

DISSERTATION

SILIBININ PHARMACOLOGY AND OPPORTUNITIES FOR THERAPY IN
HORSES

Submitted by

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ABSTRACT

SILIBININ PHARMACOLOGY AND OPPORTUNITIES FOR THERAPY IN HORSES

Milk thistle extracts have been used as a ‘liver tonic’ for centuries. In recent years, silibinin, the active ingredient in milk thistle extracts, has been studied both *in vitro* and *in vivo* to evaluate the beneficial effects in hepatic disease. It is evident from this research that silibinin significantly increases antioxidant levels and improves outcomes in diseases resulting from oxidant injury. Pharmacokinetic and pharmacodynamics evaluation of silibinin in healthy horses is a precursor to evaluation as a treatment for acute hepatic diseases in the horse. Further, liver disease is poorly characterized in horses living in the United States. Therefore, investigation of hepatic disease in horses was also necessary to evaluate the potential utility of silibinin treatment in disease. It was hypothesized that silibinin consumed orally twice daily in escalating doses over several weeks by healthy horses would have predictable serum levels, low toxicity, and result in a significant increase in antioxidant reserve. Oral silibinin demonstrated poor bioavailability, a short elimination half-life, and did not accumulate when administered twice daily over 7 days. Nonlinear pharmacokinetic behavior was observed with escalating doses, possibly due to saturable enzymatic elimination. As observed in other species, silibinin administration was considered safe and non-toxic in

horses. Increase in antioxidant reserve was an important treatment effect of silibinin identified in healthy horses. Hepatic disease in horses in the western United States had a high mortality, and fibrotic and inflammatory conditions were often encountered. Silibinin is indicated in a variety of acute and chronic diseases affecting liver function in horses, as common histologic lesions identified in horses with hepatic disease could be targeted by the multiple known treatment effects of silibinin. Future study is justified to evaluate dose, kinetics, and treatment effects in horses with hepatic disease.

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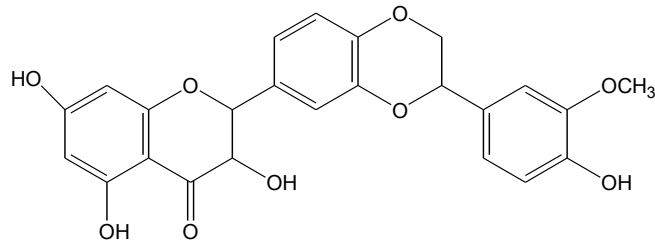
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CHAPTER 1: INTRODUCTION AND REVIEW OF THE LITERATURE

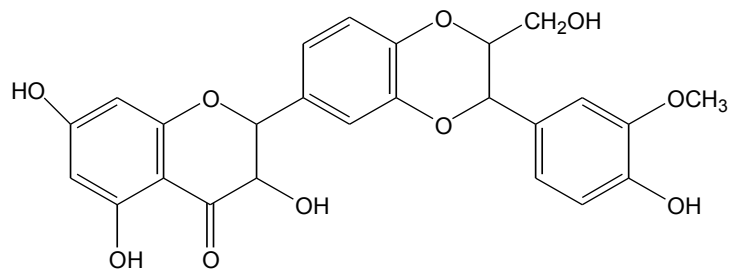
Silibinin, the active ingredient in *Silybum marianum* or milk thistle, frequently is recommended to treat humans and animals with hepatic failure. A recent study revealed that up to a third of people use milk thistle compounds to supplement therapy for chronic hepatitis.¹ Use of milk thistle seed extracts for liver protection was recorded as early as the time of the ancient Greeks and Romans.² Worldwide, researchers continue to show interest in milk thistle derivatives as a potential therapy for multiple diseases as evidenced by the over 12,000 related scientific publications produced on this subject within the last 10 years.³ This review is confined primarily to liver protective effects of milk thistle derivatives.

Milk thistle extracts have low oral bioavailability across species and, following absorption, are primarily conjugated via glucuronidation and excreted into bile and urine with minimal phase I metabolism.⁴ The chemical structures of silibinin and its major metabolite can be found in Figure 1. Extensive conjugation and biliary excretion result in short half-lives and low systemic exposure.⁵ However, due to the route of metabolism and excretion, bile silibinin concentrations are approximately 100X higher than serum concentrations.⁶ Minimal *in vitro* inhibition of CYP3A4 and CYP2C9 has not translated into significant *in vivo* decreases in specific drug metabolism and clinical interactions have not been reported.⁷⁻⁹ Despite this lack of interaction, many authors prudently

suggest caution in administering high doses of silibinin to ill patients receiving multiple medications.¹⁰



silibinin



silibinin glucuronide

Figure 1: Chemical structure of the flavolignan silibinin and its primary phase II metabolite silibinin glucuronide.

