ELSEVIER

Neurobiology of Aging 23 (2002) 737–745

NEUROBIOLOGY OF AGING

www.elsevier.com/locate/neuaging

Dietary enrichment counteracts age-associated cognitive dysfunction in canines

N.W. Milgram [a,*], S.C. Zicker [b], E. Head [c], B.A. Muggenburg [d], H. Murphey [d], C.J. Ikeda-Douglas [a], C.W. Cotman [c]

[a] *Life Science Division, University of Toronto at Scarborough, 1265 Military Trail, Scarborough, Ont., Canada M1C 1A4*
[b] *Science and Technology Center, Hill's Pet Nutrition, Inc., P.O. Box 1658, Topeka, KS 66601, USA*
[c] *Institute for Brain Aging and Dementia, University of California, 1226 Gillespie Neuroscience Research Facility, Irvine, CA 92697-4540, USA*
[d] *Lovelace Respiratory Research Institute, 2425 Ridgecrest Dr. SE, Albuquerque, NM 87108, USA*

Received 20 September 2001; received in revised form 26 November 2001; accepted 24 January 2002

Abstract

Advanced age is accompanied by cognitive decline indicative of central nervous system dysfunction. One possibly critical causal factor is oxidative stress. Accordingly, we studied the effects of dietary antioxidants and age in a canine model of aging that parallels the key features of cognitive decline and neuropathology in humans. Old and young animals were placed on either a standard control food, or a food enriched with a broad spectrum of antioxidants and mitochondrial enzymatic cofactors. After 6 months of treatment, the animals were tested on four increasingly difficult oddity discrimination learning problems. The old animals learned more slowly than the young, making significantly more errors. However, this age-associated decline was reduced in the animals fed the enriched food, particularly on the more difficult tasks. These results indicate that maintenance on foods fortified with complex mixtures of antioxidants can partially counteract the deleterious effects of aging on cognition.

Keywords: Age-dependent cognitive dysfunction; Alpha-tocopherol; Antioxidants; Ascorbic acid; Dogs; L-Carnitine; Lipoic acid; Mitochondrial function; Oddity discrimination learning; Oxidative damage

1. Introduction

Improved nutrition, disease control, and applied biotechnology have prolonged life-span in humans. But enhanced longevity comes at the cost of an increased prevalence of cognitive problems coupled with aging, which range from age-associated memory impairment to the dementia linked to neurodegenerative disorders typified by Alzheimer's disease [8,31,37]. The convergence of increased life-span and increased prevalence of cognitive dysfunction reveals a clear need for identification of mechanisms, models, and testing of interventions for treatment of age-related cognitive dysfunction. The ideal strategy for developing interventions should focus on the underlying pathophysiology in a model system that can be translated to the intended target species, humans.

At the cellular level, the aging process is accompanied by progressive accumulation of oxidative damage, decreased metabolic strategies for mitigating effects of oxidative stress, and decreased efficiency in mitochondrial function, resulting in increased production of cellular oxidants [2,4,17,40]. The consequences are particularly problematic for the nervous system, which exhibits extremely high rates of oxidative metabolism and decreased oxidative defenses, relative to other tissue [16]. A treatment strategy for age-associated cognitive dysfunction and neurodegeneration could include both counteracting the damaging effects of free radicals produced by oxidative stress and enhancing mitochondrial function. We hypothesized that intervention with a complex mixture of antioxidants and mitochondrial enzymatic cofactors should partially reverse, or slow the development of cognitive aging in canines. We chose dogs because these animals develop cognitive dysfunction, beta-amyloid pathology, and oxidative damage that parallel key features of normal and abnormal aging in humans [1,9,18,19,26,33]. We have also found that aged dogs show variability in level of cognitive function that closely resembles the aged human population in the pre-Alzheimer's disease stages, e.g., successful aging, age related memory impairment, and severe cognitive impairments [1].

Alternative models include non-human primates, aged rodents and transgenic mice. Non-human primates are, in many

* Corresponding author. Tel.: +1-416-287-7402; fax: +1-416-287-7642.
E-mail address: milgram@psych.utoronto.ca (N.W. Milgram).

0197-4580/02/$ – see front matter
PII: S0197-4580(02)00020-9

respects, the ideal animal model. However, naïve aged primates are expensive, difficult to obtain and often difficult to cognitively test. In addition, the major species of β-amyloid that accumulates in aged non-human primate brain is the shorter, more soluble species [12], which contrasts with reports in human and canine brain [10]. Rodents have a short life-span, absence of neurodegenerative changes, such as amyloid deposition, and limited cognitive abilities [42], which do not clearly model the kinds of complex cognitive deterioration seen in humans. Transgenic mouse models that over-express mutant amyloid precursor protein (APP) deposit B-amyloid, and show cognitive loss but in other respects are limited in their similarities to human brain aging and AD [25].

Previous research with these various models has implicated oxidative damage as a common factor in the development of pathology associated with brain aging. This conclusion is supported by studies indicating antioxidants can delay age-related cognitive decline in humans [36,39,45] and improve performance in aged rodents [6,22]. These findings, however, remain controversial [32,38,40]. To date the possible role of antioxidant strategies has not been evaluated in a higher animal model than the rodent. Furthermore, the combination of cellular antioxidants and mitochondrial cofactors is novel, and previously been tested.

