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**Section**: Article

**Article Title**: The Effect of Acute Exercise on Neutrophil ROS Production and Inflammatory Markers in Healthy Pre-Pubertal and Adult Males

**Authors**: Maple Liu and Brian W. Timmons

**Affiliations**: Child Health & Exercise Medicine Program, Department of Pediatrics, McMaster University, Hamilton, ON, Canada.

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THE EFFECT OF ACUTE EXERCISE ON NEUTROPHIL ROS PRODUCTION AND INFLAMMATORY MARKERS IN HEALTHY PRE-PUBERTAL AND ADULT MALES

Maple Liu and Brian W. Timmons

Child Health & Exercise Medicine Program, Department of Pediatrics, McMaster University,
Hamilton, ON

Running Title: Exercise and neutrophils

Corresponding author:
Brian W. Timmons, PhD
Child Health & Exercise Medicine Program
McMaster University
1280 Main Street West, Hamilton, ON
L8S 4K1, Canada
Email: timmonbw@mcmaster.ca
Phone: 905-521-2100, ext 77615
Fax: 905-521-1703
ABSTRACT

The adaptive effects of exercise-induced inflammation and reactive oxygen species production has been well studied in adults, but not in children. Characterizing the exercise responses in children compared to adults will start clarifying the transition from the child phenotype to that of an adult. Ten children aged 8-10 and 12 adults aged 19-21 performed 2×30min bouts of continuous cycling, separated by a 6min rest period, at a target work rate of 60% of their maximum aerobic capacity. Blood samples were collected pre- and immediately post-exercise, and analyzed for neutrophil count, systemic oxidative and inflammatory markers, and intracellular neutrophil-derived reactive oxygen species. Although post-exercise absolute neutrophils increased by approximately 2-fold in men (2.72±0.49 ×10⁹/L to 4.85±2.05 ×10⁹/L; p=0.007), boys showed no such change (3.18±0.67 ×10⁹/L to 3.57±0.73 ×10⁹/L; p=0.52). Contrary to these findings, boys did show an increase in overall intracellular neutrophil ROS production, whereas men did not. Boys also demonstrated higher overall protein carbonyl levels (0.07nmol/mg vs 0.04nmol/mg; boys vs men respectively), whereas men showed higher overall malondialdehyde (0.24µM vs 0.67µM; boys vs men respectively). The differences observed in the exercise-induced inflammatory and oxidative stress response may indicate growth-mediated adaptive responses to exercise during childhood development.
INTRODUCTION

An acute bout of exercise is a potent stimulus to the immune system in both children and adults. Interestingly, the magnitude of exercise-induced changes in some immune mediators is smaller in children compared to adults even when exercise is performed at the same relative intensity (35). This has led to the speculation that the smaller changes in inflammatory cytokines such as interleukin-6 (IL-6) and tumour necrosis factor-α (TNF-α) may be an inherent mechanism to counteract the potential for exercise-induced inflammation, which would be counterproductive to the processes of growth that are essential to children (34). While most of these studies have focused on components of the immune system, exercise also affects mediators of oxidative stress. It has been proposed that there exists a link between inflammatory cytokines and reactive oxygen species (ROS) generation (23,25), although few studies have investigated these relationships in either adults or children in the context of exercise.

ROS are a sub-population of free radical molecules originating from oxygen (5,8,19) that can affect many important metabolic processes through both direct interaction and secondary messengers (3). Typically, ROS such as superoxide anion (O$_2^-$) and hydrogen peroxide (H$_2$O$_2$), are unstable and are susceptible to react with other molecules. Although the body possesses antioxidants that counteract ROS reactivity, oxidative stress occurs when ROS activity surpasses antioxidant counteractive capacity (7,27). Chronically high levels of oxidative stress can be detrimental to health, causing negative downstream complications such as loss of membrane function, inflammation, dysregulation of protein homeostasis, and DNA mutations (5,7,10,14). However, the positive effects of oxidative stress have also been highlighted (27). ROS have been widely shown to regulate functions such as muscle development, blood flow, and contractile processes (23,27). Additionally, studies looking at oxidative stress in skeletal muscle
indicate an optimal ROS level for maximal isometric force production (23). Pediatric exercise studies have consistently shown an increase in the oxidative stress profile following acute bouts of exercise (14, 19). In light of these multi-functional effects of ROS, it seems that there exists a constant regulation of ROS to keep levels at homeostasis in order to maintain health, but the extent to which acute bouts of exercise contribute to this homeostasis remains unclear.