Silibinin is relatively insoluble in water and is not absorbed readily from the intestines.¹¹ Bioavailability can be improved by combining milk thistle extracts with solubilizing substances. Silibinin complexed with phosphatidylcholine increases oral bioavailability in healthy humans and patients with hepatic cirrhosis.^{2,12} Measurement of systemic plasma concentrations allows estimation of gastrointestinal drug absorption. Commercially available milk thistle products are highly variable in content, dissolution, and bioavailability, therefore making it important to perform pharmacokinetic testing of

products in the species of interest prior to clinical use.¹³ Silibinin does not accumulate in plasma with an 8 hour chronic dosing interval in people with non-cirrhotic chronic hepatitis.⁵ Drug exposure is increased 3 to 5 fold in people with hepatic disease, with the highest levels seen in non-alcoholic fatty liver disease and cirrhosis.¹⁴ Elevations in drug exposure due to liver disease may be related to decreased phase II conjugation and transporter proteins.¹⁴ Though silibinin exhibits linear pharmacokinetics when administered to people with normal hepatic function, nonlinear pharmacokinetics have been reported when higher doses are given to patients with hepatitis.^{5,15,16} Enterohepatic circulation, and subsequent hepatic and intestinal silibinin concentration, may contribute to this phenomenon (Figure 2). Nonlinear pharmacokinetics observed at higher doses in people with liver disease could, in part, counteract low bioavailability and improve therapeutic results in this target population.

Though milk thistle extracts have been used for centuries as a liver tonic, only recently has the mechanism of hepatic protection become better understood.⁴ Silibinin is a drug with multiple functions and targets. The mechanism of action best known is antioxidant free radical scavenging and inhibition of lipid peroxidation¹⁷. Hepatic injury has long been linked to oxidative injury.¹⁸ Silibinin is most effective in scavenging low molecular weight free radicals, such as the hydroxyl radical.¹⁹ Silibinin has been shown to be protective against oxidant injury *in vitro* both in peripheral blood,^{20,21} hepatocytes,²² and multiple other body tissues.²³ *In vivo* studies indicate that silibinin is protective against oxidant injury in multiple body tissues.^{24,25} Rats supplemented with silibinin showed a tissue specific increase in glutathione content, primarily in hepatic and

intestinal tissues.²⁶ Silibinin treated cells are more resistant to cell lysis upon exposure to oxidizing agents.²

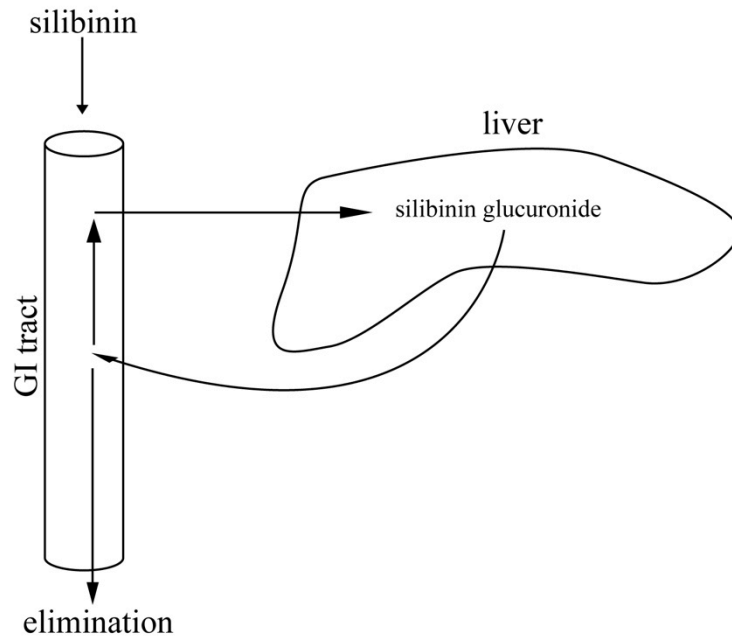


Figure 2: Schematic of silibinin disposition, including enterohepatic circulation. Its primary phase II metabolite, silibinin glucuronide, is transported via biliary flow to the intestinal tract where it undergoes cleavage, restoring silibinin parent compound.

