

## Dietary antioxidant restriction affects the inflammatory response in athletes

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The purpose of the present study was to determine the effects of dietary antioxidant restriction on plasma concentrations of carotenoids and inflammatory markers at rest and in response to exercise in endurance-trained males. Seventeen males performed two exercise trials 2 weeks apart. Participants followed their habitual antioxidant diet (H-AO) before the first exercise test, then a restricted antioxidant diet (R-AO) for 2 weeks before the second exercise test. Blood was collected pre- and post-exercise. Dietary intakes of fibre, ascorbic acid and  $\beta$ -carotene were lower ( $P < 0.05$ ) on the R-AO diet, but no other differences were observed. Pre-exercise plasma  $\beta$ -carotene concentrations were lower (H-AO, 195 (SD 92); R-AO, 123 (SD 54) ng/ml;  $P < 0.05$ ), and TNF- $\alpha$  concentrations were higher (H-AO, 16 (SD 7); R-AO, 613 (SD 325) pg/ml;  $P < 0.01$ ) on the R-AO diet compared to the H-AO diet. Most plasma carotenoid concentrations decreased with exercise, but this effect was more consistent on the H-AO diet. No differences in plasma IL-6 concentrations were observed pre-exercise, whereas post-exercise plasma IL-6 concentrations (H-AO, 30.3 (SD 16); R-AO, 15.3 (SD 5) pg/ml;  $P < 0.05$ ) were lower following the R-AO diet. Post-exercise TNF- $\alpha$  concentrations were higher on the R-AO diet. Ratings of perceived effort during submaximal exercise were higher ( $P < 0.05$ ) on the R-AO diet, but there was no difference in the time to exhaustion between diets. In conclusion, lower dietary intakes of carotenoids alter the plasma concentrations of antioxidants and markers of inflammation at rest and in response to exercise.

### Carotenoids: Cytokines: Inflammation: Oxidative stress

Elevations in oxygen consumption during exercise increase metabolic processes such as the production of reactive oxygen species (ROS), which can lead to oxidative damage of cellular lipids, proteins and nucleic acids<sup>(1)</sup>. Antioxidants defend the body against such oxidative stress and contribute to the maintenance of a healthy antioxidant:oxidant balance. There are two forms of antioxidants: enzymes such as catalase, glutathione peroxidase and superoxide dismutase; dietary antioxidants such as vitamins C and E, carotenoids, flavonoids, Zn and Se<sup>(2)</sup>. Carotenoids are found in plants, function as accessory pigments in photosynthesis and include lycopene,  $\beta$ -carotene and lutein. The major sites for storage of carotenoids in the body are adipose tissue and the liver, although they are found in other tissues including the kidney, lungs and prostate<sup>(3)</sup>. These carotenoids can be mobilised during states of increased oxidative stress such as exercise<sup>(4)</sup>.

Exercise-induced ROS are released from mitochondria in exercising muscles or from inflammatory cells such as phagocytes<sup>(5)</sup>. ROS generation can increase the secretion of pro-inflammatory mediators via the effects of NF- $\kappa$ B on the cell nucleus<sup>(2)</sup>. Consequently, exercise may increase the release of pro-inflammatory mediators<sup>(6–11)</sup>.

PUFA, which are important components of cell membranes, are vulnerable to oxidation and can attenuate a pro-inflammatory response<sup>(12–14)</sup>. Carotenoids can protect PUFA from ROS damage<sup>(15)</sup>. A change in dietary intake of carotenoids

potentially alters ROS oxidative damage to PUFA and the release of inflammatory mediators.

The aim of the present study was to examine the effect of the dietary restriction of antioxidants on the plasma concentrations of carotenoids and inflammatory mediators at rest and in response to exercise in healthy endurance-trained males.