The present experiment is part of an ongoing longitudinal study of the effects of age, cognitive enrichment, and diet on cognitive decline in beagle dogs. Approximately 1 year prior to the initiation of this study, old and young dogs were given a series of baseline cognitive tests, which were used to assign animals to cognitively equivalent groups. One of the aged groups and one of the young groups were then started on a diet enriched with a broad spectrum of antioxidants and mitochondrial enzymatic cofactors; the other groups were placed on a control diet. The animals were on the dietary intervention for approximately 6 months before starting this study. We tested the subjects on a series of four oddity discrimination learning problems. Each such task involved repeated presentation of three objects, two of which were identical, and providing reward to the subject if it responded to the odd object. We developed this test protocol in an attempt to provide a series of learning problems of sufficient difficulty to show age sensitivity. The performance of monkeys trained on a similar task has been shown to vary as a function of the extent of similarity of object used [21,43].

2. Methods

2.1. Animals

Twenty-four aged and 17 young beagles were acquired from two separate, closed colonies, with known pedigree data. Final enrolled subjects were 23 aged beagles (11 males and 12 females) and 16 young beagles (six males and 10 females). Eleven of the aged beagles and seven of the young beagles were supplied by the Lovelace Respiratory Research Institute colony whereas the rest were from the Hill's Pet Nutrition Colony. At the start of the dietary intervention, the aged dogs ranged from 8.5 to 12.5 years of age and the young beagles ranged in age from 1.95 to 4.9 years of age (see Table 1). The old animals were housed in USDA approved kennels with two dogs per kennel, hand-walked two times per week, and allowed access to toys in their kennels on a rotating basis. The young animals were housed with two to four dogs per kennel. In all other respects, the old and young dogs were treated identically.

Table 1
Age range of dogs in antioxidant and control groups

Age-range[a]	Antioxidant group	Control group
Young dogs	$N = 9$	$N = 7$
<2.00	1	1
2.01–3.99	5	4
4+	3	2
Average age (years)	3.60	3.37
Old dogs	$N = 12$	$N = 11$
8–9.99	3	1
10.0–11.99	8	8
12.0+	1	2
Average age (years)	10.61	10.97

[a] Age taken as subjects age at the start of training on the oddity study.

2.2. Diet

The two foods were formulated to meet the nutrient profile for the American Association of Feed Control Officials recommendations for adult dogs (AAFCO, 1999). Control and test diets were identical in composition, other than inclusion of a broad-based antioxidant and mitochondrial cofactor supplementation to the test diet. The control and enriched foods had the following differences in formulation on an as fed basis respectively: D,L-alpha-tocopherol acetate (120 ppm vs. 1050 ppm), L-carnitine (<20 ppm vs. 260 ppm), D,L-alpha-lipoic acid (<20 ppm vs. 128 ppm), ascorbic acid as Stay-C (<30 ppm vs. 80 ppm), and 1% inclusions of each of the following (1 to 1 exchange for corn): spinach flakes, tomato pomace, grape pomace, carrot granules and citrus pulp. The rationale for these inclusions is as follows: Vitamin E is lipid soluble and acts to protect cell membranes from oxidative damage; Vitamin C is essential in maintaining oxidative protection for the soluble phase of cells as well as preventing Vitamin E from propagating free radical production; alpha-lipoic acid is a cofactor for the mitochondrial respiratory chain enzymes, pyruvate and alpha-ketoglutarate dehydrogenases, as well as an antioxidant capable of redox recycling other antioxidants and raising intracellular glutathione levels; L-carnitine is a precursor to acetyl-L-carnitine and is involved in mitochondrial lipid metabolism and maintaining efficient function; fruits and vegetables are rich in flavonoids and carotenoids and

other antioxidants. The diet was produced by an extrusion process and was fed for no more than 6 months before a new lot was milled.

2.3. Physical exams

All animals were administered a full physical and neurologic examination prior to dietary intervention. Dogs were also examined by slit-lamp for ocular abnormalities that might have impaired visual capabilities of an animal.

2.4. Clinical chemistry

All dogs had complete blood counts, and serum chemistry analysis performed prior to diet intervention. In addition, assessment of endocrine status was performed by way of thyroid panel, and low-dose dexamethasone testing for the presence of Cushing's disease. Concentrations of Vitamin E in serum were determined by HPLC prior to the start of treatment, following 3 months of intervention and following 6 months.

2.5. Cognitive testing apparatus

As described previously [33], the test apparatus was a 0.609 m × 1.15 m × 1.08 m wooden box that was based on a canine adaptation of the Wisconsin General Test Apparatus used in cognitive tests with primates. The box was equipped with a sliding Plexiglas food tray with two lateral wells and a medial food well. Vertical stainless-steel bars cover the front of the box. The height of each bar was adjustable, so that the size of the opening to each food well could be uniquely set for each dog. The experimenter was separate visually from the dog by a screen with a one-way mirror and a hinged door on the bottom. Testing occurred in darkness, except for a light with a 60 W bulb that was attached to the front of the box. The hinged door was opened for the presentation and removal of the food tray.

2.6. Cognitive testing protocol

All subjects underwent a standard pretraining cognitive testing protocol that consisted of reward approach and object approach learning, which were procedural learning tasks designed to train animals to displace an object on a tray to obtain a food reward consisting of approximately 1 g of Hill's Prescription Diet® p/d canned food. This food served as an effective reward for all of the dogs used in the study, in the absence of imposed food deprivation. After completing the procedural learning tasks, all subjects were trained on an object discrimination learning task, which was followed by an object reversal learning task [33], an object recognition memory task [5] and delayed-non-matching-to-position task (DNMP) [7]. The initial group assignment took into consideration age, sex, and the subjects performance on the reversal learning task, the object recognition task, and the DNMP task. All animals were maintained on the control food during the pretraining period that lasted approximately 6–9 months. Beagles were maintained on dietary intervention for 6 months before behavioral testing was initiated.

After starting the dietary intervention, all of the subjects included in this study were tested on a protocol involving a series of landmark discrimination learning problems [34].

