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A Combination Cocktail Improves Spatial Attention in a Canine Model of Human Aging and Alzheimer's disease

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Abstract

Alzheimer's disease (AD) involves multiple pathological processes in the brain, including increased inflammation and oxidative damage, as well as the accumulation of beta-amyloid (A β) plaques. We hypothesized that a combinatorial therapeutic approach to target these multiple pathways may provide cognitive and neuropathological benefits for AD patients. To test this hypothesis, we used a canine model of human aging and AD. Aged dogs naturally develop learning and memory impairments, human-type A β deposits and oxidative damage in the brain. Thus, 9 aged beagles (98-115 months) were treated with a medical food cocktail containing (1) an extract of turmeric containing 95% curcuminoids; (2) an extract of green tea containing 50% epigallocatechingallate; (3) N-acetyl cysteine; (4) R-alpha lipoic acid; and (5) an extract of black pepper containing 95% piperine. Nine similarly aged dogs served as placebo-treated controls. After 3 months of treatment, 13 dogs completed a variable distance landmark task used as a measure of spatial attention. As compared to placebo-treated animals, dogs receiving the medical food cocktail had significantly lower error scores ($t(11)=4.3$, $p=0.001$) and were more accurate across all distances ($F(1,9)=20.7$, $p=0.001$), suggesting an overall improvement in spatial attention. Measures of visual discrimination learning, executive function and spatial memory, and levels of brain and CSF A β were unaffected by the cocktail. Our results indicate that this medical food cocktail may be beneficial for improving spatial attention and motivation deficits associated with impaired cognition in aging and AD.

Introduction

Alzheimer's disease (AD) is associated with progressive cognitive decline and the accumulation of senile plaques and neurofibrillary tangles (NFT) (1). Senile plaques contain beta-amyloid (A β), which is a peptide thought to play a causative role in AD (2, 3). Thus, a number of therapeutics are currently being developed to either enhance the clearance of A β in the brains of patients with AD, or reduce its production or deposition (4). However, additional pathological events occur in the AD brain that may lead to, be a consequence of, or be independent of A β or NFT accumulation and are possible targets for intervention (5). Oxidative damage and inflammation may lead to enhanced A β production, A β aggregation and NFT accumulation (6-9). Given the complexity of the numerous and interacting pathological pathways in AD, a multi-targeted approach using the combination of several compounds may be more efficacious than a single compound approach.

Oxidative damage and inflammation are pathological processes that occur in the AD brain, where increased oxidative damage to proteins, lipids, DNA and RNA (10-14) and increased neuroinflammation (15-17) are consistently reported. In combination, these modifications can lead to abnormal enzymatic function, deficits in transcription and translation and the release of toxic cytokines, all of which can cause neuron dysfunction and death. Thus, an intervention that targets these pathways and has the potential to reduce A β , NFT may provide clinical benefits for patients with AD.

In a recent study in 6-month old Tg2576 mice, a murine model of amyloid deposition at an age prior to onset of pathology, dietary supplementation with a combination of curcumin, piperine, epigallocatechingallate, alpha-lipoic acid, N-acetylcysteine, B vitamins, vitamin C and folate led to significant improvements in cortical- and hippocampal-dependent learning (18). After 6 months of treatment, soluble A β and A β oligomers (a toxic conformation of A β that can lead to synaptic dysfunction (19)) were significantly reduced. Taken together, these results suggest that a medical food cocktail may be beneficial for the treatment of AD. However, it is necessary to also test a medical food cocktail in a higher mammalian model that naturally develops cognitive and neurobiological changes similar to those seen in humans.

The current study used aged dogs as a model of human aging and AD (20). Aged dogs develop learning and memory impairments in cognitive domains sensitive to age and dementia in humans (21-23). Further, dogs naturally develop A β deposits with age (24-26) and the sequence of A β in dogs is identical to humans (27, 28). Oxidative damage to proteins and lipids is also a key feature of the aged canine brain, including mitochondrial dysfunction (29-31). In previous studies we observed cognitive improvements, reduced brain A β and oxidative damage using an antioxidant diet enriched with vitamins E and C, lipoic acid and carnitine (31-36). In the current study of aged beagles, we hypothesized that a medical food cocktail (green tea extract, piper nigrum extract, curcumin, lipoic acid, N-acetyl-cysteine) with components targeting multiple pathological processes (e.g. A β , oxidative damage, inflammation) would improve cognition and reduce neuropathology.

