Effects of dietary antioxidant supplementation before and after oral acetaminophen challenge in cats

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Objective—To determine effects of lipoic acid, vitamin E, and cysteine before and after oxidant challenge in cats.

Animals—24 sexually intact adult cats.

Procedure—Cats were allocated into 4 equal groups. For 25 weeks, group A was fed a control dry diet and groups B, C, and D received this diet supplemented with vitamin E (2,200 U/kg [dry matter basis {DMB}]) plus cysteine (9.5 g/kg [DMB]), lipoate (150 mg/kg [DMB]), or all 3 antioxidants together, respectively. Weights were measured every 3 days and venous blood obtained every 5 weeks for CBC; serum biochemical analyses; lymphocyte blastogenesis; thiobarbituric acid reactive substances concentration; and concentrations of plasma protein carbonyl, 8-OH d-guanosine, blood glutathione, plasma amino acid, lipoate, and dihydrolipoate. At 15 weeks, all cats received acetaminophen (9 mg/kg, PO, once), clinical effects were observed, and methemoglobin concentrations were measured.

Results—Lymphocyte blastogenesis increased transiently in group C and D cats. After acetaminophen administration, all groups had transient increases in methemoglobin within 4 hours and mild, brief facial edema; group C had decreased glutathione concentration and increased 8-OH d-guanosine concentration versus controls; and protein carbonyl concentration increased least for group B. Plasma lipoate and dihydrolipoate concentrations peaked by week 10 for groups C and D.

Conclusions and Clinical Relevance—Lipoate, vitamin E, and cysteine did not have synergistic effects. Lipoate supplementation (150 mg/kg [DMB]) did not act as an antioxidant but appeared to enhance oxidant effects of acetaminophen. Vitamin E plus cysteine had protective effects. (*Am J Vet Res* 2005;66:196–204)

Antioxidants are available individually or in combination in many over-the-counter nutraceutical products purchased by humans for their own use and for their pets' consumption. Lipoic acid (6,8-thioctic acid; 1,2-dithiolane-3-pentanoic acid) is a novel, endogenous acid that has become, by virtue of its effective antioxidant properties, one of the most publicized nutraceuticals on the market today. Lipoic acid was isolated and described as a naturally occurring substance in 1951.[1] Soon after, it was described as an essential cofactor for the pyruvate dehydrogenase, branched chain α-ketoacid dehydrogenase, α-ketoglutarate dehydrogenase, and glycine cleavage enzyme systems. In the 1960s, potential uses were examined for lipoic acid as a pharmacologic antioxidant because of its reversible intracellular reduction to **dihydrolipoic acid (DHLA)**. Both lipoic acid and DHLA participate in redox reactions in vivo and in vitro.[2] Although the antioxidant activities of lipoic acid and DHLA have not been tested in cats, they have been studied extensively in rats, dogs, and humans.[3–5] Results of studies[2,3,6] in these species indicate that lipoic acid may potentiate the antioxidant effects of vitamin E, cysteine, and glutathione. Because lipoic acid, vitamin E, and cysteine are used separately or in combination to treat or prevent oxidative damage in diabetic polyneuropathy[6] and acute hepatocellular damage in humans,[7] these antioxidants, if provided conveniently as dietary supplements, might also benefit the nutritional management of similar diseases in cats.

Feline hemoglobin is particularly sensitive to oxidant damage, as evidenced by Heinz body formation.[8] Acetaminophen has been used as an oxidant challenge in cats by other investigators and found to be associated with hemoglobin damage and glutathione depletion.[9,10] The purpose of the study reported here was to investigate the effects of dietary supplementation with lipoic acid, vitamin E, and cysteine on markers of oxidant damage and immune activity in healthy cats before and after an oral challenge with acetaminophen (N-acetyl-p-amino phenol). It was hypothesized that lipoic acid would act as an antioxidant and have synergistic antioxidant effects with vitamin E and cysteine in healthy cats.

To test these hypotheses, an attempt was made to determine whether lipoic acid enhanced the effects of cysteine (a cytosolic antioxidant and precursor and limiting factor in the rate of glutathione synthesis[11])

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and vitamin E (a membrane antioxidant); compare intra- and extracellular antioxidant effects of a dietary mixture of vitamin E and cysteine or lipoic acid alone or in combination with vitamin E and cysteine; and compare effects of dietary antioxidant supplementation, with and without lipoic acid, on lymphocyte activity as one marker of immune function in cats.

Materials and Methods

Twenty-four sexually intact adult cats were housed in a controlled environment that provided 14 hours of light/d and ambient temperature of 21 ± 2°C. The protocol for this study was approved by the Animal Care and Use Committee of the University of California, Davis, in accordance with the National Research Council 1985 Guide for Care and Use of Laboratory Animals. The cats ranged in age from 1 to 6 years (mean ± SD, 2.3 ± 1.3 years) and in weight from 2.6 to 5.6 kg (males, 5.1 ± 0.3 kg; females, 3.7 ± 0.6 kg). The diets were formulated and manufactured by a commercial company[a] for this study (**Table 1**). The control diet provided 72 U of vitamin E/kg of diet on a **dry matter basis (DMB)**. During 2 weeks of acclimation to the control diet and cage arrangement, all 24 cats were prefed this same dry kibble diet ad libitum and had continuous access to fresh water. During the next 25 weeks, cats were allocated into 4 groups (3 males and 3 females/group, 3 of the same sex/cage). Because these cats were healthy young adults and similar in weight within sexes, they were not grouped further by age or body weight. Group A was fed the control dry diet, and groups B, C, and D received the control diet top-dressed with vitamin E (2,200 U/kg of diet [DMB]) with cysteine (9.5 g/kg [DMB]), lipoic acid[b] (150 mg/kg [DMB]), or all 3 antioxidants (at these concentrations) combined, respectively. For clarity, the groups will be referred to as A (control), B (E + cys), C (lip), and D (E + cys + lip). The concentration of vitamin E was chosen to provide a substantially greater concentration of the vitamin than is generally found in dry cat food (typically 30 U/kg of food) but not to exceed concentrations associated with blood clotting dysfunction (> 2,000 U/kg of body weight).[12] With the assumption that the cats in this study would consume 75 to 100 g of dry food daily to maintain body weight, it was estimated that the cats that received the diets supplemented with vitamin E (diets B and D) would consume 165 to 220 U of vitamin E/d, which was approximately the same intake safely used previously for antioxidant protection in cats.[13] Cysteine concentration in the diet was determined on the basis of the moderate concentration of 10 g/kg of food in the test diet used by Fettman et al[14] and which was associated with measurable increases in blood methionine and reduced **glutathione (GSH)** concentrations but no toxic effects. After feed analysis, the actual concentration of cysteine in the test diet was 9.5 g/kg of diet (DMB). Lipoic acid concentration was determined on the basis of preliminary work in which it was determined that the maximum tolerated single dose of orally administered lipoic acid was 13 mg/kg[15] and a dietary intake minimum of 1 mg/kg was needed to achieve detectable plasma concentrations of lipoic acid with the instrumentation used in this study. Assuming that the cats in this study would consume 75 to 100 g of dry food daily to maintain body weight, it was estimated that 150 mg of lipoic acid/kg of diet (DMB) would provide each 4- to 5-kg cat with approximately 3 mg of lipoic acid/kg of body weight per day.