The anti-inflammatory effects of silibinin extend beyond inhibition of reactive oxygen species-dependent mechanisms. Many of the anti-inflammatory properties of silibinin are due to nuclear DNA/RNA mediated effects, via suppression of NF- κ B translocation and binding.²⁷ Silibinin suppressed TNF α , TNF receptor 1 and TNF receptor 1-associated apoptosis-ligand expression, as well as associated hepatic apoptosis and enzyme elevation in mice treated with fumonisin.²⁸ Silibinin also has been shown to significantly inhibit TNF and interleukin 4 expression in a model of acute hepatitis in

mice.²⁹ Silibinin strongly inhibits the 5-lipoxygenase pathway and leukotriene formation in Kupffer cells *in vitro*.³⁰ Secondary to LPS stimulation, silibinin inhibits inducible nitric-oxide synthase expression *in vitro*.³¹ Multiple *in vitro* studies in stellate cells and hepatocytes have demonstrated a reduction in monocyte chemoattractant protein 1 secondary to interleukin 1 β stimulation.^{32,33} IL-1 β and prostaglandin E2 have been reduced *in vivo* with silibinin administration, which also improved survival in a mouse LPS sepsis model.³⁴ In addition, recent evidence suggests that silibinin inhibits selectin adhesion molecule expression, necessary for leukocyte migration.³⁵

Silibinin has been shown to have an antifibrotic effect in the liver. Central to the onset of hepatic fibrosis is conversion of hepatic stellate cells to myofibroblasts, which is limited by silibinin through interruption of cell signaling. Silibinin reduces stellate cell DNA synthesis, proliferation, and migration.³² Silibinin also limits fibrous tissue production. In a hepatic injury model in rats, oral silibinin reduced liver collagen levels up to 55%.³⁶ Silibinin also reduced collagen, procollagen III, procollagen α 1, and profibrogenic mRNA expression levels in rats with biliary obstruction by 30%.^{37,38} In a study where ethanol and silibinin were administered over a period of 3 years to baboons, levels of hepatic collagen type I, procollagen mRNA, and the incidence of alcohol-induced hepatic fibrosis and cirrhosis were found to be lower than in the control group.³⁹

Hepatoprotective effects of silibinin in liver disease may be related to mechanisms of enhanced protein synthesis. In an *in vivo* study, silibinin differentially increased DNA synthesis in partially hepatectomized rats, but not rats in the healthy control group or those with hepatic neoplasia.⁴⁰ Protein synthesis is necessary for hepatic regeneration and repair following toxic and inflammatory insults. Another hepato-

protective effect of silibinin is prevention of estrogen and tauroolithocholate induced cholestasis through up-regulation of the bile salt export pump.¹¹ As bile transport across the canalicular membrane is the rate limiting step in bile flow, silibinin may aid in some forms of acquired hepatocellular cholestasis.⁴¹ Silibinin administration resulted in a dose-dependent increase in bile flow (choleresis), primarily due to stimulation of rate of bile salt synthesis *in vivo* in rats.⁴²

Multiple human clinical trials have been performed to evaluate toxicity and efficacy of silibinin treatment for hepatic disease.⁴³ Studies demonstrate that toxicity is low in humans and animals, adding to the reputation of silibinin treatment as safe and non-harmful. In a two year toxicity study in healthy rats and mice, those consuming silibinin had a significantly lower incidence of hepatic mixed inflammatory cell infiltration, bile duct hyperplasia, and spontaneous hepatocellular adenoma and carcinoma.⁴⁴ The predominant adverse events reported in people consuming silibinin are headaches and pruritus, with a lesser incidence of diarrhea and nausea.⁴⁵ Total percentage of adverse events reported in a metaanalysis of clinical trials was 2.36% with silibinin vs. 5.05% with placebo.⁴⁵ No deaths or life-threatening adverse events have been reported.⁴⁵ Laxative effects of silibinin at higher doses may be related to carrier elements or increased bile secretion.¹⁵ This implies that increases in dose may, in turn, increase risk of adverse events. Higher dose studies have not been performed in healthy people, therefore associated adverse events may be related to primary disease conditions.⁴⁶

Convincing evidence exists to support silibinin treatment for hepatic toxins. Silibinin treatment decreases the mortality rate nearly 50% in people suffering from

