Evaluation of antioxidant capacity and inflammatory cytokine gene expression in horses fed silibinin complexed with phospholipid

Eileen S. Hackett, DVM, PhD; Khursheed R. Mama, DVM; David C. Twedt, DVM; Daniel L. Gustafson, PhD

Objective—To evaluate antioxidant capacity and inflammatory cytokine gene expression in horses fed silibinin complexed with phospholipid.

Animals—5 healthy horses.

Procedures—Horses consumed increasing orally administered doses of silibinin phospholipid during 4 nonconsecutive weeks (0 mg/kg, 6.5 mg/kg, 13 mg/kg, and 26 mg/kg of body weight, twice daily for 7 days each week). Dose-related changes in plasma antioxidant capacity, peripheral blood cell glutathione concentration and antioxidant enzyme activities, and blood cytokine gene expression were evaluated.

Results—Plasma antioxidant capacity increased throughout the study period with increasing dose. Red blood cell nicotinamide adenine dinucleotide phosphate:quinone oxidoreductase I activity decreased significantly with increasing doses of silibinin phospholipid. No significant differences were identified in glutathione peroxidase activity, reduced glutathione or oxidized glutathione concentrations, or expression of tumor necrosis factor α, interleukin-1, or interleukin-2.

Conclusions and Clinical Relevance—Minor alterations in antioxidant capacity of healthy horses that consumed silibinin phospholipid occurred and suggest that further study in horses with liver disease is indicated. (Am J Vet Res 2013;74:1333–1339)

Hepatitis results in high morbidity and mortality rates in horses, and investigation of new therapeutic agents is needed. Oxidative injury is a prominent mechanism of hepatic injury, and the positive effects of the milk thistle derivative silibinin on antioxidant capacity, as well as its direct oxidant-scavenging ability, are important features in its hepatoprotective actions. The antioxidant properties of silibinin are dose dependent in mice. However, the effects of silibinin administration on antioxidant capacity in horses are unknown. Multiple assays for enzymes and substrates can be performed to evaluate antioxidant capacity in vivo. Estimation of the plasma ORAC provides information on resistance to peroxyl radical injury and has been used to estimate antioxidant capacity in horses. The GPOX enzyme is a potent detoxifier of superoxide anions and hydrogen peroxide and in this manner limits oxidative injury. Another critical enzyme in oxidant protection, NQO1, functions to catalyze the 2-electron reduction of quinones to hydroquinones in cells, limiting 1-electron reduction, which results in formation of reactive oxygen species. Glutathione is the major intracellular reducing agent, functioning to protect against oxidative stress. Shifting of GSH to GSSG indicates a shift of redox status toward oxidative stress. Maximizing antioxidant capacity is protective against oxidative stress and injury from oxidative processes. Amplification of inflammation caused by potent cytokines released by myeloid cells, such as TNFα and IL-1, promotes a cascade of events that results in sequestration of polymorphonuclear leukocytes in tissues and parenchymal dysfunction. Inflammatory responses are...
beneficial in recognition of foreign antigens and clearing infection. However, prolonged or overexuberant expression of inflammatory cytokines can be detrimental and has been linked to pathological changes associated with disease.\textsuperscript{12} Hepatocellular injury may be caused by a combination of the primary oxidative effect and the secondary inflammatory response induced by damaged hepatocytes.\textsuperscript{13} Tumor necrosis factor \( \alpha \) is directly toxic to hepatocytes and induces apoptosis.\textsuperscript{14} Interleukin-1\( \beta \) and IL-6 reduce hepatocyte protein synthesis, carbohydrate metabolism, and cytochrome P450–dependent detoxification.\textsuperscript{15} Blood chemokine concentration has been associated with severity of hepatic disease in humans.\textsuperscript{16} Derangement in the balance of pro- and anti-inflammatory serum cytokines is characteristic of alcoholic cirrhosis and is predictive of prognosis and mortality in humans.\textsuperscript{17} Inhibiting cytokine release and subsequent inflammatory cell recruitment may limit organ damage. Silibinin protects against inflammation by limiting oxidative injury, inhibiting neutrophil migration, and regulating inflammatory mediators in rats.\textsuperscript{18} Silibinin inhibits expression and synthesis of inflammatory cytokines TNF\( \alpha \), IL-1, and IL-2 in the presence of \( \alpha \) interferon.\textsuperscript{19} It was hypothesized that oral silibinin administration would increase antioxidant capacity in the blood of healthy horses because of the absence of preexisting disease or inflammation.

