{-1}) supplementation. All participants completed both trials utilising a randomised crossover design with a minimum 14 day washout period between trials. There was a significant trial × time interaction effect for plasma cortisol concentration ($P = 0.039$) which tended to be lower in the VC trial but post hoc analysis found no specific between trial differences. There was a significantly lower post-exercise neutrophilia ($P < 0.014$) in the VC trial, compared with the PLA trial. There was no trial × time interaction for measures of neutrophil function (bacteria-stimulated elastase release, fMLP or PMA-stimulated oxidative burst). However, there was a trend for higher fMLP-stimulated neutrophil oxidative burst in the VC compared with PLA trial (trial × time interaction, $P = 0.075$). These results suggest that supplementation with VC for a period of up to 2 weeks provides little to no protection against the depression of neutrophil function which typically occurs after endurance exercise.

Keywords Ascorbate · Neutrophil · Stress hormone · Cytokine · Oxidative burst

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Introduction

The incidence of infection of the upper respiratory tract may be increased in the weeks following acute bouts of heavy exertion or long-duration exercise (Bishop 2006; Nieman et al. 1990; Peters et al. 1993, 1996). Daily supplementation with large doses of vitamin C (VC) (for 7 days) reduced infection incidence following ultramarathon races (Peters et al. 1993, 1996). Increased concentrations of stress hormones of the hypothalamic-pituitary adrenal (HPA) axis (such as cortisol), adrenaline and some cytokines may contribute to the depression of immune cell function that is typically observed following prolonged exercise (Davison et al. 2005a; Li et al. 2004, 2005; Morozov et al. 2003; Nieman et al. 1997; Scharhag et al. 2002). Therefore, antioxidants possibly influence immune cell function (and thus infection incidence) by reducing the exercise-induced release of HPA stress hormones (Fischer et al. 2004; Peters et al. 2001a, b) and/or exercise-induced oxidative stress (Peters et al. 1997a, b; Robson et al. 2003). Natural antioxidant defences are enhanced in endurance-trained individuals but may still fail to prevent transient increases in oxidative stress following acute bouts of long-duration exercise (Packer 1997; Vollaard et al. 2005). However, it has been suggested that oxidative stress has little influence on immunoendocrine changes during and after prolonged exercise (Nieman et al. 1997, 2002) and that antioxidant supplementation may sometimes promote exercise-induced oxidative stress (Nieman et al. 2004; Peake 2003).

There is convincing evidence showing that daily supplementation with antioxidant mixtures for up to 2 months can significantly reduce the cortisol and interleukin-6 (IL-6) responses to prolonged exercise (Fischer et al. 2004; Vassilakopoulos et al. 2003). According to Fischer et al. (2004) IL-6 could play an important role in this process since its release from contracting skeletal muscle during exercise is reduced with antioxidant supplementation and this cytokine is

known to stimulate an increase in systemic cortisol concentration (Steensberg et al. 2003). Therefore, it may be that antioxidants actually attenuate the cortisol response to exercise by an inhibitory effect on IL-6 release. However, Nieman et al. (2004) found no effect on cortisol and greater IL-6 responses following a competitive triathlon event in athletes that were consuming vitamin E, compared with placebo, supplements daily for 2 months prior to the event.

It is also possible that increased levels of cortisol and some cytokines (e.g. IL-6, G-CSF) may have an indirect effect on neutrophil function because they contribute to exercise-induced leukocytosis (Cupps et al. 1982; Yamada et al. 2002). Increased leukocytosis (and consequently neutrophilia) may lead to immature neutrophils constituting a greater proportion of total blood neutrophils. Neutrophils which are immature or have been released from the bone marrow prematurely appear to have a lower capacity to respond to stimulation and to produce reactive oxygen species (ROS) (Berkow et al. 1986). Therefore, a lower cortisol, IL-6 and leukocytosis response to exercise may be associated with a smaller depression in immune cell (neutrophil) function. Furthermore, improved antioxidant defence may modulate the post-exercise decrease in neutrophil function by protecting these cells from oxidative attack and damage (Robson et al. 2003). Although it is now well established that antioxidant supplementation may modulate the cortisol and IL-6 responses to prolonged exercise it remains unclear as to whether such effects are also associated with a reduction in the magnitude of immunodepression. Therefore, the aim of the present study was to examine the effects of a 2-week period of oral supplementation with a high dose (1,000 mg day^{-1}) of vitamin C only, prior to a single bout of prolonged exercise, on markers of oxidative stress, hormones of the HPA axis (plasma cortisol and ACTH concentration), plasma IL-6 concentration, and measures of neutrophil functional capacity.

