Foods With Lipoic Acid and Elevated Levels of Vitamin E and Vitamin C Correlate With Whole Blood Antioxidant Concentrations and May Protect Geriatric Dogs From Oxidative Stress

Inke Paetau-Robinson, PhD
Ryan M. Yamka, PhD
Kim G. Friesen, PhD

Hill's Pet Nutrition, Inc.
Topeka, KS

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ABSTRACT

The objective of the study was to evaluate the effect of wellness foods on antioxidant status and DNA integrity in geriatric dogs. Geriatric dogs (n = 40; ≥10 years of age) were randomly assigned to 1 of 4 treatment groups and fed either an experimental wellness food with lipoic acid and enhanced vitamin E and C levels or 1 of 3 commercially available, age-appropriate foods (food A, B, and C) for a period of 90 days. Blood was collected at baseline, Day 30, and Day 90 for the analysis of serum vitamin E, serum glutathione peroxidase (GSH-Px) activity, serum fatty acids, and plasma malondialdehyde (MDA). White blood cells (WBC) were isolated from whole blood, and DNA integrity was determined by alkaline single cell gel electrophoresis (Comet assay). At Days 30 and 90, serum vitamin E concentrations were higher in the dogs fed the experimental food compared to food C. There was no difference in serum GSH-Px activity among the 4 diet groups at 30 days; however, at 90 days, the dogs receiving the experimental food had higher GSH-Px activity compared to the dogs fed foods A and B. Plasma MDA concentrations were lowest in the dogs receiving food B. After 90 days, plasma MDA concentrations were significantly lower in the experimental group compared to the dogs eating food C. DNA damage upon oxidative challenge was significantly lower in WBC from dogs fed the experimental food compared to dogs fed foods A and C. The study demonstrated that wellness foods with lipoic acid and elevated levels of vitamins E and C can enhance antioxidant status and protect geriatric animals from oxidative stress.

INTRODUCTION

Oxidative stress is believed to play a pivotal role in the pathophysiology of aging.[1] Oxidative stress is due to an imbalance of oxidants (ie, free radicals that are byproducts of normal metabolism), and antioxidants. If cells contain more oxidants than antioxidants, the cell will be under oxidative stress that can lead to damage of macromolecules such as proteins, lipids, and nucleic acids (eg, DNA). Chronic oxidative stress is asso-

ciated with the development of degenerative diseases such as heart disease, cancer, and diabetes.[2] Enhancing an animal's antioxidant status can potentially extend disease-free lifespan and improve the quality of life. Cells are equipped with endogenous antioxidant enzymes like glutathione peroxidase (GSH-Px), and also contain antioxidants derived from food. Dietary antioxidants such as vitamins E and C have been shown to maintain or improve the antioxidant status of dogs.[3,4]

Lipoic acid is a cofactor in the pyruvate dehydrogenase complex. It has 2 thiol groups that can undergo reversible oxidation to a disulfide bond. Because of its capacity to undergo oxidation-reduction reactions, lipoic acid can act as both an electron hydrogen carrier and an acyl carrier. In contrast to many antioxidants, both the reduced (dihydrolipoic acid) and the oxidized form of lipoic acid can act as an antioxidant.[5]

Vitamin E is the most effective chain-breaking lipid-soluble antioxidant; it scavenges lipid radicals during initiation and propagation of lipid peroxidation resulting in a vitamin E radical.[6,7] Vitamin E can be regenerated from its radical form by glutathione (GSH) or vitamin C (ascorbic acid) forming oxidized glutathione (GSSG) or dehydroascorbic acid, respectively.[8-10] Dihydrolipoic acid is able to reduce GSSG to GSH and dehydroascorbic acid to vitamin C.[11,12] Polyunsaturated fatty acids (PUFA) are more susceptible to oxidation, therefore, a diet high in PUFAs may require higher levels of dietary vitamin E to inhibit or slow down in vivo oxidation of fatty acids, and enhanced levels of vitamin C and lipoic acid for the recycling of vitamin E at the hydrophilic interface.

The objective of this study was to feed geriatric dogs (≥10 years of age) diets high in PUFAs but differing in levels of vitamins E and C, and lipoic acid, then assess the antioxidant status by measuring the concentrations of serum vitamin E, serum GSH-Px, and plasma malondialdehyde (MDA) at predetermined time points (Days 0, 30, and 90). Additionally, single cell gel electrophoresis (Comet assay) was performed on white blood cells (WBC) to evaluate DNA integrity (another measure of oxidative stress).[13]

MATERIALS AND METHODS

Study Population and Study Design

Forty healthy geriatric Beagle dogs (≥10 years of age) participated in the study. The dogs were determined to be healthy by physical examination and by normal reference range values on a routine serum biochemical analysis. Dogs were cared for in accordance with Institutional Animal Care and Use Committee protocols. Dogs were offered enrichment toys, received regular grooming, and had daily opportunity to socialize with other dogs and people.