**Materials and Methods**

This study was performed in conjunction with an institutional animal care and use committee–approved phase II pharmacokinetic study.\textsuperscript{20} Five horses owned by the Colorado State University Veterinary Teaching Hospital and acclimatized to their housing were used with permission, and environmental conditions were not changed. Horses were group housed in a paddock without access to grass. Horses received water ad libitum and Timothy grass hay once daily, providing for consumption of approximately 12 kg of hay/horse per day. All horses were geldings with a mean \( \pm \) SD age of 13 \( \pm \) 5 years (median, 14 years [range, 5 to 17 years]) and mean weight of 582 \( \pm \) SD 63 kg (median, 613 kg [range, 472 to 625 kg]). There were 3 Quarter Horses, 1 Arabian, and 1 Andalusian. Horses were screened prior to inclusion in the study for evidence of gastrointestinal tract or liver dysfunction by use of physical examination and serum biochemical analyses and were selected only if they readily consumed the carrier diet (400 g of pelleted feed,\textsuperscript{4} 50 g of wheat bran, and 150 mL of water [per meal]). Body weight was measured with a commercial scale, and signalment was recorded. Horses consumed each dose twice daily each day for 7 days during 4 administration periods, with progressively higher doses of silibinin phospholipid\textsuperscript{b} administered during each period and with each period separated by a washout period (minimum of 2 weeks). During week 1, twice daily, horses consumed the carrier diet without silibinin phospholipid. During week 2, horses were fed the diet plus 20 mg of silibinin phospholipid/kg of carrier diet, resulting in a 6.5 mg/kg of body weight dose of silibinin. During week 3, horses were fed the diet plus 5 mg of silibinin phospholipid/kg of diet, resulting in a 1.3 mg/kg dose of silibinin. During week 4, horses were fed the diet plus 80 mg of silibinin phospholipid/ kg of diet, resulting in a 26 mg/kg dose of silibinin. Mean \( \pm \) SD total dose of silibinin phospholipid administered in feed in week 2 was 11.6 \( \pm \) 1.3 g (median, 12.3 [range, 9.4 to 12.5 g]), in week 3 was 23.3 \( \pm \) 2.6 g (median, 24.6 g [range, 18.9 to 25.0 g]), and week 4 was 46.5 \( \pm \) 5.2 g (median, 49.2 g [range, 37.8 to 50.0 g]). All 5 horses received identical treatments, except on day 7 of week 4 (highest-dose week), when the final meal supplemented with silibinin phospholipid was incompletely consumed (1 horse) or consumed slowly (1 horse).

**Sample collection**—Blood samples (total volume, 40 mL) were obtained from horses on day 1, prior to administration of unsupplemented diet or carrier diet mixed with silibinin phospholipid, and on day 7, 1 hour following the final meal of the study diet, each week. Blood for antioxidant analysis was collected directly into EDTA tubes. Plasma was immediately separated via centrifugation at 2,500 \( \times \) g for 10 minutes, transferred to cryovials, submerged in liquid nitrogen until frozen, and stored at \( -80^\circ \)C until analysis. Following plasma removal, theuffy coat layer was transferred to a 15-mL tube, and the pelleted RBCs were transferred to a cryovial,submerged in liquid nitrogen until frozen, and stored at \( -80^\circ \)C until analysis. Theuffy coat sample was brought to a final 6-mL sample volume with PBS solution, layered onto 4 mL of a mixture of nonionic, synthetic polymer of sucrose\textsuperscript{5} and sodium diatrizoate,\textsuperscript{6} and centrifuged at 800 \( \times \) g for 30 minutes at 20\( ^\circ \)C. Following centrifugation, the fraction containing PBMCs was collected and washed twice with PBS solution. The PBMCs were counted with a hemacytometer and resuspended in PBS solution prior to freezing at \( -80^\circ \)C until analysis. Blood for cytokine gene expression analysis was collected directly into commercially available evacuated tubes containing a proprietary additive to stabilize the in vivo gene transcription profile by reduction of in vitro RNA degradation,\textsuperscript{7} maintained at 20\( ^\circ \)C for 60 minutes, and then frozen at \( -20^\circ \)C until analysis as per the manufacturer's instructions.