Amanita phalloides intoxication compared with those untreated with silibinin.⁴⁵ In addition to limiting oxidation, silibinin prevents transport of the phalloidin toxin into hepatocytes by competitive inhibition of hepatocyte-specific OATP2 transporters.² Silibinin also inhibits hepatocyte absorption of other toxins by membrane bile-salt-binding polypeptides.¹¹ In two reports, silibinin treatment improved hepatic function in workers with environmental exposure to hepatotoxic industrial solvents.^{47,48} Silibinin treatment has been shown to prevent hepatic enzyme elevation and other toxic changes in rats treated with carbon tetrachloride^{49,50} and acetaminophen.⁵¹ Silibinin also significantly prevented iron-induced hepatotoxicity in rats. Prophylactic use of silibinin may protect against iatrogenic toxins as well. In a study evaluating silibinin treatment combined with a hepatotoxic anti-tuberculosis drug, hepatic enzyme elevation was significantly reduced.² Silibinin has also been shown to be protective against radiation-induced hepatic injury and enzyme elevation.^{52,53}

Silibinin is currently recommended for use in alcoholic liver disease. Ethanol induces free radical formation through multiple pathways, resulting in steatohepatitis and cirrhosis with chronic use.⁴⁵ Patients with alcoholic liver disease undergoing 6 months of treatment with silibinin had improved cellular superoxide dismutase activity and serum glutathione peroxidase activity.⁵⁴ In one *in vitro* study performed in human hepatocytes, silibinin completely prevented ethanol-induced release of lactate dehydrogenase.⁵⁵ Double-blind placebo controlled studies have demonstrated significant decreases or normalization of hepatic enzymes in alcoholic liver disease, as well as improvement in symptoms of anorexia, nausea, and asthenia.⁴⁵ Improvement in histological scores of hepatic pathology have also been demonstrated in these patients.⁵⁶ A recent Cochrane

meta-analysis revealed all cause mortality rates were reduced 50% in patients with alcoholic liver disease concurrently treated with silibinin.⁵⁷

Silibinin is commonly recommended for use in viral hepatitis. Though silibinin has no known direct suppressive effects on viral replication, its use targets inhibition of inflammation and cytotoxic events secondary to viral infection.⁴⁵ In patients with chronic hepatitis, silibinin reduces transaminase levels due to hepatic damage and improves serum malondialdehyde levels, a marker of oxidative injury.^{3,58} Silibinin is well tolerated in human subjects with chronic hepatitis C infection.⁵⁹ Use of silibinin is accompanied by a better quality of life and reduction of symptoms in these patients.^{1,60}

Non-alcoholic fatty liver disease occurs due to metabolic stress or can accompany primary hepatitis, promoting progression of inflammatory disease to fibrosis.³ In *in vivo* models of non-alcoholic fatty liver disease, silibinin supplementation limits hepatic glutathione depletion and hydrogen peroxide production, prevents hepatic mitochondrial dysfunction, and reduces hepatic enzyme elevation.^{61,62} Silibinin treatment has undergone clinical trials in this population of human patients. A recent study demonstrated improvement in liver enzymes in patients with non-alcoholic fatty liver disease with silibinin treatment.⁶³ Silibinin also reduces C-reactive protein, inflammatory cytokines, indices of hepatic fibrosis, and degree of steatosis evaluated ultrasonographically in this population.⁶³⁻⁶⁵

Hepatic cirrhosis is a common end-result of advanced stages of alcoholic liver disease and viral hepatitis. Remodeling of hepatic architecture secondary to fibrosis can result in hepatic insufficiency, portal hypertension, and hepatic encephalopathy.²

Following onset of cirrhosis, treatments targeted at the underlying cause have limited efficacy.⁴⁵ Use of silibinin in hepatic cirrhosis targets improvement in anti-oxidant status, cytoprotection, reversal of fibrosis, and regeneration. Dose dependent decreases in hepatic enzyme levels with silibinin treatment have been documented.⁶⁶ In a placebo-controlled trial, patients with cirrhosis consuming silibinin had greater total glutathione levels and concurrent decreases in N-terminal propeptide of type III collagen values, a biomarker for hepatic fibrosis.⁶⁷ Decreased mortality rates have been documented with silibinin use in randomized controlled trials performed in patients with cirrhosis.⁶⁸ All cause mortality decreased 4.4% and mortality from liver causes decreased 7.3% in cirrhotic patients.⁴⁵