Materials and Methods

Animals—This study included 18 beagles from Ridgman Farms, Inc. (Mount Horeb, Wisconsin; Table 1). At the start of baseline cognitive testing, the ages of the animals ranged from 98-115 months (Mean=103.2 SD=5.3) or 8.2-9.6 years (Mean=8.6 SD=0.4). Dogs were housed singly in kennel buildings with indoor/outdoor runs measuring 47" × 187.5" and were fed Teklad Pioneer Lab Diets (2025 Diet; Madison, WI). Water was available at all times. All animals were thoroughly examined prior to inclusion in the study and were determined to be in good health. Health evaluations included physical examination, neurological examination, and analysis of blood biochemistry values. All procedures were conducted in accordance with University of Kentucky approved animal protocols and the NIH Policy on Humane Care and Use of Laboratory Animals.

Cognitive Testing Methods

Procedure—The cognitive testing procedures have been described previously (37). All dogs were given a series of baseline tests and ranked on the basis of error scores. Animals were placed into one of two treatment groups, balanced for cognitive test scores and age: placebo control or medical food cocktail. Two different colored capsules were formulated to allow cognitive testing technicians to be blind with respect to treatment condition and yet also be able to administer the treatment. Treatment was initiated and cognitive testing was continued for 9 months. The study design for the cognitive testing phases of the study is presented in Figure 1.

Medical Food Cocktail—The medical food cocktail was formulated to include 203 mg of green tea extract standardized to contain 50% epigallocatechingallate (203 mg epigallocatechingallate or 36.3% by weight). Piper nigrum extract (17 mg Bioperine, 95% piperine, 3.0% by weight), N-acetyl-L-cysteine (85mg, 15.3% by weight), curcumin (203 mg, 36.3% by weight) and R-lipoic acid (51 mg, 9.1% by weight) were included in the formulation. Control capsules were formulated without any of these active ingredients. Dogs were given two capsules per day, one in the morning prior to cognitive testing and one in the afternoon, after cognitive testing was completed.

Testing Apparatus

The test apparatus was a 25" × 3' × 4' wooden box constructed from plywood coated with melamine. The box was equipped with a sliding black laminate tray containing 3 food wells which are covered by objects. One teaspoon (approximately 4 mls) of wet dog food was formed into a ball and placed in one food well to serve as the food reward. Vertical stainless steel bars, which were adjusted to provide openings appropriate for individual dog sizes, served as the front of the box. The testers at behind a hinged door that allowed a sliding tray to be either pushed toward the dog or obscured from the dog's view. A 60W light was placed above the presentation tray to light the objects. Data acquisition was controlled using a customized program (Dogma, Metacog Testing Systems, New Westminster, B.C., Canada). This program controlled all randomization procedures and timing, indicated the location of the reward and stored all of the data in a SQL database. Each trial began when the tester presented the tray to the dog, and ended when the tester recorded the dog's choice of object,

thereby beginning the inter-trial interval. Each dog was given either 10 or 12 trials a day (depending on the task), with trials separated by a 30 second inter-trial interval. Dogs were tested 5 days per week.

Baseline Cognitive Testing—All animals in the study were given a battery of cognitive tests to establish baseline level of cognitive function. Dogs were given a reward and object approach learning task, followed by a simple object discrimination and reversal learning task and then a spatial non-matching to position memory task.

Reward and Object Approach Learning—All dogs received a standard four-phase pretraining protocol (21). This procedure included a phase to (1) expose dogs to the testing apparatus, (2) teach dogs that a reward was always present in one of the food wells (reward approach learning), (3) manually shaped dogs to manipulate the objects, and (4) teach dogs to visually locate the object and approach it (object approach learning). Dogs completed all of these four phases of pretraining prior to testing for object discrimination learning.

Object Discrimination Learning—The first test session was used to establish object preferences. On each trial, the hinged door separating the tester and the dog was raised and the presentation tray was pushed forward. The left and right food wells were covered by the two objects. The food reward was placed in both the left and right food wells beneath each object. Dogs were required to displace an object to expose the food reward. Ten trials were given, with two objects presented simultaneously (a yellow plastic Megablock and a blue plastic ball mounted on a blue plastic coaster) and food reward beneath both objects. The objects appeared randomly 5 times each on the left or right side. The preferred object was whichever object was chosen more frequently (i.e. 6 or more times). Subsequently, the preferred object was used as the positive stimulus. After establishing object preferences, each dog was given 10 daily trials with the food reward beneath the designated positive object. To prevent the dogs from using olfactory cues, the food odors were smeared over both wells. Further, a piece of food was pressed inside the negative stimulus such that the dog could smell it but not see it or eat it. A correct response was recorded when dogs approached and displaced the positive stimulus. An error was recorded if dogs displaced the negative object. One correction per test session was allowed, with subsequent errors resulting in immediate withdrawal of the tray and no food reward. Dogs were trained until one of two criterion levels was met: 9/10 correct on one day or 8/10 correct on two consecutive days. A maximum of 400 trials were given if a dog could not reach criterion.

