

Effects of dietary antioxidant supplementation on oxidative damage and resistance to oxidative damage during prolonged exercise in sled dogs

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Objectives—To determine effects of dietary antioxidant supplementation on plasma concentrations of antioxidants, exercise-induced oxidative damage, and resistance to oxidative damage during exercise in Alaskan sled dogs.

Animals—62 Alaskan sled dogs.

Procedure—Dogs were matched for age, sex, and ability and assigned to 1 of 3 groups: sedentary and non-supplemented (control [C]; n = 21), exercised and supplemented (S; 22), and exercised and nonsupplemented (N; 19). Dogs in group S were given 400 units of α -tocopherol acetate, 3 mg of β -carotene, and 20 mg of lutein orally per day for 1 month, then dogs in groups S and N completed 3 days of exercise. Blood samples were collected before and after 1 and 3 days of exercise and after 3 days of rest. Plasma antioxidant concentrations were determined, and oxidative damage to DNA (plasma 7,8 dihydro-8-oxo-2'-deoxyguanosine [8-oxodG] concentration) and membrane lipids (plasma hydroperoxide concentration) and resistance of plasma lipoproteins to oxidation were assessed.

Results—Supplementation increased plasma concentrations of α -tocopherol, β -carotene, and lutein. Plasma concentration of α -tocopherol increased and concentration of lutein decreased in group S with exercise. Concentration of 8-oxodG decreased in group S but increased in group N during and after exercise. Lag time of in vitro oxidation of lipoprotein particles increased with exercise in group S only.

Conclusions and Clinical Relevance—Dietary supplementation with antioxidants resulted in increased plasma concentrations of antioxidants. Moreover, supplementation decreased DNA oxidation and increased resistance of lipoprotein particles to in vitro oxidation. Antioxidant supplementation of sled dogs may attenuate exercise-induced oxidative damage. (*Am J Vet Res* 2000;61:886–891)

species (ROS) during exercise: the electron transport chain in the mitochondria, ischemia-reperfusion injuries, and activated phagocytes at sites of muscle and tissue trauma. Aerobic exercise is, therefore, a demonstrated cause of oxidative stress and oxidative damage, depending on the athlete's antioxidant status.⁴⁻⁶ Oxidative damage is not only implicated in reduced performance and acute injuries in athletes,⁷ but it also contributes to the initiation of a large number of degenerative and neoplastic diseases.⁸ Hence, study of exercise-induced oxidative stress is of particular interest to researchers attempting to prevent injuries in and enhance performance of athletes and to those attempting to understand mechanisms determining an organism's oxidant status and disease pathogenesis. Sled dogs have proven excellent models for studies of exercise-induced oxidative stress.^{9,10} The exercise they perform requires unusually high and sustained energy expenditure⁹ and, in turn, high total consumption of oxygen, because endurance exercise is aerobic in nature. In addition, sled dogs train as a team and typically have similar athletic abilities, degrees of fitness, training schedules, and work output during exercise. Moreover, dogs within 1 team are often genetically related, commonly being from 2 or 3 different litters whose sets of parents are themselves often littermates. Finally, the dogs' diet and housing are readily controlled.

Endurance exercise is associated with increases in indices of oxidative damage in sled dogs.¹⁰ Because oxidative damage is caused by oxidative stress that overwhelms antioxidant defenses, our hypothesis was that administration of dietary antioxidants would reduce indices of oxidative stress in exercising sled dogs. Specifically, the objectives of the study reported here were to determine the effects of dietary supplementation with the antioxidants α -tocopherol (vitamin E), β -carotene, and lutein on plasma concentrations of antioxidants and determine the effects of supplementation on indices of exercise-induced oxidative damage or resistance to oxidative damage in Alaskan sled dogs.