A meta-analysis of clinical trials that reviewed the evidence for silibinin use in people with liver disease specifically addressed liver-related mortality. Liver-related mortality was evaluated over all studies and found to be 10% with silibinin vs. 17.3% with placebo.⁴⁵ This reduction in liver-related mortality was significant ($p=0.01$).⁴⁵ In addition, it was found that fewer patients were hospitalized for cirrhosis related issues, with 10.0% hospitalized with silibinin use vs. 16.9% hospitalized with placebo use ($p=0.086$).⁴⁵ The incidence of development of complicating conditions, such as hepatocellular carcinoma, upper gastrointestinal bleeding, and diabetes mellitus was also lower in silibinin treated patients.^{45,68}

Randomized clinical trials have not been performed in clinically affected animals with hepatic diseases. However, much can be learned from preliminary studies in animal models and adapted from these and human clinical studies. Although marketed to horse owners, silibinin products have not been studied in horses. Silibinin is not part of core

recommendations for treatment of liver disease in horses based on a survey of veterinary large animal textbooks outlined in Table 1.

Table 1: Five current large animal veterinary textbooks were screened for therapeutics recommended in liver disease and disclosure of milk thistle derivative supplements administered in horses.

Equine Veterinary Literature Citation Evaluated	Milk thistle derivatives disclosed?
Barton, MH 'Disorders of the Liver', in Equine Internal Medicine 3 rd Edition, SM Reed, WM Bayley, DC Sellon edtrs, Saunders, St. Louis, 2010, 939-975.	No
Divers, TJ 'Liver Failure and Hemolytic Anemia', in Equine Emergencies Treatment and Procedures 3 rd Edition, Saunders, St. Louis, 2008, 237-258.	No
Divers, TJ 'Therapy of Liver Failure', in Large Animal Internal Medicine 4 th Edition, BP Smith edtr, Mosby, St. Louis, 2009, 921-923.	No
Durham A 'Management of Horses with Hepatic Disorders', in The Equine Hospital Manual, K Corley, J Stephen edtrs, Blackwell, Oxford, 2008, 524-532.	No
Lekeux, P, Kirschvink, N 'Antioxidants and Horse Health', in Current Therapy in Equine Medicine 6 th Edition, NE Robinson, KA Sprayberry edtrs, Saunders, St Louis, 2009, 73-78.	Yes
Williams, CA 'Herbs and Nutraceuticals', in Current Therapy in Equine Medicine 6 th Edition, NE Robinson, KA Sprayberry edtrs, Saunders, St Louis, 2009, 87-93.	No

In review of current veterinary textbooks, only one chapter was found that mentioned milk thistle derivatives, located in a table listing antioxidants. Specific recommendations for the use of silibinin and related compounds were not found, nor was there discussion of pros and cons of use or applications in hepatic disease.

Studies evaluating silibinin administration in large animal herbivores have thus far only been performed in dairy cattle. When silibinin was administered to peripartum dairy cows, in which subclinical fatty liver disease is common, affects included improved

lactation performance and improved body condition.⁶⁹ Though hepatic biopsies confirmed fatty liver changes in both silibinin treated and control groups, distribution of histologic change within the lobule differed.⁷⁰ In silibinin treated cows, only hepatocytes nearest the central vein were consistently affected, whereas in control cows hepatocytes throughout the lobule contained cytoplasmic vacuoles.⁷⁰ Though silibinin was not reportedly detected in the milk, plasma pharmacokinetic analyses were not performed.⁶⁹

Despite the evidence for use, silibinin is not considered a single line therapy but an adjunctive liver supportive drug indicated in a variety of acute and chronic diseases affecting liver function. Limited information is available regarding silibinin disposition and pharmacodynamics in large animal herbivores, and specifically in horses. Pharmacokinetic studies in healthy horses, utilizing an agent with potential therapeutic potential in disease, are a necessary first step prior to clinical application.

CHAPTER 2: SILIBININ PHARMACOKINETICS IN HORSES

Milk thistle derivatives are frequently recommended to treat humans and animals with hepatic conditions. The majority of beneficial effects of milk thistle extracts are attributed to the active flavolignan ingredient, silibinin. To test these effects, multiple human clinical trials have been performed to measure the efficacy of silibinin treatment for various acute and chronic hepatic diseases.⁴⁶ Human clinical trials support the use of silibinin as a treatment for viral hepatitis, alcoholic liver disease, non-alcoholic fatty liver disease, cirrhosis, and following ingestion of hepatotoxins.⁴⁵ Moreover, studies demonstrate that silibinin toxicity is low in humans and in animals. Much is known about the basic pharmacokinetics of silibinin in humans and animals.¹¹ Milk thistle extracts have low oral bioavailability across species and following absorption are primarily conjugated via glucuronidation and excreted into bile and urine with minimal phase I metabolism.⁷¹ Oral bioavailability of silibinin is known in humans,¹⁵ dogs,⁷² cats,⁷³ and rats⁷⁴. But despite its clinical use, a general lack of information about silibinin toxicity and pharmacokinetics in horses. Thus it follows that if silibinin is to be prescribed to treat equine liver disease, the basic pharmacokinetics should be evaluated first. To address this deficiency, this preliminary study attempted to determine the oral bioavailability, and single and multi-dose pharmacokinetic parameters of silibinin as a treatment in normal horses.