### Experimental methods

#### Subjects and study design

Seventeen healthy endurance-trained male adults aged 18–35 years were recruited to participate in the study. All participants were non-smokers and did not take any vitamin or mineral supplements or medications that would affect oxidative stress or inflammatory mediators. The institution's Human Research Ethics Committee approved the study and all participants provided informed written consent before participation.

Participants attended the laboratory on three separate occasions. At the first visit, participants completed a treadmill  $V_{O_{2max}}$  test, had anthropometric measurements taken and were given dietary instructions. Percentage body fat was calculated from skinfold thickness measurements (triceps, subscapula, biceps, iliac crest, super spinatus, abdominal, front thigh and midcalf) using the Womersley & Dumin equation<sup>(16)</sup>. Four-day weighed food records were completed before the second

**Abbreviations:** H-AO, habitual antioxidant diet; LTB<sub>4</sub>, leukotriene B<sub>4</sub>; ROS, reactive oxygen species; R-AO, restricted antioxidant diet.

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and third visits. For visits 2 and 3, participants arrived at the laboratory after an overnight fast (consumption of water was allowed) and provided blood samples (20 ml) before and after a treadmill exercise trial. Participants were asked to refrain from physical activity for 24 h before the exercise tests. Visits 2 and 3 were separated by 2 weeks of dietary antioxidant restriction.

#### *V<sub>O2max</sub> test*

The V<sub>O2max</sub> exercise test was conducted on a motorised treadmill (Powerjog Treadmill Model JM100, Expert Fitness, Mid Glamorgan, Wales). Ventilation (breaths/min), FE<sub>O2</sub> and FE<sub>CO<sub>2</sub></sub> were measured, and V<sub>O2</sub> and V<sub>CO<sub>2</sub></sub> were calculated from ventilation, FE<sub>O2</sub> and FE<sub>CO<sub>2</sub></sub> by a computerised online gas analysis system (SensorMedics 2900c; SensorMedics Corporation, Yorba Linda, CA, USA) calibrated to known gases. Heart rate was monitored throughout the test by electrocardiogram (SensorMedics 2900c; SensorMedics Corporation). The initial running speed of each participant was 10 km/h with a gradient of 0%. Speed increased 2 km/h every 2 min until the participant's maximum voluntary speed was achieved. If V<sub>O2max</sub> was not achieved with maximum voluntary speed, the gradient was then increased 2% every 2 min until voluntary exhaustion was achieved. V<sub>O2max</sub>, heart rate, RER, exercise time, speed and gradient were recorded. V<sub>O2max</sub> was the highest 30 s V<sub>O<sub>2</sub></sub><sup>(17)</sup> recorded together with the criteria of a plateau in oxygen uptake, RER > 1.15 and a heart rate of 220 – age ± 10 beats/min.

#### *Exercise protocols*

Participants performed the following treadmill exercise trials during the second and third visits to the laboratory. The participant's running speed commenced at the workload that elicited 60% of V<sub>O2max</sub> established during the first visit and was maintained for 30 min after which the speed was increased by 2 km/h every 2 min until the participant's maximum voluntary speed was achieved, then the gradient was increased 2% every 2 min until voluntary exhaustion. Heart rate was monitored throughout the test by electrocardiogram. The exercise tests were carried out in a thermally controlled environment (22 ± 2°C, 40–60% relative humidity). A similar exercise protocol that also involved high-intensity exercise (the incremental test to exhaustion was substituted for a 5 min run at 90% V<sub>O2max</sub>) has been shown to affect lipid peroxidation in a similar cohort<sup>(18)</sup>.

#### *Exercise performance measurements*

Ratings of perceived effort were determined using a fifteen-point Borg scale<sup>(19)</sup> and were obtained during the last minute of the 30-min submaximal portion of the exercise trial. Time to exhaustion during the incremental test following the 30-min submaximal exercise was used to measure exercise performance.

