INTRODUCTION

According to the free radical theory of aging, damage to macromolecules from the formation of reactive oxygen species (ROS), also known as free radicals, is a primary cause of age-related neuropathology. Proponents suggest that ROS formed during normal processes of aerobic respiration produce oxidative damage in cells, which leads to cumulative oxidative damage and eventual age-dependent neuropathology or cell death. The primary source of ROS is apparently mitochondrial aerobic metabolism, particularly electron transport and respiration in aged mitochondria. The production of ROS has been associated with physiologic decline of cellular functions, mutations in DNA, and damage to protein and lipids. The aldehydes produced by lipid peroxidation may act as secondary toxins by diffusing to DNA or proteins where they may form a Schiff’s base and be mutagenic, or they may inactivate certain enzymes. According to the free radical theory of aging, one would expect to see more of this oxidative damage in older individuals, as evidenced by increased oxidative lesions to DNA in old rats. Because structure determines function in biochemical reactions, it is hypothesized that the accumula-

Prior Experience, Antioxidants, and Mitochondrial Cofactors Improve Cognitive Function in Aged Beagles*

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CLINICAL RELEVANCE

Results of this study support the free-radical theory of aging and demonstrated that providing higher levels of vitamin E in food resulted in higher serum vitamin E concentrations and improved performance on landmark-discrimination tasks in aged dogs. Factors other than vitamin E also contributed to the response but remain undefined.
tion of altered structural reactions leads to decreased cellular function.

The nervous system has been proposed to be especially susceptible to oxidative stress. It has extremely high rates of oxidative metabolism (up to 20% of the body oxygen), even though it is only approximately 8% of total body weight. Nervous system susceptibility is likely exacerbated by the limited regenerative capabilities of individual neurons. Another contributing factor to this particular sensitivity to free radical–induced damage is the brain’s high content of susceptible polyunsaturated fatty acids and its comparatively low content of free radical protection. The combination of these factors may predispose the brain to damage from ROS. Direct evidence of an active role of ROS in brain aging has been shown to include existence of age-dependent increases in lipofuscin (a yellow-brown fluorescent pigment), increases in membrane lipid peroxidation, reduced glutamine synthetase, alterations in membrane lipids, and accumulation of oxidative markers with decline in antioxidant defenses, such as the reduced form of glutathione.

The free radical hypothesis of aging predicts that age-dependent neuropathology and associated cognitive decline can be attenuated or possibly reversed by reducing oxidative stress. Aged beagles have previously been shown to exhibit cognitive impairment compared with young beagles; however, the continued development of cognitive impairment may be altered by a complex mixture of antioxidants, mitochondrial cofactors, and fruits and vegetables.

The present study was designed to extend and further define factors that may have influenced the original findings. The goals were to determine whether elimination of some of the original dietary components would provide similar results and to evaluate how the results would compare with those for a similar, but different, commercially available food. Thus, treatment effects of foods having a high vitamin E content (and other dietary antioxidants and mitochondrial cofactors) but without fruits and vegetables, a moderate content of vitamin E without mitochondrial cofactors, or a lower content of vitamin E (commercially available diet) were evaluated in cognitively impaired beagles. The study also addressed the question of whether an improved response could be correlated to concentrations of vitamin E in serum. The final objective was to determine whether prior cognitive experience influenced the ability of impaired dogs to solve a novel task. The rationale for using a mixture of antioxidants was based on the hypothesis that antioxidants have specific targets and work in networks, and no single antioxidant may target all ROS; thus, the most effective strategy may involve a broad spectrum of antioxidants.

