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13. ABSTRACT *(Maximum 200 words)*

Immune responses to exercise after acute supplementation with zinc (25 mg Zn and 1.5 mg copper) and vitamin E (400 IU E) were evaluated in 10 eumenorrheic women. Subjects (age 32 ± 1 years) were studied on three occasions: once per menstrual cycle, between days 5 and 8, after supplementation with Zn, E and a placebo (P). They ran at 65 to 70% of their maximal aerobic capacity to exhaustion (94, 98, and 97 minutes for Zn, E & P, respectively). Blood levels of ACTH, cortisol, prolactin, growth hormone, vitamin E, IL-6, and selected lymphocyte subset markers (CD3, CD4, CD8, CD19 and CD56) were measured before (PRE) and immediately (POST), one hour (REC) and the day after the run. Metabolic, hormonal and immune responses were similar for Zn, E and P with one exception: percentage of CD4+ cells was significantly lower ($p<0.05$) with Zn. Under all treatments, exercise significantly ($p<0.05$) increased plasma ACTH, cortisol, prolactin, growth hormone and IL-6 concentrations. Whereas CD8 and CD56 cell percentages increased ($p<0.05$), CD4/CD8 ratio, CD3, CD4, and CD19 cell percentages decreased ($p<0.05$) at POST. These responses were temporary as most REC values were similar to PRE.

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Anita Singh 4/4/96

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Introduction

It is well recognized that both acute and chronic stress can disrupt homeostatic mechanisms and be immunosuppressive. Military training programs such as the U.S. Navy Basic Underwater Demolition School (BUDS)/ Sea, Air and Land (SEAL) and the U.S. Army Ranger programs have simulated mission scenarios where soldiers are under considerable physiological, environmental and psychological stress. We noted previously that many U.S. Navy SEAL trainees developed upper and lower respiratory tract infections, and cellulitis over the course of Hell week. Interleukin-6 concentrations increased and C-reactive protein was present in the plasma of all trainees at the end of Hell Week; these changes are characteristic of a stress response (28).

High rates of infection are also common during Ranger training and the outbreak of pneumonia in a recent Ranger class prompted a comprehensive evaluation of nutritional status and immune function during training (16). Findings indicated that cellular immunity was significantly compromised and the periods of greatest suppression corresponded with the highest infection rates. Although, women soldiers do not undergo SEAL or Ranger training, they nevertheless experience considerable stress during training, simulated missions and in combat.

Exercise is one quantifiable physiologic stressor that has been shown to alter immune function and may be used as a model for creating immunosuppression similar to that obtained with rigorous military training programs and combat

scenarios. Strenuous physical activity has been reported to temporarily suppress a variety of immune functions (13,24,29). A temporary suppression of lymphocyte proliferation in the presence of selected mitogens and changes in lymphocyte subset expression has been reported (4,8,17, 24,26). For example, a temporary decrease in the ratio of T helper (CD4) to T suppressor (CD8) cells has been noted after strenuous exercise (8,12). However, most of the work on the effects of exercise on immune responses has been done in men. Finally, prolonged strenuous running has been shown by us and other investigators to enhance the capacity of neutrophils to produce reactive oxygen anions which may be potentially harmful to cells and cell membranes (3,27,29).

It is important to consider the possibility that stress may increase the need for nutrients involved in immune function. Nutrients that serve as important cofactors in the maintenance of immune function include zinc and vitamin E. Zinc is required for the maintenance of thymulin biological activity, cell differentiation and proliferation, and serves both a mediating and regulatory role in the immune cascade (4,7,25). Vitamin E protects against damage from peroxyl radicals and products of oxidation such as malondialdehyde (19,31). The overall objective of this project was to evaluate whether acute supplementation with the micronutrients zinc and vitamin E would influence immune responses associated with physiological stress in women. A submaximal run to exhaustion on a motorized treadmill was used to elicit physiological stress. We have used this exercise paradigm previously to evoke a stress response in men (27).

Methods

Subjects: The study was conducted as a double-blinded, latin square design and each subject was tested during supplementation with zinc, vitamin E, and the placebo. The latin square design was used to control for the effect of the order of supplementation across subjects. Ten, moderately to highly trained women runners were recruited from the Washington, DC Metropolitan area. All subjects were healthy, normal weight, nonsmokers, with normal menstrual cycles, not on oral contraceptives and not taking vitamin and mineral supplements. After giving informed consent subjects underwent a maximal treadmill test to determine their maximal aerobic capacity (VO_{2max}), which was used for calculating the treadmill speed required for eliciting 65 to 70 % of their VO_{2max}. For each subject, the speed and grade of the treadmill was kept constant for all three runs.

Supplements: The zinc, vitamin E and placebo supplements were prepared at a pharmacy and the pharmacist kept the code until all data had been collected. The zinc supplement contained 25 mg of elemental zinc and 1.5 mg of copper. Copper was added to this supplement to prevent any imbalances in copper status because of the established interactions between these two micronutrients. The vitamin E supplement contained 400 IU of d ∞ tocopherol and the placebo contained dextrose. Subjects were asked to take the zinc, vitamin E or placebo twice daily once in the morning and once at night for four days (three days preceding the test and the test day and in the morning of the recovery day). Since one test was scheduled per menstrual cycle, there was at least a three to four week washout

period between supplements. Subjects also recorded their dietary intake over the 4-day period of the first test and were requested to replicate it as closely as possible during the last 2 tests.

Submaximal Run Tests: The tests were conducted in the follicular phase of the menstrual cycle between days 5 and 8 to minimize the effects of hormonal changes on endocrine and metabolic parameters; one test was conducted per menstrual cycle. Subjects reported to the Human Performance Laboratory (HPL) between 7.00 and 8.00 am after an overnight fast, weighed themselves and took their 'morning' supplement with a measured volume of deionized water (5 ml of water/kg body weight). This was done to insure uniform hydration among subjects. Next, subjects were instrumented with ECG electrodes to monitor heart rates during the run and an indwelling catheter was inserted by a physician or certified phlebotomist into a forearm vein for collecting blood samples. After the insertion of the venous catheter the subject stood up and remained standing. The submaximal run to exhaustion commenced 1 h after taking the supplement. Blood samples were collected before (PRE), immediately after (POST) and 1 h (REC) after completing the run. Blood samples for measuring lactates, glucose and stress hormones were also collected at half hourly intervals during the run when subjects stopped briefly to drink 200 ml of deionized water. For the first two hours of the run, the incline on the treadmill was set at 3%, after which, for subjects who ran more than 2 hours, the grade of the treadmill was increased to 6%. Immediately after the run, the POST blood sample was drawn and subjects were asked to rate

their perceived exertion based on the Borg Scale (2). Next, subjects walked for 5 minutes on the treadmill to cool down, then remained standing until after the REC blood was drawn. Subjects also reported to the HPL after an overnight fast on the day after the run for another recovery (24 H) blood draw. This blood sample was drawn one hour after subjects took their supplement with a cup of water: blood was drawn between 7:15 and 8:30 am.