Oxidative stress that overwhelms antioxidant defenses causes oxidative damage in cell membranes and DNA.¹⁻³ There are 3 main sources of reactive oxygen

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Materials and Methods

Animals and groups—Sixty-two sled dogs with a mean (\pm SD) age of 3.7 ± 0.3 years were used for this study. Males ($n = 34$) weighed 21.9 ± 0.4 kg, and females (28) weighed 21.3 ± 0.4 kg. After matching for sex, age, weight, and ability, dogs were assigned to 1 of 3 treatment groups: sedentary and nonsupplemented (control [C]; $n = 21$), exercised and supplemented (S; 22), and exercised and nonsupplemented (N; 19). There were no significant differences in sex, age, or weight among groups, and dogs in each group received a similar amount of training or total exercise during the 3 months prior to the study (group C, 904 ± 48 km; group S, 945 ± 43 km; group N, $1,009 \pm 50$ km). Two months prior to the exercise study, the dogs' diets were standardized to one that contained 5,777 cal/kg of food and a $\omega 6$ -to- $\omega 3$ fatty acid ratio of 5.94. On a dry-matter basis, the diet contained 35% protein, 30.8% fat, and 23.1% carbohydrates. The concentration of α -tocopherol in the diet was 57 U/kg of food. Dogs were fed to maintain their weight.

Supplementation—Beginning 1 month prior to the exercise study, dogs in group S received one biscuit containing 400 units of α -tocopherol acetate, 3 mg of β -carotene, and 20 mg of lutein with their evening meal. Dogs in groups N and C received dummy biscuits containing minimal amounts of antioxidants (0.1 mg of ascorbate, 0.1 units of vitamin E, and no β -carotene). Supplemented and dummy biscuits differed only in color and had been tested to ensure that they were accepted by the dogs. Dog handlers were masked to the treatments given.

Exercise and sample collection—Two days before exercise, all dogs were rested. Dogs in groups S and N then completed 3 days of exercise. Exercise consisted of running 64 km on day 1 of the study and 75 km on days 2 and 3 while pulling a lightly laden sled and musher at approximately 19 km/h. All dogs were then rested for 3 days.

Blood samples were collected from a jugular vein into 10-ml evacuated heparinized glass tubes^a the evening before the first day of exercise, within 2 hours after exercise on days 1 and 3, and in the evening after 3 days of rest. Blood samples were stored on ice until plasma was harvested by centrifugation for 10 minutes at $1,500 \times g$. Plasma was divided into several aliquots and stored in plastic microtubes at approximately -80 C until analyzed. During transport to the laboratory for analyses, plasma samples were kept at approximately the desired temperature by packing with dry ice and a commercial refrigerant.^b No dogs were withdrawn from the study because of injuries, fatigue, or other medical problems.

Measurement of α -tocopherol, β -carotene, lutein, and retinol concentrations—Plasma was extracted^c for analysis of α -tocopherol, β -carotene, lutein, and retinol concentrations by use of high-performance liquid chromatography. Identity of eluted compounds was confirmed by comparing absorption spectrum of eluted compounds with that of a corresponding standard, using a photodiode array detector.^c

Measurement of 7,8 dihydro-8-oxo-2'-deoxyguanosine (8-oxodG) concentration—Ultrafiltrate of plasma for measurement of 8-oxodG concentration was obtained by centrifugation of thawed plasma^d for 90 minutes at $2,000 \times g$ in a fixed-angle centrifuge.^e Concentration of 8-oxodG in the ultrafiltrate was determined by use of a competitive ELISA.^f The assay was validated for use with canine plasma by performing the assay with 6 homogenous aliquots of a plasma sample from a healthy sedentary dog. Mean concentration of 8-oxodG was 3.65 ng/ml; coefficient of variation was 4.7%. Thereafter, 12 more aliquots from the same sample were spiked with 2 concentrations of an 8-oxodG standard (80 ng/ml [$n = 6$]; 200 ng/ml [6]). Recoveries were 98.8 and

94.66%, respectively; coefficients of variation were 3.6 and 6.7%, respectively. Finally, the aliquots spiked with 200 ng of 8-oxodG/ml were serially diluted, with 6 measurements per dilution. Linear regression analysis of the results yielded an r^2 value of 0.9 with 4 dilutions.