**MATERIALS AND METHODS**

**Test Subjects**

A total of 30 beagles 9 to 13 years of age were enrolled into the baseline testing; 28 of the dogs completed a portion or all of the intervention period. The dogs came from two different sources and had different cognitive backgrounds. Eighteen dogs that were experimentally sophisticated were tested on a standard pretrained protocol that included a spatial-memory task, a size-discrimination task, and a size-discrimination reversal-learning task. These 18 dogs had approximately 2 years of testing on other cognitive tasks before enrollment in the present study. The other 12 dogs were experimentally naïve and had only been tested on the standard pretrained test protocol, which included the size-discrimination and size-discrimination reversal-learning tasks. One of the naïve dogs did not acquire the ability to perform the baseline tasks and therefore could not be assigned to a treatment group.
Dogs were examined before enrollment to assess their general health. In addition, baseline blood samples were collected from each dog and analyzed for routine clinical chemistry and hematology parameters. All dogs appeared to be in good general health, according to findings of these evaluations. The dogs were housed indoors. Dogs were each fed approximately 300 g of food daily; the amount was adjusted as needed to maintain normal body weight. Animal use and care was approved by the University of Toronto Institutional Animal Care Advisory Committee.

The Toronto General Testing Apparatus, a canine version of the Wisconsin General Testing Apparatus for primates, was used for evaluating the cognitive function of the dogs. This apparatus comprises an enclosure, a stimulus tray accessible to the animal, and a sliding panel that can be moved to either permit or prevent the animal from observing placement of a food reward under an object.

Data were collected using a customized computer program that controlled timing of all events, randomization procedures, and the location of rewards and positive objects. The program was also used to record animal responses, reaction times, and experimenter comments. Daily test sessions consisted of 10 trials separated by 30-second intervals.

**Allocation and Treatments**

Dogs that completed baseline testing (n = 29) were allocated to low-antioxidant (n = 10), moderate-antioxidant (n = 9), and high-antioxidant (n = 10) groups. Group allocation was balanced based on performance in the size-discrimination and task-reversal testing done before initiation of the intervention part of the study. Scores assigned to each dog during the initial evaluation were ranked in ascending order, and the dogs with the three lowest scores were randomly and evenly allocated to the three treatment groups.

The animals with the next three lowest scores were allocated to assure that the sum of the subject scores in each treatment group was equivalent. This ranking and allocation procedure was used for the remaining dogs ranked from 7 through 9, 10 through 12, and so forth, until all dogs were assigned from each group (naïve or experienced). Information regarding group assignment was not provided to observers until all data collection was complete to assure that all cognitive testing was done without bias.

A between-subjects design was used to determine whether performance of aged subjects in the landmark-discrimination learning task was improved for dogs fed the high-complexity antioxidant food or the moderate-antioxidant food when compared with performance of dogs fed the low-antioxidant food.

After group allocations, the animals were given a 3-day adjustment period (Days –2 through 0) to become familiar with the new (test) food. Dogs were fed a mixture of 33% new food and 67% old food on the first day of the adjustment period. The following day, the dogs were given equal proportions of new and old foods. On the final day of the adjustment period (Day 0), the entire diet consisted of new food. Days 0 through 7 served as a stabilization interval that was intended to allow the animals to adjust to the food. No behavioral testing was undertaken during this interval. The animals remained on their assigned new food from Day 0 through the end of the study.

**Food Formulations**

Three different foods were used in this study. The nutritionally complete, low-antioxidant food was a commercial canine diet (Pedigree Mealtime Small Crunchy Bites, Masterfoods USA). The moderate- and high-complexity antioxidant foods were specifically formulated to provide incremental increases in vitamin E levels and complexity of antioxi-
dant inclusion. In addition, the formulations had to be roughly similar in composition to that of the nutritionally complete, low-antioxidant food (Table 1). All formulations met American Association of Feed Control Officials recommendations for an adult food. Digestibility of the three foods in this study was not specifically tested; however, previous batches of similar formulations have been tested, and the digestibility of the three diets was determined to be similar (Table 1). The form of vitamin E used was all-rac α-tocopherol, a fat-soluble antioxidant. Vitamin C was administered as an extrusion stable form to provide a physiologic aqueous antioxidant. L-carnitine and dl-α-lipoic acid were added as mitochondrial cofactors. dl-α-Lipoic acid may also act as either a lipid soluble or aqueous antioxidant.