Methods

Ethics approval was obtained from the local Research Ethics Committee. Participants were informed of the experimental procedures (verbally and in writing) and they gave written informed consent. They also completed a medical questionnaire before participating in each test. The data of Davison et al. (2005a) were used to estimate that a sample size of nine has a 98.9% power to detect differences in the post-exercise plasma cortisol concentration, assuming a standard deviation of differences of $\sim$ 266 nM, using a post hoc paired *t* test.

Participants

Nine healthy endurance trained males (age 26 ± 2 years, body mass 71.8 ± 2.2 kg, $\dot{V}O_{2max}$ 61.6 ± 2.4 ml kg^{-1} min^{-1}, Power output at $\dot{V}O_{2max}$ 353 ± 6 W; means ± SEM) participated in this study.

Testing protocols

All participants completed four exercise bouts; two preliminary trials ($\dot{V}O_{2max}$ determination and habituation) and two main trials. Preliminary trials were separated by at least 1 week and there was a minimum of 2 weeks washout between main trials.

$\dot{V}O_{2max}$ determination

Participants performed a continuous incremental exercise test to volitional exhaustion on an electrically braked cycle ergometer (Lode Excalibur, Holland). After a 6 min warm-up (3 min at 70 W plus 3 min at 105 W) participants began cycling at 140 W. The work was increased by 35 W every 3 min, until volitional exhaustion. Expired gas was collected in Douglas bags during the final minute of each stage. Heart rate and rating of perceived exertion (RPE) were measured during this period using a telemetric device (Polar A1, Polar Electro, Kempele, Finland) and the Borg Scale, respectively. The test was ended when the participant signalled that they could continue for only 1 more minute at the required intensity. At this point a new expired gas collection was commenced and generous verbal encouragement was given. The expired gas was analysed by an O_2 and CO_2 analyser (Servomex series 1400, Crowborough, UK) and volume was measured through a gas meter allowing determination of the average $\dot{V}O_2$ at each work rate. The $\dot{V}O_2$-work rate relationship was used to determine the work rate that would elicit a $\dot{V}O_2$ equivalent to 60% $\dot{V}O_{2max}$.

Habituation ride

Participants cycled for 2.5 h at the work rate equivalent to 60% $\dot{V}O_{2max}$. At the onset 2.5 ml kg^{-1} body mass of drink (low calorie lemon flavoured squash with artificial sweetener) was consumed, every 15 min during, and on completion of the exercise bout. Expired gas (60-s sample) was collected into Douglas bags every 30 min during the exercise and measured as described above. The purpose of this trial was threefold. Firstly, to familiarise the participants with the procedures and the physical stress of the exercise. Secondly, to ensure that the selected work rate actually did elicit a relative intensity of 60% $\dot{V}O_{2max}$. Third, to ensure that the participant could maintain the selected intensity for 2.5 h after an overnight fast.

Main trials

Participants exercised under two different conditions: 2 weeks of placebo (PLA) or vitamin C (VC,

1,000 mg day^{-1} which was taken in two tablets of 500 mg, one with breakfast and one with their evening meal) in a single blind, randomised-crossover design. 2.5 ml kg^{-1} body mass of drink (low calorie lemon flavoured squash with artificial sweetener) was consumed at the onset, every 15 min during, and on completion of the exercise bout. Participants also consumed a drink (500 ml) 2 h pre-exercise. Participants arrived at the laboratory at 09:30, on the morning of the main trials, for measurement of body mass and stature. A resting (Rest) venous blood sample was taken (16 ml into vacutainer tubes: 9 ml in K_3EDTA containing tubes and 7 ml into a heparin containing tube) at 10:00, immediately before beginning to cycle for 2.5 h at 60% $\dot{V}O_{2max}$. Expired gas was collected into Douglas bags (60 s sample), during the 60th and 120th minutes of exercise, for analysis of $\dot{V}O_2$ and RER. Heart rate and RPE were recorded every 15 min during exercise. Further venous blood samples were taken immediately post-exercise (Post-Ex) and at 1 h post-exercise (1 h Post-Ex). All blood samples were obtained by venepuncture from an antecubital vein.