Measurement of lag time of in vitro oxidation of low density and very low density lipoproteins—After plasma was harvested on the day of blood collection, 0.9 ml of plasma was added to tubes containing 0.1 ml of a sucrose-EDTA solution (60 mg of sucrose/ml; 10 mg of EDTA/ml). **Low density and very low density lipoproteins (LDL+VLDL)** were oxidized in vitro, using a modification of the procedure described by Zhang et al.¹¹ Briefly, the LDL+VLDL fraction was isolated from plasma by precipitation in 2% PBS solution with a mixture of equal volumes of a 20 g of dextran sulfate (MW, 500,000)/L solution and a 2M $MgCl_2$ solution. After vortexing and centrifugation at $1,500 \times g$ for 10 minutes,^g the LDL+VLDL precipitate was reconstituted in 0.45% PBS solution; EDTA was removed from the reconstituted solution by a second precipitation. The final LDL+VLDL precipitate was reconstituted in 2% PBS solution, and concentration of LDL+VLDL cholesterol was determined by use of spectrophotometry.⁸ Cholesterol concentration in LDL+VLDL was adjusted to 75 μ mol/ml by addition of 2% PBS solution. Oxidation was initiated by addition of a $CuSO_4$ solution to a final LDL+VLDL concentration of 8 μ mol/L. Kinetics of LDL+VLDL oxidation were determined by monitoring the change in absorbance at 234 nm, using a motorized 6-cuvette spectrophotometer.^h Absorbance was recorded every 5 minutes. The change in absorbance at 234 nm over time could be divided into 4 consecutive phases: initiation, lag phase, propagation of oxidation, and decomposition.¹² Absorbances were plotted against time, and lag time was determined by linear regression of the straight portion of the curve.

Measurement of hydroperoxide concentration—Concentration of hydroperoxides in thawed plasma was determined by use of a commercially available xylenol-orange reacting substance test for lipid hydroperoxides.¹ This test is based on the hydroperoxide-mediated oxidation of ferrous to ferric ions. The generated ferric ions are captured by the chromophore xylenol orange, with an associated absorbance change at 560 nm. Pretreatment of the specimen with catalase resolves interference with hydrogen peroxide. This test was validated for use with canine plasma, using 2 homogenous aliquots of a plasma sample from a healthy sedentary dog to perform the test and repeating each measurement 3 times. Mean concentration of hydroperoxides was 8.3 μ mol/L; coefficient of variation was 4.7%. Thereafter, 2 more aliquots from the same sample were spiked with a hydroperoxide standard (50 μ mol/ml). The recovery was 75%, with a coefficient of variation of 5%. Finally, the spiked aliquots were serially diluted, and 3 readings per dilution were obtained. Linear regression analysis of the results yielded an r^2 value of 0.757 with 4 dilutions.

Statistical analyses—Data were examined graphically and analyzed by use of a 2-way repeated measure ANOVA, with time as the repeated factor. When significant differences or interactions among groups or times were found, multiple comparisons were made, using the Student-Neuman Keuls test. The null hypothesis was rejected at $P < 0.05$.

Results

Plasma concentration of antioxidants—Supplementation with α -tocopherol acetate, β -carotene, and lutein increased plasma concentrations of α -tocopherol by approximately 72%, compared with concentrations in nonsupplemented dogs

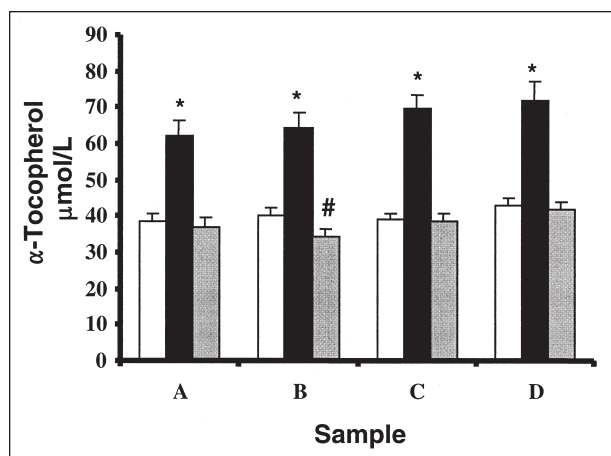


Figure 1—Mean (\pm SD) plasma α -tocopherol concentrations in sedentary, nonsupplemented dogs (open bars; $n = 21$); exercising supplemented dogs (black bars, 22); and exercising nonsupplemented dogs (gray bars, 19). Supplemented dogs received 1 biscuit containing 400 units of α -tocopherol acetate, 3 mg of β -carotene, and 20 mg of lutein orally for 1 month before the study. Plasma was obtained before exercise (sample A), after 1 and 3 days of exercise (sample B and C, respectively), and after 3 days of rest (sample D). *Significantly ($P < 0.01$) different, compared with values for other 2 groups. #Significantly ($P < 0.01$) different, compared with value for sedentary nonsupplemented group.