#### *Dietary intervention*

Between visits 1 and 2, the participants followed their habitual antioxidant diet (H-AO). Between visits 2 and 3, the participants followed a restricted antioxidant diet (R-AO) compared

to their H-AO diet for 2 weeks. Participants restricted their intake of fruits and vegetables to 1–2 servings/d as this protocol has been shown to reduce plasma antioxidant concentrations in a 2-week period<sup>(20)</sup>. Participants were also asked to avoid the consumption of other foods that are high in antioxidants including tea, cod liver oil and wheat germ oil.

#### *Dietary analysis*

Participants completed a 4-d weighed food record for two work days and two weekend days. Participants were given a set of electronic scales and instructions on how to complete the food record during visit 1, and collected food records before visit 2 while following the H-AO diet and before visit 3 while following the R-AO diet. Food records were analysed using FoodWorks program (version 2.1, Build 146, Xyris Software, Highgate Hill, QLD, Australia).

#### *Daily energy estimation*

The Cunningham equation was used<sup>(21)</sup> to estimate the daily energy requirements of participants. RMR multiplied by an activity factor of 1.8 is the best predictor of mean daily energy requirements for an athletic population<sup>(21)</sup>.

#### *Blood sample collection*

Blood samples were collected pre-exercise and immediately post-exercise. A needleless cannula was inserted into the superficial vein of the forearm before exercise and was removed after the final blood draw. Blood samples were collected into sterile EDTA tubes and stored on ice. A maximum of 20 ml whole blood was collected per blood draw. Within 2 h of collection, the whole blood was centrifuged (3000 g, 10 min, 4°C) to separate plasma, which was then stored at –80°C for later analysis.

#### *Analysis of carotenoid composition of plasma*

Carotenoids (α- and β-carotenes, lutein/zeaxanthin, lycopene and β-cryptoxanthin) were extracted from plasma and analysed via HPLC<sup>(22)</sup>. Separation was achieved using a 5 μm ODS Hypersil column (100 mm × 2.1 mm inner diameter).

#### *Analysis of inflammatory mediator composition of plasma*

All assays were performed on plasma samples according to the kit manufacturer's instructions in a single batch. IL-6 concentrations were analysed via enzyme-amplified sensitivity immunoassay with a detection limit of 2 pg/ml and 100% specificity (enzyme-amplified sensitivity immunoassay; BioSource, Nivelles, Belgium), TNF-α concentrations were analysed via ELISA with a detection limit of 3 pg/ml and 100% specificity (BioSource), leukotriene B<sub>4</sub> (LTB<sub>4</sub>) concentrations were analysed via enzyme immunoassay with a detection limit of 13 pg/ml and 100% specificity (enzyme immunoassay; Cayman Chemical Company, Ann Arbor, MI, USA).

#### *Statistical analysis*

Results are presented as means and standard deviations. Normality of plasma metabolite data was checked by skewness

and kurtosis. All plasma data that were not normally distributed were log-transformed before analysis. Differences between pre- and post-exercise values were compared using  $2 \times 2$  repeated-measures ANOVA. In all analyses, the limit for statistical significance was set at  $P < 0.05$ .

The present study was conducted according to the guidelines laid down in the Declaration of Helsinki, and all procedures involving human subjects were approved by the University of Newcastle Human Research Ethics Committee. Written informed consent was obtained from all subjects.

## Results

### Participant characteristics, dietary intake and exercise performance

Seventeen participants completed the study with one participant withdrawing before visit 2 due to injury. The characteristics of the participants are presented in Table 1 and their dietary intakes are displayed in Table 2. There were NS differences in dietary carbohydrate, protein or fat intake on either diet, whereas dietary intakes of fibre, ascorbic acid and  $\beta$ -carotene were significantly lower on the R-AO diet compared to the H-AO diet. There was NS difference in total energy intake or estimated total energy expenditure between diets. Time to exhaustion on the incremental phase of the exercise did not differ between trials (9.0 (SD 0.3) v. 9.2 (SD 0.3) min), but the rating of perceived effort was significantly higher on the R-AO diet compared to the H-AO diet (11.8 (SD 0.3) v. 12.4 (SD 0.3);  $P < 0.05$ ).