Participants completed a food record diary for the 24-h period before the first trial (habituation) and were required to follow the same diet during the 24 h prior to each main trial. They were all non-smokers and were required to abstain from alcohol, caffeine and heavy exercise for 2 days prior to each trial and to have a rest day on the day immediately before each trial. It was also stipulated that participants should not take any mineral or vitamin supplement (other than those provided) or any other antioxidant supplements during and for the 2 weeks before the study.

Analytical methods

Haematological analysis was performed on the blood samples collected into one of the K_3EDTA tubes using an automated haematology analyser (AC·T 5diff, Beckman Coulter, UK). Blood haemoglobin concentration, haematocrit, total and differential leukocyte counts were measured. A small volume of K_3EDTA anticoagulated whole blood was also stored at room temperature in an eppendorf tube for analysis of in vitro stimulated oxidative burst activity (OBA) (see below). A 1 ml aliquot of blood from the heparin tube was used for measurement of neutrophil degranulation (see below). The remaining blood (K_3EDTA and heparin tubes) was centrifuged at 1,500*g* for 10 min at 4°C and aliquots of plasma were stored at −80°C for later analysis.

In vitro stimulated-neutrophil oxidative burst activity The neutrophil OBA response to phorbol-12-myristate-13-acetate (PMA) and formyl-leucyl-methionyl-phenylalanine (fMLP) were determined according to Davison et al. (2005a) using a commercially available chemiluminescence (CL) kit (ABEL-04M, Knight Scientific, UK). Briefly, CL was measured in a microplate luminometer (Anthos Lucy 1, Salzburg, Austria) reading samples in microplate wells. Each well contained 20 µl whole blood (K_3EDTA anticoagulated) diluted 1:100 with buffer (Hank's balanced salt solution; HBSS without calcium and magnesium but with 20 µM HEPES, pH 7.4), 90 µl assay buffer (HBSS with 20 µM HEPES, pH 7.4), 50 µl Pholasin, and 20 µl adjuvant K (which enhances the luminescence of Pholasin during assays with diluted whole blood). The mixture was incubated at 37°C for 1 min (with gentle shaking) in the luminometer and 20 µl of PMA (5 µg ml^{-1}) or fMLP (10 µM) was injected manually (giving a total volume of 200 µl per well and a final concentration of 0.5 µg ml^{-1} PMA or 1 µM fMLP). When PMA was used CL was recorded in duplicate as relative light units (RLU) at 1-min intervals for 30 min and the integral of the CL curve was calculated. The integral of the baseline curve (CL response without PMA stimulation) was subtracted to calculate the PMA-stimulated CL. For fMLP, CL was measured after an initial 30 s incubation every second for 100 s and the peak difference between stimulated and unstimulated was recorded. It was assumed that the CL response depends almost entirely on neutrophils (Morozov et al. 2003) therefore the PMA-stimulated area under the curve or fMLP-stimulated peak CL responses were divided by the number of neutrophils per well to give CL per neutrophil. The unstimulated response was also expressed per litre of blood to give a measure of spontaneous CL (a snapshot of the level of ROS present in the blood at that time). The CL per neutrophil values were corrected for differences in plasma VC concentration, in accordance with Davison et al. (2005b), to account for the fact that VC competes with Pholasin for ROS, reducing the emitted light energy (measurable CL).

In vitro bacteria-stimulated neutrophil degranulation The neutrophil degranulation response to bacterial stimulant (840–15, Sigma, Poole, UK) was determined according to Davison et al. (2005a). Briefly, 1 ml aliquots of heparinised blood were added to eppendorf tubes containing 50 µl of bacterial stimulant. They were mixed by gentle inversion then incubated at 37°C for 1 h (with gentle mixing again after 30 min). After incubation, the tubes were centrifuged for 2 min at 10,000*g* and the supernatant was stored at −80°C prior to analysis of elastase concentration using an ELISA kit (BioVendor RD191021100, Heidelberg, Germany). The remaining blood (in the lithium heparin tube) was centrifuged at 1,500*g* for 10 min at 4°C. Aliquots of the plasma were stored at −80°C until analysis of elastase concentration. Neutrophil degranulation was expressed as the amount of stimulated elastase release per neutrophil.

Plasma glucose and VC concentration Plasma glucose concentration was determined on an automated analyser (Cobas Mira) using a commercially available colorimetric glucose oxidase-PAP kit (Randox, County Antrim, UK). Plasma VC concentration was determined

according to Liu et al. (1982) using a specific spectrophotometric ascorbate oxidase (E, 1.10.3.3) assay.