Biochemical Analysis:

Blood Counts: Whole blood was collected in EDTA tubes (1 ml) maintained at room temperature for determining complete blood cell counts, hematocrits, differentials, and respiratory burst activity. Complete blood cell counts were made using a Coulter Counter (Coulter Electronics, Hialeah, Fl) and differential counts were obtained from stained slides. Hematocrit and hemoglobin were determined by ultracentrifugation and the cyanomethemoglobin method, respectively.

Glucose and Lactates: Chilled sodium fluoride tubes were used for collecting samples (1 ml) for glucose and lactate determinations. These samples were centrifuged and plasma was removed and stored refrigerated until analyzed within 24 hrs of collection. Lactate and glucose concentrations were determined in duplicate (YSI SELECT 2700 Analyzer, Yellow Springs, OH).

Hormones and Vitamin E: Blood samples (10 ml) for measuring plasma concentrations of ACTH, cortisol, growth hormone, prolactin, interleukin 6, vitamin E and glutathione were collected in chilled EDTA tubes which were centrifuged at 3,000 RPM for 15 minutes in a refrigerated centrifuge. Plasma was

separated, immediately frozen on dry ice and stored at -70°C until all data collection was completed. All samples from one subject were analyzed in duplicate in one assay. Plasma adrenocortropic hormone (ACTH), growth hormone (GH) and prolactin concentrations were measured by radioimmunoassay using kits from Nichols Institute Diagnostic, Capistrano CA. Plasma estradiol, progesterone and cortisol concentrations were also measured by radioimmunoassay (Diagnostic Products Corporation, Los Angeles,CA). Interleukin 6 concentrations in plasma were measured by ELISA using Quantikine™ HS Human IL-6 Immunoassay (R and D systems, Minneapolis, MN). Plasma vitamin E (alpha and gamma tocopherol) was measured by HPLC (1) and glutathione peroxidase was measured by the automated method of McAdam et al (14).

Zinc and Copper: Blood was collected in heparinized tubes for determining plasma zinc and copper concentration. Plasma was separated by centrifugation and stored frozen for later analysis by flame atomic absorption spectrophotometry (Model 3030B AAS, Perkin-Elmer, Norwalk, CT): all samples from one subject were analyzed in duplicate in one assay.

Lymphocyte subset responses. Whole blood (1 ml) was collected in an EDTA tube maintained at room temperature. Lymphocyte subsets were identified by the two color (fluorescein and phycoerythrin; Coulter, Fl), direct staining immunofluorescence technique. Subsets included CD3 (total T cells), CD4 (helper/inducer T cells), CD8 (cytotoxic/suppressor T cells), CD19 (B cells), CD56 (natural killer cells) and CD26 (activated T cells and T cells with receptors for IL-2).

The following combinations were prepared: (1) CD3 with CD56 (2) CD4 with CD45RA (3) CD4 with CD45RO (4) CD8 with CD45 RA (5) CD8 with CD45 RO and (6) CD19 with CD26. Each combination tube contained one monoclonal antibody conjugated with fluorescein (FITC) and one with phycoerythrin (PE). Negative control and isotypic controls were prepared for each sample. The negative control, which was prepared by incubating cells without monoclonal antibody in phosphate buffered saline (PBS), was used for setting the lymphocyte gate. In order to determine the degree of nonspecific staining, an isotypic control was prepared by incubating cells with MsIgG-FITC and MsIgG-PE mouse monoclonal antibodies. MsIgG is of the same isotype as the antibodies used for the markers being studied but lacks specificity for known human antigens. The stained lymphocyte subsets were analyzed on a fluorescence activated cell scanner (FACScan, Becton Dickinson, San Jose, CA). Lymphocytes were gated live (forward versus side scatter) to exclude monocytes, granulocytes and dead cells and 10,000 cells were counted per sample. Percentages of major subset are presented in this report.

Urine: Urine samples were collected before and after exercise and stored frozen for measuring urinary malondialdehyde. A modification of the method of Halliwell et al (9) was used for determining urinary malondialdehyde. Creatinine concentrations of urine samples were measured and the data are expressed per μmol creatinine.

Statistical Analysis: The statistical analysis system (SAS) (21) computer package was used for all statistics. The data were analyzed by a two way, within

subject, repeated measures Analysis of Variance. The level of significance was set at $p<0.05$.

Results

Subject characteristics : Subjects were healthy, of normal body weight and percentage body fat and were moderately to highly physically active (Table 1). Baseline plasma estradiol and progesterone concentrations were similar for all three treatments (Figure 1). Subject compliance in taking the supplements was confirmed from plasma concentrations of vitamin E in all subjects and zinc in a sub-sample (n=4) (data not shown).

Physiologic and metabolic measures: The three running conditions were similar for all treatments as evidenced by the metabolic and physiologic measures presented in Table 2 and Figure 2. The average running times to exhaustion were not different for the zinc, vitamin E or placebo runs. Moreover, heart rates at the end of exercise were similar and subjects ratings of perceived exertion on the 20-point Borg Scale were virtually identical at the end of all three runs. Plasma lactate concentrations increased significantly at POST but returned to PRE levels at REC (Figure 2) ; lactate responses were similar for all three treatments. Plasma glucose concentrations at POST were higher (not significantly) for the vitamin E run as compared to the zinc and placebo runs, reflecting the exaggerated response of one subject. Thus, the overall physiologic and metabolic responses to exercise were similar for zinc, vitamin E and the placebo runs.