Training

A standard pretraining protocol was used to familiarize the dogs with the apparatus and train them to displace objects covering the food well to obtain a food reward that consisted of approximately 1 g of Hill’s Prescription Diet Canine p/d (Hill’s Pet Nutrition) canned food.

The size-discrimination learning task, part of the pretraining protocol in which scores were used to allocate dogs to treatment groups, consisted of presenting the dogs with red wooden blocks that differed only in height and rewarding them for responding to one deemed to be positive by the experimenter. A food reward was placed on the underside of the negative object. This was done to prevent the dog from using olfactory cues to solve the task.

The dogs were tested once a day until they reached a two-stage criterion. They had to first respond correctly on nine of 10 trials on 1 day or on eight of 10 trials on 2 consecutive days to pass the first phase. To pass the second phase, the dogs had to respond correctly on at least 70% of the next 30 trials over three consecutive sessions. Dogs were tested for a maximum of 40 days for 400 trials or until they reached a criterion, whichever came first. The dogs were tested for reversal learning by the same method, except the reward was given for the previously incorrect stimulus.

Testing

During the intervention period, dogs were tested once daily until they reached the two-stage criterion previously described. The landmark-associated objects were two identical
white lids (10 cm in diameter) and a thin rectangular yellow block of wood. The white circular lids were placed 23 cm apart covering the two lateral food wells on the presentation tray. White hook-and-loop fastener tabs were glued to the center of the top of the white lids to hold the landmark in place during the first phase of training (Figure 1). This landmark position was called L0. Dogs were required to associate the landmark on top of the lid covering the food reward. They were tested to criterion or 40 test sessions, whichever came first. Once the dogs learned to attend to the landmark in phase 1, they were required to learn the spatial elements in the landmark configuration as the distance between the landmark and the circular lid was gradually increased during the testing. The landmark was randomly positioned diagonally and medially at either 1 cm (L1) or 4 cm (L2) away from the circular lid (Figure 1). The landmark was attached to the food tray with a piece of black hook-and-loop fastener. The correct side was determined randomly, with the constraint that both sides were correct on half of the trials of each test session. The door was raised, and the tray was presented to the dog, which was allowed to respond to one side. A single correction was allowed after the first incorrect response. Dogs were tested on L1 and L2 until they either reached criterion or received 40 test sessions. Dogs were tested for a maximum of 40 days (total of 400 trials) or until they reached a criterion, whichever came first. The dogs were tested for reversal learning by the same method, except the reward was given for the previously incorrect stimulus.

Dogs that failed L0 or L1 were provided with remedial training to enable them to participate in the next phase of study. The remedial training occurred over 5 days with 15 trials each day. Each session included both single-stimulus and paired-stimulus presentations. On the single-stimulus presentations, the procedures were the same as for the regular landmark trials, with the exception that the animals were not given a choice of discriminanda, and the subject was allowed to correct all incorrect trials. The paired trials followed the same procedure of the regular landmark. The number of paired trials was increased gradually (eight on the first day to 12 on the fifth day). The number of correct right-side trials (i.e., the correct response for this type of trial is the response to the right side) for dogs with left-side...
Statistical Analysis

Data were analyzed using serum vitamin E as a continuous variable and food as a discrete variable, with \( P < .05 \) used as a standard for statistical significance. A multivariate repeated-measures analysis was performed to examine the effect of experience and task on cognitive ability on the novel task. This analysis allowed an evaluation of the effect of other parameters on cognition after the effect of circulating vitamin E was removed.

RESULTS

The analysis did not reveal any effects of food on the errors to criterion for L0, in which the landmark was placed on top of the target object. However, the experimentally naïve group performed significantly \( (P < .05) \) worse on L0 than did the experienced group (Figure 2). L1 errors to criterion were significantly \( (P < .05) \) increased in the low-antioxidant food group compared with those for both the moderate- and high-antioxidant food groups. The high-complex antioxidant group showed a

