Effect of a bioflavonoid dietary supplement on acetaminophen-induced oxidative injury to feline erythrocytes

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Objective—To determine the effect of a commercial bioflavonoid antioxidant on acetaminophen-induced oxidative injury to feline erythrocytes.

Design—Randomized controlled study.

Animals—45 healthy age-matched cats.

Procedure—Cats were assigned to 3 experimental groups. Groups 1 and 3 received a bioflavonoid antioxidant (10 mg/d) orally for 2 weeks. Groups 2 and 3 received an oxidative challenge with acetaminophen (90 mg/kg [41 mg/lb] of body weight, PO) on day 7. Packed cell volume, percentage of erythrocytes with Heinz bodies, blood methemoglobin concentration, and blood reduced and oxidized glutathione concentrations were determined at various times during the 2-week study period.

Results—Adverse effects were not associated with bioflavonoid antioxidant administration alone. Acetaminophen administration resulted in a significant increase in methemoglobin concentration in groups 2 and 3; differences were not detected between these groups. Heinz body concentrations in groups 2 and 3 increased after acetaminophen administration; however, the increase in cats that received the antioxidant was significantly less than in group-2 cats. Total blood glutathione concentrations did not change significantly in groups 2 and 3 after acetaminophen administration; however, ratio of reduced to oxidized glutathione concentration increased significantly after administration in group-2 cats, compared with group-3 cats.

Conclusions and Clinical Relevance—Oral administration of bioflavonoid antioxidants to cats at risk for oxidative stress may have a beneficial effect on their ability to resist oxidative injury to erythrocytes. (*J Am Vet Med Assoc* 2000;217:1157–1161)

Flavonoids are compounds that occur ubiquitously throughout the plant kingdom and have received attention in recent years because of their antioxidant properties.[1-3] The antioxidant activity of flavonoids is reported to result from their ability to chelate metal ions, scavenge free radicals, and inhibit lipid peroxidation, hemolysis, and hemoglobin oxidation.[4-9]

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Erythrocytes are subject to oxidative injury when intracellular reducing pathways that use **reduced nicotinamide-adenine dinucleotide (NADH)**, **reduced nicotinamide-adenine dinucleotide phosphate (NADPH)**, and **reduced glutathione (GSH)** are insufficient to meet the oxidant challenge.[10] Production of methemoglobin is induced by oxidation of ferrous (Fe^{2+}) heme to ferric (Fe^{3+}) heme; methemoglobin is usually converted to functional hemoglobin by NADH-methemoglobin reductase and, to a lesser extent, by NADPH-methemoglobin reductase. The NADPH is also necessary for the glutathione reductase-catalyzed conversion of **oxidized glutathione (GSSG)** to GSH, which is essential in preventing erythrocyte hemoglobin oxidation.[10] Oxidized hemoglobin is denatured and causes microscopically visible aggregates (Heinz bodies) to attach to the internal membrane of erythrocytes, leading to decreased deformability and shorter erythrocyte life span.[11,12]

Feline hemoglobin is more susceptible to oxidative damage than that of other mammalian species[13]; this has been attributed to 8 reactive sulfhydryl groups on the hemoglobin molecule and the ready dissociation of hemoglobin from the tetramer to dimer form.[11] Therefore, cats represent a unique model in which to study the effects of dietary antioxidants.

Acetaminophen causes oxidative injury to feline erythrocytes.[14-16] Acetaminophen is metabolized by 3 major pathways in all species studied: sulfate conjugation, glucuronide conjugation, and via the cytochrome P450 oxidase system. Because cats have limited ability to form glucuronides, the sulfate conjugation pathway becomes saturated quickly, resulting in more reactive metabolites of acetaminophen.[16] Low erythrocyte GSH content, increased methemoglobin concentrations, and the formation of Heinz bodies have been reported in cats after acetaminophen administration as well as after exposure to other oxidants.[15,17-19]

The objective of the study reported here was to determine the effects of a bioflavonoid antioxidant dietary supplement on measurable oxidative injury to feline erythrocytes induced by acetaminophen. An ancillary objective was to determine whether administration of the antioxidant would alter GSH or GSSG concentrations in the blood of clinically normal cats. We hypothesized that administration of the bioflavonoid antioxidant would increase the ability of feline erythrocytes to resist oxidative damage, and methemoglobin concentrations and Heinz bodies would be decreased, whereas blood GSH concentrations would be increased. If effective, such a supplement might be a useful nutritional support for cats at risk for oxidative injury.