**Protein assay**—Protein measurement in plasma, RBC, and PBMC samples was necessary to report protein–corrected values of glutathione and antioxidant enzymes. The RBC and PBMC samples required additional processing prior to analysis and were thawed on ice and diluted in 25mM Tris (pH, 7.4). Samples were then disrupted by sonication\textsuperscript{1} in three 2-second bursts at 30% power on ice and centrifuged at 15,000 \( \times \) g; the supernatant was collected for further analysis. Colorimetric measurement of protein was performed by use of bicinchoninic acid and standard curves of bovine serum albumin.\textsuperscript{8} Samples were added to a 96-well microplate with 200 \( \mu \)L of working reagent containing bicinchoninic acid, and incubated for 30 minutes at 37\( ^\circ \)C.
Following incubation, absorbance was measured at 562 nm with a microplate reader. Net absorbance was calculated by subtracting values of blank samples from values of bovine albumin standards and test sample replicates. Standard curves were graphed by plotting mean blank-corrected albumin standard values versus concentration and test samples estimated via linear regression. Protein concentration was reported in milligrams per milliliter.

**ORAC**—The ORAC of plasma was measured with a commercially available assay as described by Ungvari et al. The assay evaluated the ability of plasma samples to delay oxidation of a fluorescent probe by peroxyl radicals relative to known concentrations of a water-soluble vitamin E analog and has been validated in horses and other species. Plasma samples were thawed on ice, vortexed, and diluted 1:100. Fluorescein solution and either plasma or standard curve samples were thawed on ice, vortexed, and diluted 1:100. Fluorescein solution and either plasma or standard curve samples were added to a 96-well microtiter plate and incubated for 30 minutes at 37°C. The plate was read immediately following addition of 2,2'-azobis(2-methylpropionamide) hydrochloride. Fluorescence was recorded every 84 seconds for 1 hour with a fluorescent microplate reader with an excitation wavelength of 485 nm and an emission wavelength of 528 nm. Net AUC for plasma samples was calculated by subtracting blank sample AUC from test sample AUC and then compared with an antioxidant standard curve of 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid constructed by graphing the net AUC of vitamin E analog samples of known concentrations. Lower limit of quantitation was 2.5 µM vitamin E analog. Linear regression was used to estimate the vitamin E analog equivalents of plasma samples on the basis of the vitamin E analog standard curve. Plasma ORAC was compared among horses that were administered different doses of silybin phospholipid.

**NQO1 activity**—The NQO1 of PBMC and RBC samples was measured by use of the method described by Gustafson et al. The PBMC and RBC lysates were thawed on ice. A 25 mM Tris plus 0.7% bovine serum albumin solution was added to a methacrylate cuvette, followed by DCPIP (40 µM final solution) and nicotinamide adenine dinucleotide (200 µM final solution). Samples were evaluated in duplicate and mixed immediately prior to measurement of absorbance at 600 nm for 120 seconds via spectrophotometer, with and without the addition of dicumarol (20 µM final solution). The NQO1 activity was defined as the dicumarol-inhibited decrease in absorbance at 600 nm, or the difference in the change in optical density per minute between dicumarol-negative and dicumarol-positive samples. The NQO1 activity was converted to nmol of DCPIP reduced/min through calculations that used the extinction coefficient of DCPIP (21 mM⁻¹ cm⁻¹). The NQO1 was normalized for protein content and expressed as nmol of DCPIP/min/mg.

