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Oxidative Stress, Aging and CNS disease in the Canine Model of Human Brain Aging

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SYNOPSIS

Decline in cognitive functions that accompany aging in dogs may have a biological basis, and many of the disorders associated with aging in canines may be mitigated through dietary modifications that incorporate specific nutraceuticals. Based on previous research and the results of both laboratory and clinical studies – antioxidants may be one class of nutraceutical that provides benefits to aged dogs. Brains of aged dogs accumulate oxidative damage to proteins and lipids, which may lead to dysfunction of neuronal cells. The production of free radicals and lack of increase in compensatory antioxidant enzymes may lead to detrimental modifications to important macromolecules within neurons. Reducing oxidative damage through food ingredients rich in a broad spectrum of antioxidants significantly improves, or slows the decline of, learning and memory in aged dogs. However, determining all effective compounds and combinations, dosage ranges, as well as when to initiate intervention and long term effects constitute gaps in our current knowledge.

Keywords

antioxidants; beta-amyloid; behavior; cognition; mitochondrial co-factors; oxidative damage

AGING AND COGNITIVE DYSFUNCTION IN DOGS

Current estimates of the companion animal population in the US suggest that there are over 52 million senior and geriatric dogs over the age of 7 years¹. Advanced age in dogs is frequently associated with severe behavioral and cognitive deficits. Deficits in learning and memory, which are measured using systematic and controlled laboratory testing procedures, have been observed^{2–5}. In client-owned animals, behavioral signs such as disorientation, decreased social interaction, loss of prior housetraining, sleep disturbances and decreased activity are reported^{6–8}. Until recently, clinicians had regarded this constellation of behavioral observations to be indicative of senility associated with advanced age. It was also regarded in general as a progression of signs that had no plausible etiology and thus no viable intervention.

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Ruehl and collaborators^{6–9}, however, have proposed a terminology of canine cognitive dysfunction syndrome (CDS) to describe the client-owned behavioral deficits manifested in older dogs. Numerous studies, using owner based observational questionnaires, have been performed to assess the prevalence of CDS in dogs. In a study of 26 pet owners, the most commonly reported complaints associated with older dogs included destructive behaviors, inappropriate urination or defecation and excessive vocalization in older animals⁸. In other studies, approximately 18% (Proprietary market research, 1999, Pet owner sample size: 150. Data on file, Pfizer Animal Health) to 75% (Proprietary market research, 2001, Pet owner sample size: 337. Data on file, Hills Pet Nutrition) of pet owners report that their senior dogs (7 years or older) exhibited at least one clinical symptom of CDS. Further, CDS may be a progressive disease as aged dogs with impairments in one category were found within 12–18 months to have impairments in 2 or more categories^{10, 11}. Similar results have been observed by other research groups and Rofina and colleagues have studied the behavior changes of 30 dogs using three questionnaires^{6, 7, 12} in collaboration with their owners. On the basis of these questionnaires, aged dogs could be separated into being cognitively unimpaired or impaired. Questionnaires can include items measuring appetite, drinking behavior, incontinence, day/night rhythms, aimless behavior, activity or interaction, perceptual ability, disorientation, memory and personality changes. However, surveys are subjective and the use of owners as untrained evaluators of behavioral traits is subject to large variation. However, if applied appropriately by the clinician or by a researcher, as reported previously, rather than by the pet owner these questionnaires can be very useful. Owner evaluated survey-based studies measure global brain dysfunction and may be insensitive to early and subtle changes in learning and memory associated with pathological aging in dogs. An alternative method of classification involves the development of neuropsychological tests that directly provide quantitative and objective measures of cognitive function, without reliance on questionnaires^{2, 5}. These tests are labor intensive and to date have only been performed in laboratory settings. However, they may be more sensitive for identification of early cognitive impairment than questionnaires. One well-developed method of testing takes place in a modified Wisconsin General Testing Apparatus where dogs are rewarded for making a correct response. Dogs are given access to a sliding tray that contains three recessed food wells that can be covered by objects to test visual learning and memory⁵. For example, an oddity discrimination task involves presenting dogs with a choice of responding to three objects with one differing from the other two objects, which are identical¹³. To perform accurately on this task, the subject is required to learn that the odd object is associated with a food reward, and to remember the general rule when tested at a later occasion. In order to distribute the sense of smell for the solution an equal amount of food is placed in an inaccessible location of the non-rewarded objects.