Serum Analysis

Whole-blood samples were collected from each dog by jugular venipuncture 7 days before initiation of treatment and at the end of the study 3 months later. Samples were collected in plain tubes containing no anticoagulants and were centrifuged for collection of serum. Samples of serum were frozen at \(-70\)°C until analysis. The concentration of vitamin E (\( \alpha \)-tocopherol) was determined by validated high-performance liquid chromatography. Any sample having a coefficient of variation outside the range (5%) of the laboratory quality control standard was retested.
trend toward decreased errors compared with the moderate-antioxidant group; however, the difference was not significant (Table 2). L2 errors to criterion were significantly ($P < .05$) increased in the low-antioxidant group when compared with those in the high-complex antioxidant group. The high-complex and moderate-antioxidant food groups did not differ significantly on L2. Since L0 may be solved as either an object discrimination or landmark-discrimination task, the sum of L1 and L2 was used to calculate an overall assessment of total performance on the true landmark tasks. When the errors to criterion for L1 and L2 were added, there was a significant ($P < .05$) difference among all groups, with the low-antioxidant group having the most errors, followed by the moderate-antioxidant group and the high-complex antioxidant group having the least.

The change in serum vitamin E concentrations was significantly ($P < .05$) different among all groups (Table 3). The relationship between serum vitamin E concentrations and cognitive function was demonstrated in that higher concentrations of vitamin E were associated with significantly ($P < .05$) decreased errors to criterion in L1 and L2 but not in L0. In addition, because the analysis removed the effect of circulating vitamin E and there was still a significant difference between treatments, it could be concluded that other factors in the complex antioxidant food also contributed to improved cognition.

The multivariate analysis revealed no significant interaction for within-subject effects between food and experience; however, the interaction of task and experience was significant ($P < .05$). Overall, experienced dogs made signifi-

### TABLE 2. Effect of Food Containing Different Antioxidant Levels on Mean Errors (± SEM) to Criterion in Landmark-Discrimination Tasks in Beagles

<table>
<thead>
<tr>
<th>Landmark Study</th>
<th>Low-Antioxidant Food</th>
<th>Moderate-Antioxidant Food</th>
<th>High-Complex Antioxidant Food</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>28.9 ± 9.5</td>
<td>49.2 ± 14.5</td>
<td>59.5 ± 18.9</td>
</tr>
<tr>
<td>1</td>
<td>93.8 ± 18.6</td>
<td>50.9 ± 13.7</td>
<td>30.2 ± 5.9</td>
</tr>
<tr>
<td>2</td>
<td>75.7 ± 20.3</td>
<td>49.1 ± 12.9</td>
<td>34.9 ± 6.8</td>
</tr>
<tr>
<td>1+2</td>
<td>169.5 ± 25.6</td>
<td>100.0 ± 15.4</td>
<td>65.1 ± 9.2</td>
</tr>
</tbody>
</table>

$^a,b,c$ Group means within a landmark task (row) having different superscripts are significantly different using serum vitamin E as a covariate ($P < .05$).

### TABLE 3. Effect of Food Containing Different Antioxidant Levels on Serum Vitamin E Concentrations in Groups of Beagles

<table>
<thead>
<tr>
<th>Variable</th>
<th>Days after Food Change</th>
<th>Serum Vitamin E Concentration (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Low Antioxidant</td>
</tr>
<tr>
<td>Before vitamin E supplement</td>
<td>0</td>
<td>18.2 ± 1.1</td>
</tr>
<tr>
<td>After vitamin E supplement</td>
<td>90</td>
<td>17.9 ± 1.1$^a$</td>
</tr>
<tr>
<td>Change$^*$</td>
<td></td>
<td>−0.3 ± 1.2$^a$</td>
</tr>
</tbody>
</table>

$^{a,b,c}$ Values within a row having different superscripts are significantly different ($P < .05$).

$^*$ Change = Value after vitamin E − Value before vitamin E.
cantly \( (P < .05) \) fewer errors to criterion in L0 (Figure 2), whereas no such differences were detected in L1 or L2.

**DISCUSSION**

The results of this study provide further evidence that intervention with foods fortified with different levels and complexity of antioxidants can improve cognitive performance in aged beagles on a landmark discrimination-learning task. In addition, these results reveal that prior cognitive experience can influence learning of a new task. Finally, the concentration of vitamin E in serum also was correlated with performance of the task.