Cats were fed and observed daily and weighed twice weekly with the same digital laboratory scale at each weighing. After withholding food for 12 hours, morning blood samples were drawn into EDTA, heparin, or serum tubes via jugular venipuncture at the end of the prefeeding period (time 0) and then every 5 weeks. Assays performed with EDTA-anticoagulated blood samples included CBC and concentrations of malondialdehyde products (**thiobarbituric acid reactive substances [TBAR]**, protein carbonyls, and **8-OH d-guanosine [8OH-dG]**). Assays performed on heparin-anticoagulated blood samples included lymphocyte blastogenesis, **methemoglobin (MetHb)** and Heinz body percentages, and measurement of concentrations of GSH and plasma-free

Table 1—Formulations for the control diet (A) and test diets (B, C, D) fed to cats in an oxidant challenge study and expressed on a percentage dry matter basis.

Ingredient	A	B	C	D
Rice, brewers	21.5	21.5	21.5	21.5
Corn gluten meal, 60%	16.5	16.5	16.5	16.5
Poultry by-product meal	15.5	15.5	15.5	15.5
Corn, whole yellow	11	11	11	11
Soybean meal, dehulled	10.5	10.5	10.5	10.5
Chicken, whole	9.84	9.84	9.84	9.84
Corn bran	3	3	3	3
Safflower oil	3	3	3	3
Tallow with vitamin E	3	3	3	3
Fish meal	2	2	2	2
Flavor coating	2	0.65	1.99	0.64
KCl	0.5	0.5	0.5	0.5
Phosphoric acid	0.5	0.5	0.5	0.5
Mineral premix	0.3	0.3	0.3	0.3
NaCl	0.25	0.25	0.25	0.25
L-lysine	0.25	0.25	0.25	0.25
Taurine	0.1	0.1	0.1	0.1
Choline chloride	0.18	0.18	0.18	0.18
Vitamin premix	0.08	0.08	0.08	0.08
Top-coated supplement	None	—	—	—
L-cysteine	—	0.95	—	0.95
Vitamine E (50% alpha tocopherol)	—	0.4	—	0.4
Lipoic acid	—	—	0.015	0.015
Total	**100**	**100**	**100**	**100**

Diet A = Control diet. Diet B = Control diet + L-cysteine + vitamin E. Diet C = Control diet + lipoic acid. Diet D = Control diet + L-cysteine + vitamin E + lipoic acid.

Feed analysis (percentage as fed): protein, 37.2%; fat, 14.9%; carbohydrate, 34.5%; ash, 7.02%; and moisture, 6.36% for all 4 diets. In test diets (B, C, D), top-coated supplements were used in place of control-diet flavor coating. — = Ingredient not included in diet.

amino acids. Assays performed on serum samples included biochemical analyses and measurement of concentrations of lipoic acid and DHLA. Weeks 1 to 15 were referred to as the antioxidant wash-in period, the next 7 days were referred to as the acetaminophen challenge period, and the remainder of the study through 25 weeks was referred to as the post-acetaminophen challenge period.

During week 15, after withholding of food for 12 hours, a morning blood sample was taken for all assays and all cats received 1 orally administered dose of 90 mg of acetaminophen[c]/kg (mean ± SD, 90.5 ± 0.4 mg/kg), which marked the beginning of the acetaminophen challenge period. The dose was determined on the basis of studies[16–18] of effect and safety in cats performed to maximize measurable clinical effects but minimize discomfort and duration of effects. Expected adverse effects were facial edema, mild cyanosis, and methemoglobinuria. Cats were observed for adverse effects every 30 minutes for the first 12 hours, then hourly for the next 12 hours after challenge, by which time all cats appeared clinically normal. The MetHb concentrations and Heinz body percentages were measured 48 and 24 hours before and 4 and 24 hours after administration of acetaminophen. The GSH concentrations were measured just before and at 24 hours after administration of acetaminophen. These were the only measures expected to change within ≤ 24 hours of administration.

