Assessment of oxidative stress in leukocytes and granulocyte function following oral administration of a silibinin-phosphatidylcholine complex in cats

Craig B. Webb, PhD, DVM; Kelly W. McCord, DVM; David C. Twedt, DVM

Objective—To determine the effect of oral administration of a silibinin-phosphatidylcholine complex (SPC) on oxidative stress in leukocytes and granulocyte function in healthy cats.

Animals—10 purpose-bred adult cats.

Procedures—Cats were administered SPC (10 mg/kg/d) orally for 5 days; blood samples were collected prior to and immediately after the 5-day treatment period. Leukocytes were incubated with monochlorobimane for detection of reduced glutathione (GSH) via flow cytometry. Leukocytes were also incubated with dihydrorhodamine 123 and mixed with *Escherichia coli* conjugated to a fluorescent marker to measure *E coli* phagocytosis and the subsequent oxidative burst via flow cytometry. Activities of the antioxidant enzymes superoxide dismutase and glutathione peroxidase, along with the reduced glutathione-to-oxidized glutathione (GSH:GSSG) ratio and a measure of lipid peroxidation (malondialdehyde concentration [μmol/L of blood]), were measured spectrophotometrically.

Results—The mean fluorescence intensity (MFI), representing GSH content, increased significantly in feline lymphocytes and granulocytes following 5 days of oral administration of SPC. Mean ± SD lymphocyte MFI significantly increased from 27.8 ± 9.0 to 39.6 ± 6.7, and the granulocyte MFI increased from 508.6 ± 135.6 to 612.1 ± 122.9. Following 5 days of SPC administration, the percentage of phagocytic cells that were responding optimally significantly increased (from 37 ± 11.8% to 45 ± 17.5%). Other measures of oxidative stress did not change significantly.

Conclusions and Clinical Relevance—In cats, oral administration of supplemental SPC appears to increase granulocyte GSH content and phagocytic function, both of which would be potentially beneficial in cats with diseases associated with oxidative stress. (*Am J Vet Res* 2009;70:57–62)

The milk thistle extract silymarin, which is composed of multiple flavonolignans including the most active constituent silibinin (synonymous with silybin), is emerging as a treatment for a variety of diseases in human and veterinary patients.[1] One of silibinin's proposed mechanisms of action is as an antioxidant that increases intracellular reduced glutathione content.[2] It has recently been shown that oral administration of an SPC increases plasma silibinin concentrations in cats.[a] Because cats appear to be particularly susceptible to oxidative stress, administration of supplemental SPC may be a beneficial part of therapeutic regimens for a variety of diseases in this species. For example, cats with chronic liver disease have decreased concentrations of hepatic GSH.[3]

Received January 28, 2008.
Accepted March 18, 2008.
From the Department of Clinical Sciences, College of Veterinary Medicine and Biomedical Sciences, Colorado State University, Fort Collins, CO 80523.
Supported in part by Nutramax Laboratories (provision of the silibinin-phosphatidylcholine complex).
Submitted in abstract form for the 32nd Annual American College of Veterinary Internal Medicine Forum, San Antonio, Tex, June 2008.
Address correspondence to Dr. Webb.

Abbeviations

DHR	Dihydrorhodamine 123
GPx	Glutathione peroxidase enzyme
GSH	Reduced glutathione
GSSG	Oxidized glutathione
MCB	Monochlorobimane
MDA	Malondialdehyde
MFI	Mean fluorescence intensity
SOD	Superoxide dismutase
SPC	Silibinin-phosphatidylcholine complex

The production of reactive oxygen species by granulocytes (neutrophils and eosinophils) during an inflammatory reaction may contribute to the progression of disease.[4] Conversely, oxidative stress and intracellular GSH content may regulate aspects of neutrophil function such as apoptosis.[5] Neutrophil function appears decreased in several human diseases associated with oxidative stress, such as diabetes mellitus and renal failure, which are also common diseases in the feline population.[6,7]

The purpose of the study reported here was to determine the effect of oral administration of an SPC on

oxidative stress and granulocyte function in healthy cats. Phosphatidylcholine was used to increase the bioavailability of silibinin but may also have impacted the measured variables.

