Erythrocyte glutathione and plasma cysteine concentrations in young versus old dogs

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Objective—To determine whether older, otherwise healthy, client-owned dogs were deficient in glutathione or cysteine, compared with young healthy pet dogs.

Design—Cross-sectional study.

Animals—35 healthy dogs between 7 and 14 years old (older dogs) and 26 healthy dogs between 1 and 3 years old (young dogs).

Procedures—In all dogs, erythrocyte reduced glutathione concentration and plasma cysteine concentration were determined by means of high-performance liquid chromatography.

Results—Median erythrocyte reduced glutathione and plasma cysteine concentrations were not significantly different between young (1.7 mM and 8.3 μM, respectively) and older (1.7 mM and 7.6 μM, respectively) dogs. Significant differences were not identified when values for young dogs were compared with values for only those dogs ≥ 11 years old. Similarly, no differences were found between males and females overall or between males and females within age groups, although most dogs were neutered.

Conclusions and Clinical Relevance—Results suggested that otherwise healthy older pet dogs fed a variety of commercial diets do not have deficiencies in glutathione or cysteine, compared with younger dogs. Findings do not support the routine empirical use of antioxidant supplements, such as precursors of glutathione, to treat presumed circulating antioxidant deficiencies in older healthy dogs. (J Am Vet Med Assoc 2009;234:95–99)

Reactive oxygen species are molecular oxygen metabolites that are highly reactive with lipids, proteins, and DNA, causing oxidative damage to these cellular macromolecules. This damage, termed oxidative stress, is thought to accumulate over time and contribute to the aging process, and there is substantial evidence that in vivo oxidative stress and biological aging are positively correlated.1

Cellular mechanisms that exist to counteract reactive oxygen species include enzymatic stabilization and direct scavenging by molecules such as glutathione, a major intracellular antioxidant, and cysteine, a precursor of glutathione and a major extracellular antioxidant in plasma. In rodents, aging is associated with decreases in tissue and blood glutathione concentrations.2–4 In humans, glutathione concentrations have been shown to be lower in healthy elderly subjects, compared with their younger counterparts,2,4 and total plasma antioxidant capacity is lower in older humans.5

In dogs, relatively little work has been done on antioxidants and aging. Supplementation with antioxidants is popular among pet owners, but scientific evidence of the need for these antioxidants in older pet dogs has not been established. One study10 in Labrador Retrievers could not document a difference in plasma antioxidant potential between young and old dogs. Another study11 in Beagles in a research setting did identify a deficiency in erythrocyte glutathione content in older dogs that was more pronounced in males than in females, but it was unclear whether these findings were applicable to healthy pet dogs. The purpose of the study reported here, therefore, was to determine whether older, otherwise healthy, client-owned dogs of various breeds were deficient in glutathione or cysteine, compared with young healthy pet dogs. We hypothesized that older dogs, although healthy, would have lower concentrations of these antioxidants, which might warrant antioxidant supplementation.

Materials and Methods

Animals—The study was designed as a cross-sectional study of a convenience sample of healthy dogs. Dogs were recruited for the study from among pets belonging to faculty, staff, and students at the University of Wisconsin, Madison, School of Veterinary Medicine; primary care patients examined at the University of Wisconsin Veterinary Medical Teaching Hospital; and patients examined at 2 primary care veterinary clinics in Madison, Wis. Dogs were eligible for inclusion in the study only if they were healthy, as determined on the basis of history, results of a physical examination, and results of a CBC and serum biochemical profile. Physical examinations were performed by the authors. Routine hematologic testing was performed by the clinical pathology laboratory at the Veterinary Medical Teaching Hospital, and results were interpreted by one of the authors.

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the authors (LAT). Information on breed, body weight, body condition score, sex, and neuter status was recorded for all dogs, and complete dietary and medication histories for the 2 weeks prior to enrollment in the study were collected. Owners of all dogs included in the study provided informed consent.

Dogs were eligible for inclusion in the study only if their age was known and they met criteria for definition as young or older. For the present study, dogs were defined as young if they were skeletally mature and between 1 and 3 years old. Criteria for defining dogs as older were based on size of the dog because large-breed dogs have shorter life expectancies. Thus, for the present study, dogs weighing ≥ 34 kg (75 lb) were defined as older if they were ≥ 7 years old, dogs weighing ≥ 14 kg (31 lb) but < 34 kg were defined as older if they were ≥ 8 years old, and dogs weighing < 14 kg were defined as older if they were ≥ 9 years old.

Dogs were excluded from the study if the dog had any evidence of systemic disease, as determined from the history and results of the screening physical examination and hematologic testing; the dog was not within one of the defined age ranges or the dog’s age was unknown; or if the dog had been given any dietary supplements or medications other than routine heartworm preventative medication within the past 2 weeks.