Endocrine responses: Although, there was no supplement effect, exercise resulted in a significant stress response (Figure 3). Plasma ACTH and prolactin concentrations peaked at POST and decreased at REC to values that were slightly higher than PRE concentrations. Plasma growth hormone also peaked at POST but REC concentrations were below PRE. In contrast, plasma cortisol concentrations increased at POST and increased further at REC. Concentrations of all four stress hormones at 24 H were similar to PRE.

Immune responses: Supplementation with zinc and vitamin E did not affect leukocyte (WBC) counts (Figure 4) or the percentage of lymphocytes in peripheral blood (data not shown). Exercise, however, significantly increased WBC counts which remained elevated at REC and returned to PRE values at 24 H. A similar pattern was noted for the cytokine, interleukin 6 (Figure 4).

Relative counts (%) of the major subsets during the three treatments are presented in Figures 5 and 6. CD4 (helper/inducer T cells) was the only lymphocyte marker that was significantly influenced by supplementation. The percentage of cells expressing this marker was significantly lower ($p<0.05$) during zinc supplementation. Zinc and vitamin E supplementation did not significantly affect any of the other markers studied (Figures 5 and 6).

There were two distinct patterns of responses to exercise, some subsets decreased whereas others increased. CD3+ (total T cells), CD4+ (helper/inducer T cells), CD19+ (B cells) and CD45 RO+ (memory lymphocytes) cells were all significantly lower at POST (Figures 5 and 6). A similar effect was also noted for

CD26 which is a marker for activated T cells and T cells with receptors for IL-2 (data not shown). In contrast, percentages of cells that were CD8+ (cytotoxic/suppressor T cells), CD56+ (natural killer cells) and CD45 RA+ (naive lymphocytes) were significantly higher ($p<0.05$) at POST (Figures 5 and 6). The ratio of CD4+ to CD8+ cells decreased significantly ($p<0.05$) at POST to values that were 62, 61 and 69 % of PRE for zinc, vitamin E and placebo. All changes in lymphocyte subset expression were temporary and percentages of all markers at REC and 24 H were similar to PRE.

Antioxidant measures : We measured the enzyme glutathione peroxidase in plasma and excretion of urinary malondialdehyde, both of which are indexes of antioxidant function. Glutathione peroxidase activity was similar for all three treatments and did not appear to be affected by exercise (data not shown). Urinary malondialdehyde excretion tended to be higher during the placebo as compared to zinc and vitamin E supplementation; pre and post exercise values were not different (Figure 7).

Discussion

Strenuous exercise is clearly associated with transient disturbances in the immune system. Decreases in mitogen stimulated lymphocyte proliferation, immunoglobulin secretion, natural killer cell cytotoxicity and the ratio of helper/inducer to suppressor cytotoxic T cells have been reported after exhaustive exercise (8,11,13,17,22,24). However, most of this work has been conducted in men and little information is available for women. Because women in the military are exposed to considerable physiologic stress during training, deployment and combat,

it is important to characterize their immune responses to stress. In the present study we noted that exercise temporarily increased stress hormone concentrations and altered the expression of various peripheral blood lymphocyte subsets in women in a manner clearly indicative of stress. Supplementation with the antioxidant nutrients, zinc and vitamin E, did not influence the exercise-induced responses of the measures studied.

Nutritional status and immune function are closely related and nutritional deficiencies are known to compromise immune function (19,23). We chose to examine the effects of acute supplementation with zinc and vitamin E on immune responses in women since both nutrients have been reported to influence exercise-induced immune responses in men (3,20,27,31). Cannon et al (3) reported a decrease in the interleukin-6 responses in men who ran downhill on a treadmill after they had been supplemented with 800 IU of vitamin E daily for 48 days. In our study, however, the vitamin E supplementation period was short and did not decrease post exercise plasma interleukin-6 concentrations. Recently, Rokitzki et al (20) reported that five months of vitamin E supplementation significantly reduced serum malondialdehyde concentrations in elite male cyclists (20). In contrast, in our study, urinary malondialdehyde excretions, before and after exercise, were similar for zinc, vitamin E and placebo runs, indicating that lipid peroxidation was not affected by these antioxidants. However, our supplementation period was only four days.

Vitamin E supplementation did not have any effect on lymphocyte subset expression. In contrast, zinc supplementation significantly decreased the percentage of CD4+ cells whereas the ratio of CD4+ to CD8+ cells was virtually identical for all three treatments (Figure 5). The implication of this finding remains to be determined, especially, since Miller and Strittmatter (15) recently reported that zinc is essential for the growth of a subset of human CD4+ T cells that are present in all normal individuals.

The immune system is directly affected by the hypothalamic-pituitary adrenal axis and immune cells in turn modulate neuroendocrine responses. Numerous studies, mostly in men, have documented exercise-induced increases in blood concentrations of the stress hormones, ACTH and cortisol, and catecholamines; cortisol and catecholamines modulate lymphocyte subset expression (11,13). In the present study, hormonal responses to exercise were similar to those reported by other investigators (12,18). Our findings of reduced percentages of total T cells (CD3+), helper/inducer T cell (CD4) and B cells (CD19) and increased percentages of natural killer cells (CD56) and suppressor/cytotoxic T cells (CD8) in women immediately after exercise are in agreement with those reported for men (8, our unpublished observations). Changes were temporary and by one hour post recovery percentages of all subsets were similar to percentages at baseline. Finally, exercise significantly elevated plasma interleukin 6 concentrations in our subjects, a finding that has also been noted in men (6,32).

In conclusion, the present study provides evidence that a submaximal run to exhaustion evokes a stress response in women that is similar to what we and others have previously noted in men. Acute supplementation with zinc and vitamin E, however, did not have an effect on the exercise-induced hormonal or immune responses studied. That the exercise-induced hormonal and immune responses were similar for the three submaximal runs, is an indication of the repeatable nature of this stressor. All exercise-induced changes were temporary and most hormonal and immune measures returned to baseline one hour after exercise. The only measure affected by supplementation was the percentage of helper/inducer T lymphocytes (CD4+) which was significantly lower ($p<0.05$) during zinc supplementation. Future work in women should investigate the effects of exercise on functional measures of immune status, including mitogen stimulated lymphocyte proliferation, natural killer cell cytotoxicity and the production of cytokines from stimulated cells.