Following 6 months on the food intervention, the animals were tested on a series of oddity discrimination learning tasks. In each such tasks, the animal is presented with three objects, two identical and one different. To obtain reward, the animal is required to respond to the odd object. Every animal was tested on a series of four oddity tasks, referred to as oddity 1–4. The objects were selected based on similarity, with the intent of making each task more difficult than the previous one. The discriminanda used are shown in Fig. 1.

Training on each oddity task started after establishing object preferences. In the preference test session, the animals were given the opportunity of responding to either of the two different objects on 10 successive trials, with both objects associated with reward. Preference was based on the number of times the animal selected each object. If the animal had a preference for one of the objects, the non-preferred object was utilized as the odd-object in the subsequent oddity task. If no preference was determined, a coin toss decided the odd object.

The oddity discrimination testing consisted of 12 daily trials, with an intertrial interval of 30 s. On each trial, the location of the odd object was determined by random generation by the computer with the two identical objects being placed on the remaining two coasters. The computer program also assured that the odd object was located at each of the three positions on exactly four trials each session. The coasters under the two identical objects were scented with the same dog food used for the reward to prevent the animals from using olfactory cues to solve the problem. The tray was presented approximately 25 cm away from the animal for a 2 s period in order for the animal to focus on the object and process the information. The tray was then presented to the animal enabling the subject to respond to one of the three objects.

The animals were tested 6 days per week, and were allowed up to 40 days on each object pair to achieve a predetermined criterion level of accuracy. A two-stage criterion was used for passing the task to assure that animals showed consistently above average performance before learning was assumed. To pass the first phase, the animal had to score at least 11 correct; score 10 correct on two consecutive sessions; or obtain scores of 10, 9, 10 on three consecutive sessions. To successfully complete the second phase, the subject was required to subsequently achieve an average of at least 70% correct over the next three test sessions immediately following phase 1 achievement (e.g., 9, 8, 9). Thus, the minimum number of sessions required to pass the two-phase criterion was 4 days. After completing the task, the animal moved on to next problem, until four such tasks were completed.

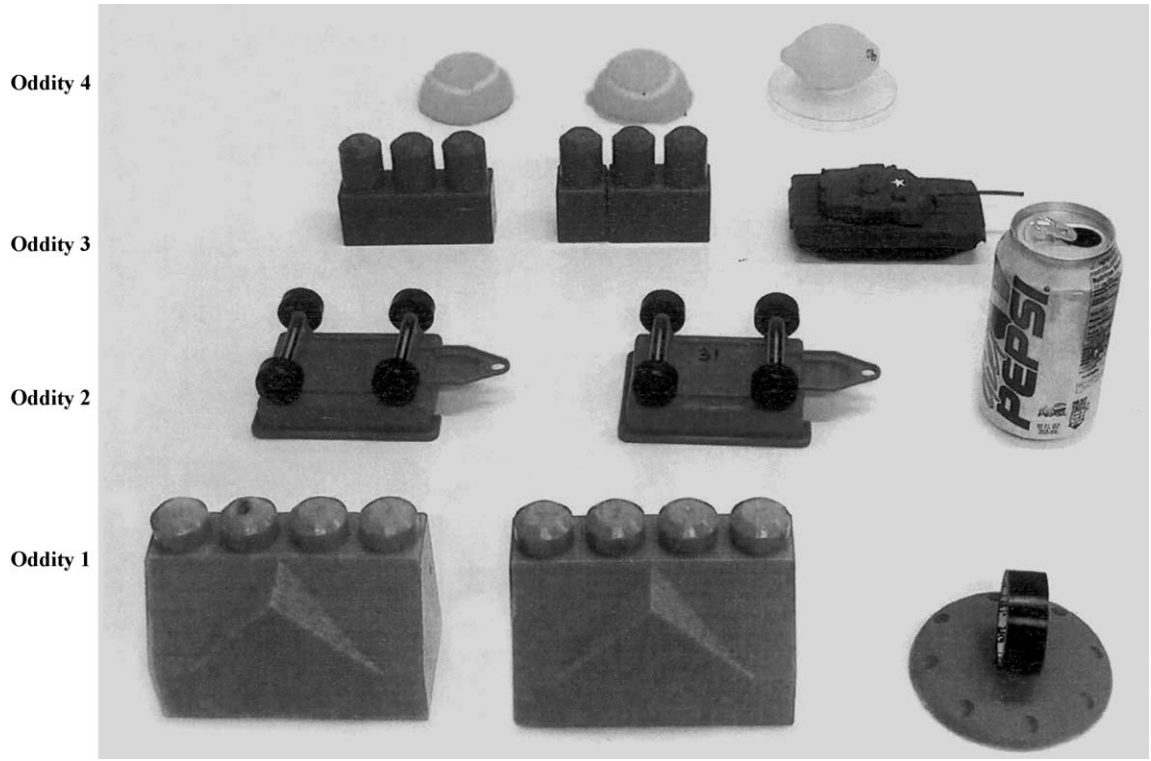

Fig. 1. Objects used in oddity discrimination learning tasks. The objects were selected on the basis of similarity in appearance. The objects used in oddity 1 (large red plastic building blocks and rolls of black hockey tape attached to a blue plastic disk), and oddity 2 (empty diet Pepsi can and bright green plastic toy cart) differed in shape, color and size. The objects used in oddity 3 (dark green plastic toy and small rectangular blue plastic building block) were similar in size, but differed in shape and color. The objects used for oddity 4 (half of yellow tennis balls and a yellow plastic lemon) were similar in size, shape and color.