The CBC and serum biochemical analyses were performed on automated analyzers.[d,e] Lymphocyte blastogenesis was evaluated by the method for tritiated thymidine uptake outlined by Tham et al,[19] as verified by Gregory et al,[20] and with the following modifications: concanavalin A[f] was the only mitogen used, the culture medium was RPMI-1640 supplemented with 1:100 penicillin-streptomycin-amphotericin and glutamine,[g] and cats had been conditioned to allow venipuncture without requiring anesthesia. In the blastogenesis plates, 5 control wells with concanavalin A and 5 test wells without concanavalin A were filled for each sample and hydrogen 3-thymidine (1 µCi/well) was added. Fresh, heparinized blood samples from all 24 cats were analyzed at the same time at each collection point. Mean **disintegrations per minute (dpm)** for each set of controls and tests was determined in a liquid scintillation counter[h] and used with lymphocyte counts from the CBC on the day of plating to calculate the stimulation factor index for each sample set on the basis of the following equations for each sampling time point:

$$\text{(1) Per group: } \frac{\text{Mean dpm/lymphocyte for test wells}}{\text{Mean dpm/lymphocyte for control wells}} = \text{stimulation factor for each cat}$$

$$\frac{\sum \text{stimulation factors for all cats in a group}}{\text{Number of cats per group}} = \text{mean group stimulation factor}$$

$$\text{(2) Index: } \frac{\text{Mean group stimulation factor}}{\text{Mean control group stimulation factor}} = \text{stimulation index per time point.}$$

We measured TBAR as a biomarker for lipid peroxidation by use of the method of Bast and Haenen.[21] Plasma protein carbonyl concentrations were determined as a marker of protein oxidation.[22–24] Briefly, for the protein carbonyl assay, each sample of blood in EDTA was centrifuged at 14,000 × *g* and the plasma removed for the assay after PCV was determined by use of microhematocrit centrifugation. By use of saline (0.9% NaCl) dilution, plasma was adjusted to obtain 1.0-mL samples of approximately 8 mg of protein/mL on the basis of the total plasma protein concentrations as determined by use of refractometry. From each diluted sample, 0.25 mL was added to each of 3 Eppendorf (1.5 mL) tubes.[i] One tube served as a control by adding 0.2 mL of 2N HCl,[i] whereas 0.2 mL of 10mM 2,4-dinitrophenylhydrazine[f] in 2N HCl was added to the other 2 tubes. Then 0.45 mL of 20% trichloroacetic acid[j] was added to all 3 tubes, which were mixed vigorously. Tubes were incubated for 1 hour at room temperature (25°C) and centrifuged at 14,000 × *g*, and the precipitated protein was saved. The precipitate was washed 3 times in an ethylacetate-ethanol[i] mixture and centrifuged at 14,000 × *g*, and the supernatant was discarded after each wash. The washed protein was dissolved in 1 mL of 6N guanidine[k] solution/tube, incubated at 37°C for 50 to 60 minutes, and mixed vigorously every 10 minutes during incubation. Each sample was read against a 6N guanidine blank in a spectrophotometer[l] at 360 and 280 nm; the differences in absorbance values between test and control values were used for calculations. The protein carbonyl concentrations were quantitated with an extinction coefficient of 22,000 per mole-centimeter and expressed as micromoles per milligram of protein. The 280-nm absorbance values were used to calculate the amount of protein in each sample on the basis of a standard bovine serum albumin curve obtained from fresh standards on the same day with the sample assay.

Heparinized plasma samples were analyzed for plasma-free amino acid concentrations by first mixing samples 1:1 with 6% sulfasalicylic acid[f] and centrifuging at 14,000 × *g* to remove protein and use of an automatic analyzer with internal and external standards.[m] Amino acid concentrations reported were taurine; asparagine; threonine; serine; glutamate; glutamine; glycine; alanine; citrulline; valine; cysteine; methionine; isoleucine; leucine; tyrosine; phenylalanine; tryptophan; ornithine; lysine; histidine; arginine; and metabolites phosphoserine, homocysteine, 1-methyl histidine, and 3-methyl histidine. Specific attention was given to methionine, cysteine, histidine, and taurine (because of effects on sulfur-containing compounds by lipoic acid[2,5]) and on the ratio of aromatic-to-branched chain amino acids (as an indicator of acute liver damage because high doses of acetaminophen[10] and lipoic acid have induced hepatotoxicosis[15] in cats). After PCV was determined by use of microhematocrit centrifugation from heparinized blood samples, GSH concentration was determined spectrophotometrically[l] from those samples by use of the method described by Prins and Loos.[25] In the assay that used an external standard and 5,5′-dithiobis-(2-nitrobenzoic acid), GSH reduced 5,5′-dithiobis-(2-nitrobenzoic acid) to 5-thio-2-nitrobenzoic acid (yellow) and the absorption was read at 412 nm.[j] Results were expressed as micromole per milliliter of RBC.

Serum lipoic acid and DHLA concentrations were measured by use of **high-performance liquid chromatography (HPLC)**. Samples (0.5 mL) were extracted and analyzed via the procedure of Sen et al.[26] Briefly, the sample and an equal volume of 20% phosphoric acid[j] were mixed well, 4 mL of hexane[k] was added, and the resulting solution was sonicated[n] for 15 seconds. Hexane was aspirated and saved for later evaporation, and another 4 mL of hexane was added to the sample tube. Each sample underwent 3 extraction cycles. The collected hexane was evaporated[o] under nitrogen gas, and 0.5 mL of resuspension solution (45% chloracetic acid,[f] 45% acetonitrile,[j] and 10% methanol[j]) was added to the dried tube. Tubes were refrigerated at 4°C overnight, mixed well, filtered (0.45-µm filter[p]) and assayed by use of HPLC with an electrochemical detector[q] with a carbon 18 column,[r] 20 µL injection volume, and a 1 mL/min flow rate. Lipoic acid and DHLA concentrations were expressed as picomoles per milligram of protein.

