

Assessment of lipid peroxidation and serum vitamin E concentration in dogs with immune-mediated hemolytic anemia

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Objective—To determine plasma malondialdehyde (MDA) and serum vitamin E concentrations in dogs with immune-mediated hemolytic anemia (IMHA) and healthy control dogs.

Sample Population—Serum and plasma samples from 36 dogs with IMHA and 40 healthy control dogs.

Procedure—Blood samples were collected from all study dogs. Plasma MDA concentrations were measured by use of a commercial colorimetric assay, and serum vitamin E concentrations (α -, γ -, and δ -tocopherol concentrations) were measured via high-performance liquid chromatography.

Results—Plasma MDA concentrations were significantly higher in the dogs with IMHA than in the control dogs. Compared with control dogs, serum α -, γ -, and δ -tocopherol concentrations were significantly lower in the IMHA-affected dogs.

Conclusions and Clinical Relevance—Results indicated a state of oxidative stress and reduced antioxidant reserve in dogs with IMHA; this finding provides support for further investigation of the potential benefits of antioxidant treatment in dogs with this disease. (*Am J Vet Res* 2004;65:1621–1624)

Immune-mediated hemolytic anemia (IMHA) is a severe disease of dogs that is associated with mortality rates of 20% to 70%¹⁻⁸; often, a specific underlying etiology cannot be definitively determined. The somewhat poor understanding of what may initiate and perpetuate this disease and its associated high complication rate contribute to the poor survival rate among dogs with IMHA. A better comprehension of the mechanisms of the disease process may provide improved methods of treatment for affected dogs.

One process that may provide a target for treatment of IMHA in dogs is oxidative injury of erythrocytes. Erythrocytes may be particularly susceptible to oxidative damage for a number of reasons. The lack of a nucleus restricts each erythrocyte to limited repair mechanisms.⁹ Additionally, erythrocytes contain iron (in the form of heme) and oxygen in close proximity⁹; iron can serve as a catalyst to form reactive oxygen species. Finally, the membrane of an erythrocyte is rich in polyunsaturated fatty acids, which can be targets of

free radical damage.⁹ In human erythrocytes, oxidative damage can result in hemolysis through a variety of mechanisms.¹⁰⁻¹² Hemolysis can generate the production of reactive oxygen species by releasing hemoglobin¹³; therefore, oxidative stress may be either an initiating or a secondary event that can perpetuate a hemolytic state. The generation of excessive reactive oxygen species in erythrocytes and the subsequent oxidative stress placed on these cells have been implicated in some forms of immune-mediated hemolysis in humans.^{11,12,14}

The role of oxidative stress in the development of IMHA in dogs is likely to be a secondary rather than a primary effect. However, oxidative stress could contribute to ongoing hemolysis and the poor outcome for dogs with this disease. If oxidative damage plays a role in the development of IMHA, this association may provide a rationale for new treatments for dogs with this disease. We hypothesized that the degree of oxidative stress would be higher and the antioxidant reserves would be lower in dogs with IMHA, compared with those values in healthy control dogs. The purpose of the study reported here was to compare plasma malondialdehyde (MDA; a marker of lipid peroxidation) and serum vitamin E concentrations in dogs with IMHA with those of healthy control dogs.

Materials and Methods

Animals—All dogs with IMHA evaluated at the Tufts University Foster Hospital for Small Animals were eligible if they met the study criteria. A CBC was performed on all dogs and included examination of a blood smear to assess RBC morphology. Study criteria included the presence of anemia (Hct < 30%) and a positive result of a Coombs' test or autoagglutination of RBCs.^{4,6} Dogs that had received immunosuppressive medications for > 24 hours before evaluation, a transfusion with blood or oxygen-carrying hemoglobin solution⁵ within the preceding 2 weeks, or iron or antioxidant supplements within the preceding 4 weeks or that had evidence of any underlying disease were excluded from the study. Dogs that met the inclusion criteria are referred to as the IMHA group.

