Oxidant stress in sled dogs subjected to repetitive endurance exercise

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Objective—To determine whether repetitive endurance exercise in sled dogs was associated with substantial lipid peroxidation, decreases in antioxidant capacity of the serum, and skeletal muscle damage.

Animals—24 lightly trained sled dogs.

Procedure—16 dogs completed a 58-km run on each of 3 consecutive days; the other 8 dogs (control) did not exercise during the study. Blood samples were collected before the first exercise run and after the first and third exercise runs. Plasma isoprostane and serum vitamin E concentrations, total antioxidant status of plasma, and serum creatine kinase activity were measured.

Results—Plasma isoprostane concentrations in dogs in the exercise group were significantly increased after the first exercise run and further significantly increased after the third exercise run. Serum vitamin E concentration was significantly decreased after the first exercise run in dogs in the exercise group, and this change persisted after the third exercise run. There was a significant linear relationship between plasma isoprostane concentration and the logarithm of serum creatine kinase activity (adjusted $r^2 = 0.84$).

Conclusions and Clinical Relevance—Results demonstrate that repetitive endurance exercise in dogs is associated with lipid peroxidation and a reduction in plasma antioxidant concentrations. We interpret these results as indicating that the antioxidant mechanisms of minimally trained dogs may, in some instances, be inadequate to meet the antioxidant requirements of repetitive endurance exercise. (*Am J Vet Res* 2000;61:512–517)

Exercise is associated with an increase in the rate of oxygen consumption, with the extent of this increase dependent on the intensity of the exercise. Because oxidative metabolism is associated with obligatory production of free radicals (reactive oxygen species),[1,2] exercise-associated increases in oxygen consumption are assumed to be accompanied by proportionate increases in free radical production.[3] Indeed, various modes of exercise in a range of species have been found to be associated with increases in markers of free radical production, lipid peroxidation, and cell injury.[4-8] Repetitive endurance exercise likely imposes a larger oxidant stress because of the repetitive, prolonged increases in oxygen consumption. However, to our knowledge, this effect of repetitive endurance exercise has not been clearly demonstrated in any species.

Free radicals are consumed by antioxidant mechanisms involving vitamin E and various antioxidant enzymes.[3] In many species, exercise, including a single bout of endurance exercise, has minimal effects on serum and tissue vitamin E concentrations, serum activities of antioxidant enzymes, and total peroxyl radical trapping capacity of plasma.[9-11] However, to our knowledge, effects of strenuous, repetitive endurance exercise on antioxidant status are not known. Because interventions and conditions that decrease the antioxidant capacity of individuals, such as vitamin E deficiency, increase markers of lipid peroxidation and skeletal muscle damage during or after exercise,[2,12] we speculate that repetitive endurance exercise may be associated with decreases in markers of antioxidant status and increases in markers of lipid peroxidation. Elite sled dogs have an exceptionally high sustained metabolic energy expenditure while racing[13] and routinely perform sustained endurance exercise on consecutive days. We speculate, therefore, that sled dogs incur high oxidant stresses in association with racing. The purpose of the study reported here was to determine whether repetitive endurance exercise in sled dogs was associated with substantial lipid peroxidation, decreases in antioxidant capacity of the serum, and skeletal muscle damage. Plasma isoprostane concentration was used as an indicator of lipid peroxidation in this study,[14,15] and sled dogs were subjected to repetitive endurance exercise by having them run 58 km a day for 3 consecutive days.

Materials and Methods

Study design—Twenty-four healthy sled dogs were used in the study. Dogs were randomly allocated to 2 groups consisting of 16 dogs (exercise group) and 8 dogs (control group). Groups were balanced for age and sex of the dogs. Dogs in the exercise group completed a 58-km run on each of 3 consecutive days. Dogs ran in 2 teams pulling an unladen sled and musher over fresh snow; mean ± SD speed was 19 ± 2.3 km/h. Runs were completed between 10 AM and 4 PM. Ambient temperatures at the time of the runs ranged from –39 to –26 C with no wind. Dogs in the control group were housed in unheated kennels (their usual housing) for the duration of the study.