2.7. Data acquisition

A customized program controlled all timing and randomization procedures, and indicated the location of each object and reward on every trial. The program also assured that on each trial, the locations of the objects were the same for every animal. Before the beginning of each trial, the computer emitted a tone that served as a cue for the dog and instructed the experimenter to present the food tray. Each trial was started when the experimenter pressed a key and simultaneously presented the tray to the subject. The dogs' responses were recorded by a key press, which also indicated the end of the trial and signaled the beginning of the inter-trial interval.

2.8. Statistics

Data for cognitive tasks were analyzed by repeated measures ANOVA with respect to source, diet, and age-group using SAS for windows with an alpha level of 0.05 for significance. Since diet and age group each had only two levels, evaluation of main effects and interactions completely explained model variability. Evaluation of the main effects of diets allowed us to detect where the control and antioxidant means were significantly different. Following the initial analysis, separation of means was performed by LSD on SAS for windows with significance set at 0.05. Data for Vitamin E and Vitamin E:triglyceride ratios were analyzed as a repeated measures analysis with respect to food, and age-group. Following initial analysis, separation of means was performed by Tukey's Studentized Range test (HSD) on SAS for windows. Data for clinical bloodwork was analyzed by individual *t*-test for each analyte.

3. Results

3.1. Physical examination

Results of physical examination did not reveal any neurologic, musculoskeletal, ocular or physical abnormalities that would have excluded participation in the study.

3.2. Clinical chemistry

Blood biochemistry profiles revealed that most dogs fell within the range of values considered normal for healthy adult dogs. No significant differences were observed between groups within the young dog category at baseline.

Table 2
Vitamin E and Vitamin E triglyceride levels in experimental and control groups at baseline and after treatment

Group/time	Vitamin E in serum (ug/ml)			Vitamin E: triglyceride in serum (ug/mg)	
	Baseline	3 Month	6 Month	Baseline	6 Month
Control: young	23.3 ± 1.1 a	24.3 ± 1.8 c	25.1 ± 2.9 b	68.2 ± 10.8 a	63.8 ± 9.5 b
Control: old	30 ± 2.2 a	29.4 ± 2.5 b, c	28.9 ± 1.5 b	28.9 ± 1.5 b	26.4 ± 4.5 c
Antiox: young	26.2 ± 1.8 a	40.8 ± 3.7 b	45.4 ± 4.3 a	63.2 ± 6.6 a	109.3 ± 9.6 a
Antiox: old	28.2 ± 2.4 a	49.6 ± 4.9 a	52.8 ± 3.8 a	33.5 ± 9.8 b	66.3 ± 8.8 b

Means of Vitamin E in serum for old ($n = 23$) and young ($n = 16$) dogs in different dietary groups prior to and at 3 and 6 months of feeding test foods. Vitamin E:triglyceride ratio in serum prior to and at 6 months of feeding test foods. Means with different letters are significantly different from each other within that time period.

There were, however, significant differences between the old and young dogs on baseline measures attributable to age. Total protein, globulin, cholesterol, triglycerides and red blood cells/ul were increased significantly compared to young animals. Conversely, albumin, creatinine, calcium, sodium, and T_3 were decreased in older animals compared to young.

Within the old dog groups, activity of alkaline phosphatase $F(1, 23) = 4.76$, $P = 0.04$ and creatine kinase $F(1, 23) = 4.49$, $P = 0.046$ were significantly higher in the control group compared to the antioxidant group, with both old groups having animals above the normal range. Considering the ages of the older dogs in the study it was anticipated that some measures would not fall within normal ranges established for young healthy dogs. None of the observed changes indicated significant health differences between the groups of old animals. The significant difference in alkaline phosphatase activity was still present at 6 months of time but the creatine kinase difference was no longer significant.

3.3. Serum Vitamin E

There was a significant effect of food $F(1, 35) = 23.07$, $P < 0.0001$ and age-group $F(1, 35) = 5.06$, $P = 0.0308$ over the entire period. There were no significant differences between concentrations of Vitamin E in serum between age or dietary groupings, at the beginning of the study. However, the older dogs had a higher serum concentration of Vitamin E than the young dogs at this and subsequent time points, which resulted in the overall age-group effect. Subsequent analysis of old versus young mean differences did not reveal any significant difference at baseline or 6 months. Following 6 months of dietary intervention, both old and young dogs

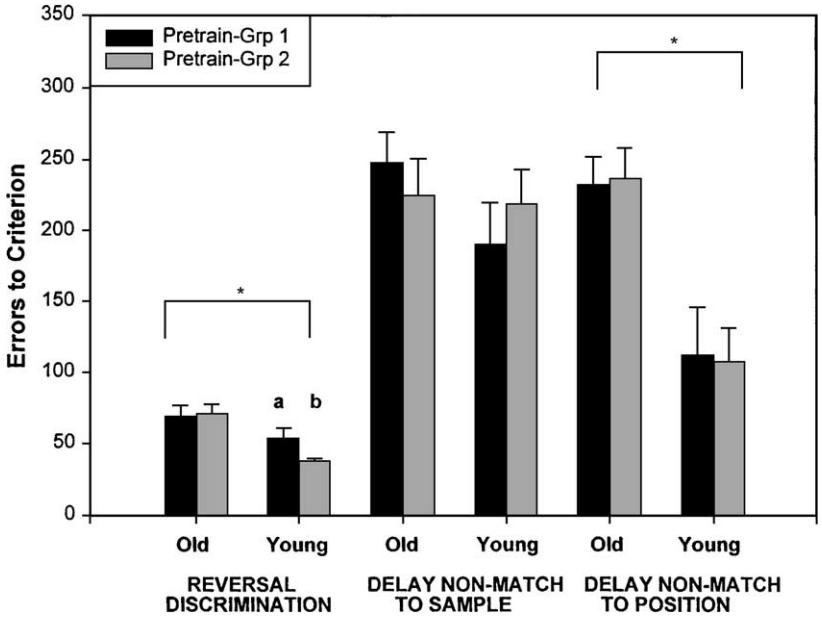

Fig. 2. Baseline cognitive data for aged and young beagles. The aged animals assigned to the antioxidant fortified test food did not differ from the aged animals assigned to the control food on any of the baseline measures, indicating that the groups were cognitively equivalent. On the other hand, we did find significant difference between the old and young groups on the reversal and DNMP tasks (*). A significant effect of treatment group was present for young dogs on the reversal learning task (a vs. b). This difference was not present on the original allocation of young dogs but appeared after one young dog was dropped for motivation concerns as detailed in the testing protocol.

on the antioxidant fortified food had significantly higher concentrations of Vitamin E in serum, compared to age-group controls (Table 2).