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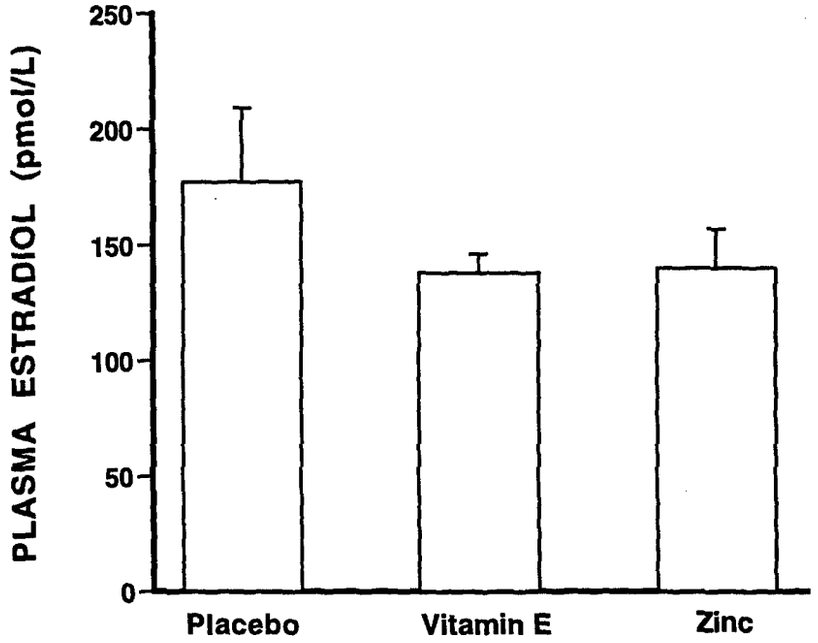
Figure 1. Baseline estradiol and progesterone concentrations for the three treatments.



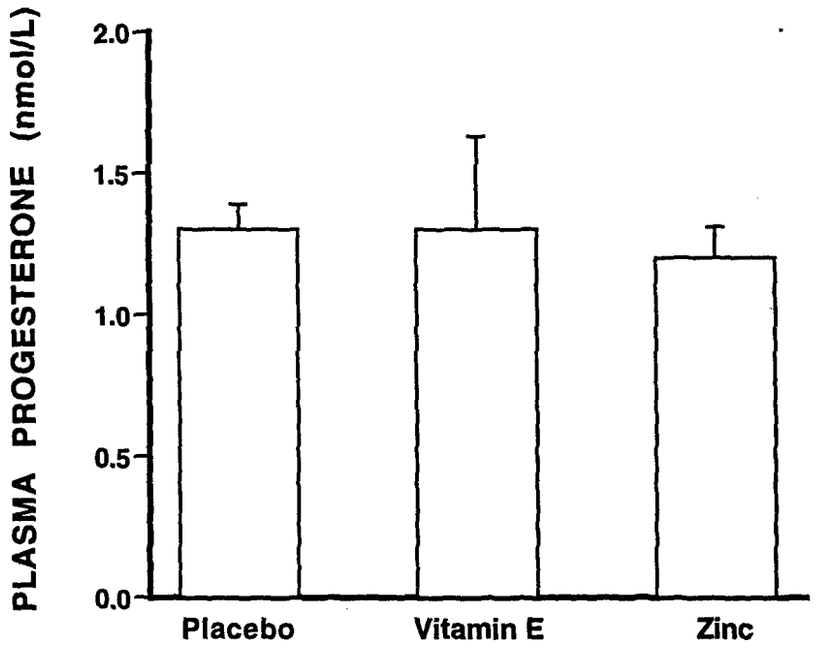


Figure 2. Plasma lactate and glucose concentrations for zinc, vitamin E and placebo conditions. *Significant exercise effect ($p<0.05$).