A previously validated, nonsubjective assay was chosen for 8OH-dG as a marker of DNA oxidant damage, which

required a 2-part procedure. First, DNA was isolated from blood in EDTA. Second, the isolated DNA was digested and analyzed for 2′-dG and 8OH-dG. The DNA isolation was started by mixing blood with lysis buffer[f] (NH_4Cl [0.155M], $KHCO_3$ [0.01M], and EDTA [0.1M] in distilled, deionized H_2O) in a polystyrene tube and discarding the supernatant after centrifugation. The pellet was resuspended in lysis buffer and centrifuged at 14,000 × *g*, and the supernatant was again discarded. The pellet was resuspended in buffer[f] (Tris-HCl [1M; pH, 8], NaCl [5M], and EDTA [0.5M] in ddH_2O), and proteinase K[s] was added to each tube, followed by 20% SDS. This mixture was incubated overnight at 65°C. Saturated NaCl solution was added to each sample, which was then shaken vigorously to foaming and centrifuged at 1,000 × *g*. The supernatant was transferred to a clean polystyrene tube; RNAse[s] was added, and the samples were incubated at 37°C for 30 minutes. Cold (4°C) 100% ethanol was added to each sample. After the DNA precipitated, the 100% ethanol was decanted and cold (4°C) 70% ethanol was added after air-drying the pellet. After brief centrifugation at 1,000 × *g*, the ethanol was decanted, the pellet air-dried, and buffer[e] (Tris-HCl [1M; pH, 8] and EDTA [0.5M] in ddH_2O) was added to the tubes. After overnight incubation to redissolve the DNA, the tubes were stored at –80°C until the HPLC assay was performed. The DNA purity was checked by use of spectrophotometric[l] absorbance of the samples at 260 and 280 nm. The DNA content was calculated as follows:

A (280 nm) / A (260 nm) = 1.65 to 1.75 for pure DNA
A (260 nm) = 1.000 for 50 µg of DNA/mL,

where A is absorbance. Assay of digested DNA samples within 48 hours was required for consistent results. Samples were acidified to a pH of 4.8 with acetic acid, nuclease P-1[s] (5 µg/mL) was added, and each tube was incubated for 30 minutes at 37°C. Tris-HCl (0.1M) was added to increase the pH to 8.5 before addition of calf intestine alkaline phosphatase[r] (1.4 U/µL), and the resulting solution was incubated for 1 hour at 37°C. The samples were transferred to spin filters[t] (capacity, 500 µL; cutoff value, 30 kd) and centrifuged at 2,000 × *g* for 20 minutes. Calf thymus DNA[f] was digested simultaneously with the cat DNA samples to serve as an external control. After filtration of the samples, 2′-dG was assayed spectrophotometrically[l] at 260 nm and the absorbance was compared with a standard curve from fresh standards (5 to 100 µg/mL). The 8OH-dG was assayed by use of HPLC[q] with a KH_2PO_4-to-methanol (90:10) mobile phase, liquid chromatography octadecyl silene base-deactivated column[r] (15 cm × 4.6 mm; 3-µm particle size), 50-µL injection volume, and 1 mL/min flow rate. Results were obtained by comparison with a standard curve (2.5 to 25 ng/mL) obtained from fresh standards. After converting 8OH-dG from nanograms per milliliter to femtomoles per milliliter and 2′-dG from micrograms per milliliter to nanomoles per milliliter and assuming 1×10^6 dG/nmol of 2′-dG, 8OH-dG concentrations were expressed as femtomoles per 10^6 dG.

Supravital new methylene blue stain was used to identify Heinz bodies and total (aggregate and punctate) reticulocytes, which were calculated as percentages of 1,000 RBCs counted.[27] For MetHb determination, blood samples were hemolyzed in dH_2O and potassium phosphate buffer[k] (pH, 6.6; 0.15M) was added. Duplicates of buffered hemolysate were analyzed by use of spectrophotometric[l] difference at 630 nm after addition of 20 mg of $K_3Fe(CN)_6$ and 5 mg of KCN[f] by use of the method of Betke et al.[28] The MetHb was expressed as a percentage of the total pigment concentration.

All values were expressed as group mean ± SD. Differences among groups at each time point were analyzed by use of ANOVA for repeated measures. All analyses were performed by use of a standard statistics package,[u] and $P < 0.05$ was considered significant. Bonferroni multiple comparisons procedures were used as post hoc correction methods because of the small sample size and multiple group comparisons.

Results

Antioxidant wash-in period—Cats in all diet groups had a mean weight increase (≤ 10%) during the first 5 weeks of the study, and differences among groups were not significant. The protein carbonyl concentrations decreased from weeks 0 to 10 (from 3.2 ± 0.2 µmol/mg to 1.9 ± 0.4 µmol/mg of protein) in all cats and by week 10 were significantly different for each group, compared with week 0 mean concentration in that group (**Figure 1**). However, protein carbonyl concentrations declined uniformly for all groups and had no significant difference among groups during this period. The GSH concentrations had no significant changes or differences among groups during this period (**Figure 2**).

Lymphocyte blastogenesis stimulation indices (**Figure 3**) for cats in groups C and D were significantly higher at week 5 (64 ± 11 and 70 ± 10, respectively), compared with cats in groups A and B (46 ± 9 and 31 ± 7, respectively). Although the stimulation index for group A appeared to increase during this time, the results were not significantly different from week 0. By week 15, the stimulation factor indices for all diet groups had a mean of 39 ± 9 with no significant differences among groups after that time. Number of lymphocytes per sample well did not vary significantly throughout the study period. Although the lymphocyte