Materials and Methods

Cats—Ten purpose-bred adult cats[b] were used in the study. All 6 neutered males and 4 sexually intact females had the same birth date and were 13 months old at the time of the study. Mean weight of the cats was 5.1 ± 1.2 kg (range, 3.4 to 5.7 kg). No abnormalities were identified in any of the cats via physical examination, and rectal temperature, heart rate and rhythm, and respiratory rate were within reference ranges. For each cat, results of a CBC indicated no remarkable abnormalities; serum biochemical analyses were not performed. The cats were housed as a group at the Colorado State University Veterinary Medical Center for the duration of the study, and all conditions and procedures were in accordance with the Colorado State University Animal Care and Use Committee guidelines and approved by that committee. All cats had ad libitum access to dry adult maintenance food that contained ≥ 56.2% protein and ≥ 7.8% fat and ≤ 4.8% crude fiber. There was no milk thistle or milk thistle extracts in the diet.

Sample acquisition—Prior to the first oral dose of the SPC in 5 cats, food was withheld from the cats for 12 hours and a central IV catheter was placed. The catheter was maintained for 12 hours during which time 7 blood samples (4 mL each) were collected for a separate pharmacokinetic bioavailability study. The catheters were then removed. A 7-day interval was allowed to elapse prior to the start of the investigation of the effects of oral administration.

On day 1, all 10 cats were administered the SPC[c] (10 mg/kg) combined with cornstarch (31% silibinin) as a capsule. Capsule administration was followed by oral administration of 3 mL of water to ensure passage of the capsule through the esophagus. Identical capsules containing SPC were administered once daily for 5 days. For purposes of the study, a blood sample (6 mL) was collected from a jugular vein of each cat by use of a standard venipuncture technique 1 day prior to administration of the first SPC capsule (ie, day 0) and approximately 2 hours after administration of the final SPC capsule on day 5.

Sample preparation—Each peripheral blood sample was divided into a tube containing EDTA (2 mL) for a CBC and a tube containing heparin (3 mL) for granulocyte function and leukocyte oxidative stress assays. The samples for the oxidative stress assays were prepared immediately following collection and stored at –70°C; these assays were run as a single batch within 25 days. The samples for the granulocyte function assays were immediately stored on ice, and the assays were completed within 6 hours.

CBC procedure—Complete blood cell counts were performed by use of an automated hematology system.[d] Plasma protein concentration was determined by use of a refractometer. White blood cell differentials (100 cell counts) and morphology assessments were performed manually by personnel of the university's Clinical Pathology Service. The absolute number of neutrophils reported as part of the CBC was used for calculation of the *Escherichia coli*-to-neutrophil ratio for the phagocytosis assay. Total numbers of leukocytes, neutrophils, and eosinophils were also recorded.

Assessment of GSH concentration via flow cytometry—Reduced glutathione is a ubiquitous intracellular thiol-containing antioxidant. The intracellular conjugation of MCB and GSH produces a fluorescent product that can be detected via flow cytometry and allows quantification of GSH concentrations in specific lymphocyte subsets.[8] Results of a previous study[8] have indicated a linear relationship (determined by use of a glutathione reductase method) between the fluorescence of MCB-stained HeLa cells and their GSH content. Erythrocytes obtained from 500 µL of heparinized whole blood were lysed with NH_4Cl, and the remaining leukocyte suspension was washed twice in Hanks balanced salt solution[e] and resuspended in fluorescent-activated cell sorter buffer (PBS solution with 2% fetal bovine serum and 0.1% sodium azide). Monochlorobimane[f] was added to a leukocyte suspension (1×10^7 cells/µL) to a final concentration of 20µM, and the cells were maintained at room temperature (approx 20°C) in the dark for 20 minutes prior to analysis. Monochlorobimane fluorescence was assessed with a 3-laser flow cytometer.[g] Ultraviolet laser excitation is required to excite MCB fluorescence. The laser was allowed to warm up for a minimum of 1 hour prior to use, and beads[h] were used to calibrate the photomultiplier tube voltage and gain prior to each session. The distinct forward-angle versus side-angle light scatter pattern produced by cat leukocytes was used to create an appropriate gating paradigm, allowing for the separate analysis of granulocytes, lymphocytes, and monocytes, as described previously.[9,10] Data were analyzed by use of commercially available software.[i]

Assessments of granulocyte phagocytosis and oxidative burst—The flow cytometric gating scheme used did not distinguish eosinophils from neutrophils. It was assumed that eosinophils contributed little if any activity to the assay of *E coli* phagocytosis, but eosinophils are capable of producing superoxide anion, which may have contributed to the oxidative burst measurements.[11]

Intracellular reactive oxygen species convert the nonfluorescent compound DHR[f] to a green fluorescent molecule rhodamine 123, which can be measured via flow cytometry.[12,13] From a working stock of 0.05mM DHR, 10 µL was added to 100 µL of heparin-treated whole blood (final concentration, 5µM DHR) and incubated for 45 minutes at 37°C.