The neuropsychological tests have been used to accomplish three objectives: 1) identify non-subjective cognitive changes as a function of age in dogs; 2) characterize the neurobiological basis of age-dependent cognitive decline; and finally 3) screen potential interventions. As such, the populations of dogs tested to date display similar characteristics to what is observed in the human population. It appears that there are old dogs that perform quite well for their age on these tests (successful agers), those that are mildly impaired (similar to age-associated memory impairments in humans) and those that are severely impaired (similar to dementia in humans).

The current terminology for classification of aged dogs based on cognitive and behavioral deficits may need refinement and/or revision as more research in this area is performed. Specifically with respect to measuring behavioral outcomes in dogs, the link between clinical measures of CDS and systematic laboratory cognitive tests is unknown. Further, it is also not clear whether results from the laboratory directly translate into the clinic. It is important to make this distinction because the link between the cognitive processing involved with laboratory-based tasks and the brain circuits compromised in the clinical signs of CDS has yet to be established. Thus it becomes difficult to classify a dog as having cognitive dysfunction

syndrome based on the neuropsychological scores since no studies have been performed where both neuropsychological tests and in-home behavioral questionnaires have been done on the same group of dogs. It may well be that dogs with the most severe cognitive deficits are those with the most noticed behavioral deficits, and therefore are truly dysfunctional. Alternatively, if there is no correlation between the behavioral deficits observed by owners and the cognitive deficits observed in the laboratory, it is possible that each is measuring different types of brain dysfunction. In either case, a full understanding of the correlates between these two types of testing is needed to arrive at a logical classification terminology for this field of study.

BIOLOGICAL BASIS FOR COGNITIVE DYSFUNCTION

There are a number of morphological features of aging in the canine brain that are similar to the hallmarks of human brain aging^{14–18}. In vivo imaging and necropsy studies show cortical atrophy¹⁹ and ventricular widening^{19–21} occur with age in dogs. More recent magnetic resonance imaging studies suggest differential vulnerabilities of specific areas of cortex to aging. For example, the prefrontal cortex loses tissue volume at an earlier age than the hippocampus in aging dogs that is correlated with cognitive dysfunction²². These results were confirmed in neurobiological experiments in a study of 30 dogs demonstrating a correlation between cortical atrophy (measured in coronal sections) and cognitive dysfunction⁷ similar to that seen in humans^{23, 24}.

The aged canine brain accumulates proteins within and around neurons with age that may be toxic^{25–27}. One form of pathology, the accumulation of diffuse proteinaceous plaques, has received the most attention in dogs because it is thought to play a causative role in the development of Alzheimer's disease in humans²⁸. Plaques contain a number of proteins but the primary constituent is the β -amyloid peptide ($A\beta$), which has identical amino acid sequences in both humans and dogs^{29, 30}. The extent of $A\beta$ plaque deposition in the canine brain increases with age^{7, 16, 31–34} and is linked to the severity of cognitive deficits^{6, 7, 35, 36}. Not only is the extent of $A\beta$ plaque formation important but also the location. For example, aged dogs that are severely impaired on a reversal learning task, which measures the ability of dogs to inhibit a previously learned behavior (sensitive to frontal lobe function), are also the same animals that show the most extensive prefrontal cortex $A\beta$ plaque pathology³⁶. As in laboratory beagles, the extent of $A\beta$ plaques varies as a function of age in companion dogs^{7, 37, 38}. Further, the extent of $A\beta$ plaques correlates with behavior changes and this association remains significant even if age is removed as a covariate^{6, 7}. $A\beta$ plaques in the cranial part of the parietal lobe correlated with behavioral changes in aged companion animals related to appetite, drinking, incontinence, day and night rhythm, social behavior (interaction with owners and other pets; personality), orientation, perception and memory⁷. However, $A\beta$ plaque deposition does not account for all the variability in individual animal test scores suggesting that other events occur that play a role in causing cognitive dysfunction. Because advancing age is one risk factor for the development of cognitive dysfunction in dogs, it is therefore reasonable to examine other age-associated pathologies to determine if these also contribute to cognition dysfunction.