Heightened metabolic demands, as occurs during an acute bout of exercise, induces an increase in systemic and cellular ROS levels (5,7). The exercise-induced mobilization of neutrophils during exercise creates the possibility for enhanced ROS generation, since neutrophils can participate in a microbicidal reaction termed neutrophil oxidative burst, where ROS molecules such as $\text{O}_2^-$ and $\text{H}_2\text{O}_2$ are released (21,30,40). However, neutrophil-generated ROS production during exercise remains relatively understudied in both children and adults. Previous work has demonstrated a relationship between the IL-6 response and neutrophil cell counts during and following exercise, suggesting that neutrophil mobilization may be linked to exercise-induced changes in IL-6 levels. We speculated that if children experience a lower IL-6 response to exercise, they may also experience less mobilization of neutrophils compared to adults. We also wanted to determine, for the first time, possible relationships between mediators of oxidative stress and inflammatory cytokines during exercise in children compared to adults to identify possible age- or maturation-related differences.

The primary objective of this study was to compare the effects of acute exercise on ROS, as measured by intracellular and systemic levels, and inflammatory markers between boys and men. To observe these changes, various methods were used to detect intracellular and systemic markers of ROS production, as well as circulating inflammatory cytokines in response to exercise. It was hypothesized that ROS production would increase as a result of exercise across
both groups; however, like the blunted exercise-induced cytokine response that is characteristicly observed in children, boys would show a blunted increase in ROS production compared to adult participants.

METHODS

Participants

Ten healthy pre-pubertal boys and 12 healthy adult men volunteered for this study. Participants were recruited from the community. Inclusion criteria included: male; age of 8 to 10 years or 19 to 21 years; and an aerobic fitness ($\text{VO}_2\text{max}$) $\geq$ 35 mL·kg$^{-1}$·min$^{-1}$. Exclusion criteria included: taking any supplements, multivitamins, or medication in the last month; allergies; and a family history of inflammatory disease. Participant characteristics are depicted in Table 1.

Experimental design and exercise testing

The study was approved by the Hamilton Health Sciences/Faculty of Health Sciences Research Ethics Board. Participants completed two visits with a span of at least 3 days between visits to allow for completion of a 3-day food record.

Visit 1: Informed consent was obtained by the parent or participant (if an adult participant) and assent obtained from the participant (if a child), followed by completion of a medical and activity questionnaire. A 3-day food record log book was also provided and explained so that it could be completed at home. Height, weight, percent body fat, and Tanner stage (for children only) were recorded, followed by the McMaster All-Out Progressive Continuous Cycling Test on a cycle ergometer to test $\text{VO}_2\text{max}$.

Visit 2: Participants were asked to refrain from doing heavy exercise 24 h prior to and from eating and drinking (other than water) 3 hours prior to this second visit. The exercise
The protocol consisted of 2×30 min bouts of cycling, with 6 min rest between bouts. The intensity was equivalent to the power output corresponding to 60% of VO$_{2\text{max}}$, as determined at Visit 1. Expired gases were analyzed periodically during the exercise to ensure each participant was working at the appropriate intensity and power output was adjusted, if necessary. Participants were allowed to drink water ad libitum.

**Three-day food record**

Participants were asked to record their diet over 3 days prior to visit 2. They were instructed to record the type and quantity of all foods consumed for 2 regular week days and one weekend day. We did not require participants to alter their normal diets, but to record it. Three-day food records were analyzed by aRegistered Dietitian using The Food Processor SQL Version 10.10.0 (Esha Research, Salem, Oregon, 2012) to provide average daily intakes (Table 2). Based on age, sex, and estimated physical activity levels, values were reported as a percent of recommended daily intake; most participants for this study were in the moderate to very active categories.

**Blood collection**

At Visit 2, an indwelling catheter was placed in a vein in the antecubital area of the arm, after the participant had rested in a supine position for 10 min. A pre-exercise blood sample was collected ~5 min before the start of exercise. A second blood sample was taken immediately post-exercise.