Methods

Experiments were performed in 2 phases, during which kinetics of single dose and multi-dose treatments of silibinin were studied. All procedures involving the use of animals in research were approved by the Colorado State University Institutional Animal Care and Use Committee as described in protocol #08-144A-01 titled 'Pharmacokinetics of silibinin in normal horses' and protocol #09-011A-01 titled 'Pharmacokinetics of silibinin in normal horses phase II: Dose escalation and impact on antioxidant status'. Horses owned by the Colorado State University Veterinary Teaching Hospital were used with permission. Horses were screened prior to inclusion in the study for evidence of gastrointestinal or liver dysfunction by physical examination and serum chemistry, and were selected only if they readily consumed the carrier diet. Body weight was measured using a commercial scale (AW-5315 Digital Indicator, Airway Scale & Manufacturing Co, Inc, Beaumont, CA). Signalment was recorded.

Phase I Dose administration

In phase I, 9 horses were administered silibinin by 3 different routes preceding single dose pharmacokinetic analyses. The routes of administration were oral, nasogastric, and intravenous. Silybin phospholipid (Siliphos^R, Indena Pharmaceuticals Inc, Milan, Italy) was administered by 2 different enteral routes, orally in feed and via nasogastric tube, at a dose of 20 mg/kg. The formulation of silybin phospholipid used in this study contained 32.7% silibinin, and at a dose of 20 mg/kg, resulted in administration of 6.5 mg/kg silibinin. An intravenous dosing solution of silibinin (SO417, Sigma-Aldrich®, St. Louis, MO) was prepared with 99% dimethyl sulfoxide as a carrier (Butler

Animal Health Supply LLC, Dublin, OH). The silibinin solution was filtered with a 0.22 µm filter (Sterivex™ GP0.22µm filter unit, Millipore Corporation, Billerica, MA) and administered intravenously at a dose of 6.5 mg/kg. A 30 day washout period was scheduled between various routes of silibinin administration. Blood samples were taken 0.5, 1, 2, 4, and 8 hours following enteral administration, and 0.83, 0.25, 0.5, 2, 4, and 8 hours following intravenous administration. Tubes filled with whole blood were allowed to clot over 20 minutes in a 37°C water bath and then centrifuged (5 minutes at 4,234 x g). The separated serum was then collected and stored at -80 C until analysis.

Phase II Dose Administration

In phase II, 5 horses consumed escalating oral doses of silybin phospholipid during 4 non-consecutive weeks. A minimum of 2 weeks washout period between dose increments was implemented. During week 1, horses consumed a blank diet. The blank diet consisted of a combination of 400 g pelleted feed (Equine Senior Horse Feed, Purina Mills, St. Louis, MO), 50 g wheat bran, and 150 mL water. During week 2, horses were fed the blank diet plus 20 mg/kg silybin phospholipid, resulting in a 6.5 mg/kg dose of silibinin. During week 3, horses were fed the blank diet plus 40 mg/kg silybin phospholipid, resulting in a 13 mg/kg dose of silibinin. During week 4, horses were fed the blank diet plus 80 mg/kg silybin phospholipid, resulting in a 26 mg/kg dose of silibinin. Blood sampling for single dose pharmacokinetics was performed on day 1 of weeks during which horses were fed 40 mg/kg and 80 mg/kg silybin phospholipid. Blood samples were taken 0.5, 1, 2, 4, and 8 hours following the first dose administered and prepared for analysis as described in phase I. Multi-dose silibinin trough concentrations were evaluated on day 7 prior to the final weekly study meal consumption.

Silibinin concentration was measured in samples taken 1 hour post final meal consumption on day 7, and compared to concentrations in 1 hour post samples from day 1, to further assess for drug accumulation secondary to repetitive dosing.