OXIDATIVE DAMAGE IN THE BRAINS OF AGED DOGS

Aging and the production of free radicals can lead to oxidative damage to proteins, lipids and nucleotides that, in turn, may cause neuronal dysfunction and ultimately neuronal death. Normally, several mechanisms are in place that balances the production of free radicals. However with age, it is possible that these protective mechanisms begin to fail. For example, superoxide dismutase, an enzyme present normally in brains that converts reactive superoxide ions to hydrogen peroxide, is one step in a series of compensatory mechanisms to reduce

dangerous free radicals. This particular enzyme system appears to decline with age in canine brain³⁹.

The brains of aged dogs accumulate oxidative damage to proteins. The accumulation of carbonyl groups increases with age^{40, 41} and is associated with reduced endogenous antioxidant enzymes such as glutamine synthetase activity or superoxide dismutase^{39, 40}. In several studies a relation between age and increased oxidative damage has been inferred by measuring the amount of end products of lipid peroxidation (oxidative damage to lipids) including by the extent of 4-hydroxynonenal (4HNE)^{7, 38, 42}, lipofuscin (LF)⁷, lipofuscin-like pigments (LFP)^{38, 42}, or malondialdehyde⁴⁰. Lastly, evidence of increased oxidative damage to DNA or RNA (8OHdG) in aged dog brain has been reported⁷.

Oxidative damage may also be associated with behavioral decline. Rofina and collaborators found that increased oxidative end products in aged canine brain^{7, 38, 41} correlates with severity of behavior changes due to cognitive dysfunction. For example a significant correlation was found between behavior changes and 4HNE, LF, 8OHdG, LFP and protein carbonyls⁴¹. One of the consequences of oxidative damage may be demyelination and additional oxidative damage was observed in the white matter of brains of aged dogs and associated with the accumulation of macrophages⁷. Demyelination is thought to play a role in cognitive dysfunctions^{43, 44}.

Oxidative damage may activate microglia/macrophages⁴⁵, which in turn produce cytokines⁴⁶ that modulate the synthesis of the A β precursor protein APP^{47, 48}. In addition, cytokines may promote the conversion of nonfibrillar A β to fibrillar A β ^{49, 50}. In combination, increased oxidative damage, the accumulation of A β plaques and possible inflammation support an association between oxidative damage and plaque pathology⁷.

These age-dependent modifications can potentially impair neuron function. The link between aging and oxidative damage suggests the hypothesis that reducing oxidative damage in the brain may lead to improved cognitive function in aged dogs. One approach to reducing oxidative damage is through the use of compounds found in foods that might have specific functional effects in specific populations. Some references refer to these substances as nutraceuticals, others as functional foods, and others as non-nutritive food based compounds. Whatever the appropriate terminology, studies for testing these compounds are numerous and sometimes difficult to interpret.

NUTRITIONAL ANTIOXIDANTS

Antioxidants are substances that have the ability to scavenge ROS and reduce the overall number of oxidants in a system^{51–53}. In general, it is now substantiated that antioxidants in biologic systems may act individually but more likely, in combination via a network of detoxification systems.

A variety of antioxidant or antioxidant defense associated molecules are derived from food sources. Vitamin E is found in high concentrations in nuts and oils, vitamin C is found in high concentrations in fruits, beta-carotene is found in certain vegetables. In addition trace minerals such as selenium, copper, zinc, and manganese which are important to enzymes that specifically detoxify free radicals (Cu/Zn SOD) or help recycle antioxidants that detoxify free radicals (glutathione peroxidase) may be acquired from different food sources. Recent research has shown that some molecules classified as mitochondrial cofactors (lipoic acid, l-carnitine) may also act to enhance function of aged mitochondrion such that there are less ROS produced during aerobic respiration. It has been shown that chronic antioxidant damage to enzymes and cell membranes may reduce the capability of these molecules to bind mitochondrial enzyme cofactors thus reducing their metabolic capacity⁵⁴. Supplementation of foods with these

mitochondrial cofactors increases the concentration within cells and restores binding to the enzymes that require them, which restores mitochondrial efficiency⁵⁵ and reduces oxidative damage to RNA⁵⁴. Further, Skoumalova et al, 2003, revealed low Vitamin E levels in the brains of aged dogs with cognitive dysfunction compared to an age-matched unimpaired group which supports the concept of adding antioxidants in foods⁴¹.