Blood samples were collected from dogs in the exercise group between 3 and 7 PM the day before the first exercise run, between 1.75 and 3.25 hours after the first exercise run,

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and between 1 and 3 hours after the third exercise run. Dogs were not fed or offered water between the end of each exercise run and collection of blood samples. Blood samples were collected from the control dogs at similar times. Control dogs were fed once daily after collection of blood samples. Blood samples were collected by means of jugular venipuncture into evacuated plain glass tubes or glass tubes containing heparin. Tubes containing heparinized blood samples were immediately placed in ice; plasma and RBC were harvested by means of centrifugation (1,500 × g at 2 C) within 30 minutes after blood collection, frozen immediately on dry ice, and shipped on dry ice to the laboratory for analysis.

The study design was approved by the Animal Care and Use Committee of the Ohio State University. The owner of the dogs provided informed consent.

Dogs—Dogs in the exercise group consisted of 8 sexually intact males and 8 sexually intact females between 1.5 and 6 years of age; mean ± SD weight was 22.3 ± 1.9 kg. Control dogs consisted of 3 males and 5 females between 1.5 and 4 years of age; mean ± SD weight was 20.2 ± 2.4 kg. All dogs were owned by a professional racing kennel and were being prepared for the racing season. Dogs in the exercise and control groups had completed 509 ± 75 miles and 475 ± 61 miles, respectively, of training in the 2 months before the study. None of the dogs had a history of recurrent rhabdomyolysis. None of the dogs had run for the 7 days immediately preceding the study, except for a 10-mile run 4 days before the study began.

Diet—Throughout the study, dogs were fed a diet composed of a proprietary dog food[a] supplemented with fat. A representative sample of the diet was collected on each of the 3 days of the study and submitted for analysis. Dogs in the exercise group consumed 4,820 calories of gross energy comprising 780 g of dry matter each day of the study. The caloric distribution was 28.3% protein, 52.8% fat, and 18.9% nitrogen-free extract. Vitamin E content of the diet, determined by means of high-performance liquid chromatography, was 136 U/kg of dry matter and was achieved by addition of α-tocopherol acetate. Dietary ω-3 fatty acid content, determined on a dry-matter basis, was 0.83%; dietary ω-6 fatty acid content was 4.18%. Dogs in the control group consumed the same diet as dogs in the exercise group. However, caloric intake of control dogs was not measured. All dogs in the study had consumed the same diet for at least 30 days prior to the study.

Biochemical analyses—Serum **creatine kinase (CK)** activity and uric acid, cholesterol, and total protein concentrations were measured by use of an automated analyzer.[b] Plasma isoprostane concentration was determined by use of a commercial assay.[c] The assay was validated by demonstrating dilutional parallelism and by measuring recovery from spiked samples; for plasma samples spiked with a standard isoprostane solution, mean difference between actual and measured concentrations was 3.8%.

Serum vitamin E concentration and **ceruloplasmin (CER)** activity and RBC **glutathione peroxidase (GPX)** and **superoxide dismutase (SOD)** activities were measured as described.[16-19] Plasma **total antioxidant status (TAS)** was determined by measuring the ability of plasma to quench absorbance of the radical cation formed by the reaction of 2,2′-azinobis-(3-ethyl) benzothiazoline-6-sulfonic acid with peroxidase and hydrogen peroxide.[d]

Statistical analyses—Data were analyzed by use of 2-way ANOVA for repeated measures. Post hoc comparisons within a group were performed by use of the Student-Newman-Keul test; post hoc comparisons between groups at each time were performed by use of the Student *t*-test for independent populations with Bonferroni correction. Serum vitamin E concentrations were adjusted for changes in serum total protein concentration, as a surrogate for changes in plasma volume, by use of the following equation:

$$\text{vitamin } E_{adj} = \text{vitamin } E_{obs} + (\text{vitamin } E_{obs} \times [(TP_1 - TP_2)/TP_1])$$

where vitamin E_{adj} is the adjusted vitamin E concentration, vitamin E_{obs} is the measured vitamin E concentration, TP_1 is the serum total protein concentration before exercise, and TP_2 is the serum total protein concentration at a subsequent sample time.