Materials and Methods

Cats—Forty-five 5- to 6-month-old cats of both sexes were obtained from Laboratory Animal Resources at Colorado State University and conditioned for at least 2 weeks. All cats were in good health, as determined by results of physical examinations and CBC. Cats were housed together and fed a commercial dry diet[a] and water ad libitum in accordance with guidelines established by the Colorado State University Animal Care and Use Committee.

Drug dosages—A commercially available bioflavonoid antioxidant[b] was administered orally (10 mg/cat/d) on the basis of the manufacturer's recommendations. A sublethal dose of acetaminophen (90 mg/kg [41 mg/lb] of body weight) that results in demonstrable erythrocyte abnormalities was chosen on the basis of previous reports.[16,20]

Experimental design—Age-matched cats of both sexes were obtained in groups of 15, which were studied consecutively. Group-1 cats received one 10-mg antioxidant capsule orally each morning from day 1 through day 14 of the study. This group did not receive an oxidative challenge and served as the control group. Group-2 cats received a single dose of acetaminophen (90 mg/kg) orally on day 7 and did not receive antioxidant. Group-3 cats received one 10-mg antioxidant capsule orally each morning from day 1 through day 14 and also received a single dose of acetaminophen (90 mg/kg) orally on day 7.

Cats were observed daily for abnormal clinical signs. Blood was collected via jugular venipuncture, using manual restraint, prior to initiation of the study and on days 7, 8, 10, 11, and 14. Baseline CBC, Heinz body count, and blood GSH and GSSG concentration were determined before day 1. Blood methemoglobin determination was performed on day 7 immediately prior to and 4 hours after acetaminophen administration in groups 2 and 3. Packed cell volume, Heinz body count, and GSH and GSSG determinations were performed on days 8, 10, 11, and 14 in all cats.

Hematologic analyses—Complete blood counts were performed on blood specimens that contained EDTA by use of an automated cell counter.[c] Packed cell volume was determined by use of microhematocrit centrifugation, and leukocyte differential count was determined by counting 100 cells on Wright-Giemsa-stained blood films. Erythrocytes that contained Heinz bodies were quantitated by counting 1,000 erythrocytes on brilliant cresyl blue-stained blood films; results were expressed as percentage values. To avoid confusion with punctate reticulocytes, only medium-sized to large Heinz bodies (≥ 1 µm in diameter) were counted. Methemoglobin concentration was determined in heparinized blood specimens by measuring the change in absorbance at 630 nm before and after addition of potassium cyanide.[d] Concentrations of GSH and GSSG in blood were determined by a dansyl chloride derivatization method[21] and analyzed by use of high-performance liquid chromatography,[e] using the procedure described by Fettman et al.[22] For this analysis, 1 ml of blood that contained EDTA was added to 1 ml of a 10% solution of perchloric acid, vortexed, and frozen at –20 C until assayed. Results (µmol per ml of blood) were adjusted for each cat's PCV and reported as µmol per ml of erythrocytes. Total blood glutathione (GSH + GSSG) concentration and the GSH:GSSG ratio were calculated.

Statistical analyses—Data were tested for normal distribution prior to analyses by use of statistical software packages.[f,g] Data for PCV, methemoglobin, and total blood glutathione were normally distributed, and group comparisons were made by use of repeated measures ANOVA. Data for Heinz bodies, GSH, GSSG, and GSH:GSSG were not normally distributed; therefore, repeated measures ANOVA was performed on ranked data. All multiple comparisons were performed by use of the method of Bonferroni and Dunn. Significance was assumed at $P \leq 0.05$ for all comparisons.

Results

All cats that received antioxidant alone (group 1) remained clinically healthy during the study period; total blood glutathione concentrations (mean ± SD) varied between 1.85 ± 0.34 and 2.39 ± 0.5 µmol/ml of RBC. Among group-2 and group-3 cats, transient cyanosis and lethargy were observed during the first 12 hours after administration of acetaminophen. Cats did not require medical intervention, and all were clinically normal within 24 hours.