CAN ANTIOXIDANTS REDUCE COGNITIVE IMPAIRMENTS AND NEUROPATHOLOGY IN AGED DOGS?

If brain aging in dogs and the progressive accumulation of oxidative damage causes cognitive dysfunction then the best test of the hypothesis is to reduce oxidative damage. This may hypothetically be accomplished through dietary enrichment with nutritional antioxidants (nutraceuticals). We completed a longitudinal investigation of the effects of dietary intervention on cognitive function of aged beagle dogs. The experimental subjects were a group of 48 aged beagle dogs (10 to 13 years of age) and 17 young dogs (3 to 5 years old). Each animal was assigned into one of two food groups using a counter balanced design based on extensive baseline cognitive testing. No differences existed between cognitive ability of groups prior to dietary intervention. The test food group was fed an antioxidant/mitochondrial cofactor with dried fruits and vegetables fortified food, while the control group was maintained on an identical base food, without the fortification, that was nutrient adequate for senior dogs. Approximately six months after starting the dietary intervention, the dogs were tested on a series of oddity discrimination learning tasks¹³. We observe both age and diet effects with old animals performing significantly worse than young dogs on all phases of the test. With respect to diet, old dogs fed the test food made significantly fewer errors on the more difficult tasks than old controls. Additional improvements in cognition in response to the antioxidant-enriched diet was observed on a test of spatial attention⁵⁶ and on repeated measures of visual discrimination and reversal learning⁵⁷. Another aspect of the longitudinal study was to measure changes in the brain that may reflect improved cognition. Aged beagles fed the antioxidant diet showed reduced protein oxidative damage and increase antioxidant activity⁵⁸. Further, the extent of A β plaque accumulation appears to be reduced in antioxidant-treated dogs⁵⁹. Other studies are still ongoing.

In addition to the laboratory testing, a behavioral field trial was performed to assess categories of signs of cognitive dysfunction which are: 1) disorientation; 2) changes in sleep patterns; 3) changes in activity; 4) changes in interactions with others; 5) loss of house training. Dogs over 7 years old that exhibited signs in two or more of those categories were recruited for the study. Half the dogs (n= 64) were fed a commercial dog food and the other half (n=61) were fed a fortified food (Hill's Prescription Diet[®] Canine b/dTM). The ingredients included vitamins E and C, docosahexaenoic (DHA) and eicosapentaenoic acid (EPA), lipoic acid, l-carnitine, dried fruits and vegetables. At the end of 60 days, the owners reported that dogs on test food were improved in all five categories while owners of dogs on control food had improved in only two. Supplemented dogs were improved in awareness of their surroundings, family and animal recognition and interaction, and enthusiasm in greeting. In addition, they circled less, soiled less in the house, and were more agile. Dogs on both the control food and test food exhibited less aimless activity, vocalized less, and slept more regularly. Overall the dogs on control food improved in 4 of 15 (27%) behaviors, whereas the experimental group improved in 13 of 15 (87%). This supports the hypothesis that reducing production of free radicals, as well as neutralizing existing ones, will improve the behavior of older dogs⁶⁰.

The most important aspect of this work is the discovery that cognitive performance can be improved by dietary manipulation. Furthermore, the effects of the dietary manipulation were relatively rapid. Antioxidants may potentially act, therefore, to prevent the development of

these age-associated behaviors, and possibly even neuropathology, by counteracting oxidative stress.

NUTRITION, AGING AND COGNITIVE DYSFUNCTION: POTENTIAL PITFALLS AND CAVEATS

There are a number of issues to consider when interpreting the rapidly growing literature describing the use of antioxidants to improve cognitive dysfunction in aged animals. Three main issues will be briefly discussed and include: (1) issues related to which compounds, dosage range of compound, route of administration and when to begin use and length of use; (2) issues related to biological outcome measures and; (3) behavioral outcome measures. The selection of compounds, dosage range, length of administration, and route of administration may vary considerably across species. The selection of specific compounds may depend on bioavailability, physiologic, genetic, and or metabolic. Supplementation with antioxidants may, or may not, increase absorption into tissues. Some antioxidants are more readily absorbed than others (vitamin E vs. grape seed extract) and may display species or meal variation differences in absorption. For example, vitamin E absorption has been shown to be highly variable based on timing of concurrent food intake⁶¹. Thus, different species may benefit from different types of antioxidant but not all may benefit from the same antioxidants. For example, canines cleave beta-carotene in their intestine into vitamin A but cats lack the metabolic enzymes to perform this function. Thus the addition of carotenoids to these two species may have differential effects. For example, cats fed canthaxanthine displayed retinal pigmentation and regional vacuolization when fed a “human-equivalent” dose of this antioxidant⁶².