Reversal Learning—After dogs had reached criterion in the object discrimination task, the reward contingencies of the positive and negative stimuli were reversed. Testing was continued on this task until the criterion described above was met. All other testing procedures were identical to those used with object discrimination learning.

Spatial Non-matching to Position—To assess spatial acquisition and memory, we first used a 2-choice non-matching to position task (38). Animals were first shown a single blue plastic Megablock covering either the left or right food wells. Animals displaced the object on one side (e.g. left) to obtain the reward. After a 5 second delay interval, animals were shown two identical blue plastic Megablocks, with the reward hidden under the object on the

side not rewarded previously (e.g. right). Dogs were given 10 trials per day with a 30 second intertrial interval and were tested until they reached criterion or 40 days (400 trials).

After learning the 2 choice task, animals were placed into a 3 choice spatial memory task. The 3 choice delayed non-matching to position task has been described previously (39). Dogs were first shown a single blueplastic Megablock covering either the left, right or center food well. Once the dog displaced the object and obtained the reward, a 5 second delay interval followed. After the delay, animals were shown 2 identical blue plastic Megablocks, one covering the well seen previously, and the other covering one of the two remaining food wells. The correct response was to select the object covering the side not rewarded previously. Dogs were given 50 days of testing consisting of 12 trials/day, with the location of the reward appearing 4 times each in the 3 food wells. During the 50 days of testing, if an animal reached criterion at the 5 second delay, the delay was increased to 10 seconds, followed by gradually increasing delay intervals. Two scores were calculated from this test: total number of errors made to reach criterion (or after 50 days of testing if criterion was not met) at the 5 second delay, and maximal memory score, which represented the maximum delay interval animals could reach criterion at in the 50 days of testing. Following incremental delay interval increases over a 50 day period, dogs were given a variable delay procedure for 20 days. Dogs were given four trials each at a 20, 70 or 110 second delay interval in a single day of testing. Accuracy scores for each delay interval were calculated as a measure of spatial working memory. Animals were tested on the spatial non-matching to position task at both baseline and after 8 months of treatment. The total errors made for reward and object approach learning, discrimination and reversal learning and acquisition of the 3 choice spatial memory task were summed and dogs were ranked according to total error scores and placed into one of two treatment groups.

Treatment Cognitive Testing—At predetermined time points during the study, animals were given tests to measure spatial attention (landmark discrimination learning), spatial memory (3-choice), oddity learning, discrimination learning and reversal learning (See Fig. 1).

Landmark Discrimination Learning—Spatial attention was measured using a landmark discrimination task (34, 40). Landmark discrimination learning was initiated 2 weeks after initiation of treatment phase with the rationale that in our previous antioxidant food studies, we observed benefits within a 2 week period of time (34). The first phase, Landmark 0, involved presenting dogs with two identical objects (plastic coasters) with a landmark object (yellow peg) placed on top of the object associated with a food reward. The correct response was to select the object closest to the landmark. Animals were trained until criterion was met, as described above. A maximum of 40 days or 400 trials was allowed on landmark 0. In the next phase of testing, the landmark was moved at successively greater distances (5, 18, or 24 cm) away from the reward object, with animals required to meet criterion before progressing to testing with a longer landmark distance. Animals that could not reach criterion at any of the longer distances within 400 trials stopped testing on this stepwise protocol. Once all animals had been tested out to a 24 cm distance on the landmark discrimination task or had reached the maximum number of trials for a single distance, all

animals were given a variable landmark distance test. In this test, animals were given 12 trials per day, with the landmark placed 5, 18, or 24 cm from the correct object. These three distances appeared for 4 trials per day with 12 trials per day in total, and dogs were given a total of 20 days of testing (i.e., 240 trials).