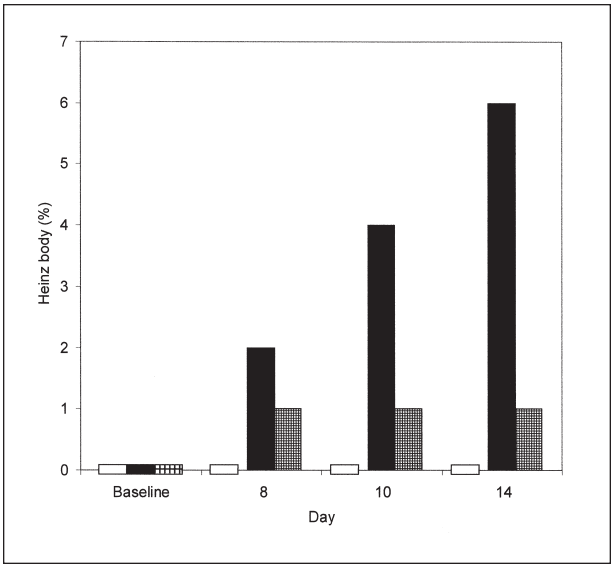

Figure 1—Median percentage of erythrocytes containing Heinz bodies in cats (n = 15/group) that received bioflavonoid antioxidant orally for 14 days (□), that did not receive antioxidant and were given acetaminophen on day 7 (■), or that received antioxidant orally for 14 days and were given acetaminophen on day 7 (⊞).

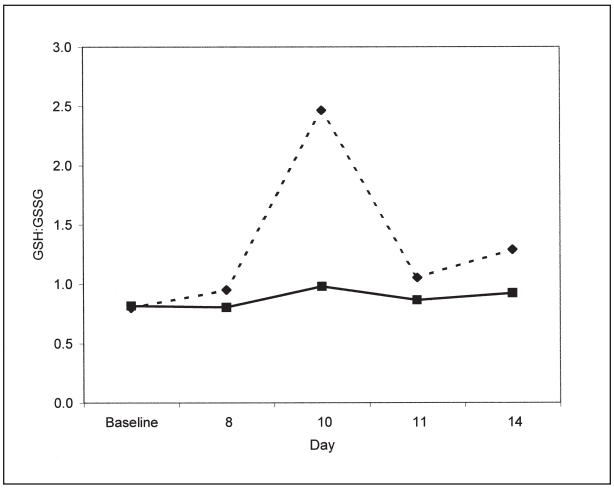

Figure 2—Median ratios of the concentrations of reduced (GSH) and oxidized (GSSG) glutathione in blood specimens of cats (n = 15/group) that did not receive antioxidant and were given acetaminophen on day 7 (dashed line) or that received oral administration of antioxidant for 14 days and were given acetaminophen on day 7 (solid line).

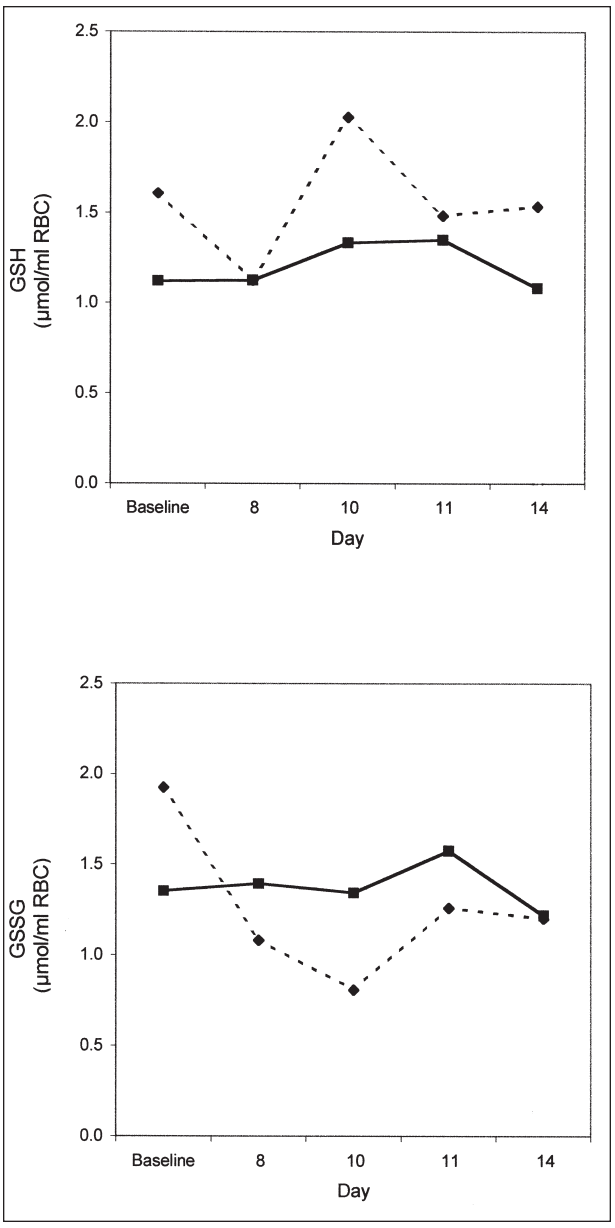

Figure 3—Median GSH (upper panel) and GSSG (lower panel) concentrations in blood specimens of cats (n = 15/group) that did not receive antioxidant and were given acetaminophen on day 7 (dashed line) or that received antioxidant orally for 14 days and were given acetaminophen on day 7 (solid line).