The association between plasma isoprostane concentration and serum CK activity was examined by use of multiple regression analysis in which dummy variables were created to account for the separate effects of each dog on the relationship. Models examined included the relationship between plasma isoprostane concentration and serum CK activity and the relationship between plasma isoprostane concentration and the logarithm (base 10) of CK activity. Models were fit to data sets that contained observations for all dogs and for dogs in the exercise group only. Similar analyses were performed to test for associations between serum vitamin E concentration and plasma isoprostane concentration and between serum vitamin E concentration and serum CK activity. The model that best fit the data was determined by examination of the multiple adjusted coefficients of determination (r^2) of each model. The null hypothesis of no difference was rejected when $P < 0.05$. Values are given as mean ± SD.

Results

Plasma isoprostane concentrations in dogs in the exercise group were significantly increased after the first exercise run and further significantly increased after the third exercise run (**Fig 1**; *P* value for group × time interaction, < 0.001), whereas plasma isoprostane concentrations were not significantly changed in the control dogs. Similarly, serum CK activity was significantly increased in dogs in the exercise group after the first exercise run, with a further significant increase after the third exercise run, but was not significantly changed in the control dogs.

Measured serum vitamin E concentration was significantly decreased after the first exercise run in dogs in the exercise group, and this change persisted after the third exercise run (**Table 1**); however, vitamin E concentration was not significantly changed over time in the control dogs. When measured vitamin E concentration was adjusted for changes in serum total protein concentration, a significant decrease in vitamin E concentration in dogs in the exercise group, but not in control dogs, was still apparent. Furthermore, the ratio of plasma vitamin E concentration to serum cholesterol concentration was significantly decreased in dogs in the exercise group, but not in control dogs (**Fig 2**).

Serum total protein concentration was significantly decreased in dogs in the exercise group, but not in control dogs, after the third exercise run (Table 1), but serum cholesterol concentration was not significantly different in either group. Serum uric acid concentration was significantly increased after the first and third exercise runs in dogs in the exercise group, but not in