Since Vitamin E is fat soluble, its concentration in body tissues may be expressed as Vitamin E per unit of fat, such as triglycerides in serum. When serum Vitamin E was expressed in this fashion, young dogs had a much higher concentration of Vitamin E per mg of triglyceride in serum at the start than older dogs. Repeated measures analysis of this ratio revealed significant effects of food $F(1, 35) = 11.69$, $P = 0.0016$ and age-group $F(1, 35) = 35.27$, $P < 0.0001$. Supplementation of Vitamin E in the food resulted in a significant increase in concentration of Vitamin E:triglyceride in both old and young dogs compared to age-group controls. However, supplementation of older dogs with Vitamin E only increased this ratio to an absolute value approximately equal to that observed in young dogs at the start of the study (Table 2).

3.4. Pretraining cognitive results

The baseline performance of the two groups of aged animals was equivalent (Fig. 2), which indicates that the groups were cognitively equivalent before starting the treatment condition. By contrast, the old group differed from the young on baseline measures in two of the three tasks, with young animals performing significantly better than old animals in both the reversal learning $F(1, 37) = 13.74$, $P = 0.0007$ and spatial memory tasks $F(1, 37) = 28.9$, $P < 0.0001$.

3.5. Oddity discrimination results

One aged control animal completed only two of the oddity problems because of time constraints. This animal's data

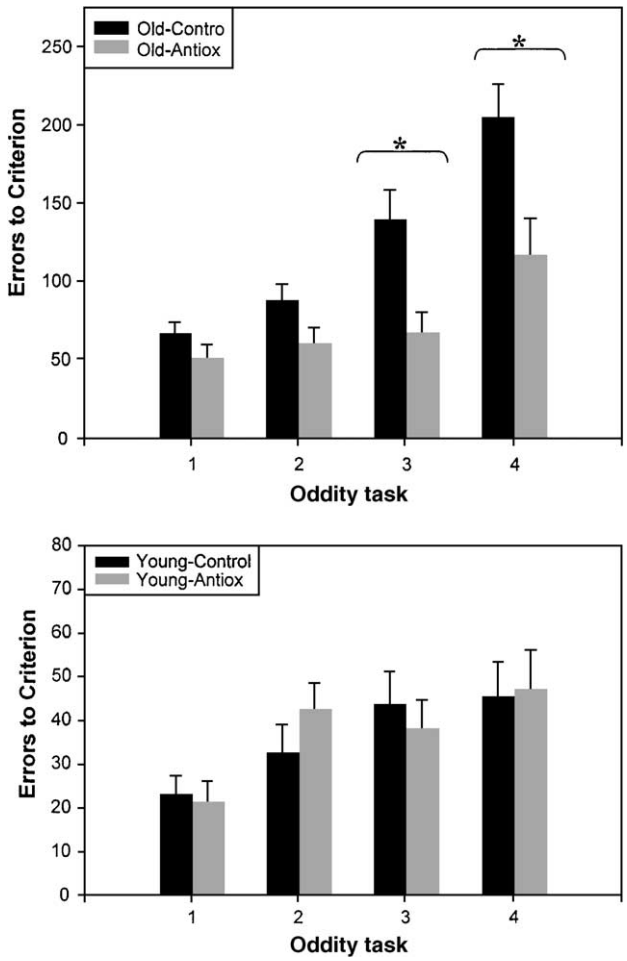

Fig. 3. Effect of age on number of errors made in learning for progressively more difficult oddity discrimination tasks. The data from the control and enriched aged groups were combined, and the data from the control and enriched young groups were combined. $^{*}P = 0.05$.

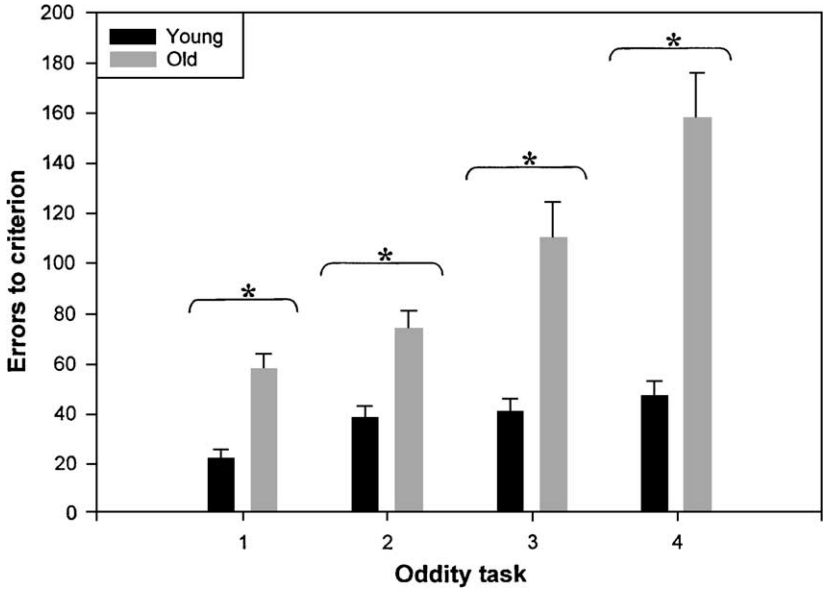

Fig. 4. Effect of food on learning a series of oddity discrimination problems in groups of old (top) and young (bottom) dogs. $^{*}P = 0.05$.