By use of an intracellular fluorophore (Alexa Fluor 488[f]), the fluorescence generated indicates *E coli* that has undergone phagocytosis. The corresponding increase in free radical production is represented by the increase in intracellular DHR fluorescence. Back-gating from the *E coli* signal allows identification of the specific DHR fluorescence associated with a particular population of intracellular *E coli*. The fluorescence is recorded as an increase in the specific cell population's MFI. Lyophilized *E coli* (strain K 12) conjugated to Alexa Fluor 488 was reconstituted with 100 µL of 2mM

sodium azide–PBS solution to produce a working concentration of 6×10^8 *E coli* particles/100 µL. *Escherichia coli* was added to the DHR-treated sample for the final 30 minutes of incubation in the proportion of 30 *E coli*/neutrophil. Immediately following incubation, erythrocytes were lysed with NH_4Cl (achieved via a 5-minute incubation period at room temperature followed by 2 washes in Hanks balanced salt solution) and the leukocyte pellet resuspended in fluorescence-activated cell sorter buffer. Ten microliters of trypan blue was added to this cell suspension to quench extracellular fluorescence (ie, that contributed by *E coli* that were adhered to the cell surface but had not undergone phagocytosis). The cell suspension was then analyzed immediately for phagocytic and oxidative burst activity via flow cytometry. Control samples (a leukocyte suspension with no additions, a suspension treated with DHR only, and a suspension treated with *E coli* only) were prepared in the same manner as described.

Assays of oxidative stress in leukocytes—The SOD enzyme catalyzes the reaction that converts the superoxide free radical to hydrogen peroxide. Glutathione peroxidase catalyzes the reaction that converts hydrogen peroxide to water. Commercially available colorimetric kits for the measurements of erythrocyte SOD and whole blood GPx activities were previously validated for use in domestic cats[14,j]; intra- and interassay coefficients of variation were determined (both approx 10% for each assay), and it was revealed that freezing samples at –80°C for 28 days did not affect results. The lower limits of detection for the SOD and GPx assays were 0.1 U/mL and 5.6 mU/mL, respectively.

Malondialdehyde is an end-product of free radical–induced cell membrane lipid peroxidation. Samples of heparinized whole blood (250 µL) were each transferred to a tube containing 2 µL of EDTA and stored at –70°C until analysis. Each whole blood sample was thawed and pipetted into a solution of 0.9mM butylated hydroxytoluene in absolute alcohol. A 0.03mM solution of thiobarbituric acid in 50% glacial acetic acid was added, and the resulting solution was heated for approximately 1 hour. The reaction mixture was cooled and acidified prior to addition of n-butanol. The MDA was extracted into the n-butanol, and the fluorescence emission at 550 nm was compared with that of standardized solutions to determine the number of micromoles of MDA per liter of whole blood. The lower limit of detection for the MDA assay was 0.5µM, and samples can be stored at –20°C for as long as 35 days before analysis without affecting assay results.[15]

Glutathione was quantified from EDTA-preserved blood by use of an enzymatic assay.[16] The assay specifications indicate linearity to a sample concentration of 2,400µM GSH, recovery of 98%, lower limit of detection (sensitivity) of 0.54µM, and no interfering effects associated with tested analogs (specificity). For oxidized disulfide glutathione, a thiol-scavenging reagent (1-methyl-2-vinyl-pyridium trifluoromethane sulfonate) is added to rapidly scavenge GSH and eliminate continued oxidation of GSH to GSSG after sample acquisition. Glutathione reductase, the reduced form of nicotinamide adenine dinucleotide, and the chromagen 5,5-dithiobis-(2-nitrobenzoic acid) were added sequentially to the sample, and the change in absorbance at 412 nm was measured spectrophotometrically. Total glutathione (GSH and GSSG) concentration was quantified in EDTA-treated blood samples without the addition of 1-methyl-2-vinyl-pyridium trifluoromethane sulfonate. Samples can be stored at –70°C for as long as 15 days before analysis without affecting assay results.[f]