Methemoglobin concentration was significantly increased 4 hours after administration of acetaminophen ($P < 0.001$) in groups 2 and 3, compared with baseline values and group-1 cats that did not receive acetaminophen. There was no difference in magnitude of methemoglobin increase between groups 2 and 3. Four hours after administration of acetaminophen, methemoglobin concentrations in groups 2 and 3 were 25.2 ± 7.2% and 25.5 ± 7.1%, respectively, whereas baseline values were 0.71 ± 0.12% and 0.85 ± 0.16%, respectively. Methemoglobin concentrations in group-1 cats that did not receive acetaminophen remained at baseline values (0.6 ± 0.12%).

Acetaminophen administration resulted in a sustained significant ($P < 0.001$) increase in percentage of Heinz bodies in groups 2 and 3, compared with values in the control group (**Fig 1**). Heinz body percentage steadily increased in group-2 cats during the study period. Although Heinz body percentage also increased in group-3 cats, the magnitude of increase was significantly ($P = 0.018$) less than that of group-2 cats and did not continue to increase after day 8.

Packed cell volume of cats in all 3 groups declined slightly during the study period, but values were never below reference range for healthy cats. Differences among groups were not detected for magnitude of decrease of PCV. Decreases in mean PCV for groups 1, 2, and 3 were 2.1, 3.4, and 4.4%, respectively.

Total blood glutathione concentration among cats that received acetaminophen (groups 2 and 3) did not change significantly after challenge; however, blood GSH:GSSG was significantly ($P < 0.001$) increased in group-2 cats, compared with group-3 cats that received antioxidant (**Fig 2**). This increase in ratio peaked at day 10, 3 days after acetaminophen administration.

When evaluated individually, neither GSH nor GSSG concentration changed significantly in any group during the study. Median GSH concentrations in group-2 cats increased and median GSSG concentrations decreased after challenge with acetaminophen, although not significantly (**Fig 3**).

Discussion

Results of the study reported here indicate that administration of the antioxidant had a significant protective effect against Heinz body formation after acetaminophen challenge, although protection against methemoglobinemia was not achieved. The Heinz body percentages detected in our study were somewhat lower than those in cats with oxidant-induced injury in other reports.[17,18,23] This may be attributable to the fact that only medium-sized to large Heinz bodies (> 1 µm in diameter) were counted in our study or may be a reflection of the degree of oxidative injury induced by the dose of acetaminophen (90 mg/kg) used here. Although information is lacking about the effects of acetaminophen on juvenile cats such as the cats used in this study, results of studies in adult cats suggest that a medium dose of acetaminophen is 60 mg/kg (27 mg/lb), and a high dose is 120 mg/kg (54 mg/lb), which is commonly associated with severe clinical signs.[16,24]

The 90-mg/kg dose was effective in inducing oxidative erythrocyte injury, as evidenced by substantial methemoglobinemia in group-2 and group-3 cats 4 hours after administration of acetaminophen. In contrast to the effect on Heinz bodies, administration of antioxidant did not have a protective effect against methemoglobinemia. This suggests that there was no effect on NADH-methemoglobin reductase (the enzyme primarily responsible for the reduction of heme to its ferrous state), at least in the initial stages of methemoglobin formation. In retrospect, it would have been useful to examine differences between groups for the time required for methemoglobin concentrations to return to baseline values. Oxidation of hemoglobin to methemoglobin develops rapidly (within hours) after

oxidative insult, so it is possible that enzyme systems were overwhelmed after administration of acetaminophen despite administration of antioxidant.