**Blood analysis**

Complete blood counts were performed from EDTA-treated whole blood to enumerate leukocytes, hemoglobin, hematocrit, the proportion of neutrophils, and neutrophil counts using
an automated Coulter counter at the McMaster University Medical Centre Core Laboratory. Changes in blood and plasma volume were accounted for according to Dill & Costill (4) using hemoglobin and hematocrit. A portion of each blood sample was allowed to clot and then centrifuged to collect serum, which was analyzed for the inflammatory cytokines IL-6 and TNF-α, and elastase using commercially-available ELISA kits (R&D systems; Minneapolis, MN). Another portion of EDTA-treated blood was centrifuged to collect plasma, which was analyzed for the ROS markers protein carbonyls and malondialdehyde, using commercially-available ELISA kits (Northwest Life Science Specialties; Vancouver, WA).

**Intracellular neutrophil ROS analysis**

Neutrophils were isolated from whole blood using both Histopaque 1077 and Histopaque 1119 according to the manufacturer’s instructions (Sigma Aldrich, Oakville, ON). Briefly, 3mL of Histopaque 1119 were overlayed with 3mL of Histopaque 1077, which was then overlayed with 6mL of whole blood. Tubes were then centrifuged at 700g for 30min. The third layer, containing granulocytes, was pooled into a separate tube, and washed with 10mL of RPMI 1640, and centrifuged at 200g for 10min. The supernatant was discarded, the pellet suspended in 2.5mL of BD Pharm Lyse lysing buffer (BD Biosciences; Ontario, CA), incubated for 5min, and centrifuged again at 200g for 5min. The cells were washed twice more, and re-suspended in 4mL of RPMI 1640 + 2.0g NaHCO₃/L RPMI1640 (Fisher Scientific; Ottawa, ON). Cell viability and count of the isolated neutrophils were assessed using a Countess cell counter. Based on previous methods (17,30,39,40), a final cell concentration of 1x10⁶ cells/mL was used, with viability >87%. Three probes were used to detect different ROS: 2’,7’-dichlorofluorescein diacetate (DCFH) as a general ROS marker, dihydrorhodamine 123 (DHR) for H₂O₂ (Sigma Aldrich; Oakville, ON), and hydroethidine or dihydroethidium (HE) for O₂⁻. Relatively small in
size and capable of diffusing across cell membranes, the probes are trapped in the cell and emit a highly fluorescent signal upon reaction with ROS (40). PMA (Fisher Scientific; Ottawa, ON) was used to stimulate neutrophils by activating the nicotinamide adenine dinucleotide phosphate oxidase enzyme resulting in an upregulation of ROS production (40). Cells were incubated for 20 min at 37˚C with each probe in concentrations determined in pilot experiments: 0.625μM DCFH, 1μM DHR, 10μM HE, and 10μM PMA. Immediately after incubation, cells were placed on ice to stop the reaction until flow cytometry analysis.

**Flow cytometry**

Flow cytometry analysis was performed using a three-laser (488nm, 633, 350nm UV), 15-color capable LSRII instrument (BD Biosciences), using the 488nm excitation, with FACsDiva software from BD Biosciences for data acquisition. A total of $10^4$ events were collected within the neutrophil gate. DCFH and DHR were measured in the FL1 channel for green fluorescence using FITC emission, and HE was measured in the FL2 channel for red fluorescence. Off-line analysis was completed using the program FlowJo version 8.7 for Macintosh (Tree Star Inc 2008), gating around the neutrophil scatter. ROS production was quantified by the median fluorescence intensity.

**Statistics**

All values are presented as means ± SD. Independent t-tests (SPSS 18.0) were used to determine differences between boys and men for chronological age, height, weight, percent body fat, and VO$_{2\text{max}}$ values, as well as percent change in ROS production by stimulated neutrophils. Two-way repeated measures ANOVAs (Statistica version 5.0) for group × time interaction (boys vs men; pre- vs post-exercise, respectively) were used to analyze the proportion of neutrophils,
neutrophil counts, IL-6, TNF-α, elastase, protein carbonyls, malondialdehyde, and fold change in DCFH, DHR, HE (stimulated values ÷ unstimulated values). A significant group × time interaction was pursued with the Tukey’s post-hoc test (Statistica version 5.0). Significance was set at \( p \leq 0.05 \). Cohen’s \( d \) equation was used to calculate effect size.