Statistical analysis—Following confirmation of a Gaussian distribution by use of the Kolmogorov-Smirnov test, the mean values for MFI, percent of phagocytic cells responding optimally and suboptimally, antioxidant enzyme activity, GSH:GSSG ratio, and MDA concentration were assessed for significant differences by use of a paired, 2-tailed Student *t* test. A value of $P < 0.05$ was considered significant for all statistical analyses performed in this study. Results are presented as mean ± SD where appropriate.

Results

All of the cats tolerated capsule administration on each of the 5 days. None of the cats developed any treatment-related adverse effects during the study period.

Leukocyte GSH concentration determined via flow cytometry—The fluorescence generated by the intra-

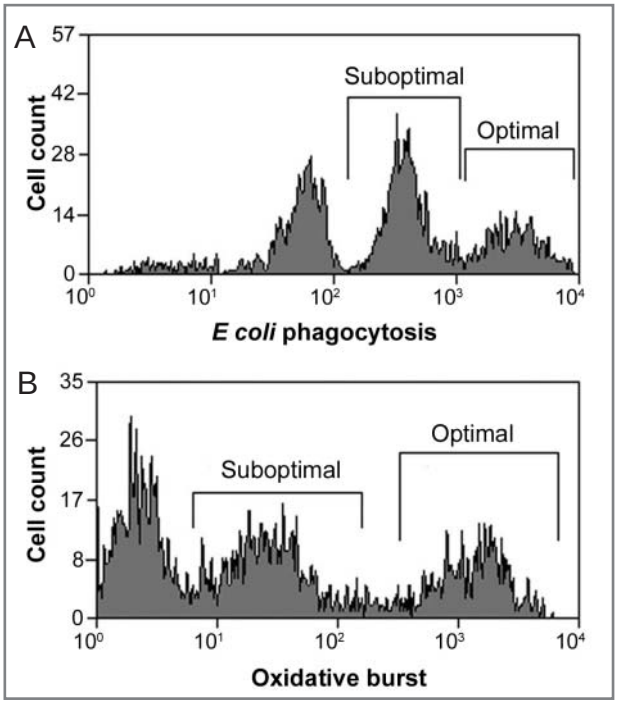

Figure 1—Function (determined via flow cytometry) of granulocytes obtained from 10 healthy cats after oral administration of an SPC (10 mg/kg/d) for 5 days. Fluorometric assessments of *Escherichia coli* phagocytosis (A) and oxidative burst (B) were used to evaluate granulocyte function. The MFI of Alexa 488 represented phagocytized intracellular *E coli,* and the corresponding increase in free radical production was represented by the increase in DHR fluorescence. Cell count represents the number of cells. Notice that there are 2 distinct populations of phagocytic cells; a population with a greater intracellular *E coli* MFI and a correspondingly greater DHR signal (representing cells with an optimal response) and a population with a lesser *E coli* MFI and a correspondingly smaller DHR signal (representing cells with a suboptimal response).