were, therefore, excluded from the initial repeated measures ANOVA. To include the data from this dog, subsequent separate analysis were done for each of the four oddity tasks. The initial analysis revealed highly significant effects of age $F(1, 30) = 65.149$, $P < 0.0001$, diet $F(1, 30) = 10.098$, $P = 0.0034$, and task $F(3, 90) = 19.79$, $P < 0.0001$. We also found significant interactions between diet and age $F(1, 30) = 11.09$, $P = 0.002$, task and age $F(3, 90) = 9.257$, $P < 0.0001$, and between task and diet $F(3, 90) = 3.256$, $P = 0.025$.

Fig. 3 illustrates that age effect was due to the young animals committing fewer errors than the old animals. The age differences also varied as a function of task. For the old animals, performance on the first task did not differ from performance on the second. All other task comparisons were statistically significant, Fig. 3 illustrates that these results are due to the animals making more errors on each successive task than they had on the previous task ($P < 0.025$). For the young animals, by contrast, there were no significant differences in performance between any two tasks.

The results of the dietary manipulation are shown in Fig. 4. The top panel shows that the significant overall effect of diet was due exclusively to superior learning shown by the old animals on the antioxidant diet, when compared to the old animals on the control diet. The effect of dietary treatment also varied as a function of task. Diet did not significantly affect performance on oddity 1, the first task. On the second task, the interaction between age by diet was marginally significant $F(1, 35) = 3.904$, $P = 0.056$. On task three the diet effect was highly significant $F(1, 34) = 12.32$, $P = 0.0013$ as was the diet by age interaction $F(1, 34) = 9.715$, $P = 0.004$. Task 4 also had a significant diet effect ($F(1, 34) = 4.78$, $P = 0.035$) and diet by age interaction $F(1, 34) = 5.118$, $P = 0.030$. There was no significant effect of source.

4. Discussion

These results indicate first, that the oddity discrimination task provides a sensitive measure of age-dependent cognitive deterioration in dogs, and second, that this age-dependent effect can be at least partially reduced by maintenance on a food fortified with a complex mix of antioxidants and mitochondrial enzymatic cofactors.

The general utility of any animal model in evaluating the effect of interventions on age-dependent cognition depends on the extent to which the model reflects age-related cognitive dysfunction. The oddity learning task used in the present experiment can be solved by the animals' learning to associate one of two stimuli with reward, which involves visual discrimination learning. Visual discrimination learning is often insensitive to age in animal models [3,27,35,38]. This was not the case in the present experiment: we found highly significant age differences in favor of the young animals. There are two possible reasons why discrimination learning is age-sensitive in some instances only. First, the effect of age may depend on the difficulty of the discrimination [20]. Task difficulty was clearly a factor in the present experiment; the harder the problem, the greater the age-difference. Aged non-human primates are deficient in acquiring some types of visual based discrimination learning, but not others [44]. Second, age differences in discrimination learning could reflect differences in strategies used to solve each of the oddity problems. The subjects could potentially use either an associative (stimulus-reward), or a more cognitive strategy. An associative strategy requires the subject to learn to associate the correct object with reward through repeated pairing of the two, and depends on repetition. A more cognitive strategy involves learning the general rule that only one of the objects is correct—in this case the odd item. Task complexity was manipulated by varying the similarity of the test objects. We assumed that increased difficulty would result from increased similarity, and this proved to be the case for the old animals. They showed progressively more errors on each successive task, which is consistent with their using an associative strategy. The young animals performance, by contrast, did not differ significantly on any of the tasks, suggesting the use of a cognitive strategy. In fact, some of the animals learned each successive task progressively faster, despite the increase in task difficulty.

The use of a series of problems of graded difficulty is a novel innovation of the present study, which to our knowledge has not previously been used in assessing cognitive interventions in animal models. The protocol revealed that both age and diet effects are amplified by increasing the difficulty of the task. Had we used only a single level of task difficulty, we may not have seen clear effects because of the task being either too easy, or too difficult. Thus, we did not find a significant effect of diet on the first and easiest of the oddity discrimination problems.

The most important result of this study was clearly the superior performance of the animals on the enriched diet compared to controls. A number of factors probably account for the strong dietary effects seen in this study, including use of aged subjects, 6 month maintenance on the diet, use of a test protocol with progressively more complex problems, and the particular components of the diet. The importance of using aged subjects was illustrated by the absence of any diet effect in the young dogs. Because the young dogs performed at a much higher overall level than the old, creating a possible ceiling effect. But we also would not expect to see an effect of diet on cognition in young dogs for theoretical reasons; namely because oxidative stress is not likely to induce substantial neuronal dysfunction until relatively late in life. The importance of duration of time on the diet is more difficult to evaluate, and needs more systematic study. We have found positive effects of an antioxidant diet after a shorter maintenance period [34], but the effects were less robust.

With respect to dietary constituents, to our knowledge, this is the first study to have combined substances that target enhancement of mitochondrial function with antioxidants

that suppress the action of free radicals. Our results build upon and extend the findings that antioxidants or mitochondrial cofactors alone decrease age related cognitive decline in other species [14,15,23,24,41,46]. Our results may be attributable to two different synergistic strategies: first, a complex mixture of antioxidants that supports a network of antioxidants requiring several components to act together for effective function; and second, improved mitochondrial metabolic function that decreased free-radical production while improving mitochondrial energetics and efficiency.