Oddity Discrimination—After approximately 3 months of treatment (Fig. 1), dogs were tested for oddity discrimination learning (41). This task involves 4 sets of 3 objects, with two identical objects and one novel per set. Animals were presented with the first set of 3 objects simultaneously, with a food reward hidden under the novel object. Dogs were given 12 trials per day, with the location of the food reward appearing four times each of the 3 food wells. Once dogs reached criterion and learned the first task, the next set of objects was used. The dogs were required to reach criterion with each object set prior to moving to the more difficult object sets. Animals that failed to learn one of the oddity discrimination tasks within 40 days of testing were stopped. Not all animals were able to learn all 4 phases of the oddity discrimination task. The total number of errors accumulated during the trials to either reach criterion or complete 40 days of testing for each oddity task was used to examine treatment effects.

Size Discrimination and Reversal Learning—After animals were treated for 6 months, they were given a size discrimination and reversal task. The procedures were identical to those used during baseline object discrimination learning (described in the baseline testing procedures). In this test, dogs were shown two objects simultaneously and the objects differed only in size (42). Dogs were first given 10 trials with both objects baited to establish a preference. Subsequently, for size discrimination learning, the preferred size of object was consistently rewarded. After criterion was met, size reversal learning was initiated, the reward contingencies were reversed, and dogs were rewarded for selecting the previously incorrect sized object. Dogs were given a total of 40 days to learn the size discrimination task. If criterion was met during this time, the dogs were immediately subjected to size reversal learning, which was identical to the discrimination task but the reward contingencies were reversed. Animals were given an additional 40 days to reach criterion in the size reversal task. The total number of errors accumulated in the trials to either reach criterion or complete 40 days of testing was used as dependent measures for both tasks.

Spatial Non-matching to Position—After approximately 8 months of treatment, dogs were again given the 3-choice spatial memory task. Dogs were given a variable delay procedure (20, 70 or 110 seconds) for 20 days, identical to that completed during baseline testing. Accuracy scores for each delay interval were calculated as a measure of spatial working memory.

Blood Biomarkers

At baseline and after 3, 6 and 9 months of treatment, blood was drawn for basic blood biochemistry. Panels for basic metabolism, liver and kidney function and hematology were completed at each time point.

Cerebrospinal Fluid Samples and Brain Tissue Collection

At the end of the study, animals were administered propofol (3.0-6.0 mg/kg, IV), orally intubated and maintained on isoflurane in 100% O₂. CSF was collected sterilely via cisterna magna puncture. Animals were then exsanguinated via right jugular vein-atrium cannulation and administered Beuthanasia-D[®] Special (Schering-Plough, Union, NJ, 1.0 ml/10 lb). The brain was removed. The left hemisphere was placed in 4% paraformaldehyde at 4°C for 72 to 80 hours prior to transfer to phosphate buffered saline (PBS), pH 7.4, with 0.02% NaN₃ and storage at 4°C. The right hemisphere was coronally sectioned and flash frozen to -80°C.

Immunohistochemistry

A fixed coronal block containing the parietal cortex was subdissected and 50 µm free-floating sections were taken using a vibratome. Aβ immunostaining has been described previously (37). Aβ was detected with anti-Aβ1-17 (mouse monoclonal 6E10 antibody, 1:2000, Covance, Princeton, NJ) and with anti-Aβ1-42 antibody (rabbit polyclonal, 1:3000, Invitrogen, Carlsbad, CA). Briefly, the protocol consisted of pretreatment with 90% formic acid (FA) (43) prior to overnight incubation with primary antibody, incubation with anti-mouse/rabbit secondary antibodies, amplification and detection with an ABC peroxidase kit and visualization with a DAB substrate kit (both from Vector Laboratories., Burlingame, CA). Negative controls omitting primary or secondary antibody were included, resulted in negligible staining. A similar protocol was used to detect gliosis with an anti-glial acidic fibrillary protein antibody (rabbit polyclonal anti-GFAP; 1:20,000; Millipore, Billerica, MA), although FA pretreatment is unnecessary with this antibody. Sections were mounted on glass slides and coverslipped with Depex mounting media.