Analysis

Standard dilutions of silibinin (2.5 – 1000 ng/ml) were prepared in acetonitrile and control serum collected from healthy horses that were not exposed to milk thistle derivatives. Each standard contained 200 ng of naringenin as the internal standard. Naringenin stock solution was prepared in methanol at 10 µg/ml. Serum (500µl) samples were extracted with 2.5 ml of acidified ethyl acetate (0.1% formic acid) with vortexing for 10 minutes. Organic and aqueous layers were separated by centrifugation (10 minutes at 3000 x g) and the organic layer removed, evaporated and reconstituted in 250 µl of beginning mobile phase. Samples were then centrifuged (10 minutes at 10,600 x g) and 200 µl of supernatant transferred to HPLC vials for liquid chromatography – mass spectrometry (LC/MS/MS) analysis.

Negative ion electrospray ionization (ESI) mass spectra were obtained with a triple quadrupole mass spectrometer with a turbo ionspray source interfaced to an HPLC system (Applied Biosystems 3200 Q Trap®, Foster City, CA, Shimadzu LC 20A, Columbia, MD). The column used was YMC ODS-AQ 5µm 120 Å 50 x 2 mm with Phenomenex C18 pre-column. The LC elution gradient began with 20% acetonitrile/80% ammonium acetate + 0.1% acetic acid going to 90% acetonitrile/10% ammonium acetate + 0.1 acetic acid over 3.5 minutes with a flow rate of 500 µl/min, sample injection volume of 10 µl, and a total run time of 5.5 minutes. The mass spectrometer settings

were: turbo ion spray temperature 500°C, spray needle -4500 V, declustering potential (DP) -55 V, entrance potential (EP) -6 V, collision energy (CE) -37, collision gas N₂ (CAD) low, curtain gas (CUR) 10, ion source gas 1 60, ion source gas 2 80, collision exit potential (CXP) 0, and needle position 6. Samples were quantified by the internal standard reference method in the MRM mode by monitoring the transition m/z 481 to m/z 125 for silibinin and m/z 271 to m/z 119 for naringenin (internal standard).

Quantitation of silibinin was based on standard curves in prepared matrix using the ratio of silibinin peak area to naringenin peak area. Pharmacokinetic parameters were determined by non-compartmental analysis and expressed as mean and standard deviation. Peak serum concentration (C_{max}) was reported in μM . The area under the curve (AUC) for 0 to 8 hours for enteral methods was calculated using the trapezoidal method and reported in $\mu\text{M}\cdot\text{hr}$. The clearance over bioavailability fraction (CL/F) was calculated as dose divided by $\text{AUC}_{0-8\text{hr}}$ and reported in L/hr. Pharmacokinetic parameters for intravenous dosing were calculated using commercial software (WinNonlin Version 4.1, Pharsight Corporation, St Louis, MO). Bioavailability (%) was determined by dividing the enteral $\text{AUC}_{0-8\text{hr}}/\text{dose}$ by the intravenous $\text{AUC}_{0-8\text{hr}}/\text{dose}$ and multiplying by 100. Relative bioavailability (%) between differing routes of enteral administration was calculated by dividing the oral $\text{AUC}_{0-8\text{hr}}/\text{dose}$ by nasogastric $\text{AUC}_{0-8\text{hr}}/\text{dose}$ and multiplying by 100.

Results

Nine healthy horses with normal physical exam parameters were studied in phase I. All horses were geldings with a mean age of 15 years, Range 4 – 29 years. Breed of

horses included 7 Quarter Horses, 1 Arabian, and 1 Andalusian. Mean weight was 589 kg, Range 500 – 640 kg. Mean total dose of silybin phospholipid administered by enteral methods was 11.8 grams, Range 10.0 – 12.8 grams. Silibinin IV dose solution contained 166 mg/mL. Horses were administered a mean volume of silibinin solution of 23.1 mL, Range 19.6 – 25.1 mL. Both oral and intravenous forms were well tolerated.

Five healthy horses with normal physical exam parameters were studied in phase II. All horses were geldings with a mean age of 13 years, Range 5 – 17 years. Breed of horses included 3 Quarter Horses, 1 Arabian, and 1 Andalusian. Mean weight was 582 kg, Range 472 – 625 kg. Mean total dose of silybin phospholipid administered in meals in week 2 was 11.6 grams, Range 9.4 – 12.5 grams, in week 3 was 23.3 grams, Range 18.9 – 25.0 grams, and week 4 was 46.5 grams, Range 37.8 – 50.0 grams. Seven days of twice daily feeding of silybin phospholipid and blank diet was well tolerated. On day 7 of week 4 (highest dose week), the meal was incompletely consumed (1 horse) or consumed slowly (1 horse).