RESULTS

Participant characteristics

As expected, our participants were significantly different in chronological age, height, and weight \( (p<0.05) \). However, there were no differences observed in percent body fat or \( \text{VO}_{2\text{max}} \) between boys and men. From dietary records (Table 2), energy \( (\text{kcal/kg}) \), carbohydrate and fat intake \( (\text{g/kg}) \), Vitamin A \( (\text{RAE/kg}) \) and Vitamin C \( (\text{mg/kg and % recommended}) \) were significantly lower in the men compared with the boys.

Immune cell response (Table 3)

Exercise induced a significant increase in total leukocyte count post-exercise for both boys and men \( (p=0.016; \ p=0.004, \ \text{respectively}) \). Boys showed a lower proportion of neutrophils compared to men \( (\text{main group effect: } p=0.03) \) with an effect size \( (d) \) of 0.88. Exercise did not induce significant changes in the proportion of neutrophils from pre- to post-exercise. Post-exercise neutrophil counts in men were approximately two-fold higher than pre-exercise levels \( (\text{main time effect: } p=0.007) \) with a \( (d) \) of 0.90, an effect that was not observed in boys.

Protein carbonyls, malondialdehyde, and elastase (Table 4)

Boys showed overall higher concentrations of protein carbonyls than men \( (\text{main group effect: } p<0.001) \) with a \( (d) \) of 2.39. However, men showed overall higher concentrations of malondialdehyde compared to boys \( (\text{main group effect: } p<0.001) \) with a \( (d) \) of 1.88. There were
no significant exercise effects on either protein carbonyls or malondialdehyde for boys or men. There were no significant changes in elastase levels either between groups or due to exercise.

**IL-6 and TNF-α inflammatory markers**

Boys showed overall higher concentrations of TNF-α than men (main group effect: \( p<0.001 \)) with a \((d)\) of 1.59. Men also showed an increase in TNF-α levels from pre- to post-exercise (\( p=0.008 \)); however, no increase was observed in boys (Figure 1A). Exercise induced a 2.5-fold increase in IL-6 levels from pre- to post-exercise (main time effect: \( p<0.001 \)) with a \((d)\) of 1.84 (Figure 1B). It was determined that post-exercise IL-6 concentrations were significantly higher than baseline in both boys and men, and that boys showed significantly lower post-exercise IL-6 levels compared to men (exercise effect: boys \( p<0.001 \), men \( p<0.001 \); post-exercise boys vs post-exercise men \( p=0.01 \) with a \((d)\) of 1.07.

**Intracellular ROS markers: DCFH, DHR, HE (Figure 2)**

Exercise resulted in a higher fold-change of stimulated to unstimulated values for DCFH post-exercise in boys (\( p=0.018 \)) but not in men (\( p=0.71 \)). A time effect was seen for DHR, with a higher post-exercise fold change compared to pre-exercise (\( p=0.02 \)) with \((d)\) of 0.48. Although post-exercise DHR fold-changes were not significantly different to pre-exercise values in men (\( p=0.13 \), boys tended to have a higher fold-change (\( p=0.097 \)). Exercise also induced a higher fold-change for HE (main time effect: \( p=0.05 \)) with a \((d)\) of 0.59.

**DISCUSSION**

Despite the large body of evidence describing physiological adaptations gained from exercise in adults, such literature is scarce within the pediatric population. On the one hand, this is surprising given the role that physical activity and exercise may play at this crucial time of
development; on the other hand, limitations in biological samples preclude an in depth understanding of biological processes. To address gaps in our understanding, the main objective of this study was to identify the age-related transition in the exercise-induced production of ROS and inflammatory markers, comparing boys to men.

The changes in neutrophils from before to after exercise showed an approximately 1.5-fold increase in men, but no change in boys. In contrast, the proportion of neutrophils remained constant with neither group showing an exercise-induced change, indicating that acute exercise induces a more rigorous mobilization of neutrophils into the circulation in men than that observed in boys. These results are consistent with the limited existing research directly comparing children to adults, where acute exercise generally elicits a smaller neutrophil response in children (28,34,37).