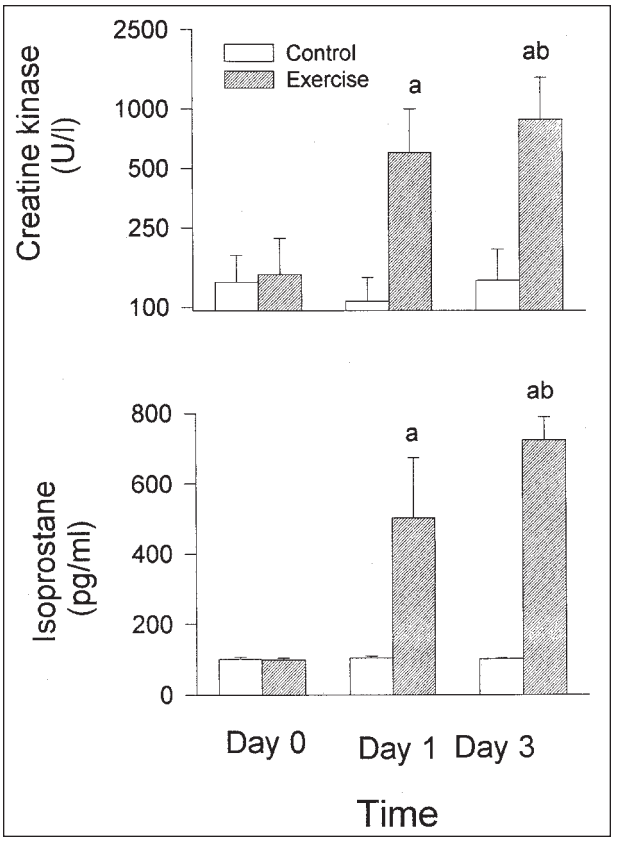

Figure 1—Mean serum creatine kinase activity (top) and plasma isoprostane concentration (bottom) of Alaskan sled dogs before exercise and after the first and third of 3 consecutive daily bouts of strenuous exercise (shaded bars; n = 16) and of control dogs that were not exercised (white bars; n = 8). Error bars represent SD. a = Significantly ($P < 0.05$) different from value obtained before exercise for that group. b = Significantly ($P < 0.05$) different from value obtained after the first exercise bout (day 1) for that group.

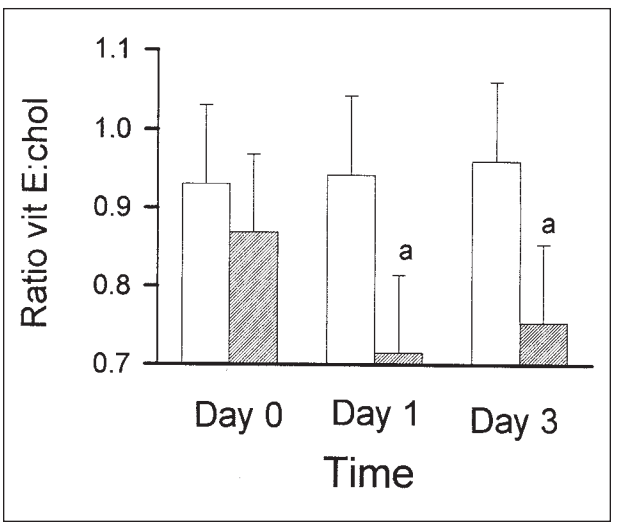

Figure 2—Mean ratio of plasma vitamin E concentration to serum cholesterol concentration in Alaskan sled dogs before exercise and after the first and third of 3 consecutive daily bouts of strenuous exercise. *See* Figure 1 for key.

control dogs. Total antioxidant status of plasma and RBC SOD and GPX activities were not significantly different between groups or over time. Plasma CER activity was significantly decreased after the third exercise run in dogs in the exercise group.When data for all dogs were analyzed, there was a significant ($P < 0.001$) linear relationship between plasma isoprostane concentration and the logarithm of serum CK activity (**Fig 3**; adjusted $r^2 = 0.84$). With the logarithm of serum CK activity as the dependent variable, the regression slope was 0.0012 ± 0.0001, and the intercept was 1.81 ± 0.9. A significant linear relationship (adjusted $r^2 = 0.80$) was also detected when only data for dogs in the exercise group were included. Plasma isoprostane concentration was also related to serum CK activity, but the relationship was not as strong (adjusted $r^2 = 0.70$).

There was a significant ($P < 0.001$) inverse relationship between plasma isoprostane concentration and serum vitamin E concentration (adjusted $r^2 = 0.49$). With plasma isoprostane concentration as the dependent variable, the regression slope was –64.4 ± 9.9 and the intercept was 1,270 ± 213 pg/ml

Table 1—Plasma, serum, and RBC constituents (mean ± SD) of Alaskan sled dogs before exercise and after the first and third of 3 bouts of strenuous exercise (exercise group) and of control dogs that were not exercised (control group)