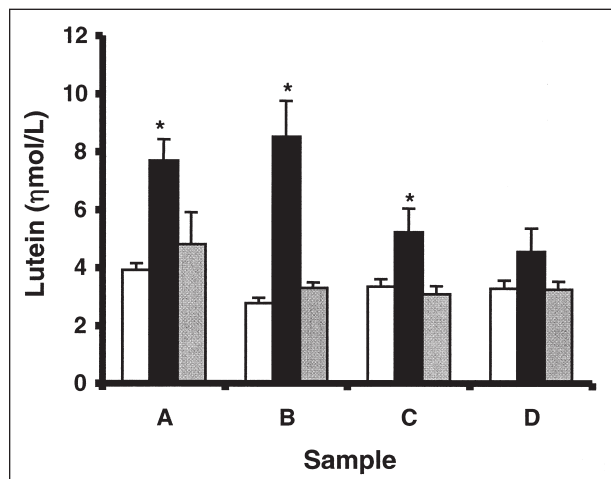


Figure 2—Mean (\pm SD) plasma lutein concentrations in sedentary nonsupplemented dogs; exercising supplemented dogs; and exercising nonsupplemented dogs. See Figure 1 for key.

(Fig 1). Likewise, supplementation increased plasma concentration of β -carotene to approximately $0.003 \mu\text{mol/L}$, whereas β -carotene was not detectable in nonsupplemented dogs. Finally, supplementation increased plasma lutein concentration by approximately 91% (Fig 2). There was, however, no effect of supplementation with carotenoids on plasma retinol concentration, which averaged $3.6 \mu\text{mol/L}$ for all groups.

In group S, there was an increase in plasma concentration of α -tocopherol associated with exercise, whereas in group N, there was a transient decrease in α -tocopherol concentration after day 1 of exercise (Fig 1). Exercise had no effect on plasma β -carotene concentration. However, lutein concentration in group

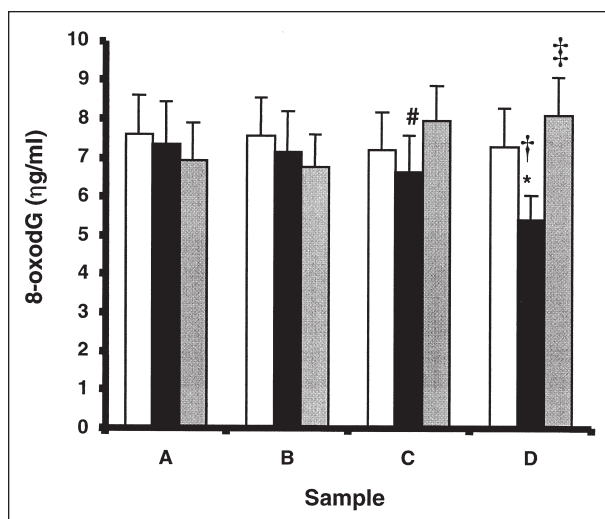


Figure 3—Mean (\pm SD) plasma concentrations of 7,8 dihydro-8-oxo-2-deoxyguanosine (8-oxodG) in sedentary nonsupplemented dogs, exercising supplemented dogs, and exercising nonsupplemented dogs. *Significantly ($P < 0.01$) different, compared with values for other 2 groups. #Significantly ($P < 0.05$) different, compared with value for exercising nonsupplemented group. †Significantly ($P < 0.05$) different from values for same group determined at times A, B, and C. ‡Significantly ($P < 0.05$) different from value for same group determined at time B. See Figure 1 for key.