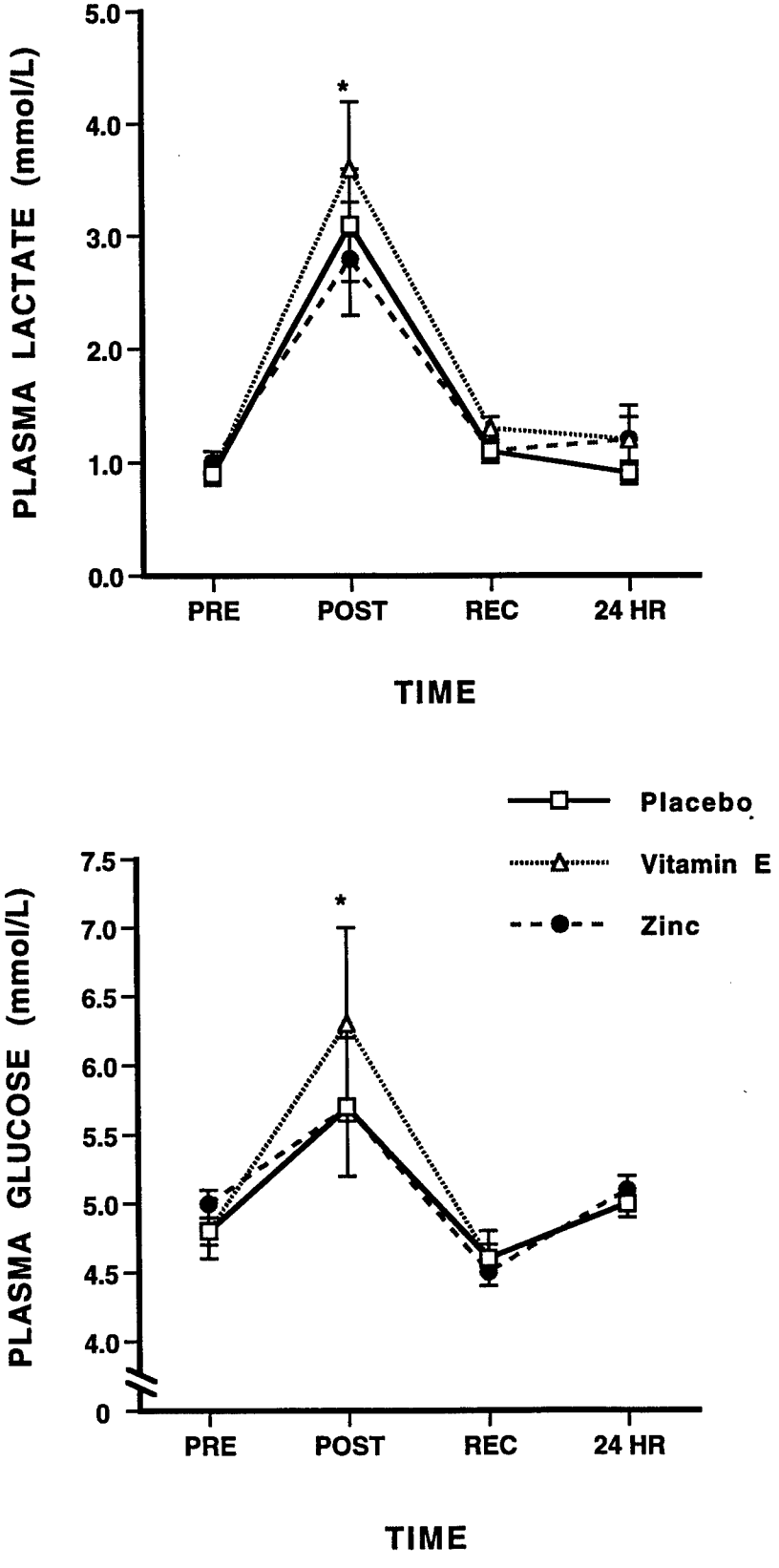


Figure 3. Plasma adrenocorticotropin hormone, cortisol, growth hormone and prolactin concentrations for zinc, vitamin E and placebo conditions. *Significant exercise effect ($p<0.05$).

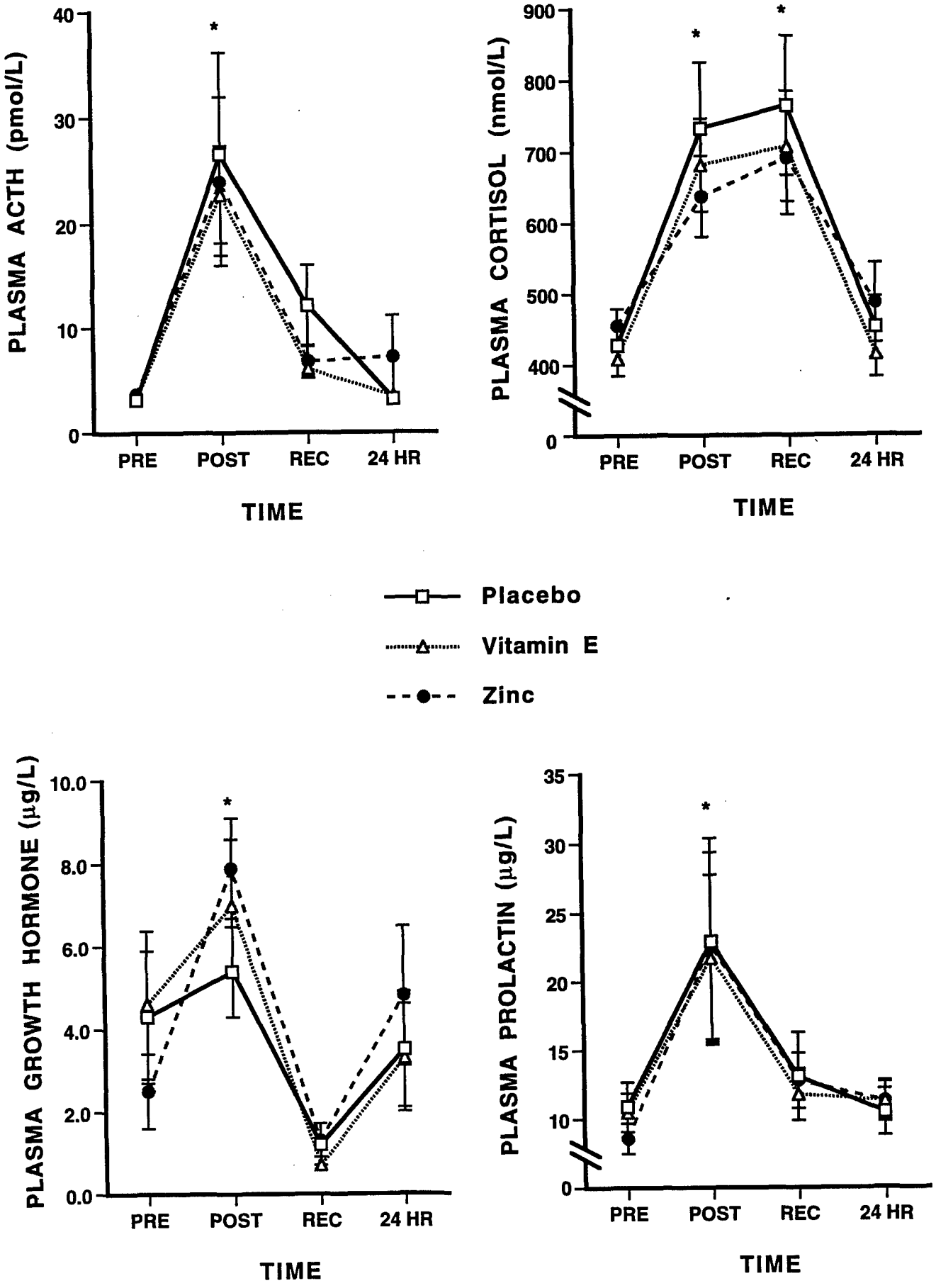


Figure 4. Peripheral blood leukocyte (WBC) counts and plasma interleukin 6 (IL-6) concentrations for zinc, vitamin E and placebo conditions. *Significant exercise effect ($p<0.05$).