Aβ ELISA

Aβ was extracted from frozen tissue measured as previously described (44, 45). Briefly, frozen parietal cortex samples were extracted in ice cold PBS (pH 7.4) containing a protease inhibitor cocktail (PIC -Amresco, Solon, OH) and centrifuged at 20,800 × g for 30 min. at 4°C. Following centrifugation, the supernatant was collected and the pellets were sonicated (10 × 0.5 sec pulses at 100W, Sonic Dismembrator, Fisher Scientific, Pittsburgh, PA) in 2% SDS with PIC and centrifuged at 20,800 × g for 30 min. at 14°C. The supernatant was again collected and the remaining pellets were sonicated in 70% FA, followed by centrifugation at 20,800 × g for 1 hour at 4°C. Aβ was measured in tissue samples using a standard, well-characterized two-site sandwich ELISA, as described previously [47]. Briefly, an Immulon 4HBX plate was coated with 0.5 µg antibody per well, incubated overnight at 4°C and blocked with a solution of Synblock (AbD Serotec, Raleigh, NC), as per manufacturer's instructions. Aβ40 was measured using monoclonal antibody Ab42.5 (against human Aβ1-16) for capture, and biotinylated antibody 13.1.1 (end-specific for human Aβ40) for detection. Aβ42 was measured using monoclonal antibody 2.1.3 (end-specific for human Aβ42) for capture, and biotinylated 4G8 (against human Aβ17-24; Covance) for detection.

FA-extracted material was initially neutralized by a 1:20 dilution in TP buffer (1 M Tris base, 0.5 M Na₂HPO₄), followed by a further dilution (1:5) in Antigen Capture (AC) buffer (20mM Na₃PO₄, 0.4% Block Ace (AbD Serotec), 0.05% NaN₃, 2mM EDTA, 0.4M NaCl, 0.2% BSA, 0.05% CHAPS, pH 7) for a final dilution of 1:100. SDS soluble fractions were

diluted 1:20 in AC buffer alone. PBS fractions were diluted 1:1 in AC buffer alone. A peptide standard curve of A β was run on the same plate for comparison, and standards and samples were run at least in duplicate; A β values were determined by interpolation relative to the standard curve. Plates were washed 2-4 times between steps with standard PBS containing 0.05% Tween-20, followed by PBS. Plates were developed with TMB reagent (KPL, Inc., Gaithersburg, MD), stopped with 6% *o*-phosphoric acid and read at 450 nm using a multiwell plate reader (BioTek, Winooski, VT). CSF A β 40, A β 42 and total A β were measured using the same ELISA procedure; CSF total A β was measured using antibody Ab42.5 for capture, and biotinylated 4G8 for detection. CSF samples were diluted 1:5 in AC buffer for measurement by ELISA.

Data and Statistical Analysis

For each learning task (e.g. discrimination, reversal), the total errors made to reach criterion in the two groups were compared using a t-test. For landmark discrimination and oddity discrimination, a repeated measures univariate ANOVA was used to test for treatment effects. For the variable landmark and spatial memory tests, a repeated measures ANOVA was used to determine whether further distances or longer delay intervals were differentially improved with treatment. A repeated measures ANOVA was used to detect changes in blood biochemistry with age. The extent of A β plaque accumulation was determined based on a scale of 0 to 5 with increasing intensity and groups were compared using Mann-Whitney U tests. All statistics were conducted using IBM SPSS Statistics 19.

Results

At baseline, all dogs were matched into two groups (Figure 2) and no statistical differences in age or cognition were noted prior to the start of treatment. For reward approach learning ($t(15)=0.62$ $p=0.54$), object approach learning ($t(14)=1.61$ $p=0.13$), object discrimination learning ($t(13)=0.82$ $p=0.43$), object reversal learning ($t(13)=0.65$ $p=0.52$), spatial learning ($t(13)=0.16$ $p=0.88$) and spatial memory ($t(12)=0.73$ $p=0.48$), the two groups were equivalent. Of the 18 dogs given baseline testing, 2 dogs in the placebo group and 2 dogs in the cocktail group would not perform consistently and could not be utilized for the cognitive testing portion of the study. However, they were maintained on the study to obtain biological outcome measures. After less than one month of treatment, one female in the placebo group required euthanasia for unmanageable pain due to arthritic elbows.

Spatial Attention

Landmark discrimination learning was initiated after a 2 week wash in of the cocktail. Acquisition of the serial individual landmark tasks (0, 5, 18, or 24 cm) did not show significant treatment effects, although as the testing proceeded, dogs in Group 2 (cocktail) were trending towards fewer errors (Figure 3A-D). Variable distance landmark testing was completed in 6 placebo treated and 7 cocktail treated dogs for a period of 20 days after the initial learning phase with either 5, 18, or 24 cm distances appearing each day, four times perday for a total of 12 trials perday. The total number of errors made during the 20 days of testing was significantly decreased in the cocktail group ($t(11)=4.34$ $p=0.001$; Figure 3E). Next, the accuracy was calculated for individual distances for each dog, given that landmark

testing is more difficult with increasing distance between the landmark and the object. A repeated measures general linear models analysis indicated a significant main effect of distance ($F(2,18)=15.94$ $p<0.0005$) and of treatment group ($F(1,9)=20.7$ $p=0.001$). The interaction, however, was not significant, suggesting that overall the treated dogs performed at higher levels of accuracy but increasing distance affected performance equally across groups (Figure 3F).