		Sample time		
Variable	**Group**	**Before**	**Day 1**	**Day 3**
Plasma				
Vitamin E (μg/ml)				
Measured				
	Exercise	19.9 ± 1.2	14.8 ± 0.9*	15.0 ± 1.0*
	Control	20.1 ± 2.6	19.7 ± 2.0	19.8 ± 2.0
Adjusted†				
	Exercise	19.9 ± 1.2	15.4 ± 0.9	16.5 ± 0.9
	Control	20.1 ± 2.6	19.6 ± 2.0	19.8 ± 2.0
Ceruloplasmin (U/L)				
	Exercise	38.8 ±16.8	36.9 ± 16.3	24.7 ±16.7*
	Control	37.8 ± 8.7	28.3 ± 13.6	36.3 ± 12.5
Total antioxidant status of plasma (mmol/L)				
	Exercise	1.7 ± 0.2	1.7 ± 0.2	1.7 ± 0.2
	Control	1.7 ± 0.5	1.7 ± 0.5	1.7 ± 0.2
Serum				
Cholesterol (mg/dl)				
	Exercise	229 ± 25	207 ± 25	199 ± 23
	Control	216 ± 35	209 ± 34	206 ± 34
Total protein (g/dl)				
	Exercise	6.3 ± 0.1	6.0 ± 0.1	5.7 ± 0.1*
	Control	6.1 ± 0.1	6.1 ± 0.1	6.1 ± 0.1
Uric acid (mg/dl)				
	Exercise	0.3 ± 0.1	0.5 ± 0.1*	0.5 ± 0.1*
	Control	0.3 ± 0.1	0.3 ± 0.1	0.3 ± 0.1
RBC				
SOD (U/mg protein)				
	Exercise	173 ± 15	172 ± 20	180 ± 22
	Control	185 ± 35	205 ± 30	198 ± 21
GPX (U/g Hb)				
	Exercise	2.72 ± 0.64	3.18 ± 0.55	2.89 ± 0.82
	Control	2.36 ± 0.64	3.20 ± 0.34	2.25 ± 0.23

*Significantly ($P < 0.05$) different from value for control group at the same time. †Adjusted for changes in serum total protein concentration.

SOD = Superoxide dismutase. GPX = Glutathione peroxidase.

Dogs in the exercise group raced 58 km a day for 3 consecutive days; blood samples were collected before dogs were given access to food or water after that day's run.

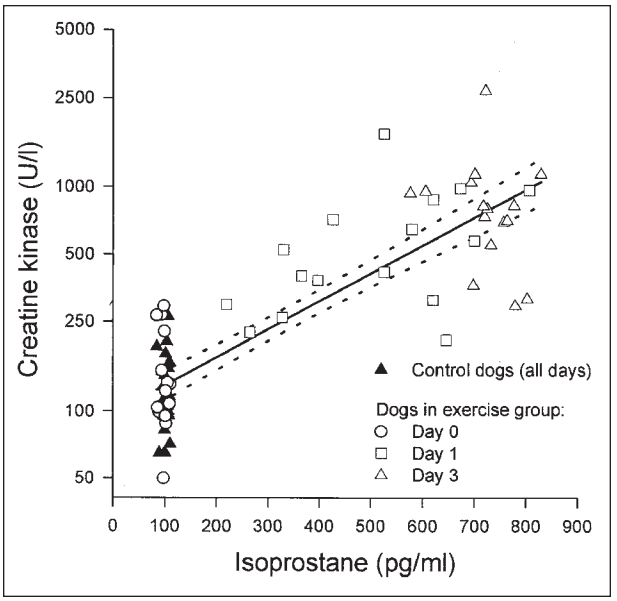

Figure 3—Scatterplot of serum creatine kinase activity versus plasma isoprostane concentration for Alaskan sled dogs before exercise and after the first and third of 3 consecutive daily bouts of strenuous exercise and for control dogs that were not exercised. Solid line represents best fit.

when data from all dogs were considered. Similarly, there was a significant ($P < 0.001$) inverse relationship between the logarithm of serum CK activity and serum vitamin E concentration (adjusted $r^2 = 0.64$). With logarithm of the serum CK activity as the dependent variable, the regression slope was –0.090 ± 0.012 and the intercept was 3.56 ± 0.26 when data from all dogs were considered.