**GPOX activity**—The GPOX activity of PBMC and RBC samples was measured by use of the method described by Gustafson et al. A GPOX assay has been validated in horses. The reaction mixture consisted of 2.59 mL of 50 mM potassium phosphate buffer (pH 7.0) with 1 mM EDTA, 10 µL of 5 mM sodium azide, 100 µL of 150 mM GSH, 100 µL of 2.2 mM hydrogen peroxide, 5 µL of glutathione reductase, and 100 µL of 8.4 mM NADPH in a methacrylate cuvette. Test samples were added after a linear rate was established at 340 nm absorbance for 240 seconds in a spectrophotometer. The GPOX activity was defined as the rate of NADPH oxidation in the presence of glutathione and glutathione reductase, or the difference in linear rates following sample addition. The GPOX activity was converted to pmol of NADPH reduced per minute through calculations that used the extinction coefficient of NADPH (6.2 mM⁻¹ cm⁻¹). The GPOX was normalized for protein content and expressed as pmol of NADPH/min/mg.

**GSH and GSSG concentration**—The GSH and GSSG concentration assays used in this study have been validated in horses and were performed according to instructions from a commercially available assay. The PBMC and RBC lysates were thawed on ice and precipitated with 5% sulfosalicylic acid. The PBMC samples were diluted 2-fold or did not require dilution for analysis. The RBC samples were diluted 10- to 100-fold for analysis. Samples were added to a 96 well microtiter plate with a working solution containing 3,3’-dithiobis(2-nitrobenzoic acid) and glutathione reductase. Following 5 minutes of incubation at 20°C, NADPH solution was added and the plate was read immediately. A microplate reader recorded absorption of samples every 60 seconds for 5 minutes at 412 nm. The resulting slope was plotted from the change in absorbance at 412 nm/min. Standard curves of GSH and GSSG were analyzed, and linear regression of the change in absorbance at 412 nm/min was used to estimate the concentration of GSH and GSSG in test samples. Lower limit of quantitation of the assays was 0.5 ng/mL. Estimated concentration of GSH and GSSG was normalized for protein content and expressed in nanomoles per milligram.

**RT-PCR assay**—An RT-PCR assay was used to quantify cytokine gene expression in blood samples. Samples were thawed, and total RNA was extracted by use of a commercial kit and manufacturer’s instructions. Samples were converted to cDNA by RT by use of 1.0 µg of RNA sample and RT master mix, with incubation at 42°C for 15 minutes and 95°C for 5 minutes as described. Equine-specific intron-spanning primer and probe sets were used. Cytokines evaluated included TNFα, IL-1, and IL-2. Reaction mixtures composed of 5 µL of cDNA, 6.25 µL of nuclease-free water, 1.25 µL of 20X assay mix for the primer-probe set, and 12.5 µL of master mix were incubated at 95°C for 10 minutes and underwent 40 cycles in a sequence detection system. β-glucuronidase was used as the housekeeping gene. Changes in gene expression (ΔΔCt) were calculated by use of the following formula:

\[ \Delta\Delta C_t = \left( \frac{\text{Cytokine threshold cycle – β-GUS threshold cycle}_{\text{SAMPLE 1}}}{\text{Cytokine threshold cycle – β-GUS threshold cycle}_{\text{SAMPLE 2}}} \right) \]

Results were reported as relative cytokine gene expression calculated by use of 2-ΔΔCt, calibrated to samples from day 1 prior to administration of the blank diet for each individual gene.
Statistical analysis—Changes in plasma antioxidant capacity, glutathione, antioxidant enzymes in RBCs and PBMCs, and quantity of cytokine mRNA relative to silibinin dose, were analyzed by use of repeated measures ANOVA with Bonferroni multiple comparisons for post hoc pairwise comparisons. Values of P < 0.05 were considered significant.

Results

All 5 horses completed the study. Each 7-day period of twice-daily feeding of silibinin phospholipid was well tolerated.

ORAC—Plasma ORAC was measured for all time points (Table 1). Plasma samples delayed oxidation of the fluorescent probe to a greater extent than did blank samples. Plasma ORAC increased significantly (P = 0.005) throughout the study period.