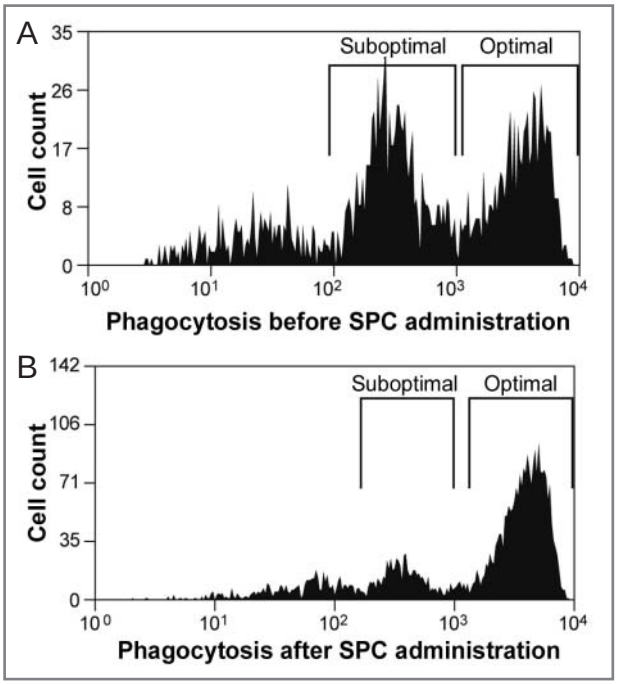

Figure 2—Phagocytosis of *E coli* by granulocytes obtained from 1 of 10 healthy cats before (A) and after (B) oral administration of an SPC (10 mg/kg/d) for 5 days. The MFI of Alexa 488 represented phagocytized intracellular *E coli*. Cell count represents the number of cells. In this cat, 42% and 43% of granulocytes had optimal and suboptimal responses, respectively, prior to administration of SPC; following treatment with the SPC for 5 days, 67% and 19% of granulocytes had optimal and suboptimal responses, respectively.

cellular conjugation of MBC with GSH was assessed as the MFI for distinct populations of leukocyte subsets. Compared with values at day 0, the MFI increased significantly in lymphocytes and granulocytes following 5 days of oral administration of SPC; lymphocyte MFI increased from 27.8 ± 9.0 to 39.6 ± 6.7 ($P = 0.001$), and granulocyte MFI increased from 508.6 ± 135.6 to 612.1 ± 122.9 ($P = 0.009$). The monocyte MFI did not change significantly (576.0 ± 160.6 at day 0 and 562.1 ± 140.3 at day 5 [$P = 0.067$]).

Granulocyte function determined via flow cytometry—Although eosinophils are not phagocytic cells, they generate superoxide anions and may contribute to the oxidative burst activity recorded by this flow cytometry assay.[16] From CBC data obtained from the study cats, the mean number of leukocytes, neutrophils, or eosinophils prior to SPC administration did not differ from the number of those cells after the final dose of SPC. At days 0 and 5, the mean number of lymphocytes was 7.1 ± 3.2 × 10^3/µL and 7.4 ± 2.2 × 10^3/µL, respectively; mean number of neutrophils was 4.3 ± 1.6 × 10^3/µL and 4.0 ± 1.3 × 10^3/µL, respectively; and the mean number of eosinophils was 0.47 ± 0.32 × 10^3/µL and 0.57 ± 0.47 × 10^3/µL, respectively. Neutrophils accounted for 61% to 54% of the total leukocyte count, whereas eosinophils accounted for 6.6% to 8.6% of the total leukocyte count.

Two distinct populations of phagocytic cells were identified: a population with a greater intracellular *E coli* MFI and correspondingly greater DHR signal (termed the optimal-response cell group) and a population with a lesser *E coli* MFI and correspondingly lesser DHR signal (termed the suboptimal-response cell group; **Figure 1**). Following 5 days of oral SPC administration, there was a significant ($P = 0.049$) increase in the percentage of phagocytic cells that were responding optimally (37 ± 11.8% at day 0 to 45 ± 17.5% at day 5; **Figure 2**). The percentage of phagocytic cells that were performing suboptimally did not change significantly (27.6 ± 8.1% at day 0 to 22.8 ± 4.7% at day 5; $P = 0.18$).

Oxidative stress assays—To assess the oxidative stress in leukocytes, SOD activity, GPx activity, GSH:GSSG concentration ratio, and MDA concentration were evaluated. In the study cats, values before and after administration of SPC for 5 days did not change significantly. At days 0 and 5, SOD activity was 168.55 ± 25.21 U/mL and 157.78 ± 39.4 U/mL, respectively ($P = 0.48$); GPx activity was 19,730 ± 3,949 U/L and 22,675 ± 4,215 U/L, respectively ($P = 0.14$); GSH:GSSG concentration ratio was 228.9 ± 118.4 and 204.9 ± 181.8, respectively ($P = 0.74$); and MDA concentration was 20.36 ± 7.2 µmol/L and 18.18 ± 4.3 µmol/L, respectively ($P = 0.26$).