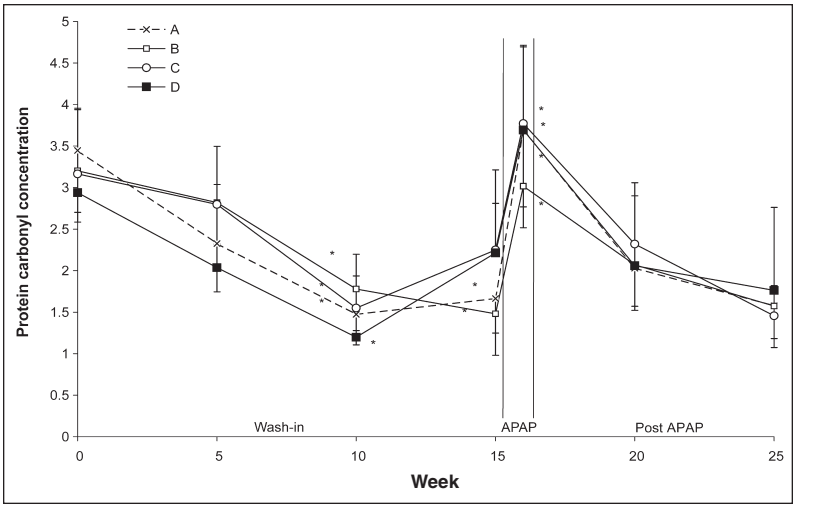

Figure 1—Protein carbonyl concentrations (mean ± SD [µmol/mg of protein]) for cats (n = 6/group) during an oxidant challenge study of 25 weeks. A = Control cats. B = Cats that received vitamin E and cysteine. C = Cats that received lipoate. D = Cats that received vitamin E, cysteine, and lipoate. APAP = Acetaminophen. Wash-in = Period before acetaminophen administration. Post-APAP = Period after acetaminophen administration. *Within each group, significantly ($P < 0.05$) different from value at start of week 0.

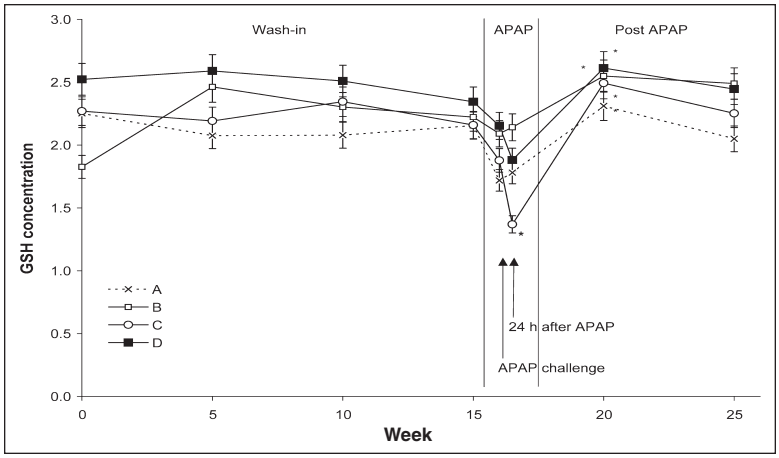

Figure 2—Reduced glutathione (GSH; mean ± SD [mmol of GSH/mL of RBCs]) concentrations for cats in an oxidant challenge study of 25 weeks. *Within each group, significantly ($P < 0.05$) different from value at previous time point. *See* Figure 1 for remainder key.

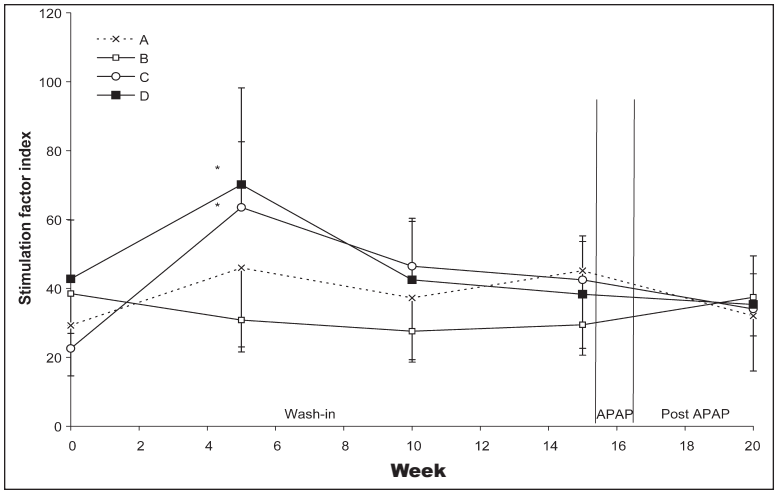

Figure 3—Lymphocyte blastogenesis stimulation factor indices (mean ± SD) for weeks 0 through 20 in an oxidant challenge study in cats. *Significantly ($P < 0.05$) different, compared with control value. *See* Figure 1 for remainder key.

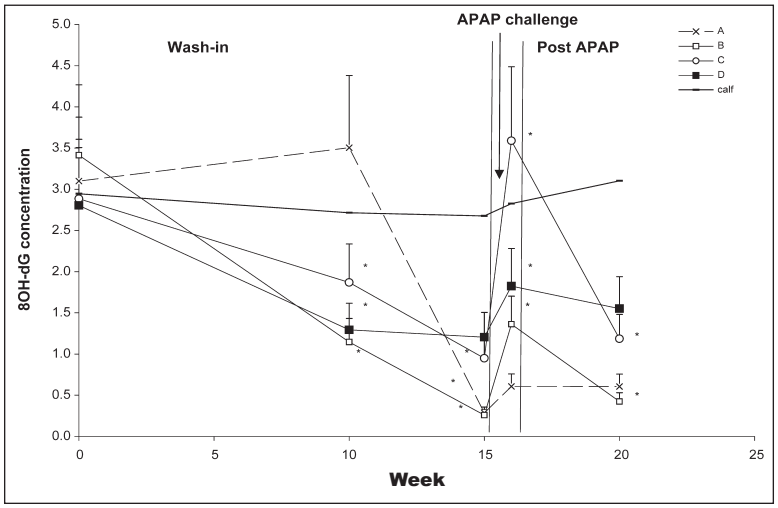

Figure 4—8-hydroxy-d-guanisine (8OH-dG) concentrations (mean ± SD [fmol of 8OH-dG/106 dG and compared with concentration in calf DNA as external standard]) in an oxidant challenge study in cats. *Within each group, significantly ($P < 0.05$) different from value at previous time point. *See* Figure 1 for remainder key.

count appeared to decline in group C cats, the differences between time points were not significant.