Table 1—Plasma ORAC (mean ± SD [median {range}]) of horses orally administered various doses of silibinin phospholipid.

<table>
<thead>
<tr>
<th>Silibinin dose (mg/kg)</th>
<th>Sample day</th>
<th>Sample type</th>
<th>ORAC*</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1</td>
<td>PBMC</td>
<td>(9.7 ± 7.8)</td>
</tr>
<tr>
<td>6.5</td>
<td>1</td>
<td>RBC</td>
<td>(10.1 ± 6.5)</td>
</tr>
<tr>
<td>13</td>
<td>2</td>
<td>RBC</td>
<td>(12.0 ± 7.0)</td>
</tr>
<tr>
<td>26</td>
<td>1</td>
<td>RBC</td>
<td>(12.0 ± 7.0)</td>
</tr>
</tbody>
</table>

*Vitamin E analog equivalents per milliliter of plasma protein.

RT-PCR ASSAY

In 1 horse, the RT-PCR assay housekeeping gene was amplified too late, invalidating the cytokine data. Therefore, this horse was excluded and only 4 horses were included in the final analysis. Differences in TNFα, IL-1, and IL-2 expression were not detected among groups. Relative quantities of gene expression calibrated to the first sampling period, day 1 of the

Table 2—Mean ± SD (median {range}) values (corrected for sample protein concentration) of endogenous antioxidant enzyme activities and glutathione concentrations in PBMCs and RBCs of horses orally administered various doses of silibinin phospholipid.

<table>
<thead>
<tr>
<th>Silibinin dose (mg/kg)</th>
<th>Sample day</th>
<th>Sample type</th>
<th>NQO1 activity (nmol of DCPIP/min/mg)</th>
<th>GPOX activity (pmol of NADPH/min/g)</th>
<th>GSSG (nmol/mg)</th>
<th>GSH (nmol/mg)</th>
<th>GSSG:GSH</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1</td>
<td>PBMC</td>
<td>(22.5 ± 1.8)</td>
<td>(10.0 ± 8.0)</td>
<td>14.2 ± 4.8</td>
<td>15.3 ± 8.5</td>
<td>1.00</td>
</tr>
<tr>
<td>6.5</td>
<td>1</td>
<td>RBC</td>
<td>(10.1 ± 6.5)</td>
<td>(12.0 ± 7.0)</td>
<td>12.0 ± 7.0</td>
<td>12.0 ± 7.0</td>
<td>1.00</td>
</tr>
<tr>
<td>13</td>
<td>2</td>
<td>RBC</td>
<td>(12.0 ± 7.0)</td>
<td>(12.0 ± 7.0)</td>
<td>12.0 ± 7.0</td>
<td>12.0 ± 7.0</td>
<td>1.00</td>
</tr>
<tr>
<td>26</td>
<td>1</td>
<td>RBC</td>
<td>(12.0 ± 7.0)</td>
<td>(12.0 ± 7.0)</td>
<td>12.0 ± 7.0</td>
<td>12.0 ± 7.0</td>
<td>1.00</td>
</tr>
</tbody>
</table>

*Within a column and sample type, values with different superscript letters are significantly (P < 0.05) different.
unsupplemented diet week, were determined (Table 3).

**Discussion**

The primary antioxidant effect identified in the healthy horses fed diets supplemented with silibinin in the present study was alteration in plasma ORAC. Temporal effects and those of the carrier diet may also have been sources of alterations in plasma ORAC in this study. Modest increases in plasma ORAC have also been associated with decreases in plasma lipid hydroperoxides in horses following exercise-induced stress. In a study of rats that were administered silibinin via the intraperitoneal route, increases in GSH concentration were tissue specific and occurred primarily in the liver and intestines. The authors attributed the tissue-specific effects to the basic pharmacokinetics of silibinin, which undergoes predominantly biliary excretion and is maintained in high local concentrations by enterohepatic circulation. It is presumed that the antioxidant protective effects of silibinin are similarly concentrated in the tissues of the liver and intestines. Therefore, it is expected that the changes evident in the blood of horses consuming silibinin would be greater in the liver, which is the target organ in horses with liver disease. Hepatic antioxidant effects of silibinin are well documented. Less is known regarding the relative antioxidant effect on blood versus hepatic tissues. In experimental carbon tetrachloride-induced hepatitis, hepatic and RBC GSH were both measured and silibinin administration improved GSH concentration in both tissues, but to a greater degree in hepatic tissue.