Discussion

In the present study, oral administration of SPC for 5 days resulted in an increase in granulocyte GSH content and an increase in granulocyte phagocytosis of *E coli* in healthy cats. Silibinin, the most active flavonoid from the milk thistle extract silymarin, is frequently administered as a nutraceutical to animals with a variety of diseases. One proposed mechanism of action of silibinin is that it increases the intracellular concentration of the endogenous antioxidant GSH.[2] Glutathione is the predominant low–molecular-weight thiol in mammalian cells, which acts as a reducing agent, an antioxidant free radical scavenger, and a key component of the cellular thiol-disulfide redox state.[17] In a study[18] of 74 humans with chronic diseases (including gastrointestinal tract and cardiovascular diseases and cancer), > 36% of patients had decreased concentrations of GSH, compared with findings in healthy individuals. Decreased antioxidant defenses and GSH depletion may increase the susceptibility of humans with diabetes mellitus to oxidative injury, and GSH concentrations are reduced in the mononuclear cells of humans with hepatic cirrhosis.[19–21] In cats, glutathione is particularly important as an endogenous antioxidant; the concentration of glutathione is significantly decreased in the livers of cats with naturally occurring hepatic disease, compared with the value in the livers of healthy cats.[3]

The increase in intracellular GSH content of lymphocytes and granulocytes following administration of SPC for 5 days in cats perhaps reflects 1 mechanism of action through which silibinin exerts its antioxidant properties. Neutrophils produce free radicals as part of their oxidative burst activity; that free radical production can have cellular, local, and systemic consequences. The increase in granulocyte GSH content following SPC administration may reflect a predisposition for those particular leukocytes to readily incorporate antioxidants.[22,23] The increase in the GSH content of lym-

phocytes (nonphagocytic cells) and the lack of significant increase in GSH content of monocytes (cells that are capable of phagocytosis) as a result of SPC administration are more difficult to explain, but these findings emphasize the differences in responses of subsets of cells to treatment with antioxidants and likely to oxidative stress. Although global measures of oxidative stress (eg, urine concentrations of isoprostanes) provide important information, an advantage of flow cytometry is that it can be used to examine individual cell types.[24] It would be particularly interesting to investigate the GSH content of different types of feline liver cells (eg, hepatocytes vs Kupffer cells) in response to administration of silibinin.

Results of the present study indicated that the GSH contents of granulocytes and monocytes were quite similar and that these values were markedly different from the GSH content of lymphocytes. These findings are similar to those of other studies[8,10] in which glutathione concentrations in feline or human leukocytes were investigated. Via flow cytometry, neutrophil subpopulations have been identified on the basis of differential binding and ingestion of zymosan particles.[25] Although the gating scheme used in the present study did not distinguish between neutrophils and eosinophils and activated eosinophils are known to produce superoxide anion, it is assumed that neutrophils are responsible for the phagocytosis of *E coli* measured in the assay. This assay identified 3 subpopulations of neutrophils in healthy cats in our investigation: a subset that did not appear to ingest any bacteria, a subset that responded optimally under these conditions, and a subset that responded suboptimally. The corresponding increase in granulocyte GSH content and increase in the percentage of phagocytic cells performing optimally would imply that intracellular GSH concentration is related to the increase in phagocytic function. If an increase in GSH concentration acted as a buffer that neutralized intracellular free radicals, then perhaps individual neutrophils were able to deal with greater numbers of bacteria. The converse is that neutrophils may be less able to kill bacteria once they are internalized because of truncated burst activity. Results of a bacterial killing assay performed on blood samples collected from cats before and after administration of SPC or GSH depletion would address this question. However, the increase in GSH content of nonphagocytic cells (lymphocytes) and the absence of a significant increase in a separate population of leukocytes that are able to perform phagocytosis (ie, monocytes) argues against a causal connection between GSH content and phagocytic function. The ongoing development of cell-specific fluorescent markers for neutrophils will further the ability to define differences in the behavior of various types of leukocytes.