### Plasma carotenoids

The changes in carotenoid concentrations are shown in Fig. 1. Pre-exercise plasma  $\beta$ -carotene and lutein/zeaxanthin concentrations were lower ( $P < 0.05$ ) on the R-AO diet compared to the H-AO diet; there were NS differences in the pre-exercise concentrations of the other carotenoids. On the H-AO diet, all plasma carotenoid concentrations except lutein/zeaxanthin decreased ( $P < 0.05$ ) in response to exercise. On the R-AO diet, lycopene and  $\beta$ -cryptoxanthin concentrations decreased ( $P < 0.05$ ), whereas  $\alpha$ -carotene concentrations increased ( $P < 0.05$ ) in response to exercise. Post-exercise lycopene concentrations were lower ( $P < 0.05$ ) on the R-AO diet, but no other significant differences in carotenoid concentrations were observed between diets.

**Table 1.** Characteristics of participants ( $n$  17) (Mean values and standard deviations)

	Mean	SD
Age (years)	24	1
Ht (cm)	179	2
Wt (kg)	75	2
Estimated DEE (MJ/d)	13.4	0.1
$V_{O_{2max}}$ (ml $O_2$ /kg per min)	57.7	1.4
Percentage body fat (%)	14.8	1.1

DEE, daily energy expenditure.

**Table 2.** Dietary intakes of participants ( $n$  17) on their habitual antioxidant (H-AO) and reduced antioxidant (R-AO) diets (Mean values and standard deviations)

	Diet				$P^*$
	H-AO		R-AO		
	Mean	SD	Mean	SD	
Energy intake (MJ/d)	14	1	14	1	
Protein (g)	138	10	148	9	
Carbohydrate (g)	423	28	413	7	
Fat (g)	109	8	114	7	
Polyunsaturated (g)	15	1	16	1	
Monounsaturated (g)	40	3	40	2	
Saturated (g)	40	4	48	4	
Fibre (g)	36	4	24	2	$< 0.01$
Ascorbic acid (mg)	139	14	49	8	$< 0.001$
$\beta$ -Carotene ( $\mu$ g)	5093	931	1142	142	$< 0.001$

\* Differences in means between diets (ANOVA).