In this study, we assessed neutrophil production of intracellular ROS, as measured by flow cytometry. Three fluorescent markers were used to gauge different types of ROS; DCFH is a general ROS marker, DHR is specific to H$_2$O$_2$, and HE is specific to O$_2^-$ (40). Compared to men, boys demonstrated a significantly higher fold-change in neutrophil-stimulated ROS production after exercise (measured by DCFH) with other markers following the same trend. The higher stimulated neutrophil ROS production, taken together with no change in the neutrophil response to exercise in boys, may be explained in part, if the boys demonstrated a more sensitized oxidative response to exercise. Smith et al attributed neutrophil priming and activation, to growth hormone produced after a bout of exercise, since in vitro studies have shown the presence of growth hormone receptors on neutrophils (29). Exercise studies involving children have shown increases in growth hormone after an acute bout of exercise in healthy participants (16,18,38). Although the exercise-induced growth hormone response in adults is
higher in absolute concentrations, children typically show a relatively larger percent change from baseline with exercise, which is most likely important for the anabolic processes during their development (35). Given the results of our study, a relatively larger growth hormone response to exercise may also contribute to a sensitized neutrophil ROS production in boys compared with men.

A possible sensitizing effect of exercise on neutrophils may also be attributed to the type of neutrophils mobilized with exercise. Exercise can induce recruitment of neutrophils from circulation in the blood, or induce mobilization from the bone marrow (20) or other marginated pools. An earlier study revealed varying levels of intracellular \( \text{H}_2\text{O}_2 \) produced per neutrophil after PMA-stimulation, and this was used to distinguish two subpopulations of neutrophils, thought to originate from either circulation or from capillary beds and blood vessel walls (29). Two recent studies tested adult male participants on treadmill protocols of various intensities (20,22). Peake and colleagues determined that the decrease in the specific neutrophil CD16 receptor after an acute bout of high intensity exercise could be explained by a shift toward the release of newly recruited neutrophils from bone marrow (20). This is supported by previous studies showing an increase in immature neutrophils after exercise (1,31,32), and that neutrophils shed their CD16 receptors when they are mobilized from the bone marrow (9). Because boys generally experienced a higher fold-change in ROS after exercise than men, which cannot be attributed to increase in absolute neutrophils, it is possible that exercise-induced neutrophil recruitment in boys originates from the bone marrow opposed to circulating pools in the blood. It is also important to note that, although the men reported lower dietary levels of Vitamins A and C, this did not appear to translate into higher levels of oxidative stress. Self-report diet
records are subject to recall bias, and more work is needed to understand the effect of dietary antioxidants on exercise-induced oxidative stress in children.

To complement our neutrophil data, we measured the systemic levels of inflammatory mediators and markers of oxidative stress. Acute exercise did not affect systemic TNF-α concentrations in boys, but induced a small increase in men, which is consistent with existing literature especially for children, where TNF-α either does not change or has been seen to decreases due to exercise (16,26,37). Adults do not generally demonstrate an increase in TNF-α levels after acute exercise; increases are mostly seen as a result of long duration exercise protocols (13,35). We also found that acute exercise significantly increased systemic IL-6 levels from baseline in both boys (2-fold increase) and men (4-fold increase), and post-exercise IL-6 concentrations were lower in boys compared to men. These results are also consistent with the literature whereby although children generally do show an increase in IL-6 after acute exercise, it is of much smaller magnitude compared to adults (16,26,35,37). Our findings are consistent with a review of 67 exercise trials with various modes of testing that showed acute exercise cycling stimulates an increase in IL-6 by 5-fold from baseline in adult participants (6). In contrast to our cytokine findings, an acute bout of cycling exercise did not induce significant increases in protein carbonyls or malondialdehyde post-exercise in either boys or men. To our knowledge, only two pediatric studies have tested the effect of acute exercise on circulating protein carbonyl levels, with conflicting results. Nikolaidis et al. found that protein carbonyls were approximately two-fold higher in both boys and girls after intermittent swimming exercise (19) whereas our previous work did not find changes in protein carbonyls following a 1hr exercise protocol set at an intensity of 70%VO2max (36). The adult literature on protein carbonyl levels after a bout of acute exercise is also conflicting, despite a greater number of studies done to date (5,7).
available studies suggest that protein carbonyls increase in an intensity dependent fashion, with a sustained elevated level several hours after exercise (7,11). The current study showed no change in plasma protein carbonyl levels in men as a result of an acute bout of cycling exercise (Table 3), which is consistent with results obtained from exercise studies using similar protocols such as graded exercise tests or short testing periods (within 1hr) (2,7,12). It is possible that additional blood samples collected during the recovery period may have detected increases in protein carbonyl levels, compared to baseline (2). To our knowledge, there have not been any previous studies looking at the effects of acute exercise on malondialdehyde in children. However, three studies tested the effect of acute exercise on markers of lipid peroxidation (19,24,41). Only one of the three studies showed a significant increase in thiobarbituric acid-reactive substances, testing trained pre-pubescent swimmers with an intermittent high-intensity swimming protocol (19). Control subjects from the other two studies did not show any significant changes in F2-isoprostanes (common marker for lipid peroxidation) after an acute intermittent bout of 30 min cycling exercise, or an incremental cycling exercise to exhaustion (24,41). Our results parallel the latter two studies, which is not unexpected due to the similarity in the nature of exercise protocols.