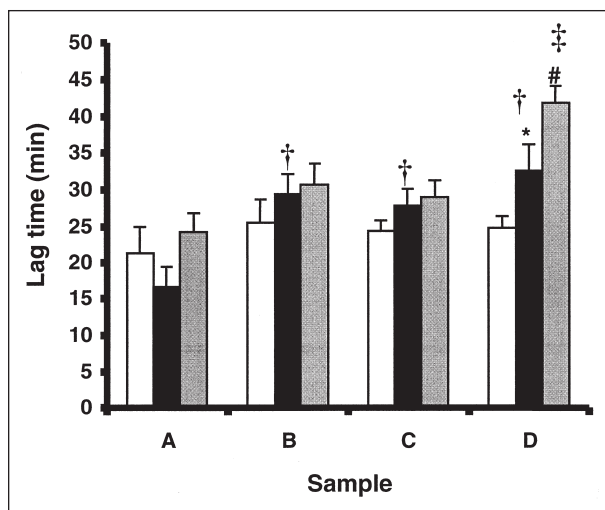


Figure 4—Mean (\pm SD) lag time of in vitro oxidation of plasma low density lipoprotein and very low density lipoprotein particles in sedentary nonsupplemented dogs, exercising supplemented dogs, and exercising nonsupplemented dogs. *Significantly ($P < 0.01$) different, compared with values for other 2 groups. #Significantly ($P < 0.05$) different, compared with value for exercising nonsupplemented group. †Significantly ($P < 0.01$) different from value for same group determined at time A. ‡Significantly ($P < 0.01$) different from values for same group determined at times A, B, and C. See Figure 1 for key.

S decreased after 3 days of exercise and after 3 days of rest (Fig 2). Exercise did not affect plasma retinol concentration, although retinol concentration increased after 3 days of rest in all groups, with higher concentrations detected at this time in the groups that were exercised (ie, S and N), compared with the control group.

Indices of oxidative damage or resistance to oxidative damage—Plasma concentration of 8-oxodG

significantly decreased ($P < 0.01$) during and after exercise in group S. Conversely, concentration of 8-oxodG significantly ($P < 0.05$) increased during and after exercise in group N (Fig 3).

Supplementation and exercise affected lag time of in vitro oxidation of LDL+VLDL particles. Specifically, exercise resulted in an increase in lag time in group S but not in group N, whereas lag time increased significantly in both groups after 3 days of rest. Lag time after 3 days of rest was significantly ($P < 0.01$) greater in group N, compared with the other groups (Fig 4). Supplementation or exercise had no effect on rate of in vitro oxidation of LDL+VLDL particles or on plasma concentration of hydroperoxides.

Discussion

Results of the present study indicate that oral supplementation of Alaskan sled dogs with α -tocopherol, β -carotene, and lutein increased plasma concentrations of these antioxidants. In addition, the increase in plasma concentrations was associated with a significant increase in resistance of lipoproteins to oxidative damage and a reduction in exercise-induced oxidative damage of DNA.

Supplementation with α -tocopherol acetate increased plasma concentration of α -tocopherol before exercise. It is likely that this increase was not limited to plasma concentration but was associated with an increase in muscle concentration of α -tocopherol¹³ and was, therefore, indicative of an increase in whole body stores of vitamin E. We were unable to explain the exercise-induced increase in plasma α -tocopherol concentration in group S, although results of several studies in humans indicated similar increases in response to exercise.^{14,15} The exercise-associated increase of α -tocopherol concentration in systemic circulation may be attributable to release of tocopherol from adipose tissue associated with the release of free-fatty acids during exercise.¹⁴ Measured concentrations of plasma constituents are often adjusted for changes in plasma volume to estimate changes in body or plasma content of the constituent. However, in this and other studies,^{9,10} such an adjustment would not have changed the increase of plasma concentration of α -tocopherol, as total plasma protein concentration decreased with exercise (present study: group C, 6.3 ± 0.2 g/dl, exercised groups before exercise, 6.3 ± 0.05 g/dl; exercised groups after 1 day of exercise, 6.1 ± 0.04 g/dl; exercised groups after 3 days of exercise, 5.9 ± 0.06 g/dl). Therefore, the observed increase in plasma α -tocopherol concentration during exercise cannot be attributed to hemoconcentration. In group N, the transient decrease in plasma concentration of α -tocopherol after 1 day of exercise was followed by an increase after 3 days of rest to a value that was significantly higher than that obtained after 1 day of exercise and similar to values observed in group S. This pattern suggests an oxidative stress-associated depletion of plasma α -tocopherol after 1 day of exercise in group N¹⁶ and resistance to α -tocopherol depletion during exercise in group S.