Much has been learned from in vitro and in vivo experiments with respect to the peripheral anti-inflammatory effects of silibinin administration. Silibinin reverses increases in serum TNFα, IL-1β, and IL-6 expression in rats with sepsis induced by cecal ligation and perforation. A decrease in acute lung and brain injury accompanies this anti-inflammatory effect. In rats with experimental nonalcoholic fatty liver disease, silibinin decreases plasma TNFα expression concurrent with improvements in liver inflammation and fatty infiltration evident via histologic examination.

In rats with experimental renal ischemia and reperfusion, silibinin decreases plasma TNFα and IL-1 expression associated with partial hepatectomy and the resultant inflammatory response. Release of TNFα and cytotoxicosis secondary to toxic damage are decreased by silibinin administration in perfused livers and isolated Kupffer cells. In canine hepatocytes, silibinin ameliorates the proinflammatory influence of IL-1β, including production of chemotactic cytokines, and reduces hepatocyte damage. Silibinin also dimin-

### Table 3—Blood cytokine gene expression (mean ± SD [median {range}]) of horses orally administered various doses of silibinin phospholipid.

<table>
<thead>
<tr>
<th>Silibinin dose (mg/kg)</th>
<th>Sample day</th>
<th>TNFα</th>
<th>IL-1</th>
<th>IL-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Referent</td>
<td>1.17 ± 0.56</td>
<td>1.12 ± 0.21</td>
<td>1.73 ± 1.51</td>
</tr>
<tr>
<td>1.4</td>
<td>1.04 ± 0.21</td>
<td>1.39 ± 0.20</td>
<td>1.41 ± 0.62</td>
<td></td>
</tr>
<tr>
<td>2.8</td>
<td>1.27 ± 0.51</td>
<td>1.04 ± 0.31</td>
<td>1.32 ± 0.20</td>
<td></td>
</tr>
<tr>
<td>5.6</td>
<td>1.20 ± 0.50</td>
<td>1.21 ± 0.45</td>
<td>1.31 ± 0.50</td>
<td></td>
</tr>
</tbody>
</table>

Gene expression values indicate relative quantity of gene calibrated to day 1 of the unsupplemented diet (0 mg/kg) week.
ishes the proinflammatory influence of IL-1 in human hepatic stellate cells.33

Gene expression of the inflammatory cytokines TNFα, IL-1β, and IL-2 did not change in the present study of healthy horses fed diets supplemented with silybin phospholipid. In the absence of an active inflammatory stimulus, this was not surprising. However, the effects of silybin on inflammatory cytokines have been detected in the blood of patients with naturally occurring diseases, especially those diseases in which oxidative injury is prominent. In chronic hepatitis C virus infection, silybin administration inhibits TNFα production by PBMCs.14 Production of TNFα by blood lymphocytes in humans with end-stage diabetic nephropathy is significantly reduced following silybin administration.39 Silybin also reduces inflammatory cytokines and disease severity in humans with non-alcoholic fatty liver disease.43–47

Results of the present study provide baseline data on the effects of silybin in healthy horses. Minor changes were observed in antioxidant capacity, which was consistent with previous observations in healthy cats.33 Because of the pharmacokinetics of silybin,33 despite low bioavailability, its antioxidant effects will likely be most prominent in the liver and intestinal tract of horses.

References


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35. Das SK, Vasudevan DM. Protective effects of silymarin, a milk thistle (Silybum marianum) derivative on ethanol-induced oxidative stress in liver. Indian J Biochem Biophys 2006;43:306–311.