Plasma antioxidant capacity Plasma antioxidant capacity (PAC) was determined using a commercially available chemiluminescent test (ABEL-21M, Knight Scientific, UK) for measuring the capacity of plasma to scavenge the superoxide radical. Briefly, 10 µl of heparinised plasma was added to 50 µl of Pholasin, 100 µl of "solution A" and 15 µl assay buffer in a microplate well. Superoxide is generated instantaneously when "solution B" is added to "solution A" and light is emitted (in the presence of Pholasin). Therefore, if antioxidants are present they compete with Pholasin for superoxide and less light is emitted. Twenty-five microlitre of "solution B" was injected into the well (via an automated dispenser) while in the luminometer (Anthos Lucy 1, Salzburg, Austria) and the luminescence was measured every second thereafter. There is a linear relationship between peak CL and antioxidant capacity of the sample. Therefore, an ascorbate standard curve was run as part of the assay allowing expression of the antioxidant capacity of the sample in ascorbate equivalent antioxidant units (µM).

Plasma cortisol, ACTH and IL-6 concentrations Aliquots of K_3EDTA plasma were analysed to determine the concentrations of cortisol, ACTH and IL-6 using commercially available ELISA kits (DRG EIA-1887 v6.0, Germany; Biomercia 7023, Ca, USA; and Diaclone, Besancon, France, respectively).

Plasma malondialdehyde (MDA) concentration A specific colorimetric method, based on that used by Satoh (1978), for quantitative analysis of plasma MDA concentration was used. Briefly, plasma samples were incubated at room temperature for 10 min in 20% trichloroacetic acid. The mixture was then centrifuged at 3,000*g* for 10 min and the supernatant decanted. 1 ml of a weak solution (0.05 M) of H_2SO_4 was added to the precipitate which was mixed thoroughly then centrifuged again and the supernatant decanted. Both liberation of MDA and colour reaction were then performed simultaneously by heating (30 min in a boiling water bath) the precipitate with another 1.25 ml of H_2SO_4 and the sodium sulphate/thiobarbituric acid (Na_2SO_4/TBA) reagent mixture (the concentration of TBA was 0.2% and Na_2SO_4 was 2 M; the solution also contained 20 µM Fe^{2+} to aid colour development). Resulting chromogen was extracted with butyl alcohol, the absorbance of which was read immediately at 535 nm against butyl alcohol.

Data analysis

To compare the two trials, PLA and VC, a two-way repeated measures ANOVA (trial × time) was used. Post hoc analysis was carried out, where appropriate, using paired samples *t* tests with the Holm-Bonferonni correction. The Greenhouse Geisser correction was applied to all ANOVA *P* values. All results were presented as mean ± SEM. Plasma volume changes were estimated in accordance with Dill and Costill (1974) and used to correct blood variables for any haemoconcentration (or dilution). However, the functional neutrophil measures (CL and elastase release per neutrophil) were not corrected in this way as they are expressed per neutrophil and are therefore related to the number of neutrophils present in the stimulated sample.

Results

The average dietary composition for the 24 h period prior to each trial was as follows: total energy intake of 11.22 ± 0.56 MJ, percentage energy derived from carbohydrate, protein and fat was 58.5 ± 1.8, 15.8 ± 1.2, and 26.1 ± 2.0%, respectively. This was equivalent to an average intake of 5.8 ± 0.6 g kg^{-1} body mass (g kg^{-1} BM) of carbohydrate and 1.5 ± 0.1 g kg^{-1} BM of protein. The average dietary VC intake was 74 ± 13 mg. There was no difference between the PLA and VC trials for any of the variables related to physiological demand of the exercise (Table 1).

Markers of oxidative stress and antioxidant capacity

A main effect of time was observed for PAC ($P = 0.043$) but post hoc analysis found no differences across time. There was also a main effect of trial ($P = 0.013$) but no interaction indicating higher PAC in the VC trial (Table 2). For plasma VC concentration, there was a main effect of trial ($P < 0.001$) but absence of any trial × time interaction indicating higher plasma VC concentrations in the VC trial. Plasma MDA concentration was unaffected by the exercise and the temporal response was not influenced by the VC supplementation (Table 2). Spontaneous CL (spCL) per litre of blood was increased post-exercise ($P = 0.02$) but the temporal response was not influenced by the VC supplementation (Table 2).