Alternatively, many of the antioxidants utilized in this study also have anti-inflammatory properties [11,28,29]. There has been an association of non-steroidal anti-inflammatory intake and decreased incidence of dementia in humans, which suggests that inflammation is a contributor to neurocognitive decline [30]. As such, the antioxidants included in this dietary fortification may have acted via an anti-inflammatory path, or synergistically, with antioxidant mechanisms to elicit the profound effect observed.

We suggest that the combination of antioxidants with mitochondrial enzymatic cofactors may work together synergistically to enhance mitochondrial function leading to a decrease in both the production and consequences of reactive oxygen species [13]. Taken together our data supports the hypothesis that oxidative damage and mitochondrial function is a fundamental mechanism contributing to age-associated cognitive dysfunction and underscores the need to conduct similar trials in humans.

Acknowledgments

This project was sponsored by funds provided by Hill's Pet Nutrition, Inc. Topeka, KS, the National Institute of aging (Grant AG12694) and by the US Department of the Army, Contract No. DAMD17-98-1-8622. The content of the information does not necessarily reflect the position or the policy of the government, and no official endorsement should be inferred. The investigators adhered to the "Guide for the Care and Use of Laboratory Animals" prepared by the Committee on Care and Use of Laboratory Animals of the Laboratory Animal Resources, National Research Council (NIH Publication No. 86-23, Revised 1985). Research was conducted in facilities fully accredited by the AAALACI. Additional funding was provided by Science & Technology Center, Hill's Pet Nutrition, Inc., P.O. Box 1658 Topeka, KS, 66601, USA. We thank Christina Siwak and Dwight Tapp for assistance in collecting blood samples, Krista Witty for her help in selecting the oddity objects, and Jennifer Lau, Maggie Lau and Joanne Castillo for help in cognitive testing.

References

[1] Adams B, Chan A, Callahan H, Siwak C, Tapp D, Ikeda-Douglas CJ, et al. Spatial learning and memory in the dog as a model of cognitive aging. Behav Brain Res 2000;108(1):47–56.

[2] Ames BN, Shigenaga MK, Hagen TM. Oxidants, antioxidants, and the degenerative diseases of aging. Proc Natl Acad Sci USA 1993; 90:7915–22.

[3] Bartus RT, Dean RL, Fleming DL. Aging in the rhesus monkey: effects on visual discrimination learning and reversal learning. J Gerontol 1979;34:209–19.

[4] Beal MF. Aging, energy, and oxidative stress in neurodegenerative diseases. Ann Neurol 1995;38(3):357–66.

[5] Callahan H, Ikeda-Douglas CJ, Head E, Cotman CW, Milgram NW. Development of a protocol for studying object recognition memory in the dog. Progr Neuro-Psychopharmacol Biol Psychiatr 2000;24(5):693–707.

[6] Carney JM, Starke-Reed PE, Oliver CN, Landum RW, Cheng MS, Wu JF, et al. Reversal of age-related increase in brain protein oxidation, decrease in enzyme activity, and loss in temporal and spatial memory by chronic administration of the spin-trapping compound *N-tert*-butyl-α-phenylnitrone. Proc Natl Acad Sci USA 1991;88:3633–6.

[7] Chan ADF, Nippak P, Murphey H, Ikeda-Douglas CJ, Muggenburg BA, Head E, Cotman CW, Milgram NW. Visuospatial impairments in aged canines: the role of cognitive-behavioral flexibility. Behav Neurosci (in press).

[8] Crook III TH, Larrabee GJ. Diagnosis, assessment and treatment of age-associated memory impairment. J Neural Transm Suppl 1991;33:1–6.

[9] Cummings BJ, Head E, Ruehl WW, Milgram NW, Cotman CW. The canine as an animal model of human aging and dementia. Neurobiology of Aging 1996;17:259–68.

[10] Cummings BJ, Satou T, Head E, Milgram NW, Cole GS, Savage MJ, et al. Diffuse plaques contain C-terminal ABeta 1–42 and not ABeta 1–40: evidence from cats and dogs. Neurobiol Aging 1996;17(4):653–9.

[11] Fryer MJ. Vitamin E status and neurodegnerative disease. Nutr Neurosci 1998;1:327–51.

[12] Gearing M, Tigges J, Mori H, Mirra SS. Aβ40 is a major form of β-amyloid in non-human primates. Neurobiol Aging 1996;17:903–8.

[13] Hagen TI, Lykkesfeldt RT, Liu J, Wehr CM, Vinarsky V, Bartholomew JC, et al. (R)-Alpha-lipoic acid-supplemented old rats have improved mitochondrial function, decreased oxidative damage, and increased metabolic rate. FASEB J 1999;13(2):411–8.

[14] Hagen TM, Ingersoll RT, Wehr CM, Lykkesfeldt J, Vinarsky V, Bartholomew JC, et al. Acetyl-L-carnitine fed to old rats partially restores mitochondrial function and ambulatory activity. Proc Natl Acad Sci USA 1998;95:9562–6.

[15] Hager K, et al. Alpha-lipoic acid as a new treatment option for Azheimer type dementia. Arch Gerontol Geriatr 2001;32(3):275–82.

[16] Halliwell B. Reactive oxygen species and the central nervous system. J Neurochem 1992;59(5):1609–23.

[17] Harman D. Aging: a theory based on free radical and radiation chemistry. J Gerontol 1956;11:298–300.

[18] Head E, Thornton PL, Tong L, Cotman CW. Initiation and propagation of molecular cascades in human brain aging: insight from the canine model to promote successful aging. Progr Neuro-Psychopharmacol Biol Psychiatr 2000;24(5):777–86.