Control dogs were owned by hospital faculty, staff, and students. All control dogs were considered to be clinically normal on the basis of results of physical examination and CBC and had an absence of underlying diseases. Dogs that had received iron or antioxidant supplements within 4 weeks of the study were excluded. The study was approved by the Tufts University Institutional Animal Care and Use Committee, and owners of all dogs signed an informed consent form.

Study design—For dogs with IMHA, blood was collected within 4 hours of admission. Serum and plasma (with EDTA as an anticoagulant) were separated within 30 minutes

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of collection, and samples were stored at -70°C until analysis. Notation was made if the plasma or serum had visible hemolysis. Plasma MDA concentration was determined by use of a commercial colorimetric assay^b; to assess serum vitamin E concentration, α -, γ -, and δ -tocopherol concentrations were determined by use of high-performance liquid chromatography.

Statistical analyses—Data are reported as mean \pm SD or as median (range) values. Data that were not normally distributed were transformed. For the IMHA-affected dogs and control dogs, categorical data were compared by use of χ^2 analyses. Continuous variables were compared between the 2 groups by use of independent *t* tests or a Mann-Whitney *U* test if the data could not be normalized. Comparison between 2 continuous variables was performed by use of Pearson correlation tests. Multiple regression analysis was performed to test for the effects of age, hemolysis, and group on MDA concentrations. All analyses were performed with commercial statistical software,^c and a *P* value of < 0.05 was considered significant.

Results

Thirty-six dogs with IMHA and 40 healthy control dogs were enrolled in the study. More female dogs than

male dogs were included in the IMHA group, but there was no difference ($P = 0.343$) in the ratio of males to females between the IMHA (27 females and 9 males) and control groups (26 females and 14 males). The IMHA group was significantly ($P < 0.001$) older (7.9 ± 2.5 years) than the control group (5.5 ± 2.7 years). The most common breeds represented in the IMHA group included Cocker Spaniel ($n = 8$; 2 English and 6 American), mixed breed (5), Beagle (3), Shih Tzu (2), Dalmatian (2), Poodle (2), and Boxer (2). The mean Hct of the IMHA group ($17.9 \pm 5.6\%$) was significantly ($P < 0.001$) lower than that of the control group ($47.8 \pm 5.0\%$). Among the IMHA-affected dogs, other abnormalities included autoagglutination (32/36 dogs), positive result of a Coombs' test (21/27; 9 dogs were not tested), and spherocytes (19/36).

Plasma MDA concentrations were significantly ($P < 0.001$) higher in the IMHA group, compared with the value in the control group (Figure 1). Plasma MDA concentrations were significantly ($P = 0.004$) correlated with age when both groups were analyzed ($r = 0.376$). However, there was no significant correlation between plasma MDA concentration and age for the IMHA group alone ($r = 0.068$; $P = 0.718$) or the control group alone ($r = -0.120$; $P = 0.552$). Because hemolysis might alter the performance of the colorimetric assay, plasma samples that were hemolyzed ($n = 22$) were compared to those that were not hemolyzed (54). Hemolysis was significantly ($P < 0.001$) more likely to occur in plasma samples obtained from dogs in the IMHA group (18/36 dogs) than in samples obtained from dogs in the control group (4/40). Hemolyzed plasma samples had a significantly ($P < 0.001$) higher MDA concentration, compared with that of nonhemolyzed samples. However, results of multiple regression analysis including group (IMHA or control), age, and hemolysis indicated that group ($P < 0.001$) and hemolysis ($P < 0.001$) were significant predictors of plasma MDA concentrations.