Table 1 Mean heart rate, RPE, % $\dot{V}O_{2max}$, $\dot{V}O_2$ and RER during exercise

	PLA	VC
Heart rate (beats min^{-1})	146 (3)	145 (4)
RPE	12 (1)	12 (0)
% $\dot{V}O_{2max}$	60.9 (0.7)	61.4 (1.1)
$\dot{V}O_2$ (l min^{-1})	2.67 (0.06)	2.70 (0.09)
RER	0.87 (0.01)	0.86 (0.01)

Values are mean (SEM)
RPE rating of perceived exertion; *RER* respiratory exchange ratio

Table 2 Plasma VC concentration, antioxidant capacity and markers of oxidative stress

	Rest	Post-Ex	1 h Post-Ex	Main effects *P* values (trial; time; interaction)
Plasma VC (μM)				
PLA	47 (4)	52 (4)	52 (4)	0.000; 0.413; 0.552
VC	92 (7)	91 (5)	93 (5)	
PAC (μM)				
PLA	595 (35)	590 (52)	637 (31)	0.013; 0.043; 0.888
VC	790 (69)	719 (49)	853 (74)	
Spontaneous CL ($\times 10^3$ RLU l^{-1}[a]) ($n = 8$)				
PLA	82 (10)	112 (6)	116 (17)	0.572; 0.034; 0.155
VC	84 (14)	100 (21)	91 (23)	
Plasma MDA (μM)				
PLA	2.1 (0.2)	2.0 (0.2)	2.2 (0.1)	0.150; 0.229; 0.588
VC	1.8 (0.1)	1.8 (0.1)	1.9 (0.2)	

Values are means (± SEM). Results from two-way ANOVA are presented in main effects *P* values column

VC vitamin C; *PAC* plasma antioxidant capacity, ascorbate equivalent antioxidant units; *CL* chemiluminescence; *RLU* relative light units; *MDA* malondialdehyde

[a] Values are expressed as the integral of the 30 min CL response per l of blood

Plasma glucose, ACTH, cortisol and IL-6 concentrations

Plasma glucose concentration was significantly decreased post-exercise compared with rest ($P < 0.001$). There was also a main trial × time interaction ($P = 0.048$) but post hoc analysis found no between trial differences. Plasma ACTH concentration was significantly increased post-exercise ($P = 0.001$) and returned to near baseline by 1 h post-exercise ($P = 0.102$) but there was no difference between the PLA and VC trials. Plasma IL-6 concentration increased post-exercise ($P = 0.002$) and remained elevated at 1 h post-exercise ($P = 0.001$) but there was no difference between the PLA and VC trials. There was a significant trial × time interaction effect for plasma cortisol concentration ($P = 0.039$) however, post hoc analysis found no between trial differences. There was a tendency for values to be lower post-exercise ($P = 0.08$) and 1 h post-exercise ($P = 0.074$) in the VC trial. Furthermore, one-way ANOVA analysis on each trial independently revealed a main effect of time ($P = 0.002$) in the PLA trial with the post-exercise and 1 h post-exercise values significantly increased ($P = 0.004$ and 0.009, respectively) whereas the main effect of time in the VC trial was not quite significant ($P = 0.052$) (Table 3).

Circulating leukocyte number and function

The number of circulating leukocytes and neutrophils increased immediately post- and 1 h post-exercise ($P < 0.001$). A significant trial × time interaction was observed for both variables ($P = 0.013$ and 0.01, respectively) (Fig. 1). The post-exercise ($P = 0.01$ and 0.014, respectively) and 1 h post-exercise ($P = 0.006$ and 0.004, respectively) increases were significantly smaller with VC supplementation for total leukocyte and neutrophil counts, respectively.

fMLP-stimulated CL per neutrophil (Fig. 2b) was significantly decreased at 1 h post-exercise ($P < 0.001$) but there was only a trend for a trial × time interaction ($P = 0.075$). No main effects were observed for PMA-stimulated CL per neutrophil (Fig. 2a) but there was a trend for a main effect of time ($P = 0.059$). Neutrophil degranulation (Fig. 3), as measured by bacteria-stimulated elastase release per neutrophil, was significantly decreased post-exercise ($P < 0.001$) and 1 h post-exercise ($P = 0.001$). The temporal pattern was similar for both trials (interaction, $P = 0.575$).