In the cats of the present study, oral administration of an SPC daily for 5 days failed to significantly change the measured indicators of oxidative stress. One possible explanation is that the large SDs associated with those values and small number of cats available for the study resulted in insufficient power to identify significant differences in the oxidative stress measures. Another explanation is that a 5-day treatment period was not sufficient for changes to develop, and monitoring these oxidative stress variables over an extended treatment period appears warranted. It may be important that the cats included in the present study were healthy and undergoing no apparent oxidative stress. Perhaps if the balance between endogenous oxidants and antioxidants is not challenged by disease, cells are unlikely to incorporate exogenous antioxidants and disrupt their redox homeostasis. Finally, it is possible that significant changes in the oxidative stress variables did develop but only in certain cell populations. Changes that only occurred in a relatively small number of leukocytes could go undetected by assays that measured variables in whole blood, wherein the number of erythrocytes is comparatively greater by several orders of magnitude. Evaluation of these variables in leukocytes, hepatocytes, or other cell populations that are likely undergoing oxidative stress in cats with naturally occurring disease might reveal processes amenable to antioxidant intervention. Another limitation of the present study was the lack of a placebo control group. However, assignment of some of the study cats to such a group would have reduced the number of cats in the treatment group; thus, paired comparisons of variables before and after SPC administration in a single group of cats were made with the assumption that oxidative stress measures and granulocyte function would not change significantly over a 5-day period of placebo administration.

In cats, the bioavailability of silibinin is increased when it is administered as an SPC. It is possible that the phosphatidylcholine itself had some effect on the variables measured in our study. Neutrophils can produce phosphatidylcholine oxidation products during phagocytosis.[26] In a study[27] in humans, dietary supplementation with linoleic acid as soy phosphatidylcholine for 3 days resulted in a 2-fold increase in the percentage of polymorphonuclear leukocyte killing of *Candida albicans*, presumably as a result of a change in the membrane lipid composition of those leukocytes. Administration of soybean phosphatidylcholine for 2 weeks resulted in a slight but significant increase in human neutrophil superoxide production, but it was concluded that changing neutrophil function through manipulation of cell membrane composition was difficult at best.[28] There does not appear to be much direct evidence for an increase in leukocyte GSH content following administration of supplemental phosphatidylcholine, although it has been suggested that citicoline, a phosphatidylcholine intermediate, increases glutathione content in gerbils with experimentally induced transient cerebral ischemia.[29] Determination of the impact of each of the individual components in a complex has clinical relevance when they are used independently.

a. Webb CB, Gustafson D, Twedt DC. Bioavailability following oral administration of a silibinin-phosphatidylcholine complex in cats (abstr), in *Proceedings*. Am Coll Vet Intern Med Forum 2008;22:812.
b. Cedar River Laboratories, Mason City, Iowa.
c. Nutramax Laboratories Inc, Edgewood, Md.
d. Siemens Healthcare Diagnostics, Deerfield, Ill.
e. Sigma-Aldrich, St Louis, Mo.
f. Invitrogen, Chicago, Ill.
g. DakoCytomation, Fort Collins, Colo.
h. SpectraAlign Beads, DakoCytomation, Fort Collins, Colo.

i. GraphPad, Prism Software, San Diego, Calif.
j. Randox Laboratories Ltd, Oceanside, Calif.