Compared with values in the control dogs, serum α -, γ -, and δ -tocopherol concentrations all were significantly ($P < 0.001$, 0.006, and 0.001, respectively) lower in the IMHA-affected dogs (Figure 2). Age was significantly correlated with serum concentrations of α -tocopherol ($r = -0.433$; $P < 0.001$) and γ -tocopherol ($r = -0.320$; $P = 0.022$) but not with serum δ -tocopherol concentration ($r = -0.143$; $P = 0.275$). Hemolysis was associated only with serum α -toco-

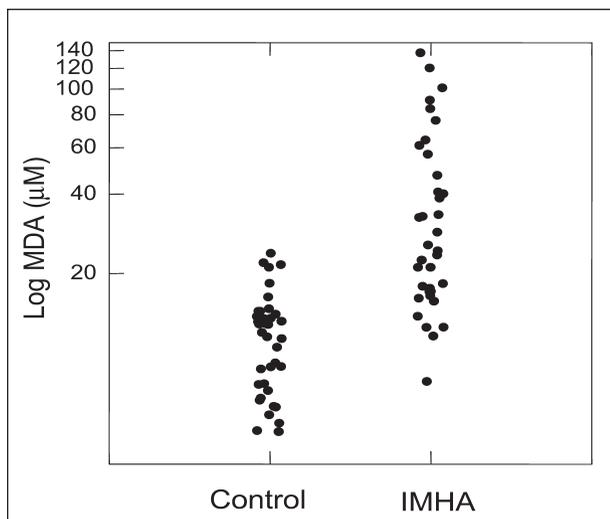


Figure 1—Plasma malondialdehyde (MDA) concentrations (log scale) in 36 dogs with immune-mediated hemolytic anemia (IMHA) and 40 healthy control dogs. Median plasma MDA concentration was significantly ($P < 0.001$) higher in the IMHA-affected dogs than it was in the control group.

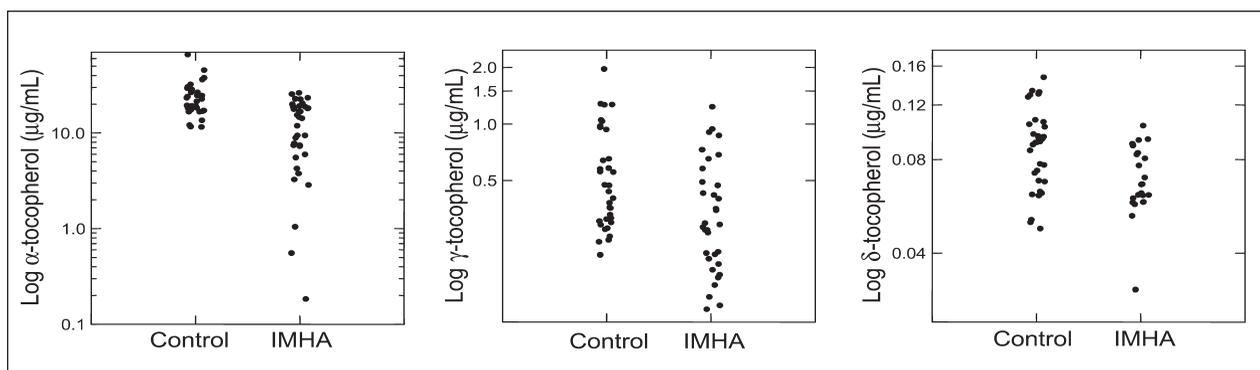


Figure 2—Serum α -, γ -, and δ -tocopherol concentrations (log scale) in 36 dogs with IMHA and 40 healthy control dogs. Median serum α -, γ -, and δ -tocopherol concentrations were significantly ($P < 0.001$, 0.006, and 0.001, respectively) lower in the IMHA group, compared with values in the control group.

pherol concentrations ($P = 0.004$). Results of multivariate analysis indicated that only group (IMHA or control) was significantly associated with α - ($P = 0.006$), γ - ($P = 0.006$) and δ - ($P = 0.002$) tocopherol concentrations.