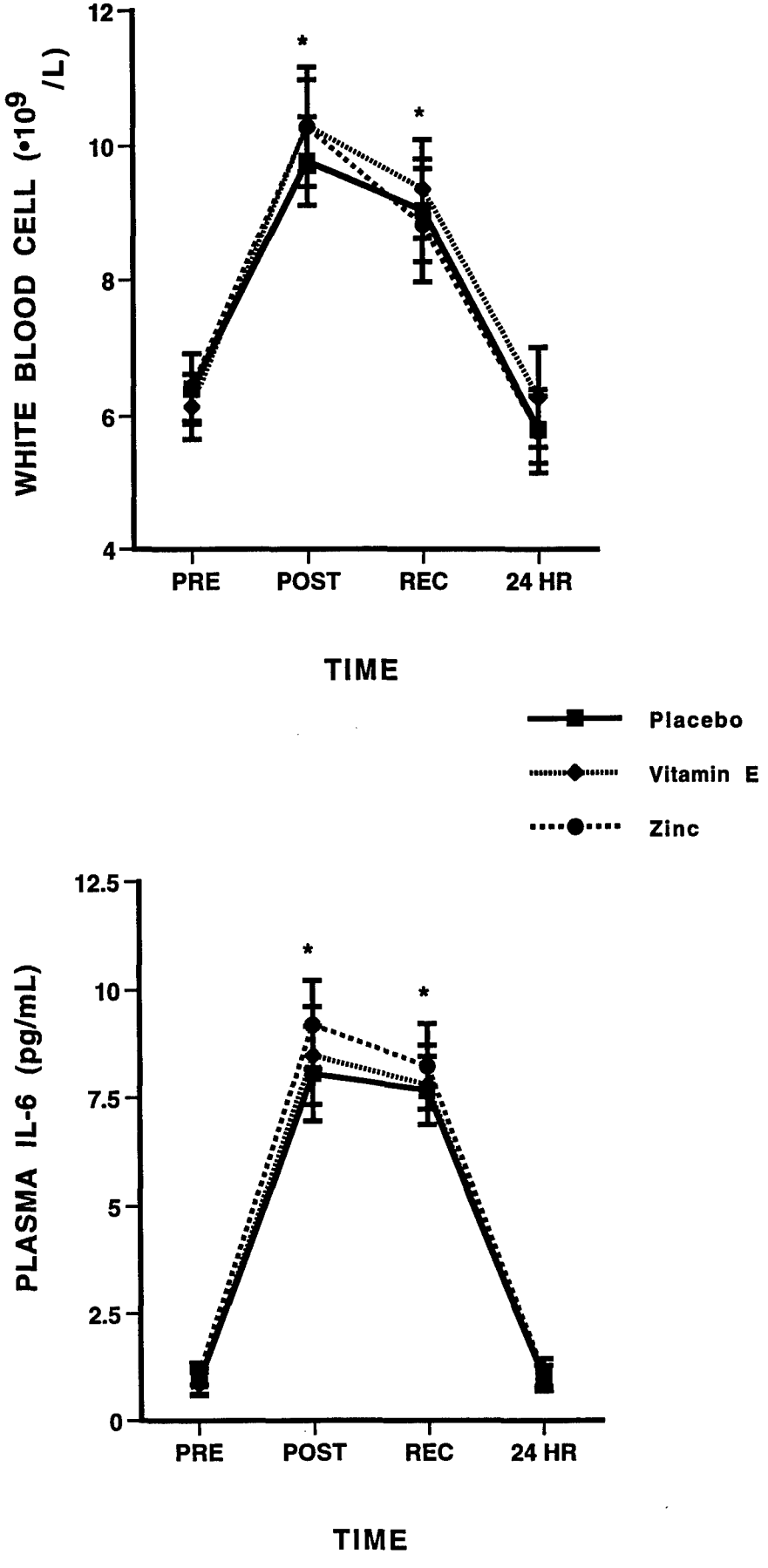


Figure 5. Responses of T lymphocyte markers to exercise after supplementation with zinc (code 3), vitamin E (code 2) and placebo (code 1). *Significant exercise effect ($p<0.05$). Note: A significant ($p<0.05$) supplementation effect was noted for CD4+ cells.