Oddity Discrimination

After approximately 3 months of treatment, dogs were given the oddity discrimination tasks. For oddity 1, 6 control dogs and 8 treated dogs reached criterion. For oddity 2, 5 of the control dogs and 7 of the treated dogs reached criterion. For oddity 3, 5 control and 6 treated dogs reached criterion. For oddity 4, 5 control and 5 treated dogs reached criterion. There was no main effect of treatment overall, nor was the interaction between oddity task and treatment significantly different. However, when we analyzed only those dogs that completed all 4 oddity tasks, we found that there was a significant main effect of the oddity task on error scores ($F(3,27)=6.7$ $p=0.002$), suggesting increasing task difficulty with each new set of oddity objects.

Size Discrimination, Reversal Learning and Spatial Memory

After 6 months of treatment, dogs were given a new discrimination and reversal task that was based on distinguishing differences in size. A comparison of error scores between the two groups showed no significant treatment effects. For reversal learning, 6/8 treated dogs and 3/5 control dogs were able to solve the task. For reversal learning, the total error scores between the two groups were not significantly different.

Spatial Memory Retest

After approximately 8 months of treatment and prior to the end of the study, dogs were retested for spatial working memory. A total of 13 dogs were able to finish this last set of testing ($n=6$ control and $n=7$ treated dogs). Overall, the total number of errors made over 20 days of testing was not significantly different between the two groups.

Blood Biochemistry

Four time points were used to compare blood biochemical changes with treatment over time: baseline, 3 months, 6 months and 9 months. Of all the measures, the most consistent change with treatment was in creatine phosphokinase (CPK) values, although all were within the normal range. Blood CPK decreased over time in both groups ($F(3,45)=11.2$ $p<0.0005$), suggesting no toxicity to the heart or skeletal muscle. Treated dogs had a more rapid decrease in CPK as compared to controls ($F(3,45)=3.71$ $p=0.018$).

A β Outcomes

Potential treatment effects on CSF A β 40, A β 42, and total A β were tested using t-tests for each measure. CSF taken during the euthanasia process was available from 8 control and 9 treated dogs. Overall, the treated dogs showed no difference in CSF A β 42, A β 40, or total A β (Figure 4A). In addition, brain A β was measured in the parietal cortex, given that

treatment effects were observed for landmark discrimination learning and the parietal cortex is thought to be critically involved with performance on this task (46). Brain A β was measured using two approaches. First, immunohistochemistry was used to determine the extent of A β in plaques in the parietal cortex and the prefrontal cortex and was not different between the two groups. Second, ELISA was used to investigate differences in PBS soluble A β 40, PBS soluble A β 42, SDS soluble A β 40, SDS soluble A β 42, FA soluble A β 40 or FA soluble A β 42 in the parietal cortex as a function of treatment. Parietal cortex A β was not significantly affected by the cocktail (Figure 4B, C, D). We did not observe differences in the extent of anti-GFAP labeling in the two treatment groups (data not shown).

Discussion

Treatment with a medical food cocktail in aged beagles, as a model of human aging and AD, resulted in an improvement in spatial attention, but not in other measures of cognition. Interestingly, the number of animals that stopped responding as the cognitive tests became more difficult was higher for control-treated dogs, suggesting a possible improvement in motivation level in treated animals. Neurobiological studies show no changes in either the extent of A β plaque accumulation or the biochemical measures of soluble and insoluble A β 1-40 and A β 1-42.