For 30 days prior to study initiation, all dogs were fed a food that met the required nutritional needs for dogs as established by the Association of American Feed Control Officials (AAFCO). Following this 30-day wash-out period, the dogs were stratified by age, gender, and body fat percentage, then assigned to 4 different treatment groups. Each group of dogs was randomly assigned to receive either the experimental food or 1 of 3 commercially available age-appropriate products for 3 months. The experimental food was formulated in accordance with the AAFCO nutrient guide for dogs and balanced to meet maintenance requirements. The food was supplemented with alpha-tocopherol and ascorbic acid. The nutrient composition of each food is presented in Table 1.

Analyses

Whole blood was collected from each dog on Days 0, 30, and 90 during the study period and immediately placed on ice and then centrifuged to separate serum or plasma. Serum for the analysis of vitamin E, GSH-Px, and fatty acids was separated and stored at -70°C until analyzed. Another whole blood sample was collected from each dog into an evacuated tube containing potassium EDTA. A 500-µL fraction of the whole blood was separated for immediate use in the single

Table 1. Analyzed nutrient profiles of the 4 foods utilized in the study.

Nutrients, 100% Dry Matter Basis	Experimental Food	Commercial Food A	Commercial Food B	Commercial Food C
Crude protein, %	20.10	27.65	27.76	29.39
Fat, %	16.45	13.52	11.08	13.59
Calcium, %	0.71	0.79	1.28	1.35
Phosphorus, %	0.61	0.68	0.93	1.14
Eicosapentaenoic acid, %	0.32	0.10	<0.01	0.10
Docosahexaenoic acid, %	0.22	0.09	<0.01	0.08
Linoleic acid, %	4.00	2.92	1.90	2.60
Total n-3 fatty acids, %	1.30	0.48	0.13	0.41
Total n-6 fatty acids, %	3.96	3.10	1.79	2.66
Methionine, %	1.00	0.49	0.51	0.66
Manganese, ppm	87	77	71	69
Alpha-tocopherol, IU/kg	1492	594	894	421
Vitamin C, ppm	127	288	86	21
Lipoic acid, ppm	136	NA	NA	NA

Experimental food ingredient list: corn meal, poultry meal, soybean meal, animal fat, pal enhancer A, flaxseed, soybean oil, fish oil, beet pulp, corn gluten meal, DL-methionine, pal enhancer B, potassium chloride, dicalcium phosphate, calcium carbonate, L-carnitine, choline chloride, vitamin E, L-lysine, vitamin premix, iodized salt, taurine, L-tryptophan, L-threonine, mineral premix, preservative, manganese sulfate.

cell gel electrophoresis assay (also called Comet assay) before the remaining blood was centrifuged to separate the plasma. Plasma for the analysis of MDA was stored at -70°C until analysis. Serum vitamin E was analyzed using the method by Hoehler et al.[14] Serum GSH-Px activity was determined using the method described by Lawrence et al.[15] Plasma MDA was analyzed using a modified method by Nielsen et al.[16] Alkaline Comet assay to determine DNA integrity in WBC was performed following the protocol by Singh et al.[17] Serum fatty acids were analyzed using a modified method described by Rodriguez-Palmero et al.[18]

Statistical Analysis

Data were analyzed using General Linear Models procedure of SAS19 to determine treatment means. The experimental unit was dog and Day 0 was used as a covariate. The experimental food was then compared to the 3 commercially available foods. Differences were considered significant when $P < 0.05$ and trends were determined when $P < 0.10$.

RESULTS

All 40 dogs remained healthy during the entire study period. Tables 2 and 3 present the results at Day 30 and 90, respectively, for serum GSH-Px, serum vitamin E, plasma MDA, serum fatty acids, and DNA integrity. At day 30 serum vitamin E concentrations were significantly higher in the dogs fed the experimental food compared to food C ($P = 0.01$). At Day 90 serum vitamin E concentrations were higher in the experimental food group compared to food C; however, the difference did not reach statistical significance ($P = 0.08$). Serum GSH-Px activity was not different among the 4 diet groups at Day 30; however, at Day 90, serum GSH-Px activity was higher in the dogs receiving the experimental food compared to the dogs fed foods A ($P = 0.07$) and B ($P = 0.05$). Plasma MDA concentrations were significantly lower in

Table 2. Antioxidant markers and fatty acids measured in the blood in dogs at Day 30 fed 4 different foods.