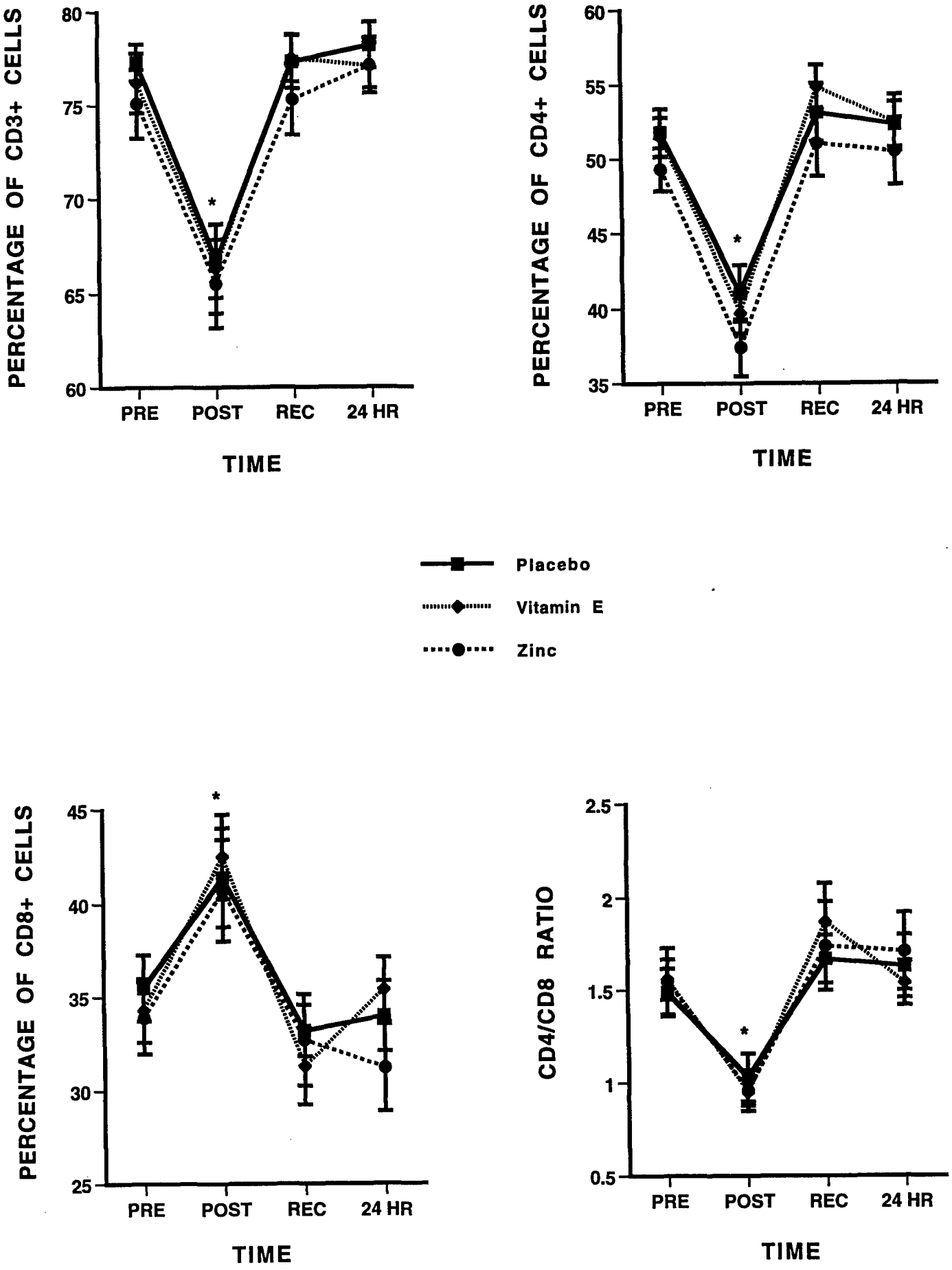
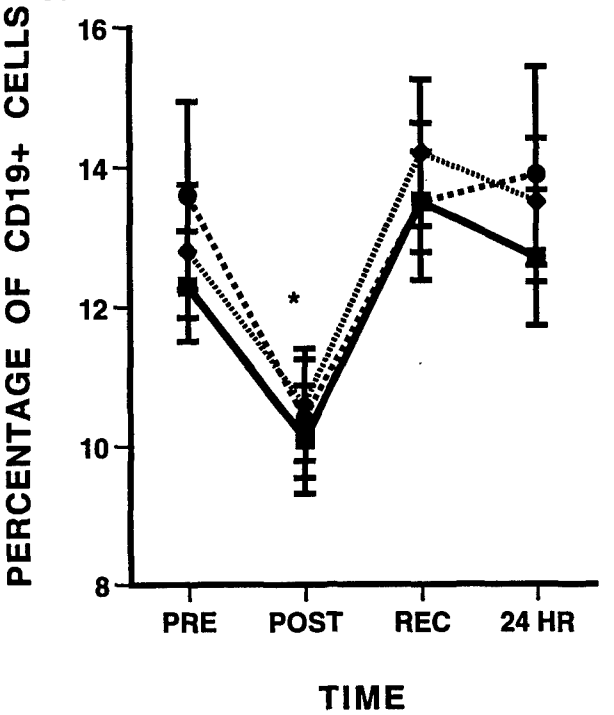
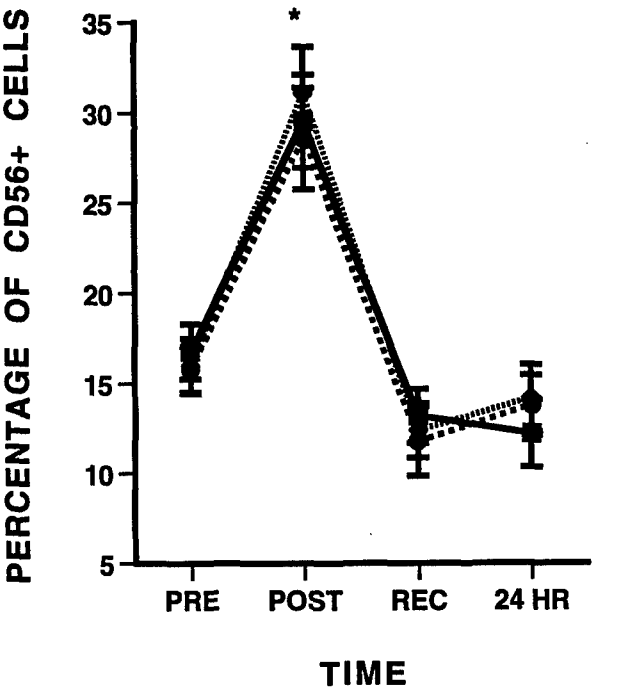
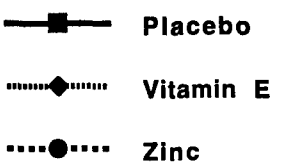
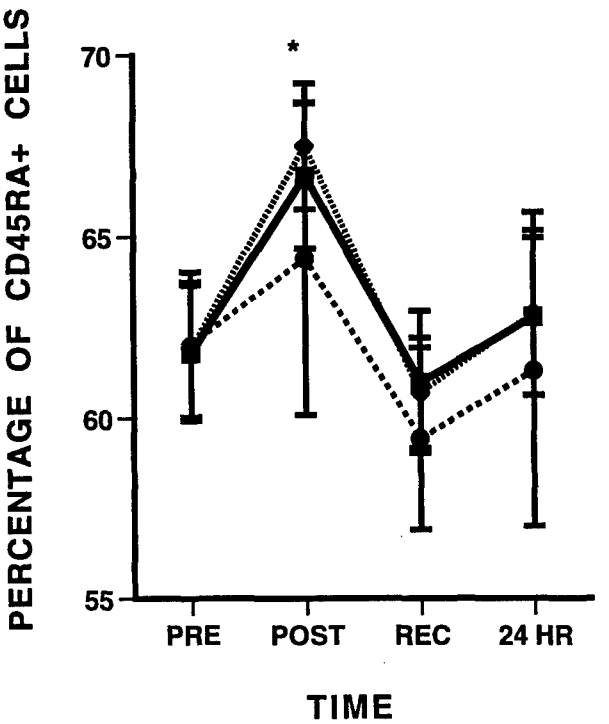


Figure 6. Responses of B lymphocytes (CD 19+ cells), natural killer cells (CD56+) and naive (CD45 RA+) and memory (CD45 RO+) lymphocytes to exercise after supplementation with zinc, vitamin E and placebo. *Significant exercise effect ($p<0.05$).