Discussion

The main findings of the present study are that vitamin C supplementation for 2 weeks prior to an acute bout of prolonged exercise (2.5 h at 60% $\dot{V}O_{2max}$) resulted in slightly lower leukocytosis, neutrophilia and cortisol responses. However, there was no effect on plasma IL-6 or ACTH concentrations and no physiologically significant effect on the reduction of neutrophil functional capacity (in vitro-stimulated oxidative burst or degranulation) that typically occurs following such exercise.

It has been shown that antioxidant supplementation can reduce the cortisol (Fischer et al. 2004) and IL-6 (Fischer et al. 2004; Vassilakopoulos et al. 2003) responses to endurance exercise when consumed daily for 1 week or more and VC alone may reduce the cortisol response (Peters et al. 2001a, b). The findings of the present study are in line with those of Peters et al. (2001a, b) who showed a significant reduction in post-exercise plasma cortisol in participants that were supplemented with 1,000–1,500 mg VC daily for 7 days prior to exercise. There is also agreement with the findings of Fischer et al. (2004) in regard to the cortisol response, but not the IL-6 response, to prolonged exercise. It is unlikely that adrenal cortisol and VC release occur together in response to oxidative stress (Peake 2003). Therefore, a reduced cortisol response with prolonged, high dose VC supplementation is likely caused by some other mechanism(s). According to Fischer et al. (2004) IL-6 could be important since its release from contracting skeletal muscle is reduced with antioxidant supplementation and this cytokine is known to stimulate an increase in systemic cortisol concentration (Steensberg et al. 2003). However, the present results show that when VC was supplemented alone rather than with other antioxidants there was a trend for lower cortisol responses but no effect on the systemic IL-6 response. Therefore, the present results suggest that there may be some small effects of VC on adrenal cortisol synthesis and/or release. This, however, appears to be independent of changes in plasma IL-6 concentration or oxidative stress and the precise mechanism(s) remains unclear. In fact the

Table 3 Plasma cortisol, ACTH and IL-6 responses

	Rest	Post-Ex	1 h Post-Ex	Main effects *P* values (trial; time; interaction)
ACTH (pg ml^{-1})				
PLA	24 (2)	170 (47)	44 (11)	0.303; 0.002; 0.276
VC	28 (2)	106 (25)	29 (4)	
IL-6 (pg ml^{-1})				
PLA	0.6 (0.1)	7.2 (1.4)	5.8 (1.1)	0.818; 0.000; 0.795
VC	0.6 (0.2)	7.0 (1.5)	5.5 (1.0)	
Cortisol (nM)				
PLA	326 (34)	644 (74)	622 (91)	0.097; 0.004; 0.039
VC	337 (42)	513 (65)	418 (52)	

Values are means (± SEM). Results from two-way ANOVA are presented in main effects *P* values column
ACTH adrenocorticotrophic hormone; *IL-6* interleukin-6

observations of those studies using both vitamins C and E (Fischer et al. 2004; Vassilakopoulos et al. 2003) may actually be caused by an additive effect of vitamin C and vitamin E or be related to the fact that vitamin E is lipid soluble. This seems feasible because in the study by Fischer et al. (2004) there was no difference between the placebo and supplemented groups in IL-6 gene expression or the level of IL-6 protein within the exercised muscle fibres. This suggests that the reduced systemic IL-6 concentration is a result of reduced translocation from the tissue into the circulation. Therefore, the site of action could well be the plasma membrane, which could explain the need for the lipid soluble vitamin E in addition to vitamin C. Furthermore, in the study by Fischer et al. (2004) there was a larger increase of systemic IL-6 post-exercise (in the region 20 pg ml^{-1} in the placebo group as compared with 7.2 pg ml^{-1} in the present study) which is where the greatest difference between groups was observed. The values observed at 2 h during exercise were similar between groups and in line with the values observed in the present study. Therefore, it may be that the slight methodological differences between that and the present study, such as duration of exercise (3 h compared with 2.5 h) explain the discrepancy. Furthermore, Fischer et al. (2004) observed a significant post-exercise increase in the marker of oxidative stress plasma 8-iso-PGF$_{2\alpha}$. There was no evidence of a significant increase in oxidative stress during the exercise undertaken by participants in the present study, which may go some way to explaining this difference. It should also be noted that in a study by Nieman and colleagues (2004) 2 months of vitamin E supplementation actually resulted in a greater IL-6 response to prolonged exercise (with no effect on the cortisol response). However, the exercise undertaken in that study was extreme by comparison (a competitive event of approximately 12 h duration) with post-exercise IL-6 values more than 12-fold higher than in the present study (in the region of 80 pg ml^{-1}).