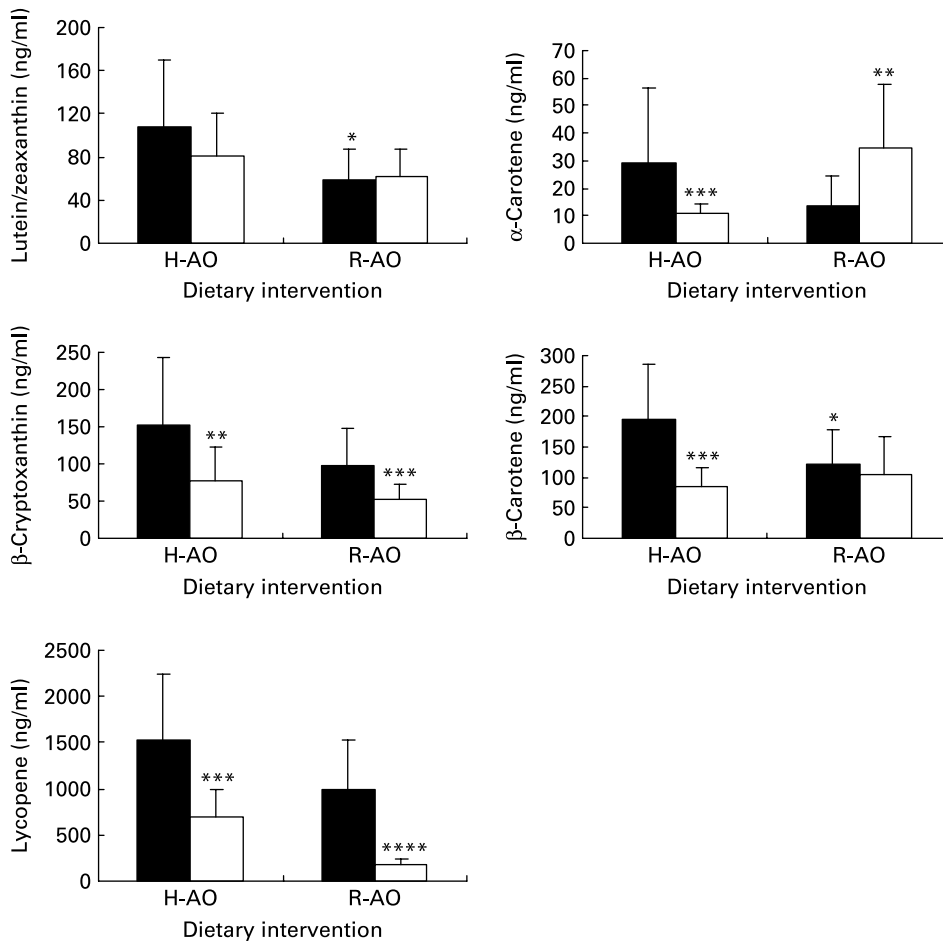
### Plasma inflammatory mediators

The changes in inflammatory mediators are shown in Fig. 2. Pre-exercise plasma IL-6 concentrations were similar on both diets. IL-6 concentrations on both the H-AO and the R-AO diets were not affected by exercise. Although not statistically significant, IL-6 concentrations tended to increase ( $P = 0.1$ ) with exercise on the H-AO diet but did not change on the R-AO diet ( $P = 0.6$ ). Post-exercise IL-6 concentrations were significantly higher ( $P < 0.05$ ) on the H-AO diet compared to the R-AO diet. Pre-exercise TNF- $\alpha$  concentrations were 38-fold higher ( $P < 0.01$ ) and post-exercise TNF- $\alpha$  concentrations were 14-fold higher ( $P < 0.001$ ) on the R-AO diet compared to the H-AO diet; there was no statistically significant response to exercise on either diet. Pre-exercise plasma LTB<sub>4</sub> concentrations were similar on both diets. Plasma LTB<sub>4</sub> concentrations decreased ( $P < 0.001$ ) with exercise on the H-AO diet but did not change significantly on the R-AO diet; post-exercise plasma LTB<sub>4</sub> concentrations were lower ( $P < 0.05$ ) on the H-AO diet compared to the R-AO diet.

## Discussion

The present study has found that restricting the dietary intake of fruit and vegetables to 1–2 serves/d reduces the plasma concentrations of carotenoids, an important group of antioxidants, alters the plasma carotenoid responses to exercise and increases the plasma levels of the inflammatory marker TNF- $\alpha$ . These findings suggest that a diet rich in fruit and vegetables is an important defence against oxidative stress for athletes.

Analysis of the participants' 4-d weighed food records found that their intakes of fibre, ascorbic acid and one marker of dietary carotenoid intake,  $\beta$ -carotene, were reduced on the R-AO diet, suggesting that they adhered to the recommendations to restrict their intake of fruit, vegetables and other major food sources of antioxidants during the study. Plasma concentrations of carotenoids are reflective of the previous several days dietary intake<sup>(23)</sup>, and as the pre-exercise plasma concentrations of carotenoids were lower on the R-AO diet compared to the H-AO diet, this indicates that the participants made the requested dietary changes for the 2 weeks of fruit and



**Fig. 1.** The effect of exercise on plasma carotenoid concentrations in endurance-trained male athletes consuming a habitual antioxidant (H-AO) or restricted antioxidant (R-AO) diet (*n* 17). Mean values were significantly different at \**P*<0.05 (H-AO v. R-AO), \*\**P*<0.05 (pre-exercise v. post-exercise), \*\*\**P*<0.01 (pre-exercise v. post-exercise), \*\*\*\**P*<0.001 (pre-exercise v. post-exercise, H-AO v. R-AO). ■, Pre-exercise; □, Post-exercise.