Regarding the 8OH-dG assay, although samples were collected at week 5, more than half the samples were lost because of operator error and the remaining results were not analyzed statistically. At 10 weeks, cats in groups B, C, and D had significantly lower concentrations of 8OH-dG, compared with cats in group A at weeks 0 and 10 (**Figure 4**). At week 15, group A cats had a significant decrease in 8OH-dG concentration to a mean value < 50% of the value at week 0. Values for groups B and C decreased significantly from weeks 10 to 15.

Plasma lipoic acid concentrations were significantly higher in groups C and D at week 5, compared with week 0. Increased mean values were maintained for the remainder of this first 15-week period (**Figure 5**). Plasma DHLA concentrations were significantly higher than control for group C at week 5 and for groups C and D at week 10. Mean values of 6.0 ± 0.7 pmol of DHLA/mg and 5.5 ± 0.7 pmol of DHLA/mg of protein, respectively, were maintained for the remainder of this first 15-week period.

Acetaminophen challenge period—Clinically, all cats had mild facial edema and cyanosis within 2 hours of acetaminophen administration; all clinical signs resolved within 24 hours. Subjectively, these clinical signs were most severe in group C cats and least severe in group B cats. Following acetaminophen challenge, body weight in the C and D group cats decreased slightly, compared with weights in the other 2 groups. Group C females had reduced weight (mean, 11 ± 2%), which was significant, compared with weights of females in the other 3 groups. Although PCV values decreased in all groups after administration of acetaminophen, these values decreased no more than 10% within any group and there were no significant differences in PCV among the groups. Weight decrease could have been caused by acutely reduced food or water intake with resulting dehydration, which suggested that the true PCV values may have decreased > 10%. However, acute changes in plasma protein and osmolarity were not measured. Putative methemoglobinuria was visually evident in the litter boxes within the 24-hour period after acetaminophen administration, but identification of specific cats affected and quantitation was not possible because 3 cats used the 2 litter boxes in each cage and because of the variable absorbency of the shavings.

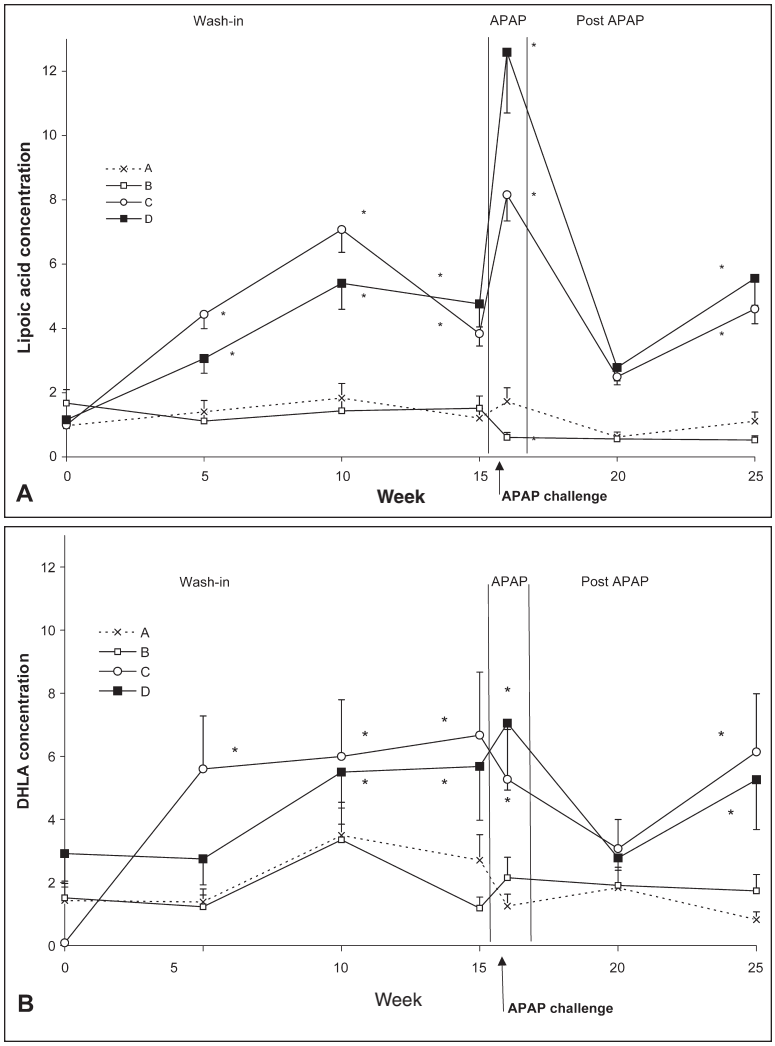

Figure 5—Plasma lipoic acid (A) and dihydrolipoic acid (DHLA; B) concentrations (mean ± SD [pmol/mg of protein]) in a 25-week oxidant challenge study in cats. *Significantly ($P < 0.05$) different, compared with control value.

The MetHb percentage increased significantly in all 4 groups at 4 hours after acetaminophen administration, with highest mean peak values of 44 ± 6% for C group cats **(Figure 6)**. At 24 hours after acetaminophen administration, Heinz body percentage had increased significantly from 4.1 ± 3.3% (mean at week 15 for all groups combined) to 43.9 ± 21.5% in group C and 23.3 ± 23.1%, 15.9 ± 7.9%, and 30.3 ± 6.7% in groups A, B, and D, respectively. Although PCV transiently decreased for all cats during this period, there was no significant difference among groups and PCV values had returned to baseline by the 20-week sampling point for all cats.

Plasma protein carbonyl concentrations increased sharply and significantly for all diet groups at the end of the challenge period, compared with concentrations at the end of the antioxidant wash-in period (Figure 1). During the first 24 hours after acetaminophen administration, GSH concentrations remained unchanged for group B cats, decreased slightly for group A and D cats, and decreased significantly for group C cats (Figure 2).

Mean 8OH-dG concentration was increased significantly at week 16 for cats in all 3 antioxidant groups, compared with concentrations at the end of the antioxidant wash-in period (Figure 4). The group C cats had the highest increase in 8OH-dG concentration 7 days after acetaminophen administration (278% of preadministration concentration), compared with ≤ 100% increase in the other 3 groups.