Medical Food Cocktail Improves Spatial Attention

During the acquisition phase of a spatial attention task, as the task became increasingly difficult, cocktail-treated animals trended toward improved performance. In a previous study we showed that an antioxidant diet could improve spatial attention after a 2 week treatment period (34) and this formed the rationale for the short wash in period. However, treatment effects did not become significant until the variable distance task, in which cocktail-treated dogs outperformed placebo dogs at all distances. These results suggest that spatial attention overall was improved with the cocktail, which is consistent with our previous reports of improved spatial attention in aged beagles maintained on an antioxidant diet containing vitamins E and C, and lipoic acid (34). However, in this previous study, aged dogs on the antioxidant diet performed similarly to aged dogs on the control diet on the landmark task at short distances (i.e. the less difficult component to the task). Treatment differences were apparent at the longest distance, suggesting a selective effect involving task difficulty. Taken together, these two studies suggest that the medical food cocktail improves spatial attention, regardless of the distance animals are required to evaluate when selecting the correct side.

Interestingly, similar possible effects on motivation were noted for both this study and for the previously published study of aged beagles fed an antioxidant diet (summarized in (20)). On the landmark task, the number of animals that failed to learn the sequential set of increasing distances was higher in the control group than the antioxidant-fed group (34). Similar observations were noted in the current study, in that fewer control treated animals learned the task. When data from these two studies are taken into consideration, antioxidants and/or mitochondrial co-factors may have an overall effect on motivation or interact with task difficulty. Given that both formulations contain lipoic-acid (current study = 102 mg

perday; previous study = 2.7 mg/kg/day = ~270 mg per day for a 10 kg beagle), some of the consistent outcomes may be related to this compound, although the dose in the current study was less than half that of the previous study. Further, each formulation contained additional compounds that could affect efficacy, therefore, future studies dissecting the individual contribution of each compound to cognition, in addition to possible dose effects, would be beneficial.

Although significant improvements were observed in landmark discrimination learning in treated dogs in the current study, these results did not extend to other measures of cognition including complex learning, spatial learning and memory, discrimination learning, and reversal learning. There may be several explanations for the subsequent negative findings. First, the medical food cocktail may have short term benefits on the brain that are subsequently overcome as neurons compensate or as the aging process continues. Given the short duration of the study, the aging process is unlikely a factor. Second, the medical food cocktail may selectively improve spatial attention but not function in other cognitive domains. The landmark discrimination task used in the canine study was directly translated from work in nonhuman primates (46). In monkeys with a posterior parietal cortex lesion, landmark discrimination learning is disrupted. However, monkeys with a prefrontal cortex lesion can still perform the task, suggesting that the landmark task may depend on a cortical circuit that involves the parietal cortex. If a similar circuitry is also engaged in dogs, the medical food cocktail may selectively improve a cortical circuit involving a contribution from the parietal cortex. It is possible that as animals become increasingly sophisticated with the cognitive tests that the treatment effects cannot be detected against extensive training. However, our previous longitudinal studies in aging dogs suggest that continuous cognitive training does not obscure treatment effects and indeed, some cognitive benefits become larger (35).

Medical Food Cocktail Improves Attention Independently of A β

Several components in the medical food cocktail have been linked to A β modifying or lowering effects. EGCG may increase alpha-secretase processing of APP (47) or decrease BACE protein level (48), thus reducing A β production and converting A β fibrils into smaller less toxic aggregates (49). Further, curcumin has been shown to bind A β plaques in transgenic mice (50) and aged dog brain (51, 52). In mice, curcumin is linked to A β reduction through several possible mechanisms including the disaggregation of plaques (50, 53, 54) and attenuating the maturation of the APP (55).

The parietal cortex in aged dogs shows significant A β deposition that typically begins after 10 years of age. In contrast, the prefrontal cortex in dogs can show plaques as early as 8 years of age (26, 56). A β reduction was not noted in measures of plaque accumulation or by ELISA in the current study. The relatively selective effect of the medical food cocktail on cognition in this study may be related to improved cortical function in the parietal cortex prior to significant A β deposition (e.g. preventive), as opposed to improved function that relies on a prefrontal cortical circuit with A β pathology already. In support of this hypothesis, parietal cortex A β was not reduced in treated animals, most of which had diffuse plaque accumulation at the end of the study. Two animals in the treated group had the

highest levels of parietal A β and yet improved on the landmark task relative to untreated dogs. Thus, the medical food cocktail may have improved spatial attention independently of the effects of A β , suggesting possible metabolic improvements. Interestingly, the flavonoid, EGCG, can reduce A β induced mitochondrial dysfunction (57, 58), which may provide neuronal benefits in the absence of A β reduction. Curcumin can reduce A β -associated inflammation (59) and A β -induced tau phosphorylation (60). Although piperine was included in the cocktail primarily to increase the bioavailability of curcumin and EGCG (61, 62), it has anti-oxidant activity (63, 64). Additionally, N-acetyl-cysteine is also an antioxidant that has had some beneficial outcomes in AD patients in clinical trials (65). Thus, multiple other pathways could be modified in response to the medical food cocktail that do not involve A β reduction.