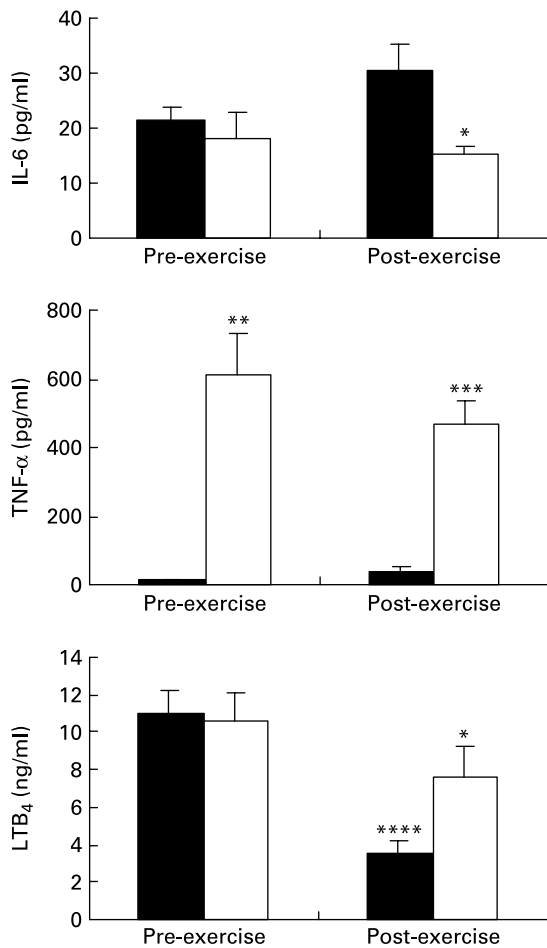
vegetable restriction. It also suggests that regular consumption of fruits and vegetables is required to maintain appropriately high levels of carotenoids in the circulation. Furthermore, this suggests that the habitual diet of these trained male endurance athletes has a desirably higher intake of dietary antioxidants sourced from fruit and vegetables.

IL-6 is a major inflammatory mediator released from skeletal muscle during exercise<sup>(24)</sup>. Dietary restriction of carotenoids did not effect the pre-exercise plasma concentrations of IL-6, but plasma IL-6 concentrations are typically low in endurance-trained adults<sup>(8)</sup> and in the present study baseline concentrations were comparable to healthy adults<sup>(25,26)</sup>. Exercise can increase the production of IL-6 in healthy trained and untrained adults<sup>(6,8,27,28)</sup>, although the extent of any changes is dependent on the exercise characteristics. There were NS changes in plasma IL-6 concentrations in response to exercise in the present study, although the post-exercise concentrations of IL-6 appear higher on the H-AO diet compared to the R-AO diet. IL-6 was reduced following the R-AO diet post-exercise only. R-AO was expected to increase IL-6; however, it is likely that exercise may have contracted the effect of R-AO diet on IL-6 concentrations. This does merit further examination. Notably, there was no difference in IL-6 concentrations between H-AO

and R-AO groups pre-exercise. The implications of this apparent difference, or whether the magnitude of this difference is physiologically significant, are not clear.