Supplementation with β -carotene also increased the plasma concentration of this antioxidant. However,

dogs in group S had a mean plasma concentration of only $0.003 \mu\text{mol/L}$, and dogs in group N had concentrations of β -carotene in plasma too low to be detected by use of HPLC. Although the difference in concentrations was significant between groups S and N, plasma concentrations of β -carotene in dogs that received supplements were only 6 to 14% of the concentration range observed in humans.¹⁷ It is reasonable to doubt the antioxidant function of β -carotene at such low plasma concentrations. Dogs and other animals with white-fat lack a carotenoid transport protein, and unless administered at pharmacologic doses, carotenoids absorbed in the intestinal tract are metabolized to retinol in intestinal mucosal cells before being absorbed into blood.¹⁷ In humans, 10 to 30% of ingested carotenoids are absorbed,¹⁸ so plasma carotenoid concentrations in healthy nonsupplemented dogs are less than in humans (humans, 0.21 to $0.52 \mu\text{mol/L}$).¹⁷ In addition, there is a preferential uptake of lutein in the presence of large amounts of β -carotene in the intestinal lumen.^{19,20} Therefore, supplementation with lutein may have further impaired absorption of β -carotene, which may help explain the low plasma β -carotene concentrations that we detected in supplemented dogs.

Lutein, another carotenoid, was given to the dogs at a higher dosage than β -carotene (20 mg of lutein vs 3 mg of β -carotene per day), so this alone may explain why mean plasma concentration of lutein ($0.007 \mu\text{mol/L}$) was greater than that of β -carotene ($0.003 \mu\text{mol/L}$) in group S. Nevertheless, competition for intestinal absorption between carotenoids in favor of lutein may have contributed to the higher concentration of lutein in plasma.^{19,20} In addition, lutein is transported mainly by **high-density lipoprotein (HDL)** particles, the major lipoprotein particle in dogs, whereas β -carotene is transported mainly by LDL particles. Therefore, higher concentrations of HDL particles relative to LDL particles in canine plasma may indirectly have accounted for higher lutein concentrations. The decrease in plasma lutein concentration after 3 days of exercise and throughout a period of rest in group S suggests an exercise-associated consumption of this antioxidant. Lutein, when present in higher concentrations relative to other antioxidants, may be preferentially used to prevent oxidative damage. This would explain why a decrease in plasma lutein concentration was not detected in group N during exercise. In addition, lutein may have had a sparing effect on α -tocopherol in group S; lutein concentration decreased and α -tocopherol concentration increased with exercise in this group.

Plasma retinol concentration did not significantly differ among groups despite administration of β -carotene, a precursor of retinol in many species, to dogs in group S. The only significant change in retinol concentration was an increase in all 3 groups detected at the end of the study (ie, after 3 days of rest for groups S and N). At this time, plasma retinol concentration in the exercised groups was significantly greater than concentration in the control group, suggesting a delayed effect of exercise.