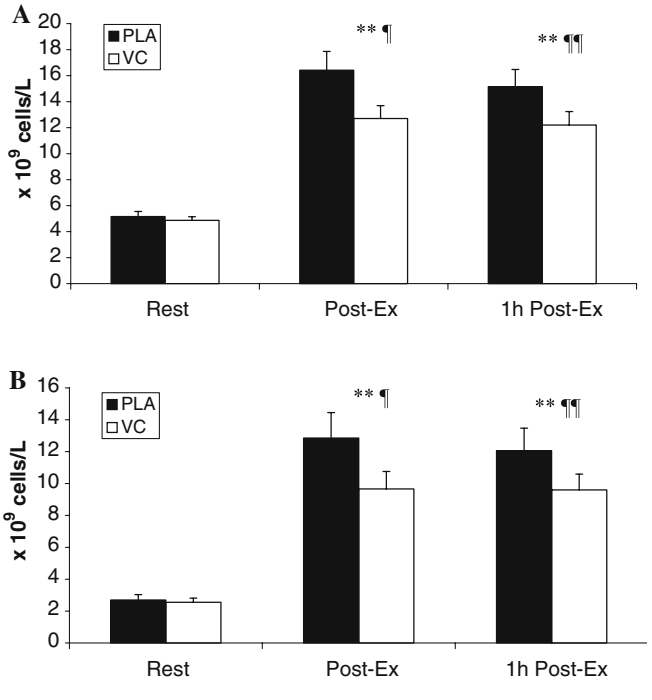

Fig. 1 Blood leukocyte (**a**) and neutrophil (**b**) counts. Values are means (± SEM). *Two-way ANOVA* significantly different from Rest (*$P < 0.05$; **$P < 0.01$), Significantly different from PLA ($^{¶}P < 0.05$; $^{¶¶}P < 0.01$)

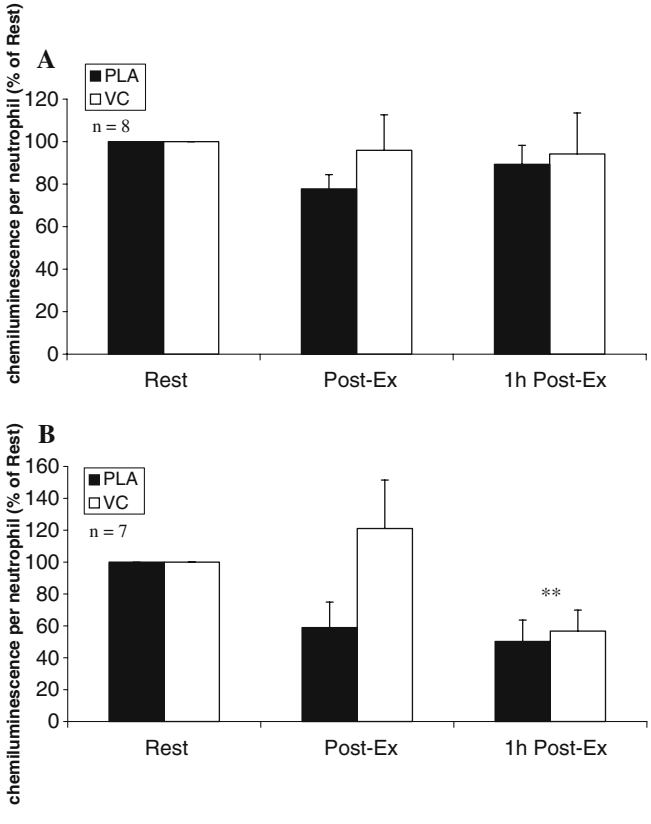

Fig. 2 Capacity of neutrophil to generate reactive oxygen species in response to in vitro stimulation with PMA (**a**) and fMLP (**b**). Values are means (± SEM). *Two-way ANOVA* significantly different from Rest (*$P < 0.05$; **$P < 0.01$)