References

1. Gazák R, Walterová D, Kren V. Silybin and silymarin—new and emerging applications in medicine. *Curr Med Chem* 2007;14:315–338.
2. Garrido A, Arancibia A, Campos R, et al. Acetaminophen does not induce oxidative stress in isolated rat hepatocytes: its probable antioxidant effects is potentiated by the flavinoid silybin. *Pharmacol Toxicol* 1991;69:9–12.
3. Center SA, Warner KL, Erb HN. Liver glutathione concentrations in dogs and cats with naturally occurring liver disease. *Am J Vet Res* 2002;63:1187–1197.
4. Melley DD, Evans TW, Quinlan GJ. Redox regulation of neutrophil apoptosis and the systemic inflammatory response syndrome. *Clin Sci (Lond)* 2005;108:413–424.
5. Watson RW. Redox regulation of neutrophil apoptosis. *Antioxid Redox Signal* 2002;4:97–104.
6. Chonchol M. Neutrophil dysfunction and infection risk in end-stage renal disease. *Semin Dial* 2006;19:291–296.
7. Calvet HM, Yoshikawa TT. Infections in diabetes. *Infect Dis Clin North Am* 2001;15:407–421.
8. Scott RB, Collins JM, Matin S, et al. Simultaneous measurement of neutrophil, lymphocyte, and monocyte glutathione by flow cytometry. *J Clin Lab Anal* 1990;4:324–327.
9. Byrne KM, Kim HW, Chew BP, et al. A standardized gating technique for the generation of flow cytometry data for normal canine and normal feline blood lymphocytes. *Vet Immunol Immunopathol* 2000;73:167–182.
10. Webb C, Bedwell C, Guth A, et al. Use of flow cytometry and monochlorobimane to quantitate intracellular glutathione concentrations in feline leukocytes. *Vet Immunol Immunopathol* 2006;112:129–140.
11. Someya A, Nishijima K, Nunoi H, et al. Study on the superoxide-producing enzyme of eosinophils and neutrophils-comparison of the NADPH oxidase components. *Arch Biochem Biophys* 1997;345:207–213.
12. Walrand S, Valeix S, Rodriguez C, et al. Flow cytometry study of polymorphonuclear neturophil oxidative burst: a comparison of three fluorescent probes. *Clin Chim Acta* 2003;331:103–110.
13. Kampen AH, Tollersrud T, Lund A. Flow cytometric measurement of neutrophil respiratory burst in whole bovine blood using live *Staphylococcus aureus*. *J Immunol Methods* 2004;289:47–55.
14. Marshall MD, Samantha LW, Skinner ND, et al. Feline antioxidant enzyme activity: the effect of sample storage on stability. *J Nutr* 2002;132:1733S–1734S.
15. Wasowicz W, Neve J, Peretz A. Optimized steps in fluorometric determination of thiobarbituric acid-reactive substances in serum: importance of extraction pH and influence of sample preservation and storage. *Clin Chem* 1993;39:2522–2526.
16. Tietze F. Enzymatic method for quantitative determination of nanogram amounts of total and oxidized glutathione: applications to mammalian blood and other tissues. *Anal Biochem* 1969;27:505–522.
17. Jones DP. Redox potential of GSH/GSSG couple: assay and biological significance. *Methods Enzymol* 2002;348:93–112.
18. Lang CA, Mills BJ, Mastropaolo W, et al. Blood glutathione decreases in chronic disease. *J Lab Clin Med* 2000;135:402–405.
19. Sakamaki H, Akazawa S, Ishibashi M, et al. Significance of glutathione-dependent antioxidant system in diabetes-induced embryonic malformations. *Diabetes* 1999;48:1138–1144.
20. Altomare E, Vendemiale G, Albano O. Hepatic glutathione content in patients with alcoholic and non alcoholic liver diseases. *Life Sci* 1988;43:991–998.
21. Yamauchi A, Tsuyuki S, Inamoto T, et al. Liver immunity and glutathione. *Antioxid Redox Signal* 1999;1:245–253.
22. Scott RB, Matin S, Hamilton SC. Glutathione, glutathione S-transferase, and transmembrane transport of glutathione conjugate in human neutrophil leukocytes. *J Lab Clin Med* 1990;116:674–680.
23. Ghoti H, Amer J, Winder A. Oxidative stress in red blood cells, platelets and polymorphonuclear leukocytes from patients with myelodysplastic syndrome. *Eur J Haematol* 2007;79:463–467.
24. Montuschi P, Barnes P, Roberts LJ II. Insights into oxidative stress: the isoprostanes. *Curr Med Chem* 2007;14:703–717.
25. Nuutila J, Lilius EM. Flow cytometric quantitative determination of ingestion by phagocytes needs the distinguishing of overlapping populations of binding and ingesting cells. *Cytometry A* 2005;65:93–102.
26. Jerlich A, Schaur RJ, Pitt AR, et al. The formation of phosphatidylcholine oxidation products by stimulated phagocytes. *Free Radic Res* 2003;37:645–653.
27. Jannace PW, Lerman RH, Santos JI, et al. Effects of oral soy phosphatidylcholine on phagocytosis, arachidonate concentrations, and killing by human polymorphonuclear leukocytes. *Am J Clin Nutr* 1992;56:599–603.
28. Guarini P, Bellavite P, Biasi D, et al. Effects of dietary fish oil and soy phosphotidylcholine on neutrophil fatty acid composition, superoxide release, and adhesion. *Inflammation* 1998;22:381–391.
29. Adibhatla RM, Hatcher JF. Citicoline mechanisms and clinical efficacy in cerebral ischemia. *J Neurosci Res* 2002;70:133–139.