In the dogs with IMHA, there was a significant ($P < 0.001$) negative correlation between plasma MDA and serum α -tocopherol concentrations ($r = -0.655$). A significant relationship was not found between plasma MDA concentration and any of the serum tocopherol concentrations in the control dogs. In the IMHA group, there was no relationship between plasma MDA concentration and Hct, autoagglutination of RBCs, presence of spherocytes, or a positive result of Coombs' test; similarly, there was no relationship between any of the serum tocopherol concentrations and those variables.

Discussion

In our study, the evaluation of plasma MDA concentration as a marker of lipid peroxidation revealed that oxidation was significantly higher in the dogs with IMHA than it was in healthy control dogs. This finding is similar to data obtained from human studies, which indicated that median plasma MDA concentration was high in humans with anemia secondary to β -thalassemia major¹² and in individuals infected with hepatitis C virus who were treated with ribavirin (for which the major adverse effect is reversible hemolytic anemia).¹¹ However, results of 1 study¹⁰ in humans did not indicate a difference in plasma MDA concentration between humans with and without sickle cell anemia. In addition to differences in plasma MDA concentration between dogs with IMHA and control dogs in our study, serum α -, γ -, and δ -tocopherol concentrations were significantly lower in the IMHA group. These findings and the negative correlation between plasma MDA and serum α -tocopherol concentrations suggest that an imbalance between oxidation and antioxidant protection may be associated with IMHA in dogs. Whether this imbalance plays a role in the pathophysiology of the disease or is merely a secondary finding is not known. Further studies would be needed to examine this relationship.

There were a number of limitations to our study. The serum concentration of only 1 antioxidant, vitamin E, was measured. Various enzymatic antioxidants and free radical scavengers are involved in limiting endogenous oxidant damage in dogs. In addition to financial considerations, the rationale for evaluating serum vitamin E concentration was that it is fat-soluble and thereby protects polyunsaturated fatty acids in membranes from oxidation. In addition, our group has data which indicate that dogs with cardiac disease have reduced serum vitamin E concentrations despite increased oxidant stress.¹⁵ Although serum concentrations of all 3 forms of tocopherol (α , γ , and δ) were lower in dogs with IMHA in our study, compared with values in control dogs, only serum α -tocopherol concentration was negatively correlated with plasma MDA concentrations in the IMHA group. This was not surprising because α -tocopherol has the highest antioxidant activity, followed by γ - and δ -tocopherol (in decreasing order of activity).¹⁶

Another limitation of the present study was that dogs in the IMHA group were older than the control dogs. This was a consequence of the difficulty enrolling older dogs with no health problems and that were receiving no nutritional supplements into the study. Although the significantly higher plasma MDA concentration in dogs with IMHA was independent of age statistically, this statistical correction for age differences is inferior to having age-matched groups. To ensure that any differences detected are the results of group differences alone, researchers in future studies should carefully match control dogs for age.

Finally, hemolysis of plasma samples was more common among those obtained from the IMHA group (as a result of the underlying disease) than it was among those obtained from the control group. This may have falsely increased the plasma MDA concentrations detected in IMHA-affected dogs (in fact, median plasma MDA concentration was significantly higher in the hemolyzed samples than in the nonhemolyzed samples). However, even if all hemolyzed plasma samples were excluded and the 36 nonhemolyzed samples obtained from the control dogs were compared with the 18 nonhemolyzed samples obtained from the IMHA-affected dogs, the differences in plasma MDA and all 3 serum tocopherol concentrations between the groups were still significant. Thus, although hemolysis may have falsely increased the plasma MDA concentration in some of the IMHA-affected dogs in the present study, the effect of group (ie, IMHA vs control) appeared to have an independent effect on plasma MDA concentration.

Without doubt, additional studies would be needed to determine the role of oxidant stress in the development of IMHA in dogs and whether the imbalance between oxidation and antioxidant protection may provide a potential target for new treatments.

^aOxyglobin, Biopure, Cambridge, Mass.

^bBioxytech LPO-586, R & D Systems Inc, Minneapolis, Minn.

^cSystat 10.0, SPSS, Chicago, Ill.

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