While this study presents novel data related to exercise-induced changes in markers of oxidative stress, it is limited by a small sample size. It is possible that a low statistical power led to non-significant results. For example, Elastase (ng/ml) decreased from pre- to post-exercise by 15% in boys and 12% in men. With a larger sample size, such differences may have been significant. The exercise induced increase in ROS production reported in Figure 2 could also have suffered from low statistical power. Therefore, our results can be interpreted with caution. Importantly, however, the findings from this study confirm previous age-related differences in
the IL-6 response to exercise and provide information necessary to design future trials to examine the relationship between ROS and exercise in health and disease.

In conclusion, the novel findings from this study point towards exercise as a potential stimulant for a sensitized neutrophil response in boys, which has not been previously characterized. This was contrary to the exercise response in men; despite a greater neutrophil mobilization, exercise did not induce the same sensitized neutrophil response as in boys. Further examining the exercise-induced adaptive mechanisms in children compared to adults will lend a better understanding of healthy growth and development in children.

ACKNOWLEDGEMENTS

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REFERENCES


Figure 1. TNF-α and IL-6 values at baseline and after an acute bout of exercise in boys (n=10) and men (n=12). A. TNF-α: pg/mL, B. Interleukin-6 (IL-6: pg/mL). Values are expressed as means ± SD. * post-exercise values are significantly higher at p<0.05 compared to pre-exercise, **post-exercise values are significantly different at p<0.05 between boys and men.
Figure 2. Fold change in intracellular ROS production (stimulated ÷ unstimulated), measured by A. DCFH, B. DHR, C. HE fluorescence intensity in boys (n=10) and men (n=12). Values are expressed as means ± SD. * post-exercise values are significantly higher at $p<0.05$ compared to pre-exercise.
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Table 1. Participant characteristics

<table>
<thead>
<tr>
<th></th>
<th>Boys (n=10)</th>
<th>Men (n=12)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chronological age (years)</td>
<td>9.5 ± 1.2</td>
<td>20.9 ± 1.0*</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>140.1 ± 9.4</td>
<td>178.2 ± 9.8*</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>34.7 ± 6.9</td>
<td>78.3 ± 18.8*</td>
</tr>
<tr>
<td>% Body fat</td>
<td>14.7 ± 7.4</td>
<td>12.8 ± 4.0</td>
</tr>
<tr>
<td>VO2max (mL/kg/min)</td>
<td>51.4 ± 8.1</td>
<td>49.5 ± 13.3</td>
</tr>
<tr>
<td>Tanner</td>
<td>1 (n=8)</td>
<td>2 (n=2)</td>
</tr>
</tbody>
</table>

*, significantly different from boys. %body fat: Percent body fat was calculated using bioelectrical impedance analysis; Tanner stage was self-assessed using five diagrams depicting stages of pubic hair development for boys.
Table 2. Daily intake of macronutrients per kilogram body weight and major antioxidants from 3-day food record.