The results of the current study are in general agreement with prior results indicating positive effects of antioxidant intervention on landmark discrimination.\(^\text{26}\) The current study was performed in part to replicate those findings as well as to extend the understanding of which components of the antioxidants in food were important for the outcome. However, some important differences exist between this study and the prior report.

No significant effect of dietary intervention was noted in the current study on the initial test phase (L0). This failure may have resulted from combining naïve and experienced animals. A primary factor affecting L0 performance in the current study was previous test experience, which may have confounded detection of any food effect. Prior cognitive experience has been shown to be associated with rate of cognitive decline in humans.\(^\text{27}\)

In the previous report, a significant \( (P < .05) \) effect of antioxidant intervention was noted on L0, L1, and L2 based on chi-square analysis of pass rates. The present analysis was performed as a complement to the errors to criterion, which showed a significant effect of antioxidants only at L0. The different statistical response between the two studies is attributed to the failure of several dogs to pass criterion in the historical study, which would lead to a progressive decrease in the power of the analysis of the errors to criterion. By contrast, all dogs on the current study solved the initial landmark task (L0) and only two dogs failed any subsequent landmark tasks. This suggests that the dogs in the current study were less impaired overall than the group from the previous report. This may have provided a kind of floor effect on detecting beneficial consequences of the dietary intervention in the current study. That is to say, the task may have been too easy, relative to the degree of cognitive impairment, to demonstrate a benefit of antioxidants in L0 for the impaired dogs in the present study, as had been demonstrated in the prior study.

The combination of antioxidants with experience may provide a synergistic effect. Cognitive enrichment and physical enrichment have been shown to enhance neuronal plasticity and to be protective against age-related cognitive decline in humans.\(^\text{28–30}\) As such, this present short-term study suggests that extensive prior experience on other neuropsychologic tasks had carry-over effects into a novel task, which is of importance in developing strategies to slow age-related cognitive decline in beagles and, possibly, in humans.

The present investigation intentionally used a very short intervention adjustment time to mimic the conditions of the previous study. Other animal studies\(^\text{30,31}\) that have shown beneficial effects of antioxidants on cognition used longer adjustment periods than were used in the present study. Rats kept on antioxidant-enriched food for 8 weeks\(^\text{30}\) or 8 months\(^\text{31}\) before cognitive assessment gained significant \( (P < .05) \) cognitive improvement. Rats treated for 4 to 5 months with daily intraperitoneal antioxidant injections before initiating testing on the Morris water maze displayed improved performance.\(^\text{32}\) Children 6 to 11 years of age were
tested on a series of cognitive tests (verbal learning, visual memory, arousal, attention, retrieval, and eye-hand perception and coordination) after they had been maintained on antioxidant-fortified biscuits for 43 weeks. In one recent study, the rate of cognitive decline was reduced by 36% for older people after 3 years of increased levels of vitamin E intake when compared with rates for control subjects. A 6-month course of food enriched with antioxidants was shown to partially counteract the deleterious effects of aging on cognition, as well as to increase the ability of aged canines to learn.

All three groups in the current study had significant ($P < .05$) changes in vitamin E serum concentrations after 90 days of dietary antioxidant intervention. A recent study by Milgram et al compared serum vitamin E concentrations from dogs older than 7 years of age with those from dogs 2 to 5 years of age. In that study, old dogs had reduced vitamin E when compared with concentrations in young dogs, when vitamin E concentration was expressed per serum triglyceride. Both groups (old and young dogs) showed an increase in vitamin E after supplementation, but in the case of the old dogs, the increase only brought the levels to approximately the baseline value for the young dogs. This suggests a need for enhanced dietary vitamin E supplementation in the geriatric dog. In the current study, the relationship between serum vitamin E concentrations and cognitive function suggests there is a positive relationship between antioxidant supplementation (vitamin E concentration) and cognitive function.