Blood Markers	Experimental Food	Commercial Food A	Commercial Food B	Commercial Food C	Vs A*	Vs B*	Vs C*
Serum glutathione peroxidase, μg/10^6	5.85	5.68	5.68	5.96	0.28	0.25	0.50
Serum vitamin E, μg/mL	39.4	40.7	39.6	31.4	0.64	0.95	0.01
Serum vitamin E:total cholesterol ratio	0.19	0.18	0.20	0.15	0.37	0.09	<0.01
Plasma malondialdehyde, μM	1.12	1.02	0.67	1.20	0.36	<0.01	0.45
Comet head DNA, %	94.6	93.3	94.6	93.4	0.03	0.98	0.04
Linoleic acid, mg/dL	62.3	59.3	50.3	50.2	0.40	<0.01	<0.01
Eicosapentaenoic acid, mg/dL	9.80	3.96	1.53	3.68	<0.01	<0.01	<0.01
Docosahexaenoic acid, mg/dL	16.6	10.0	3.9	11.7	<0.01	<0.01	<0.01
Total n-6 fatty acids, mg/dL	107	126	104	114	<0.01	0.56	0.27
Total n-3 fatty acids, mg/dL	28.5	15.2	6.4	17.0	<0.01	<0.01	<0.01

*Probability of greater F value.

the dogs receiving food B compared to the experimental food at Day 30 ($P < 0.01$). After 90 days on the study food, plasma MDA concentrations were significantly lower in the experimental group compared to the dogs eating food C ($P < 0.01$). DNA damage upon hydrogen peroxide challenge as assessed by the Comet assay was significantly lower in the experimental diet group, indicated by a higher percentage of head DNA, compared to foods A ($P = 0.03$) and C ($P = 0.04$) at Day 30. After 30 and 90 days of eating the experimental food, dogs had significantly higher serum concentrations of eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), and total n-3 fatty acid compared to dogs receiving any of the 3 commercial foods ($P < 0.01$). Serum concentrations of EPA reflected dietary levels of that fatty acid. Total n-6 fatty acid concentrations were significantly lower in the dogs receiving the experimental food compared to dogs receiving foods A ($P < 0.01$) and C ($P = 0.05$) at Day 90.

DISCUSSION

Oxidative stress is the result of an imbalance in oxidants to antioxidant defense systems to repair systems within the cell.[20,21] The generation of oxidants in cells occurs during normal metabolism such as mitochondrial electron transport and peroxisomal beta-oxidation of fatty acids. Phagocytes, for example, macrophages and neutrophils, may

Table 3. Antioxidant markers and fatty acids measured in the blood in dogs at Day 90 fed 4 different foods.

Blood Markers	Experimental Food	Commercial Food A	Commercial Food B	Commercial Food C	Vs A*	Vs B*	Vs C*
Serum glutathione peroxidase, µg/10^6	5.09	4.83	4.82	5.11	0.07	0.05	0.93
Serum vitamin E, µg/mL	40.7	43.1	40.6	33.0	0.56	1.00	0.08
Serum vitamin E:total cholesterol ratio	0.22	0.20	0.23	0.15	0.35	0.44	<0.01
Plasma malondialdehyde, µM	1.05	1.22	0.87	1.34	0.13	0.11	<0.01
Linoleic acid, mg/dL	62.7	60.3	55.8	53.0	0.60	0.14	0.05
Eicosapentaenoic acid, mg/dL	9.02	3.70	1.51	3.52	<0.01	<0.01	<0.01
Docosahexaenoic acid, mg/dL	17.4	12.1	3.9	14.5	<0.01	<0.01	<0.01
Total n-6 fatty acid, mg/dL	108	134	112	124	<0.01	0.59	0.05
Total n-3 fatty acid, mg/dL	28.5	17.7	6.9	20	<0.01	<0.01	<0.01

*Probability of greater F value.

generate oxidants as part of their host defense system.[1] Endogenous antioxidant systems in the cells include vitamins E and C, lipoic acid, catalase, superoxide dismutase, GSH-Px, and metallothionein.[1] Dietary vitamin E, vitamin C, and lipoic acid would be considered exogenous antioxidants. The body also has several repair systems that are able to repair oxidative damage. These include lipases, proteolysis, lysosomal turnover, and DNA repair enzymes.[1] Overall, positive and negative feedback between the generation of oxidants, antioxidants defenses, and oxidative damage repair determines the outcome of aging.