<table>
<thead>
<tr>
<th>Nutrient</th>
<th>Total or % Rcmd</th>
<th>Boys (n=8)</th>
<th>Men (n=11)</th>
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</thead>
<tbody>
<tr>
<td>Energy (kcal)</td>
<td>Total</td>
<td>2041.7 ± 152.9</td>
<td>2805 ± 1143</td>
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<td></td>
<td>%Rcmd</td>
<td>78.4 ± 13.3</td>
<td>84.1 ± 33.2</td>
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<tr>
<td>Energy (kcal/kg)</td>
<td>Total</td>
<td>58.8 ± 12.1</td>
<td>36.5 ± 14.9*</td>
</tr>
<tr>
<td></td>
<td>%Rcmd</td>
<td>270.7 ± 65.3</td>
<td>241.8 ± 96.3</td>
</tr>
<tr>
<td>Protein (g/kg)</td>
<td>Total</td>
<td>2.6 ± 0.6</td>
<td>1.9 ± 0.8</td>
</tr>
<tr>
<td></td>
<td>%Rcmd</td>
<td>270.7 ± 65.3</td>
<td>241.8 ± 96.3</td>
</tr>
<tr>
<td>Carbohydrates (g/kg)</td>
<td>Total</td>
<td>7.2 ± 2.1</td>
<td>4.4 ± 1.9*</td>
</tr>
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<td></td>
<td>%Rcmd</td>
<td>69.8 ± 18.9</td>
<td>73.8 ± 32.8</td>
</tr>
<tr>
<td>Fat (g/kg)</td>
<td>Total</td>
<td>2.3 ± 0.7</td>
<td>1.3 ± 0.6*</td>
</tr>
<tr>
<td></td>
<td>%Rcmd</td>
<td>97.1 ± 27.8</td>
<td>95.6 ± 45.1</td>
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<tr>
<td>Vitamin A (RAE/kg)</td>
<td>Total</td>
<td>22.2 ± 9.5</td>
<td>10.8 ± 8.4*</td>
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<td></td>
<td>%Rcmd</td>
<td>147.7 ± 75.2</td>
<td>90.9 ± 71.6</td>
</tr>
<tr>
<td>Vitamin C (mg/kg)</td>
<td>Total</td>
<td>3.7 ± 2.2</td>
<td>1.7 ± 1.5*</td>
</tr>
<tr>
<td></td>
<td>%Rcmd</td>
<td>315.6 ± 141.6</td>
<td>141.4 ± 119.8*</td>
</tr>
<tr>
<td>Vitamin E (mg/kg)</td>
<td>Total</td>
<td>0.082 ± 0.022</td>
<td>0.081 ± 0.072</td>
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<td></td>
<td>%Rcmd</td>
<td>29.2 ± 7.1</td>
<td>41.3 ± 35.6</td>
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</table>

*, significantly different than boys, p<0.05. %Rcmd: Percent recommended based on age, sex, and physical activity levels (most participants were in the moderate to very active categories), RAE: retinol activity equivalents for vitamin A.
Table 3. Complete blood counts and systemic markers of oxidative stress pre- and post-exercise in boys and men.

<table>
<thead>
<tr>
<th></th>
<th>Boys (n=10)</th>
<th></th>
<th>Men (n=12)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pre</td>
<td>Post</td>
<td>Pre</td>
<td>Post</td>
</tr>
<tr>
<td>Leukocytes (×10⁹/L)</td>
<td>6.3±1.3</td>
<td>7.2±1.3*</td>
<td>5.0±0.7</td>
<td>7.7±2.5*</td>
</tr>
<tr>
<td>Neutrophils (%)</td>
<td>50.3±2.8</td>
<td>48.9±5.7</td>
<td>54.5±7.6</td>
<td>56.6±9.6</td>
</tr>
<tr>
<td>Neutrophils (×10⁹/L)</td>
<td>3.2±0.7</td>
<td>3.5±0.7</td>
<td>2.7±0.5</td>
<td>4.5±2.0*</td>
</tr>
<tr>
<td>Protein carbonyls (nmol/mg)</td>
<td>0.070±0.012</td>
<td>0.070±0.012</td>
<td>0.046±0.010</td>
<td>0.041±0.011</td>
</tr>
<tr>
<td>Malondialdehyde (μM)</td>
<td>0.2±0.2</td>
<td>0.3±0.2</td>
<td>0.7±0.3</td>
<td>0.7±0.3</td>
</tr>
<tr>
<td>Elastase (ng/mL)</td>
<td>88.7±43.3</td>
<td>75.7±27.5</td>
<td>94.6±23.7</td>
<td>85.2±19.0</td>
</tr>
</tbody>
</table>

*, significantly different than Pre.