Sample collection and preparation—Blood samples were collected from dogs included in the study between June 2006 and August 2007. At the time blood samples were collected for the screening CBC and serum biochemical panel, an additional sample (2 mL) was collected in a tube containing sodium heparin. To prevent oxidation of glutathione and cysteine, 200 μL of 27mM monobromobimane was immediately added, and the tube was covered with foil and placed on ice. Samples were centrifuged at 4°C, and plasma was separated from RBCs. Plasma and RBC samples were frozen at –80°C until assayed. All samples were assayed within 1 week after acquisition. Blood samples were collected from both young and older dogs throughout the study period to control for possible seasonal differences in antioxidant status.

Measurement of glutathione and cysteine concentrations—Erythrocyte reduced glutathione and plasma cysteine concentrations were determined by means of high-performance liquid chromatography, as described. For determination of glutathione concentration, 1.25 μL of thawed RBCs was placed in an amber tube containing 7.5 μL of 180mM monobromobimane and 142.5 μL of phosphate-buffered saline solution (0.9% NaCl). After incubation in the dark at 37°C for 10 minutes, RBCs were lysed with 25 μL of 9.1M perchloric acid, and the sample was centrifuged at 9,850 X g for 15 minutes. Two hundred microliters of the RBC lysate supernatant was diluted with 480 μL of cold phosphate-buffered saline solution. Proteins were precipitated with 55 μL of 50% 5-sulfosalicylic acid in 500μM dithiothreitol, and the sample was centrifuged at 9,850 X g for 10 minutes. A 380-μL aliquot of the supernatant was incubated with 60 μL of 5% 5-sulfosalicylic acid in 500μM dithiothreitol, 100 μL of 1M N-ethylmorpholine, and 20 μL of acetonitrile at 37°C for 5 minutes in the dark. Prior to chromatography, 20 μL of trichloroacetic acid was added to neutralize the alkaline pH of the N-ethylmorpholine. Samples were analyzed for reduced glutathione content with a C18 column (4.6 mm × 25 cm) with fluorescence detection (excitation, 394 nm; emission, 480 nm). Elution was performed with a gradient starting at 100% mobile phase A (0.05% triethylamine and 1.0% glacial acetic acid in water) and transitioning to 80% mobile phase B (acetonitrile) over 20 minutes at 2 mL/min, resulting in elution of glutathione at 6.4 minutes. The limit of quantitation for erythrocyte glutathione concentration was 30μM, with intra-assay coefficients of variation ranging from 2.4% to 8.5% and interassay coefficients of variation ranging from 8.7% to 13.1%.

For measurement of plasma cysteine concentration, 600 μL of thawed plasma was combined with 9.0 μL of monobromobimane (180mM) in acetonitrile diluted with 51 μL of phosphate-buffered saline solution. Proteins were precipitated with 72.6 μL of 50% 5-sulfosalicylic acid in 500μM dithiothreitol. After centrifugation at 9,850 X g for 15 minutes, 350 μL of the supernatant was mixed with 4.2 μL of 50% 5-sulfosalicylic acid in dithiothreitol, 8.96 μL of N-ethylmorpholine, and 14 μL of acetonitrile. Samples were incubated in the dark for 5 minutes at 37°C, then 14 μL of trichloroacetic acid was added. The samples were assayed as described for determination of glutathione content, with elution of cysteine at 5.2 minutes. The limit of quantitation for plasma cysteine concentration was 1μM, with intra-assay coefficients of variation ranging from 0.9% to 7.1% and interassay coefficients of variation ranging from 7.6% to 14.6%. Standards containing known concentrations of glutathione and cysteine were run concurrently with each assay.

Statistical analysis—Data are reported as medians and ranges. Antioxidant concentrations were compared between groups by means of the Mann-Whitney U test. Standard software was used for all analyses; values of P ≤ 0.05 were considered significant.

Results

Twenty-six healthy young dogs (median age, 2 years; range, 1 to 3 years) and 35 healthy older dogs (median age, 10 years; range, 7 to 14 years) were enrolled in the study. Sex, breed, and body weight distributions were similar between groups (Table 1), although median body condition scores were significantly higher in older dogs than in young dogs (Table 2).