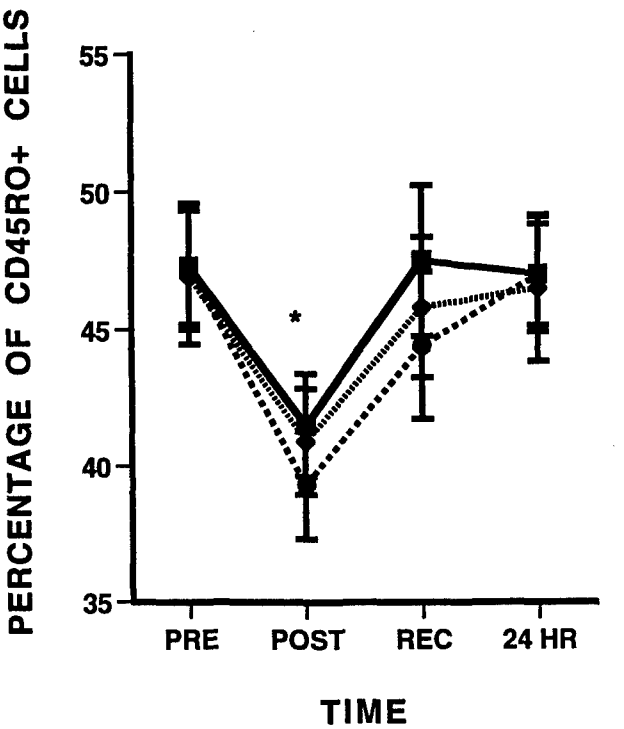


Figure 7. Urinary malondialdehyde (MDA) concentrations in PRE and POST exercise samples for all three treatments.

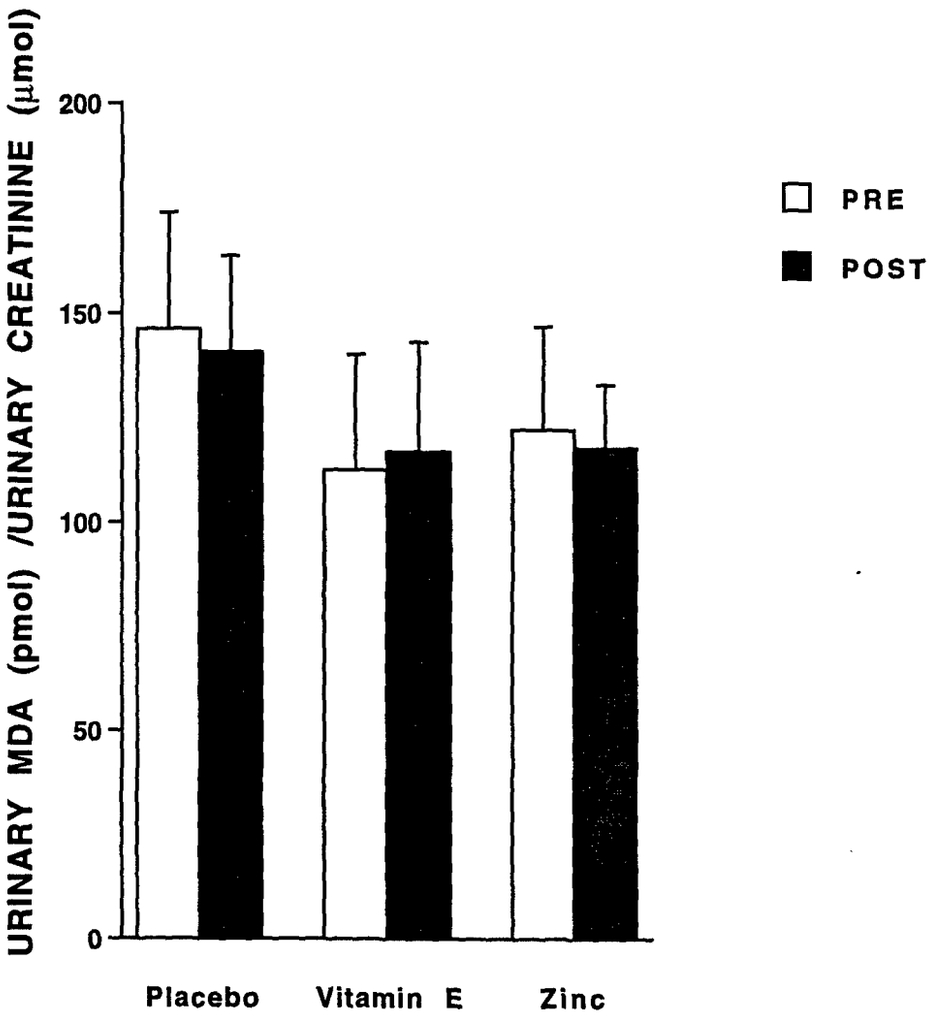


Table 1. General characteristics of the subjects

Characteristic	Mean (± SEM)	Range
Age (years)	32 ± 1	28 to 36
Height (inches)	65 ± 1	60 to 69
Weight (Kg)	60.6 ± 1.8	52.5 to 69.5
Body fat (%)	21.1 ± 1.2	15.2 to 27.6
Maximal aerobic capacity (L/min)	2.6 ± 0.1	2.1 to 3.4
Maximal aerobic capacity (ml/kg/min)	43.9 ± 2.1	32.6 to 51.7

Table 2. Selected physiological and metabolic measures for the three running conditions

	Zinc run	Vitamin E run	Placebo run
Cycle day	6.0 ± 0.3	6.0± 0.3	6.0 ± 0.4
Time to exhaustion	94 ± 11	98 ± 11	97 ± 10
Borg Scale[1]	18 ± 1	18 ± 1	18 ± 1
Heart rate (bpm)[1]	173 ± 3	173 ± 3	174 ± 4

[1]Measured immediately at the end of exercise

Appendix

None

Abstracts and Publications

None

Contributors

Contributors to this project include: Eric Clark, Elise Galliven, Demetria Sapienza, Park Lockwood, Dr. Anita Singh, Dr. Elzbieta Zelazowska, Dr. Patricia Deuster and MAJ Linda Lawrence, MC, USAF. Several current and former members of the Human Performance Lab, Dept of Military and Emergency Medicine, USUHS received pay for this research effort.

DEPARTMENT OF THE ARMY
U.S. ARMY MEDICAL RESEARCH AND MATERIEL COMMAND
FORT DETRICK, FREDERICK, MD 21702-5012

REPLY TO
ATTENTION OF:

MCMR-RMI-S (70-1y) 23 Jan 97

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