The dose of individual antioxidants may also vary as a function of species and dose levels determined to be efficacious in rodents may not be translatable to other higher mammalian species attributable to intrinsic metabolic differences. Another important consideration is the metabolic changes with age of animals – many pharmaceuticals and likely nutraceuticals show different absorption, elimination, and toxicity profiles in older animals when compared to younger animals. Interactions of administered compounds may also important; single compound supplementation may not be as efficacious as multi-compound administration within the food, similar to what is observed with amino acid nutrition.

To further complicate the issue, when is the best time to begin antioxidant supplementation? It is likely that young animals will not require nor respond to antioxidant interventions. This leaves the question of - what age would supplementation be prophylactic? It is likely that the age at which benefits from nutraceuticals, such as antioxidants, are observed will vary in different species.

In order to critically evaluate the therapeutic potential for antioxidants (and other nutraceuticals) for the aging process is to consider the outcome measures used to determine efficacy. A variety of new laboratory methods have been developed that purportedly measure the effects of ROS in biologic systems and potentially the results of an intervention. An increase in markers is presumed to be attributable to increased ROS being produced and thus more oxidative damage. A decrease is presumed to indicate less production of ROS and less cellular damage. Markers are specific for different biomolecules such as DNA, 8-oxodeoxyguanosine), lipids (alkenals, MDA, TBARS), prostaglandins (isoprostanes) protein (nitrotyrosine, protein carbonyls), and advanced glycation endproducts (AGE). The utility of these measures has been discussed in the literature and there currently is no consensus as to their specific predictive usefulness for health outcomes. Further, less evidence is available indicating how systemic measures of oxidative damage reflect central nervous system damage. In addition, the methodology to perform these measures is sometimes complex and may be sensitive to sample preparation. Standard protocols are not available for all measures to be used across different

laboratories. Nonetheless, some associations have been made between reduction of these markers of oxidative damage and improved health outcomes. Behavioral outcome measures are even more vulnerable to a lack of standardized testing procedures and variability across laboratories or clinics. For example, in the clinical setting, it is critical to rule out physical causes that may cause behavior changes in aging dogs including sensory decay (e.g. blindness, deafness), or other systemic illnesses (e.g. liver and renal problems). Another point is that questionnaires should be used as a tool by the clinician who in turn, obtains information from the owner. This approach should reduce any bias or misinterpretation of the questions by pet owners. Laboratory behavioral studies are more limited because they are difficult to perform as the expense, length of time required for intervention, and ability to control the dietary intake of subjects is problematic. Nonetheless, this is where the ultimate proof of efficacy must be shown on health outcomes. The study of animals with shorter lifespans than humans, and with potential for more dietary control such as dogs, is useful for developing nutritional strategies that may prove beneficial in determination of outcomes. However, as stated previously, translating results from studies of one species to must be done with caution because of issues discussed previously related to doses and bioavailability.

SUMMARY

Decline in cognitive functions that accompany aging in dogs may have a biological basis, and many of the disorders associated with aging in canines may be mitigated through dietary modifications that incorporate specific nutraceuticals. Based on previous research and the results of both laboratory and clinical studies – antioxidants may be one class of nutraceutical that provides benefits to aged dogs. Brains of aged dogs accumulate oxidative damage to proteins and lipids, which may lead to dysfunction of neuronal cells. The production of free radicals and lack of increase in compensatory antioxidant enzymes may lead to detrimental modifications to important macromolecules within neurons. Reducing oxidative damage through food ingredients rich in a broad spectrum of antioxidants significantly improves, or slows the decline of, learning and memory in aged dogs. However, determining all effective compounds and combinations, dosage ranges, as well as when to initiate intervention and long term effects constitute gaps in our current knowledge.

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