By 1 week after acetaminophen administration, lipoic acid concentrations in group B cats were slightly but significantly lower, compared with group A cats (Figure 5). In contrast, a sharp and significant increase in lipoic acid concentration occurred in the C and D groups from 3.8 ± 0.5 pmol/mg of protein to 8.2 ± 0.9 pmol/mg of protein and from 4.8 ± 0.7 pmol/mg of protein to 12.6 ± 2.1 pmol/mg of protein, respectively. During this time, DHLA concentrations were not significantly different from the values obtained at the end of the antioxidant wash-in period.

Post-acetaminophen challenge period—By week 25, cats in all diet groups had returned to their week 0 body weight. Although mean weight of group C males was 3% less than the males in the other 3 groups, differences were not significant. After the acetaminophen challenge period increase, plasma protein carbonyl concentrations returned to baseline (week 0) concentrations by week 20 and through the end of the study (Figure 1).

After the initial decrease during the acetaminophen challenge period, mean GSH concentrations in all groups increased significantly, especially in group C cats, and returned to antioxidant wash-in period values by the end of the study (Figure 2). By week 20 (5 weeks after acetaminophen challenge), 8OH-dG concentrations had returned to values determined at the end of the antioxidant wash-in period in all groups (Figure 4) and were not assayed again.

Within 5 weeks of the oxidant challenge, lipoic acid had returned to week 0 concentrations and then increased back to the values obtained at the end of the antioxidant wash-in period at week 25. The DHLA concentrations in group C and D cats decreased to week 0 values at week 20 but again increased significantly to values greater than group A concentrations at week 25 (Figure 5).

Lymphocyte stimulation index for all groups at week 20 had returned to week 0 values, and there were no significant differences among groups. Although lymphocyte blastogenesis was assayed throughout the study, mechanical difficulties with the cell-harvesting apparatus invalidated the results for the week 25 samples.

Additional results—Results of the plasma amino acid measurements did not reveal changes specific to any time period in the study and so were reported

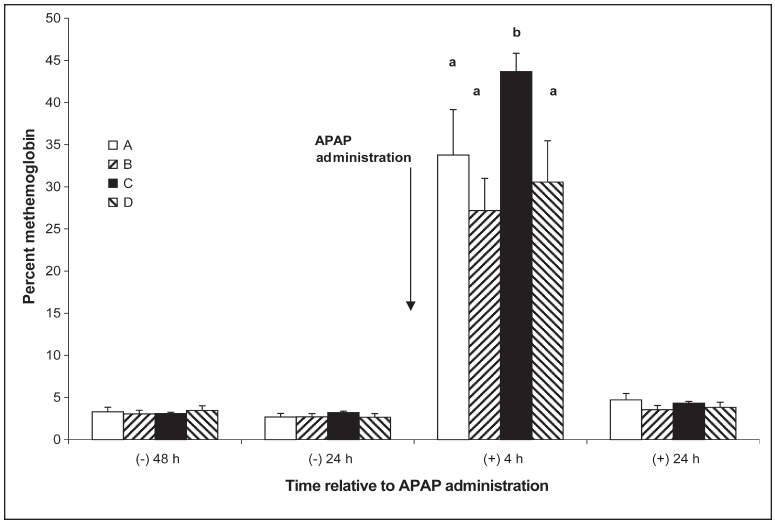

Figure 6—Methemoglobin concentration (mean ± SD [percentage of total pigment]) before and after APAP administration in an oxidant challenge study in cats. [a]Significantly ($P < 0.05$) different, compared with (-) 24-hour value for that group. [b]Significantly ($P < 0.05$) different, compared with (-) 24-hour value for that group and with (+) 4-hour values for all other groups. *See* Figure 1 for remainder of key.

together. Ratios of branched chain-to-aromatic amino acid concentrations stayed in the range of 3 to 4 for all groups throughout the study. From week 15 onward, mean plasma concentrations of threonine, serine, glycine, leucine, ornithine, and lysine in group C cats were slightly but significantly decreased, compared with concentrations in control group cats. These amino acid concentrations were also decreased slightly but significantly in group D cats at weeks 20 and 25. Mean plasma arginine concentrations were significantly lower at week 5 in group C cats and at week 10 in group D cats, compared with concentrations in control cats (94 ± 10 nmol/mL, 97 ± 7 nmol/mL, and 125 ± 19 nmol/mL, respectively). These decreases remained unchanged throughout the rest of the study period. In group B cats, mean plasma arginine concentrations (144 ± 17 nmol/mL) were significantly higher than mean control concentrations (125 ± 19 nmol/mL) at week 5, and the significant difference between these groups remained through week 25. Results for sulfur amino acids revealed no significant changes among groups throughout the study.

There were no significant differences in TBAR or serum biochemical values among groups at any sampling point; although some fluctuations in values were apparent over the course of the study, all values stayed within the reference ranges for our laboratory. All but 1 cat remained healthy throughout the study period. One male cat in group A developed cystitis at week 5 and was treated for 10 days with orally administered amoxicillin (15 mg/kg, q 12 h). This cat did not stop eating or lose weight, and treatments were completed and cystitis resolved before the acetaminophen challenge point, so the cat remained in the study.

Discussion

Contrary to our hypothesis, lipoic acid appeared to act as more of a pro-oxidant than an antioxidant in the healthy young cats in this study. Instead of synergy among the 3 antioxidants, the vitamin E and cysteine mixture seemed to provide some protection against the unexpected oxidizing effects of lipoic acid.