Table 1—Demographic characteristics of dogs enrolled in a study to determine whether healthy older dogs were deficient in glutathione or cysteine, compared with healthy young dogs.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Young dogs</th>
<th>Older dogs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (y)</td>
<td>2 (1–3)</td>
<td>10 (7–14)</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>27 (18.3–54.6)</td>
<td>27.1 (21–52.3)</td>
</tr>
<tr>
<td>BCS</td>
<td>5.0 (4.0–7.0)</td>
<td>6.0* (3.0–7.0)</td>
</tr>
<tr>
<td>Breed</td>
<td>9</td>
<td>11</td>
</tr>
<tr>
<td>Mixed</td>
<td>17</td>
<td>24</td>
</tr>
<tr>
<td>Purebred</td>
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<td></td>
</tr>
<tr>
<td>Sex†</td>
<td>13</td>
<td>18</td>
</tr>
<tr>
<td>Male</td>
<td>13</td>
<td>19</td>
</tr>
<tr>
<td>Female</td>
<td></td>
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Data are given as median (range) or as number of dogs. *Significantly (P < 0.003) higher than value for young dogs. **Significantly (P < 0.033) higher than value for young dogs. †2 young female and 3 young male dogs were sexually intact; all other dogs were neutered.

BCS = Body condition score (scored on a scale from 1 to 9).
score was significantly \( (P = 0.033) \) higher in older dogs than in young dogs. Twelve of the 35 older dogs were ≥ 11 years old, and only 2 were 7 years old (a German Shepherd Dog and an English Mastiff). Overall, there were 20 mixed-breed dogs and 41 purebred dogs. Golden Retrievers \( (n = 5) \), German Shepherd Dogs \( (4) \), Airedales \( (4) \), and Malamutes \( (4) \) were the most common purebred dogs.

Dogs were being fed a variety of high-quality commercial diets, with young dogs being fed diets manufactured by Purina \( (n = 8) \), Royal Canin \( (4) \), Iams \( (4) \), Natura \( (3) \), Hills \( (2) \), and others \( (5) \). Older dogs were being fed diets manufactured by Iams \( (n = 9) \), Purina \( (7) \), Hill’s \( (7) \), Royal Canin \( (3) \), Natura \( (2) \), and others \( (3) \). Two older dogs were being fed homemade vegetarian diets, and the remaining 2 older dogs were being fed a combination of 2 commercial diets.

For most of the commercial diets being fed, information on antioxidant content could be obtained from the manufacturer’s Web site. Seventeen of the 26 young dogs were being fed diets supplemented with vitamin E, and 10 were being fed diets supplemented with vitamin C. Nineteen of the 35 older dogs were being fed diets supplemented with vitamin E, and 10 were being fed diets supplemented with vitamin C. Vitamin C content in diets fed to young dogs (median, 85 mg/kg of diet as fed) was not significantly \( (P = 0.63) \) different from vitamin C content of diets fed to older dogs (median, 70 mg/kg of diet as fed). In contrast, vitamin E content in diets fed to young dogs (median, 460 U/kg of diet as fed) was significantly \( (P = 0.008) \) higher than vitamin E content in diets fed to older dogs (median, 277 U/kg of diet as fed).

Figure 1—Scatterplots of erythrocyte reduced glutathione concentrations in 26 healthy dogs between 1 and 3 years old (young) and in 35 healthy dogs ≥ 7 years old (older). Horizontal lines represent medians.

Figure 2—Scatterplots of plasma cysteine concentrations in 26 healthy dogs between 1 and 3 years old (young) and in 35 healthy dogs ≥ 7 years old (older). Horizontal lines represent medians.

Figure 3—Scatterplots of erythrocyte reduced glutathione concentrations in 26 young healthy dogs and in 35 older healthy dogs grouped on the basis of sex. Horizontal lines represent medians. Open symbols represent neutered dogs; filled symbols represent sexually intact dogs. F = Female. M = Male.
Erythrocyte reduced glutathione concentration in older dogs (median, 1.7 mM; range, 1.2 to 2.5 mM) was not significantly ($P = 0.83$) different from concentration in young dogs (median, 1.7 mM; range, 1.0 to 2.3 mM; Figure 1). Similarly, plasma cysteine concentration in older dogs (median, 7.6 µM; range, 4.1 to 19.2 µM) was not significantly ($P = 0.19$) different from concentration in young dogs (median, 8.3 µM; range, 1.8 to 37.7 µM; Figure 2). When values for only those 12 dogs ≥11 years old were included in the analysis, glutathione (median, 1.8 mM) and cysteine (median, 7.5 µM) concentrations in older dogs were still not significantly ($P = 0.25$ and 0.60, respectively) different from concentrations in young dogs.

Erythrocyte glutathione and plasma cysteine concentrations were also not significantly different between male and female dogs overall or within age groups (Figures 3 and 4). Although the number of sexually intact dogs was small and all sexually intact dogs were young, there were no discernible differences in glutathione or cysteine concentration between sexually intact and neutered dogs.