Plasma TNF-α concentrations were comparable to healthy adults before exercise<sup>(11,29)</sup> and were not influenced by exercise, the same was observed with IL-6, but unlike IL-6, TNF-α concentrations were substantially higher both before and after exercise on the R-AO diet compared to the H-AO diet. Previous studies have shown varying effects of exercise on plasma TNF-α concentrations<sup>(6,8,10,11,30)</sup>. Dietary restriction had no effect on pre-exercise plasma LTB<sub>4</sub> concentrations, which were comparable to healthy adults<sup>(31)</sup>. LTB<sub>4</sub> concentrations increased in response to exercise on the H-AO diet but not the R-AO diet. Previously, varying responses in plasma LTB<sub>4</sub> concentrations have been observed with exercise including increases in trained and untrained adults<sup>(30,32)</sup> and no change in trained athletes<sup>(30,33)</sup>. Post-exercise plasma LTB<sub>4</sub> concentrations were lower on the H-AO diet than the R-AO diet. Plasma cytokine changes with exercise are varied, with increases in IL-6 of up to 8000-fold in athletes after a spartathlon<sup>(34)</sup>, increases in TNF-α of up to 2.6-fold<sup>(8)</sup> and increases in LTB<sub>4</sub> of up to 3-fold<sup>(32)</sup>. This indicates that there is great variability in plasma cytokine responses to exercise. Together, the higher





**Fig. 2.** The effect of exercise on plasma IL-6, TNF- $\alpha$  and leukotriene B<sub>4</sub> (LTB<sub>4</sub>) concentration in endurance-trained male athletes consuming a habitual antioxidant (H-AO) or restricted antioxidant (R-AO) diet ( $n$  17). Mean values were significantly different at \* $P$ <0.05 (H-AO v. R-AO), \*\* $P$ <0.01 (H-AO v. R-AO), \*\*\* $P$ <0.001 (H-AO v. R-AO), \*\*\*\* $P$ <0.001 (pre-exercise v. post-exercise). ■, H-AO; □, R-AO.

TNF- $\alpha$  and LTB<sub>4</sub> concentrations on the R-AO diet indicate an elevated inflammatory state when deprived of valuable dietary antioxidants.

Plasma carotenoids have the potential to provide protection from oxidative stress during exercise<sup>(3)</sup>. The plasma concentrations of some but not all carotenoids were lower before exercise on the R-AO diet compared to the H-AO diet, suggesting that carotenoids would be less capable of contributing to antioxidant protection on the R-AO diet. Intense aerobic exercise in fit adults decreases plasma antioxidants<sup>(35)</sup>. Most plasma carotenoid concentrations decreased in response to exercise on the H-AO diet, but this response was markedly attenuated on the R-AO diet again suggesting that plasma carotenoids contributed less to providing protection from oxidative stress during exercise on the R-AO diet. Although  $\alpha$ -carotene levels increased post-exercise following the R-AO diet, exercise was expected to decrease  $\alpha$ -carotene on the R-AO diet. There was no difference in  $\alpha$ -carotene levels between H-AO and R-AO pre-exercise. This merits further investigation.

Dietary restriction of carotenoids did not alter exercise performance. Participants' times to exhaustion did not differ

between diets, but there was an increase in the rating of perceived effort during submaximal exercise on the R-AO diet compared to the H-AO diet. This indicates that dietary carotenoid restriction does not affect short-duration exhaustive exercise performance, but it can increase the participant's perception of effort possibly via an increase in exercise-induced oxidative damage while performing the submaximal exercise typical of endurance activities. The impact of a longer duration of dietary restriction of carotenoids on exercise performance or perceived effort is not known.

In summary, dietary restriction of fruits and vegetables to 1–2 serves/d in healthy endurance-trained men can increase plasma inflammatory mediators and decrease plasma carotenoid concentrations at rest. Post-exercise inflammatory mediators are elevated with dietary carotenoid restriction compared to a diet rich in fruit and vegetables. Exercise performance although unchanged was perceived to be harder when performed after dietary carotenoid restriction. The impact of a longer duration of dietary restriction of carotenoids on oxidative stress and inflammation and whether this has detrimental effects on exercise performance and perceived effort are not known. The present study suggests that a diet rich in carotenoids may be beneficial to combat exercise-induced oxidative stress in athletes performing exercise.

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