Discussion

In the present study, sled dogs that underwent repetitive strenuous exercise had progressive increases in plasma concentration of isoprostane, a sensitive and specific marker of lipid peroxidation in rats. Degree of lipid peroxidation, as indicated by plasma isoprostane concentration, was well correlated with serum CK activity, an indicator of skeletal muscle cell damage in dogs. However, exercise was not associated with a decrease in TAS, a crude indicator of plasma antioxidant status, although there was a significant decrease in concentration of vitamin E, a fat-soluble antioxidant.

Numerous studies have demonstrated that exercise is associated with an increase in free radical formation, oxidative damage to cell membranes, and skeletal muscle cell damage.[5-9,20] The degree of free radical formation is believed to be proportional to the rate of oxygen consumption, with more free radicals produced and greater oxidative stress as more oxygen is consumed.[3] Once the amount of oxidative stress overwhelms the antioxidant capacity of the body, oxidative damage to tissues may occur.[3] We have previously demonstrated that while racing, elite sled dogs have rates of sustained energy expenditure that exceed those reported for any other large mammal.[13,21] Thus, sled dogs would be expected to incur a substantial oxidative stress during consecutive days of strenuous exercise, such as racing. Indeed, dogs in the present study had a marked (600%) and progressive increase in plasma isoprostane concentration during the 3 days of exercise and this increase was correlated with an increase in serum CK activity, an indicator of muscle cell damage. Results were consistent with those reported for cyclists, in whom there is a significant increase in serum concentration of conjugated dienes, a crude indicator of lipid peroxidation, during a 5-day race.[20] In contrast, in healthy young human males, 3 days of exercise for 90 minutes at 65% of maximum oxygen consumption was not associated with any indication of oxidative damage to RNA.[22] Discrepancies among results of these studies may be attributable to the different indices of oxidant damage measured in each study. We believe that results of our study demonstrate the cumulative effect of oxidative stress induced by repetitive, strenuous endurance exercise.

Despite the evidence of a progressive increase in lipid peroxidation in the dogs of this study, we were unable to demonstrate significant changes in plasma antioxidant status or in activity of RBC antioxidant enzymes, although there was a decrease in plasma CER activity in response to exercise. The lack of change in TAS in these dogs stands in contrast to results reported for humans and horses, in which exercise increases the antioxidant capacity of plasma.[9,23,24] The TAS assay yields quantitative information regarding the antioxidant capacity of plasma, and is associated primarily with the effects of vitamin E, ascorbic acid, protein sulfhydryl groups, and an unidentified antioxidant.[25] The exercise-induced increase in plasma antioxidant capacity in humans is attributed to an increase in plasma vitamin E concentration.[9,26,27] In contrast, plasma vitamin E concentration decreased in response to exercise in dogs in the present study. The lack of change in TAS suggests that the decrease in vitamin E concentration may have been offset by increases in other antioxidants, as evidence by the increase in serum uric acid concentration. The fact that exercise did not have an effect on RBC SOD and GPX activities is consistent with previous reports.[26]

Serum vitamin E concentrations in dogs in the present study were similar to those previously reported for domestic dogs but higher than those reported for horses.[28-30] Serum vitamin E concentrations less than approximately 5 µg/ml in dogs are associated with pathologic changes in smooth muscle, the CNS, skeletal muscle, and the retina.[31,32] We are not aware of other studies of the effect of endurance exercise on serum vitamin E concentration in dogs; however, serum vitamin E concentration in dogs is not affected by 1 hour of running on a treadmill at 4 m/s.[33]