Glutathione peroxidase is one of the predominant endogenous antioxidant defense systems in the animal.[1,21] All dogs in this study presented with high concentrations of serum GSH-Px. At Day 90, serum GSH-Px activity was higher in the dogs receiving the experimental food compared to the dogs fed foods A and B. The serum concentration of GSH-Px can be enhanced by supplemental selenium and vitamin E. The high concentrations of GSH-Px at baseline are likely due to the well-balanced antioxidant-supplemented diets the dogs were fed over the past years prior to entering into the study.

Malondialdehyde is a peroxidation product of PUFAs and as such can serve as a biomarker of lipid peroxidation in vivo.[22] Studies in humans have shown that MDA concentrations increase and GSH-Px concentrations decrease in the elderly (age 86 ± 6 years) compared to the young (age 29 ± 4

years).[23,24] In another study that compared a group of 21- to 40-year-old healthy subjects to elderly subjects age 61-85 years, the former group had higher plasma concentrations of MDA and protein carbonyls.[25] Endogenous and hydrogen peroxide-induced DNA damage were increased in lymphocytes of the elderly subjects. Cigarette smoke contains an array of oxidants,[26] therefore, smoking creates an environment in the body that is likely to result in oxidative stress. Vitamin E has been shown to successfully reduce lipid oxidation and MDA concentrations in smokers and non-smokers.[27,28] Malondialdehyde can react with DNA and form MDA-DNA adducts, which, if not repaired by the body's own repair enzymes, can interfere with normal gene expression.[29]

In the study presented here, we measured plasma MDA concentrations as a biomarker of lipid peroxidation and oxidative stress. After 90 days on the experimental food, plasma MDA concentrations were significantly lower in the experimental group compared to the dogs eating food C. It appears that the MDA concentrations were inversely related to the vitamin E content in the diet. Additionally, lipoic acid, which was present in the experimental food, plays a role in the regeneration of vitamin E from its radical form, thus making more vitamin E available for scavenging radical products from lipid peroxidation. When interpreting the results, it is important to consider the high levels of PUFAs in the experimental diet. After 30 and 90 days of eating the experimental food, dogs had significantly higher serum concentrations of EPA, DHA, and total n-3 fatty acid compared to dogs receiving any of the 3 commercial foods. Serum concentrations of EPA reflected dietary levels of that fatty acid. Total n-6 fatty acid concentrations were significantly lower in the dogs receiving the experimental food compared to dogs receiving foods A and C at Day 90. Polyunsaturated fatty acids are very susceptible to in vivo oxidation and can increase oxidative stress in the animal that, theoretically, can increase plasma MDA concentrations. We did not observe an increase in plasma MDA concentrations in the experimental diet group; in fact, MDA concentrations decreased over the course of the study in this group; this is likely due to the high level of vitamin E and lipoic acid in the diet. The low concentration of plasma MDA in dogs eating food B is most likely associated with the extremely low dietary levels of PUFAs and high dietary level of vitamin E.

During oxidative stress, reactive oxygen species can interact with and damage DNA.[29] DNA damage may occur in many forms, for example, oxidized bases, strand breaks, or sister chromatid exchange. The type of DNA modification depends on the type of reactant attacking, its concentration, and the cell's ability to protect or repair its DNA from the oxidative attack. With increasing age, the efficacy of the cell's own defense system may be diminished.[23,24,30-32] A widely accepted method to detect DNA damage in a single cell is the Comet assay, a rapid and sensitive fluorescent microscopic method to assess DNA strand breaks from a single cell.[17] The DNA fragments will migrate in the electric field and the intensity of the fluorescent stain will give an indication with regard to the extent of DNA damage. In the study presented, we demonstrated that elevated levels of dietary vitamin E and lipoic acid maintained DNA integrity of WBC in geriatric dogs. The initial DNA damage was low in this study population; this is likely due to the well-balanced antioxidant-supplemented diet the dogs were fed over the past years. Based on the high plasma vitamin E concentrations at enrollment into the study, it can be assumed that the body pool of vitamin E was high. The washout period included in the study design was too short to reduce vitamin E concentrations significantly. It is difficult to achieve additional protection through dietary antioxidants in a healthy study population. DNA damage may serve as a marker for cancer risk.[33,34] We decided to isolate WBC to evaluate the extent of DNA damage in these cells assuming that processes occurring in WBC reflect processes that occur in other tissues at risk for cancer development.

This study confirms that food supplemented with vitamins E and C, and lipoic acid, based on objective biomarkers of antioxidant status and oxidative stress (ie, serum vitamin E, serum GSH-Px, plasma MDA, and DNA integrity in WBC), help to maintain the antioxidant status of geriatric dogs.

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