If oxidative stress contributes to age-related declines, then antioxidants should be able to at least partially slow or reverse these declines. There are no studies that demonstrate a reversal of signs with the provision of antioxidants, but it is theoretically possible because cellular function is a balance of damage versus repair. Thus, if a cell can slow ROS production to a rate that allows for cellular repair or replication, function may be restored.

Many studies have focused on the effect of fruit and vegetable extracts with high antioxidant activity. The antioxidant activity of extracts has been identified via oxygen radical absorbance capacity (ORAC) assay. Joseph et al examined the effect of long-term feeding of rats with either a control food or a food supplemented with strawberry extract, spinach extract, or vitamin E for prevention of age-related decrements in cognitive or neuronal function. The results indicated that ingestion of foods with high ORAC values can delay the onset of age-related deficits of signal-transduction processes (dopamine release) and performance on learning tasks. In another study, aged rats fed strawberry, blueberry, or spinach extract exhibited better motor learning and cerebellar function. The phytochemicals contained in fruits and vegetables may also produce effects other than antioxidant protection, including changes in membrane fluidity and reduction of the inflammatory response. In addition, several studies have shown improvement in the rate of decline of cognitive function or neuroprotective effects with more defined source interventions with lipoic acid, vitamin E, and fatty acids.

Antioxidants benefit animals with age-related cognitive decline and those suffering from neurodegenerative diseases like Alzheimer’s disease (AD) or cognitive dysfunction syndrome (CDS). An effect on deposition of Aβ amyloid protein may represent a common underlying mechanism. Yamada et al showed that oxidative stress is involved in the mechanism of Aβ-induced neurotoxicity. In cultured cells there is evidence that Aβ increases the levels of hydrogen peroxide and lipid peroxides and that α-tocopherol combats Aβ neurotoxicity. Cotman et al re-
viewed the relationships among geriatric cognitive function decline, oxidative damage, and Aβ deposition. They theorized this deposition is part of a series of cascading neuropathologic events, with oxidative stress playing a major initiating role by damaging lipids, proteins, and nucleic acids, which results in elevation of amyloid precursor protein, Aβ peptide deposition, synapse loss, DNA damage, cell dysfunction, and ultimately cell death. This cycle of events fuels itself because Aβ is able to generate lipid and protein oxidative damage.

Head et al studied the link between oxidative damage, aging, and Aβ in the canine brain, using assays of malondialdehyde (MDA), an indicator of lipid peroxidation; protein carbonyl, an indicator of oxidative damage to proteins; and glutamine synthetase (GS), an enzyme vulnerable to oxidative damage. Their studies revealed elevation of MDA in the prefrontal cortex and serum (but not the cerebrospinal fluid), elevated carbonyl formation in the brain, and an age-dependent decline in GS activity. Comparison of these results with the amount of Aβ deposited revealed a lack of correlation; however, they surmised that oxidative damage and Aβ deposition may accumulate in parallel with age.

An alternative to the hypothesis formed in the present study is that many of the antioxidants utilized in this study also have antiinflammatory properties. One study indicated there is an association between NSAID intake and decreased incidence of dementia in humans, which suggests that inflammation is a contributor to neurocognitive decline. As such, the antioxidants included in this dietary fortification may have acted via an antiinflammatory path or synergistically with antioxidant mechanisms to elicit the effects observed.

Regardless of the specifics of the cause-and-effect relationship of oxidative stress, Aβ, AD, and CDS, research indicates vitamin E, other antioxidants, and mitochondrial cofactors may be beneficial in the battle against age-related cognitive decline and possibly other neurodegenerative disorders. In addition, prior cognitive experience and training could also impact the ability to solve cognitive tasks. The results of this study provide additional substantiation of these hypotheses and support the free radical theory of aging while showing that vitamin E and antioxidant supplementation in food results in higher serum vitamin E and improved performance on landmark discrimination tasks in the aged canine.

**REFERENCES**

12. Halliwell B: Reactive oxygen species and the central


41. Sano M, Ernesto C, Thomas RG, et al: A controlled trial of selegiline, alpha-tocopherol, or both as treat-