Dietary supplementation with lipoic acid alone appeared to potentiate the immediate oxidative effects of acetaminophen because clinical signs were more severe, methemoglobin and Heinz body percentages were significantly higher, and protein carbonyl and 8OH-dG concentrations were significantly higher in group C cats after acetaminophen administration; all these changes were in the intracellular markers of oxidant damage. In addition, dietary supplementation of lipoic acid at least partially reduced the antioxidant effectiveness of the vitamin E and cysteine mixture because 8OH-dG concentrations were higher in group D than in group B cats from weeks 10 through 20. Vitamin E and cysteine appeared to protect against the effects of lipoic acid, as judged on the basis of the less severe changes in GSH, 8OH-dG, and methemoglobin concentrations in group D versus group C cats and the earlier decrease in 8OH-dG concentration in group B cats. Heinz body percentage differences suggested greater antioxidant protection of the cats' hemoglobin by the E + cys mixture because the A group and both groups that consumed lipoic acid had higher Heinz body concentrations after acetaminophen administration than did the B group.

The GSH concentrations were significantly lower in group C cats, compared with other groups, within 24 hours of acetaminophen administration. The GSH may have been used to help reduce excess intracellular lipoic acid. However, redox cycling with lipoic acid also may have then helped replenish GSH concentrations because group C and D cats had faster return to preacetaminophen GSH concentrations. Interpretation of GSH values included consideration of recent concerns about measurement artifact possibly introduced by thiol oxidation during in vitro GSH assay procedures and about the variability of circulating GSH concentrations in humans and rodents.[29] At each collection time, by assaying GSH immediately from all samples collected as a group and performing the assays on each sample in triplicate, we found little variation in GSH among cats within each group.

Decreased protein carbonyl concentrations in all cats during the first 15 weeks suggested that the control diet alone was protective against protein damage, compared with previous diets fed to these cats. The addition of antioxidants to the control diet contributed to protection against DNA oxidative damage, as indicated by significant decreases in 8OH-dG concentrations by week 10 in the groups that received supplements, compared with the control group. After oxidant challenge, we expected an increase in 8OH-dG concentrations, reflecting DNA damage, and expected the concentrations would be highest in the control cats (the group without supplemental antioxidant protection). However, the higher 8OH-dG concentrations in

the C and D groups (although not significant), compared with the control group after challenge, suggested that lipoic acid (or DHLA) may have acted as a pro-oxidant and also counteracted potential protective effects of the vitamin E and cysteine combination. The DHLA exerts pro-oxidant effects in tissue cell culture,[30] and plasma concentrations reached in this study may have been high enough to exert such pro-oxidant effects.

In the group C cats, DHLA reached plateau concentrations in plasma by week 5, whereas lipoic acid increased until week 10. Lipoic acid concentrations increased similarly in group D cats, but DHLA concentrations did not plateau until the 10-week point in that group. This delay could be attributed to either an increase in the rate of intracellular metabolism that converted lipoic acid to DHLA or a delay in the export of DHLA from the cells in the group D cats. Substrate competition for binding sites is one potential explanation for this delay because lipoic acid and cysteine (found together in the D group diet) can both be metabolized by glutathione reductase. In the C and D groups, lipoic acid and DHLA concentrations reached an approximate 1:1 ratio by week 10, which may be the time needed to reach equilibrium between the parent compound and this metabolite with dietary supplementation. Because DHLA was also increased in groups A and B at week 10, endogenous production of DHLA could have contributed to a DHLA increase in all the groups at this time point. The increase in lipoic acid and DHLA concentrations in group C and D cats after acetaminophen administration could be attributable to either their reduced biliary excretion or hepatocellular damage in association with oxidative damage. Although serum liver enzyme activities remained within reference ranges in all cats, 8OH-dG concentrations in the C and D groups did not fully return to pre-acetaminophen concentrations, suggesting that lipoic acid supplementation hindered cellular recovery. Decreases in plasma amino acid concentrations that appeared first in group C cats and later in group D cats may have been indications of early cellular damage[31] attributable to lipoic acid supplementation, whereas vitamin E plus cysteine supplementation to group D cats may have delayed the toxic effects of lipoic acid in that group. Decreases in arginine, essential in cats[32,33] and integral to T-lymphocyte responses and wound healing,[34,35] may also indicate early immune system suppression in lipoic acid-supplemented cats.

The TBAR results had marked interindividual variations such that no conclusions could be drawn regarding antioxidant effects on lipid peroxidation. These results were similar to those from a previous study[14] in cats that received 10 g of cysteine/kg of diet, and the authors concluded that malondialdehyde concentrations in healthy cats would be unaffected by antioxidant supplementation.

Results of our study indicated that lipoic acid did not provide protection against and actually potentiated oxidant damage when provided to cats at 150 mg/kg in the diet over 25 weeks. In contrast, vitamin E and cysteine provided some beneficial effects, including apparent protection against pro-oxidant effects of lipoic acid.

a. Nestle-Purina Co, St Louis, Mo.
b. Chemco Industries, Los Angeles, Calif.
c. Tylenol, McNeil Laboratories Inc, Fort Washington, Pa.
d. Advia, Bayer HealthCare LLC, Tarrytown, NY.
e. Hitachi 717, Roche, Indianapolis, Ind.
f. Sigma, St Louis, Mo.
g. Medium mix, Life Technologies, Grand Island, NY.
h. Tricarb liquid scintillation counter, Packard Instrument Co, Downers Grove, Ill.
i. Brinkman Industries, Westbury, NY.
j. EM Science, Gibbstown, NJ.
k. Fisher Scientific, Fair Lawn, NJ.
l. Spectronic Genesys 5, Milton-Roy, Rochester, NY.
m. Beckman 7300, Beckman Instruments, Fullerton Calif.
n. Model W375, Heat Systems Ultrasonics Inc, Plain View, NJ.
o. N-Evap Organomation Associates Inc, Berlin, Mass.
p. Acrodisc syringe filters, Gillman Laboratory, Pall Corp, Ann Arbor, Mich.
q. ESA Coulochem II Model 5010, 5011 analytical cell, Chelmsford, Mass.
r. Supelcosil, Supelco, St Louis, Mo.
s. Roche, Indianapolis, Ind.
t. Millipore-MC, Millipore, Burlington, Mass.
u. Analyze-it for Excel, Analyze-it Software Co, Leeds, UK.

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