We detected a clear effect of supplementation on

plasma concentration of 8-oxodG during and after exercise. Oxidative damage of DNA during exercise results in breakage of chromatid strands, formation of baseless sugars, and oxidation of bases. A useful indicator of DNA damage by reactive oxygen species is detection of an increase in plasma 8-oxodG concentration. Repair of DNA containing 8-oxodG is not by the classical excision-repair pathway, which involves excision of only damaged bases.³ Instead, 8-oxodG is excised as a whole nucleotide, and it can, therefore, be measured in urine or plasma by use of immunoassays. In humans, marathon running has been associated with a 1.3-fold increase (corrected for variations in creatinine concentration) in the ratio of blood 8-oxodG to 2'-deoxyguanosine (the intact nucleotide) concentration.⁴ Furthermore, vigorous exercise for approximately 10 h/d results in a 33% increase in the rate of urinary excretion of 8-oxodG.²¹ Vitamin E has been shown to decrease nonspecific DNA damage in humans after a single bout of exhaustive exercise.²² In the present study, the effect of antioxidants on preventing DNA damage was apparent in group S; plasma concentration of 8-oxodG during exercise was significantly less in this group than in group N. In addition, plasma concentration of 8-oxodG after 3 days of rest was also significantly less in group S than in the other groups and less than before and during exercise in the same group. In group C there were no significant changes in plasma 8-oxodG concentration throughout the study, and in group N, concentration of 8-oxodG increased between day 1 and 3 of exercise and after 3 days of rest, compared with the concentration determined before exercise. When plasma concentrations of antioxidants are increased by dietary supplementation, there may be enhanced use of these antioxidants and decreased DNA damage as a consequence of exercise-induced oxidative stress. Results of the present study suggest that dietary supplementation with antioxidants conferred increased resistance to oxidative damage in exercising sled dogs.

Measurement of lag time of in vitro LDL+VLDL oxidation gives a crude estimate of the antioxidant status of these lipoprotein particles. Low-density lipoprotein particles are the major carriers of lipid hydroperoxides in plasma, which makes them particularly susceptible to oxidative damage.^{23,24} In vitro oxidation of lipoprotein particles by copper sulfate occurs in 4 phases: initiation, lag phase, propagation of oxidation, and decomposition. In the initiation phase, antioxidants, particularly tocopherols, reduce copper (II) ions to copper (I) ions. This, in turn, retards the oxidation of lipoproteins by copper (II) ions.¹² During the lag phase, oxidation of lipoproteins produces radicals that are scavenged by antioxidants. The depletion of antioxidants marks the end of the lag phase and the beginning of the propagation phase. Therefore, the greater duration of the lag phase, the greater the antioxidant status of the lipoproteins. Results of studies in humans indicate that 30 minutes of moderate exercise in non-supplemented individuals is sufficient to decrease oxidation lag time and increase oxidation rate.²⁵⁻²⁷ In the present study, however, lag time of in vitro LDL+VLDL

oxidation did not decrease in group N. We could not explain why lag time after exercise was greater in group N than group S, but the increase of lag time after day 1 and 3 of exercise in S but not N suggests a protective effect of antioxidants. The failure of lag time to decrease during exercise in group N may be related to a training effect from which all dogs benefited. Indeed, LDL particles from trained humans have prolonged lag phases when oxidized in vitro (ie, greater antioxidant status), compared with LDL particles from untrained humans.²⁸ This observation, however, could not be directly explained by differences in antioxidant concentration of LDL particles.^{26,28}

Dietary supplementation or exercise did not affect rate of in vitro LDL+VLDL oxidation. This is in contrast to findings of studies that evaluated humans.^{26,29} We also did not detect an effect of supplementation or exercise on plasma concentration of hydroperoxides. We believe that this result could be explained by the fact that the dogs were exercising at intensities below maximum effort. The type of endurance exercise the dogs performed may not have triggered an increase in oxidative damage resulting in the formation of hydroperoxides. Similar lack of exercise-induced production of lipid hydroperoxides was reported in rats and humans.⁴

^aSherwood Monoject Division, St Louis, Mo.

^bTechni-ice USA Inc, Las Vegas, Nev.

^cAlliance 2690 HPLC system, Waters, Milford, Mass.

^dCentrifree micropartition devices, 30,000 MW cut-off, Amicon, Beverly, Mass.

^eT422 floor model centrifuge, Jouan, Winchester, Va.

^f8-OHdG Elisa kit, Genox Corp, Baltimore, Md.

^gTotal cholesterol kit and certified cholesterol calibrator, Sigma Chemical Co, St Louis, Mo.

^hUV-160U recording spectrophotometer, Shimadzu Corp, Kyoto, Japan.

ⁱBioxytech LPO560, OXIS Health Products, Portland, Ore.

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