Precipitation of oxidized hemoglobin into Heinz bodies has been associated with decreased erythrocyte GSH concentration and decreased erythrocyte survival in cats. In 1 study, cats with Heinz bodies had decreased erythrocyte GSH concentration and lower PCV.[23] However, low levels of oxidant stress have also been associated with increased erythrocyte GSH concentration,[17] and Heinz body percentages as high as 50% have been reported in cats after ingestion of onion powder, without any evidence of GSH depletion.[18]

In the study reported here, median blood GSH concentration in group-2 cats increased after acetaminophen challenge, whereas median blood GSSG concentration simultaneously decreased. This resulted in significantly increased median blood GSH:GSSG for group-2 cats, which did not receive antioxidant, compared with group-3 cats, which did receive antioxidant. Because there was no change in total blood glutathione concentration in either group, the increase in GSH:GSSG suggests an alteration in the glutathione redox cycle in favor of regenerating GSH. One explanation for this finding may be that administration of acetaminophen induced glutathione reductase activity in group-2 cats and thus increased GSH concentration. Alternatively, glutathione peroxidase may have been inactivated, decreasing GSSG concentration. Inactivation of glutathione peroxidase as a result of hemoglobin oxidation has been detected in human erythrocytes.[25] However, GSH concentrations observed in our study were not sufficient to prevent increased Heinz body formation. Cats in group 3 were apparently able to meet the oxidant challenge without altering their glutathione redox cycle, and Heinz body formation was minimal. Suppression of Heinz body formation in group-3 cats, without an increase in blood GSH concentration, suggests that the antioxidant effectively scavenged free radicals produced by the oxidant, thus precluding stimulation of the glutathione redox cycle. Free radical scavenging may have been sufficient to suppress Heinz body formation without preventing methemoglobinemia, because Heinz bodies form at a much slower rate than methemoglobin.

Increased GSH:GSSG observed in group-2 cats after oxidant challenge was unexpected but not without precedent. Alterations in this ratio may represent a response to oxidative injury in which enzymes of the glutathione redox cycle are induced, but the resulting increased enzyme activities may not be sufficient to afford complete protection. The interactions that occur as erythrocyte glutathione and enzyme systems act to protect the cell from oxidative injury are complex. Although GSH is usually thought of as protective, there are reports of dogs with hereditarily high GSH concentration in erythrocytes that are more susceptible to certain types of oxidants.[26,27] One study of human erythrocytes found that NADPH concentration, but not GSH concentration, correlated with the oxidant sensitivity of hemoglobin.[28] A study investigating species variations in the antioxidant capacity of erythrocytes was unable to predict the susceptibility of erythrocytes to oxidative stress on the basis of glutathione concentration or activity of antioxidant enzymes.[29] In humans with chronic renal disease, increased erythrocyte GSH concentration, which decreases after dialysis, is thought to be a compensatory protective mechanism against toxins associated with uremia.[30] Acceleration of the glutathione redox cycle, accomplished by stimulating either glutathione reductase, glutathione peroxidase, or both, protects rat myocytes from free radical-induced damage.[31] In that study, a vitamin E analogue accelerated the peroxidase reaction and led to lower GSH:GSSG, whereas NADPH supplied by glucose accelerated the reductase reaction and resulted in higher GSH:GSSG. The best protection was achieved by stimulation of both enzymes, thus activating the complete redox cycle.

Anything that affects glutathione synthesis or the enzymes of the glutathione redox cycle may affect the ability to respond to oxidant-induced injury. Drugs such as N-acetylcysteine and captopril increase GSH or GSSG concentration, respectively, with no stimulation of glutathione synthesis.[32] Dietary supplementation of cats with cysteine increases blood GSH and GSSG concentrations.[22] Many other therapeutic agents, including various bioflavonoids, are valuable for their ability to scavenge free radicals without acting directly on glutathione.[2,4,6,7,33]

Cats are uniquely sensitive to oxidative injury to erythrocytes, and Heinz body formation, as the result of exogenous oxidant exposure as well as endogenous disease processes such as lymphoma, diabetes mellitus, and hyperthyroidism, has been well-documented.[19,23,24,34] Dietary treatment that would minimize oxidative injury could be of benefit to sick cats and may be a useful protective supplement in healthy cats. Results of the study reported here suggest that daily oral supplementation of cats with a bioflavonoid antioxidant provides therapeutic and protective support, at least when supplementation begins prior to exogenous oxidative stress.

[a]Science Diet Feline Maintenance, Hill's Pet Nutrition Inc, Topeka, Kan.
[b]Proanthozone, Animal Health Options, Golden, Colo.
[c]Coulter Model S Plus IV, Coulter Electronics Inc, Hialeah, Fla.
[d]OSM 3 Hemoximeter, Radiometer America, Westlake, Ohio.
[e]Waters HPLC pumps and radial column, Millipore Corp, Milford, Mass.
[f]BMDP Statistical Software, Los Angeles, Calif.
[g]Statview Abacus Concepts Inc, Berkley, Calif.

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