Relative to the previous study of a similar dietary cocktail published in the triple transgenic mouse model of AD (3 $\times$ Tg), some similarities and differences are noted. First, transgenic mice show significant improvements in spatial memory and reduced A β when treated with a similar cocktail (18). In contrast, the canine study showed selective improvements in spatial attention, along with possible improvements in motivational level. Further, A β remained unaffected by treatment in dogs. A key difference in the two studies is the age at which animals were started on treatment. In the transgenic mouse study, treatment was initiated when animals were 6 months of age, when cognitive deficits and A β neuropathology had not yet developed. In the canine study, animals were started on treatment at 8-10 years of age, when cognitive deficits and prefrontal A β pathology can both be present. Thus, the mouse study was preventive whereas the dog study may be described as both preventive and treatment targeted (given the varying ages of A β deposition in different cortical regions). In dogs, either earlier initiation or longer duration of treatment may prove to be beneficial across a broader range of cognitive tasks.

High doses of EGCG in beagles can lead to toxicity (66) and the combination of various compounds may lead to unexpected interactions. Thus, multiple measures were monitored from blood in the study animals. However, blood biochemistry parameters did not reveal any liver, kidney or hematological toxicity of the formulation, suggesting that the cocktail may be safe for use in human clinical trials.

A multi-faceted treatment that includes components targeting multiple pathological cascades in the brain has promise for improving brain health and reducing AD-associated cognitive deficits and neuropathology. The current formulation shows promise in terms of improving spatial attention and motivation in patients with AD, and that 9 months of dosing did not lead to abnormal blood biochemistry in aged beagles. Initiating treatment using a prevention approach, increasing the duration of the treatment or modifying the doses of individual components to the cocktail may further benefit cognition and reduce neuropathology associated with aging. It is also interesting that the dietary background of these animals was well-balanced and considered “healthy” for a senior dog. The possibility of further cognitive benefits of aged animals or humans who have a less healthy diet or perhaps a “bad diet” would be an exciting area to address.

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Figure 1.

Study Design. Dogs were given a series of baseline cognitive tests and then balanced into two groups, control and treatment. Both groups followed the same protocol through out the study but with subsets of animals learning individual tasks.

Figure 2.

Baseline cognitive test scores as a function of group prior to treatment. Dogs were matched into two groups equivalent for reward approach, object approach, object discrimination, object reversal, and spatial learning. In addition, overall memory scores were also matched between the two groups. Bars represent group means and error bars are standard errors of the mean.

Figure 3.

Landmark discrimination learning as a function of treatment. Dogs in both groups underwent a systematic training protocol of learning to select one of two identical objects solely on its location. In landmark 0 (A), dogs performed equivalently. As the landmark was moved progressively further away from the correct object (B, C, D) the treated dogs on average performed with fewer errors, although differences were not statistically significant. On the variable distance landmark phase of the task, overall, dogs treated with a medical food cocktail showed significantly fewer errors (E). Further, accuracy overall across 3 distances (5, 18, 24 cm) was improved in cocktail treated dogs (F). Bars indicate group means and error bars are standard errors of the mean.

Individual data points are also shown by circles in graphs A-E.

Figure 4.

Aβ in CSF and parietal cortex as a function of treatment. Levels of CSF Aβ40, Aβ42, and total Aβ were not reduced in response to treatment (A). Similarly, in parietal cortex, levels of PBS soluble Aβ (B), SDS soluble Aβ (C), and FA soluble Aβ (D) were not reduced in medical food cocktail treated dogs. Bars represent group means and error bars are standard errors of the mean.

Individual data points are also shown by open (Aβ40) or closed (Aβ42) circles. Total Aβ is shown as a black square.

Table 1
Animal Demographics*

Group	Age at Start of Study in Months (SE)	Age at Start of Treatment in Months (SE)	Age at Death in Months (SE)	Time on Treatment in Months (SE)
Treatment (n=8)	104.8 (2.2)	114.4 (2.2)	123.7 (2.2)	9.4 (0)
Control (n=7)	101.6 (1.1)	111.1 (1.1)	119.5 (1.3)	8.4 (0.9)

* SE = standard error of the mean. No statistical differences in the groups were observed.