[19] Head E, McCleary R, Hahn FF, Milgram NW, Cotman CW. Region-specific age at onset of β-amyloid in dogs. Neurobiol Aging 2000;21(1):89–96.

[20] Itoh K, Izumi A, Kojima S. Object discrimination learning in aged Japanese monkeys. Behav Neurosci 2001;11:259–70.

[21] Iversen SD, Humphrey NK. Ventral temporal lobe lesions and visual oddity performance. Brain Res 1971;30(2):253–63.

[22] Joseph JA, et al. Age-related neurodegeneration and oxidative stress: putative nutritional intervention. Neurol Clin 1998;16(3):747–55.

[23] Joseph JA, et al. Reversals of age-related declines in neuronal signal transduction, cognitve, and motor behavioral deficits with blueberry, spinach, or strawberry dietary supplementation. J Neurosci 1999;19:8114–21.

[24] Joseph JA, et al. Oxidative stress protection and vulnerability in aging: putative nutritional implications for intervention. Mech Ageing Dev 2000;116(2/3):141–53.
[25] Kawarabayashi T, Younkin LH, Saido TC, Shoji M, Ashe KH, Younkin SG. Age-dependent changes in brain, CSF, and plasma amyloid β protein in the Tg2576 transgenic mouse model of Alzheimer's disease. J Neurosci 2001;21(2):372–81.
[26] Kiatipattanasakul W, Nakamura S, Kuroki K, Nakayama H, Doi K. Immunohistochemical detection of anti-oxidative stress enzymes in the dog brain. Neuropathology 1997;17:307–12.
[27] Lai ZC, Moss MB, Killiany RJ, Rosene DL, Herndon JG. Executive system dysfunction in the aged monkey: spatial and object reversal learning. Neurobiol Aging 1995;16:947–54.
[28] Li Y, Liu L, Barger SW, Mrak RE, Griffin WS. Vitamin E suppression of microglial activation is neuroprotective. J Neurosci Res 2001;66:163–70.
[29] McGahon BM, Martin DSD, Horrobin DF, Lynch MA. Age related changes in LTP and antioxidant defenses are reversed by an α-lipoic acid-enriched diet. Neurobiol Aging 1999;20:655–64.
[30] McGeer PL, Schulzer M, McGeer EG. Arthritis and anti-inflammatory agents as possible protective factors for Alzheimer's disease: a review of 17 epidemiologic studies. Neurology 1996;47:425–32.
[31] McKhann G, Drachman D, Folstein M, Katzman R, Price D, Stadlan EM. Clinical diagnosis of Alzheimer's disease: report of the NINCDS-ADRDA work group under the auspices of Department of Health and Human Services task force on Alzheimer's disease. Neurology 1984;34:939–44.
[32] Mendelsohn AB, et al. Use of antioxidant supplements and its association with cognitive function in a rural elderly cohort: the MoVIES Project. Monongahela Valley Independent Elders Survey. Am J Epidemiol 1998;148(1):38–44.
[33] Milgram NW, Head E, Weiner E, Thomas E. Cognitive functions and aging in the dog: acquisition of non-spatial visual tasks. Behav Neurosci 1994;108:57–68.
[34] Milgram NW, Estrada J, Ikeda-Douglas CJ, Drozdz J, Castillo J, Head E, et al. Landmark discrimination learning in aged dogs is improved by treatment with an antidoxidant enriched diet. Soc Neurosci Abstr 2000;26(Part 1):531.
[35] Moss MB, Rosene DL, Peters A. Effects of aging on visual recognition memory in the rhesus monkey. Neurobiol Aging 1988;9 (5/6):495–502.
[36] Paleologos M, Cumming RG, Lazarus R. Cohort study of Vitamin C intake and cognitive impairment. Am J Epidemiol 1998;148:45–50.
[37] Petersen RC, Smith GE, Waring SC, Ivnik RJ, Tangalos EG, Kokmen E. Mild cognitive impairment: clinical characterization and outcome. Arch Neurol 1999;56(3):303–8.
[38] Rapp PR. Visual discrimination and reversal learning in the aged monkey (*Macaca mulatta*). Behav Neurosci 1990;6:876–84.
[39] Sano M, Ernesto C, Thomas RG, Klauber MR, Schafer K, Grundman M, et al. A controlled trial of selegiline, alpha-tocopherol, or both as treatment for Alzheimer's disease. New Engl J Med 1997;336: 1216–22.
[40] Shigenaga MK, Hagen TM, Ames BN. Oxidative damage and mitochondrial decay in aging. Proc Natl Acad Sci USA 1994; 91:10771–8.
[41] Socci D.J. Crandall B.M. Arendash G.W. Chronic antioxidant treatment improves the cognitive perfomance of aged rats. Brain Res Sep 25, 1995; 693(1/2):88–94.
[42] Thomas RK. Investigating cognitive abilities in animals: unrealized potential. Brain Res Cogn Brain Res 1996;3(3/4):157–66.
[43] Thomas RK, Frost T. Oddity and dimension-abstracted oddity (DAO) in squirrel monkeys. Am J Psychol 1983;96:51–64.
[44] Voytko ML. Impairments in acquisition and reversals of two-choice discriminations by aged rhesus monkeys. Neurobiol Aging 1999;20:617–27.
[45] Warsama Jama J, Launer LJ, Witteman JCM, den Breeijen JH, Breteler MMB, Grobbee DE, et al. Dietary antioxidants and cognitive function in a population-based sample of older persons. Am J Epidemiol 1996;144(3):275–80.
[46] Youdim KA, et al. Short-term dietary supplementation of blueberry polyphenolics: beneficial effects on aging brain performance and peripheral tissue function. Nutr Neurosci 2000;3:383–97.