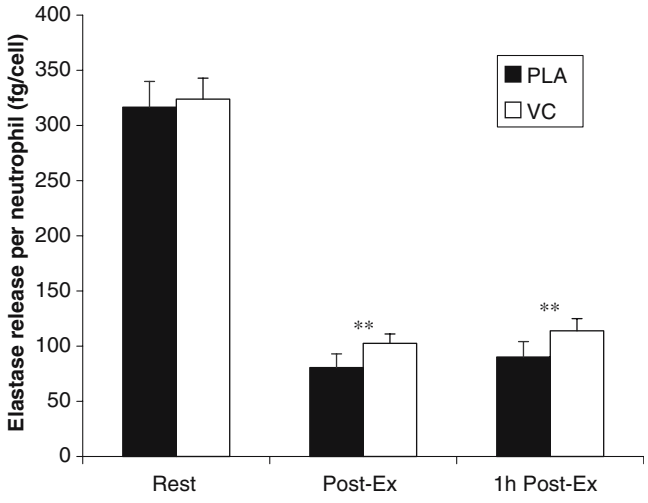

Fig. 3 Neutrophil bacteria-stimulated degranulation response. Values are means (± SEM). *Two-way ANOVA* significantly different from Rest (*$P < 0.05$; **$P < 0.01$)

Following exercise there was a significant decrease in the bacteria-stimulated elastase release (degranulation) per neutrophil and fMLP-stimulated CL per neutrophil. PMA-stimulated CL per neutrophil showed a trend towards a decrease post-exercise, especially in the PLA trial. These findings are in line with previous findings (Davison et al. 2005a; Li et al. 2004, 2005; Morozov et al. 2003; Nieman et al. 1997; Scharhag et al. 2002) suggesting that exercise decreases the killing capacity of neutrophils. Berkow et al. (1986) demonstrated that immature (or prematurely released) neutrophils have lower activity of NADPH oxidase and a lower capacity to produce ROS compared with fully matured neutrophils. Therefore, the post-exercise neutrophilia may contribute to post-exercise decreases in the functional capacity of neutrophils.

Regardless of the mechanism, supplementation with VC was not effective at reducing the magnitude of this apparent decrease in neutrophil function. This suggests that the magnitude of the decreases in neutrophilia and cortisol observed in the present study, with VC supplementation, are not great enough to have a significant physiological effect on the actual capacity of neutrophils to respond to stimulation. However, it is worthy of note that there was a tendency for fMLP-stimulated CL to be higher post-exercise in the VC trial ($P = 0.075$ for trial × time interaction effect) and that there was only $n = 7$ for this measure owing to problems with the assay which must be performed on fresh (diluted) whole blood. Robson et al. (2003) observed that 7 days of supplementation with antioxidants (18 mg β-carotene, 900 mg VC and 90 mg vitamin E) prior to a 2 h treadmill run at 65% $\dot{V}O_{2max}$ significantly enhanced post-exercise neutrophil OBA, compared with placebo, even though there was no effect on leukocyte count or plasma cortisol concentration. This suggests that there may be a direct effect on neutrophils, maybe by reducing (auto)oxidative damage or modification. The trend for a blunted decrease in fMLP-stimulated CL (but lack of effect with PMA-stimulated CL), in the present study, in the VC trial also support this idea as receptor dependent activation (fMLP-stimulated) may be more sensitive to oxidative modification than receptor independent activation (PMA-stimulated) because the receptors are more likely to be exposed to external attack/damage by ROS. This would suggest that greater effects might have been evident if the magnitude of oxidative stress experienced by the current participants was greater. However, there was no trial × time interaction effect for bacteria-stimulated neutrophil degranulation (requiring receptor-dependent activation) which does not support this contention.

In conclusion, 2 weeks of supplementation with 1,000 mg day^{-1} (2 × 500 mg) of VC was effective at increasing antioxidant defence, modulating the leukocytosis and neutrophilia responses and possibly had some small effects on the plasma cortisol response. However, there was no effect on plasma ACTH or IL-6 concentrations. This suggests that there may be some direct effect on the adrenals from the VC supplementation, possibly via the mechanisms suggested by Peake (2003). Nonetheless, there was no physiologically significant effect on the measures of neutrophil functional capacity. This suggests that supplementation with VC alone for a period of up to 2 weeks provides very limited, or no protection against the depression of neutrophil function which is typically observed after prolonged exercise. However, the magnitude of oxidative stress experienced by participants was relatively modest in the present study. It may be possible that there would be greater decreases in neutrophil function under conditions of greater physical and/or oxidative stress, the magnitude of which could be blunted to a relatively greater degree with VC supplementation and further research is required to clarify this.

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