Concentrations of plasma constituents are often adjusted for exercise-induced changes in plasma or blood volume when examining the effects of exercise on plasma constituents. The assumption is that exercise-induced increases or, more frequently, decreases in plasma volume will decrease or increase, respectively, the measured concentration of the constituent when there has been no change in true plasma concentration

of the constituent. Use of changes in serum (or plasma) total protein concentration as an indicator of changes in plasma volume of dogs performing endurance exercise is problematic, because total protein concentration may decrease not only because of plasma volume expansion but also because of loss of protein from the vascular space or, less likely, decreased protein production.[34,35] The validity of adjusting serum vitamin E concentration for changes in serum total protein concentration is therefore uncertain. However, regardless of whether serum vitamin E concentration was adjusted or not, the decrease in plasma vitamin E concentration was significant. Similarly, the ratio of serum vitamin E concentration to serum cholesterol concentration decreased. Together, these results suggest that there was a true decrease in serum vitamin E content.

Although a direct causal link between the decrease in serum vitamin E concentration and the increases in plasma isoprostane concentration and serum CK activity was not established in this study, the consistent statistical associations between serum vitamin E concentration, serum CK activity, and plasma isoprostane concentration were strongly suggestive of at least a common mechanism for the changes observed in each. The strong association may indicate a causal relationship, in which increased oxidant stress reduces antioxidant capacity (ie, serum vitamin E concentration), resulting in lipid peroxidation and skeletal muscle cell damage. Serum vitamin E concentration was decreased after the first and third exercise runs, suggesting an exercise-induced consumption of vitamin E and raising the possibility of greater decreases in serum vitamin E concentration during more prolonged or more frequent exercise. However, to our knowledge, neither the efficacy of supplementation with antioxidants in preventing the exercise-induced decrease in serum vitamin E concentration, nor the effect of running farther or more often on serum vitamin E concentration has been studied in dogs.

Isoprostanes are a series of prostaglandin-like compounds formed in vivo by free radical-catalyzed peroxidation of arachidonic acid.[14] Production of isoprostanes is nonenzymatic and is not inhibited by drugs that inhibit phospholipase α_2 or cyclooxygenase.[15] Isoprostanes are sensitive and specific indicators of lipid peroxidation, and plasma isoprostane concentration is 20 times as sensitive as serum concentrations of malondialdehyde or thiobarbituric acid derivatives for detecting oxidative damage in rats.[14,15] The marked increases in plasma isoprostane concentration among dogs in the exercise group, but not among control dogs, in the present study suggests that the technique used to collect and store plasma prior to measurement of isoprostane concentration did not result in substantial ex vivo isoprostane production.

The high correlation between serum CK activity and plasma isoprostane concentration may be explained in several ways. There may be a direct and causal link, with lipid peroxidation resulting in damage to skeletal muscle cells and release of CK into plasma. This explanation is plausible, given results of studies in other species,[1,2,4,7] but results of the present study do not allow us to conclude that this definitely took place. It is possible that injuries to skeletal muscle cells that result in release of CK may also cause lipid peroxidation, so that concurrent increases in serum CK activity and plasma isoprostane concentration are not causally related. It is also possible that an undetected factor resulted in independent increases in plasma isoprostane concentration and serum CK activity. Finally, it is possible that the relationship between serum CK activity and plasma isoprostane concentration observed in the present study may have been a result of chance alone, although this is unlikely given the low P value ($P < 0.001$).

In summary, we document that repetitive endurance exercise in dogs decreases serum vitamin E concentration, and this change is associated with marked increases in markers of lipid peroxidation. We interpret these results as indicating that the antioxidant mechanisms of minimally trained dogs may, in some instances, be inadequate to meet the antioxidant requirements of repetitive endurance exercise.

[a]Eukanuba, The Iams Co, Dayton, Ohio.
[b]Cobas-Mira Clinical Analyzer, Hoffman LaRoche, Nutley, NJ.
[c]8-isoprostane ELISA, Assay Designs, Oxford, Mich.
[d]TAS assay, Alexis Corp, San Diego, Calif.

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