**Discussion**

Results of the present study suggested that otherwise healthy older pet dogs fed a variety of commercial diets do not have deficiencies in glutathione or cysteine, compared with younger dogs. This is in contrast to results of several studies involving humans, which have shown age-associated decreases in erythrocyte glutathione concentrations, with patients 60 to 65 years of age having significantly lower concentrations than young adults in their twenties. It is also in contrast with results of a previous study involving Beagles housed in a research setting, in which erythrocyte glutathione concentration was significantly lower in old (≥9 years old) dogs than in young (<1 year old) ones. In the latter study, dogs were purpose bred and were fed a single diet, so there were fewer confounding factors. In addition, none of the dogs in that study had been neutered and all were in kennels, such that their lifestyle was different from the lifestyles of the privately owned dogs included in the present study.

We also did not identify any significant differences in erythrocyte glutathione or plasma cysteine concentrations between male and female dogs in the present study. In humans and sexually intact Beagles, glutathione deficiency is more pronounced in older males than females. In contrast, most dogs in the present study had been neutered, which should have eliminated any effects of reproductive hormones on antioxidant status. All 5 sexually intact dogs (2 females and 3 males) in the present study were also young, and too few sexually intact dogs were included to compare antioxidant concentrations between neutered and sexually intact dogs.

We chose to measure plasma cysteine concentration in the present study because of its important role as an extracellular antioxidant and to measure erythrocyte glutathione concentration because of its high intracellular concentrations. In addition, studies in humans have found differences between young and old subjects in regard to thiol concentrations in other tissues; however, both erythrocyte and hepatic glutathione concentrations increase in cats given antioxidant supplements. We did not measure markers of lipid peroxidation or DNA damage in these dogs. In laboratory Beagles, brain concentrations of malondialdehyde were higher in older (≥8 years old) dogs than in young dogs. In these same dogs, however, brain malondialdehyde concentration was significantly correlated with blood malondialdehyde concentration, suggesting that redox status in the blood may be a reasonable surrogate marker of redox status in tissues such as the brain in dogs.

One important limitation of the present study was the relatively small sample population. The sample size was sufficient, however, to detect a 25% reduction in glutathione concentrations in older dogs, compared with young dogs, with 95% power and was selected on the basis of preliminary data from our laboratory on erythrocyte glutathione concentrations in healthy dogs. Median antioxidant concentrations and their distributions were quite similar between young and older dogs in the present study, so it appears unlikely that our inability to detect significant differences between groups was a result of insufficient power.

We intentionally included dogs of many different breeds in the present study to reflect the range of patients typically examined by veterinarians. We did not establish individual breed criteria for defining dogs as older, even though there are breed differences in expected lifespan, even among dogs of similar stature and body weight. There may be breed differences in antioxidant status that we had inadequate numbers to evaluate. For example, Newfoundlands have been found to have lower glutathione and cysteine concentrations than do Beagles. It is possible that breed differences may have obscured the influence of age on antioxidant status in the present study. It is also possible that older dogs in the present study were not representative of truly geriatric dogs. Although we allowed large-breed dogs as young as 7 years old to...
participate, only 2 such dogs were enrolled. When we compared antioxidant concentrations between young dogs and only those dogs ≥ 11 years old, we still did not identify any difference between age groups.

Importantly, dogs in the present study were not fed a uniform diet before antioxidant concentrations were measured. This is because we wanted to evaluate dogs as if they had been brought to a veterinarian for routine care and preventative medicine advice. When we compared food labels for antioxidant vitamin E and vitamin C contents, we found that vitamin C content was not significantly different between age groups but that vitamin E content was significantly higher in the diets fed to the young dogs. Vitamin E has been shown to increase erythrocyte glutathione concentrations in humans. Thus, the higher dietary vitamin E content in diets fed to younger dogs could have biased our results by artificially increasing glutathione concentrations in young dogs, which would have made it more likely for us to detect a difference between age groups.

Although median body condition score was significantly higher in older dogs than in young dogs, the ranges were similar, and no dog in either group was obese (body condition score > 7.0). This was important because obesity has been associated with oxidative stress in humans. In any case, higher body condition scores in older dogs would have biased our results by artificially decreasing antioxidant concentrations in older dogs, which again would have made it more likely for us to detect a difference between age groups.

Importantly, dogs in the present study were not under any stress associated with food withholding, illness, or hospitalization. Prolonged (4 to 7 days) fasting can deplete glutathione in humans, and withholding food for as short as 24 hours has been shown to have differential effects on glutathione status in young versus old mice. Thus, it is possible that collecting blood samples after food had been withheld from dogs in the present study may have revealed age-associated differences in the antioxidant responses to food deprivation.

Finally, we cannot conclude from results of the present study that aging has no effect on antioxidant status in dogs. However, we can conclude that otherwise healthy older pet dogs fed a variety of commercial diets do not have deficiencies in glutathione or cysteine, compared with younger dogs. Thus, our findings do not support the routine empirical use of anti-oxidant precursors of glutathione to treat presumed circulating antioxidant deficiencies in older healthy dogs. Additional work is needed to determine the interaction, if any, between age and antioxidant status in clinically ill and anorectic dogs.

References