Terminal elimination was 2.1 hours (Range 1.5 – 3.3 hours). Maximum serum concentration and total exposure were higher during intravenous administration compared to enteral administration. Pharmacokinetic parameters for differing routes of administration of identical doses of silibinin evaluated in phase I are given in Table 2. When enteral routes were compared, maximum serum concentration and total exposure were enhanced by nasogastric, as illustrated graphically in Figure 3. Bioavailability for the oral fed silibyn phospholipids was 1.4% and nasogastric bioavailability was 5.0%. Relative oral bioavailability of silybin phospholipid compared to nasogastric administration was 28%.

Table 2: Pharmacokinetics of single dose silibinin in horses given by enteral and intravenous routes. Results reported as mean and standard deviation.

Route	n	Dose of Silibinin (mg/kg)	C _{max} (μM)	AUC _{0-4hr} (μM·hr)	AUC _{0-8hr} (μM·hr)	CL/F (L/hr)
Oral in feed	9	6.5	0.10±0.11	0.09±0.07	0.12±0.14	182795±169276
Nasogastric	9	6.5	0.32±0.24	0.34±0.27	0.40±0.29	26555±13421
Intravenous	9	6.5	19.3±3.58	-	7.45±1.18	0.0009±0.0002

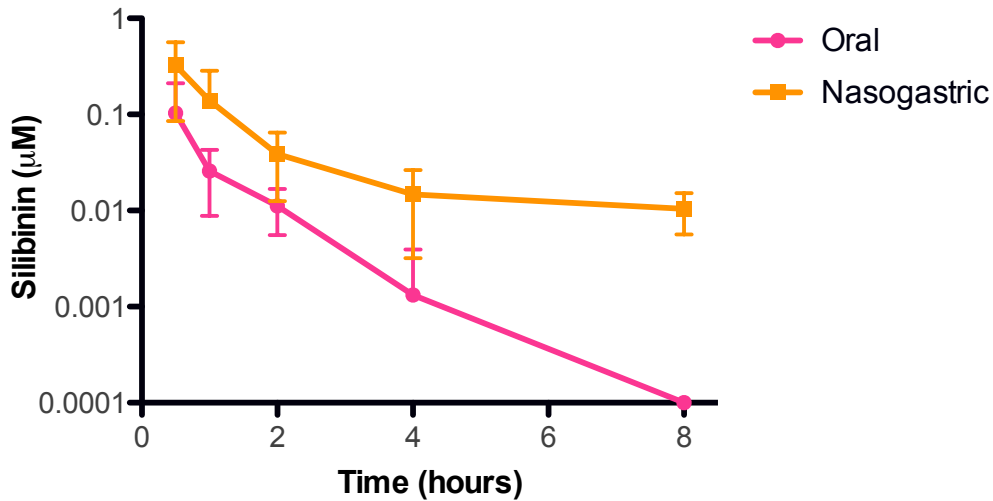


Figure 3: Cumulative mean serum drug elimination of single dose silybin phospholipid administered by nasogastric tube and fed orally in 9 healthy horses. Measured silibinin concentration (y axis) in μM is reported in log scale. Error bars represent standard deviations. Eight hours after feeding silybin phospholipid orally, serum silibinin levels were below the lower limit of quantitation of the assay (2.5 ng/mL).

Pharmacokinetic parameters for differing doses of silibinin given with feed are given in Table 3.

Table 3: Pharmacokinetics of single dose silibinin in horses given with feed. Results reported as mean and standard deviation.

Dose of Silibinin (mg/kg)	C_{max} (μM)	AUC_{0-4hr} (μM·hr)	AUC_{0-8hr} (μM·hr)	CL/F (L/hr)
6.5	0.10±0.11	0.09±0.07	0.12±0.14	182795±169276
13	0.17±0.11	0.17±0.10	0.24±0.10	79631±38383
26	0.84±0.51	1.11±0.41	1.40±0.39	24385±7571

Apparent oral clearance (CL/F) of silibinin in horses, reported in Table 3, decreased with increasing dose. A more than proportionate increase in silibinin maximum serum concentration as well as the area under the serum concentration-time curve (AUC) was found to occur at the highest silibinin dose administered. These disproportionate increases are illustrated in Figure 4. To further investigate the linearity of silibinin oral pharmacokinetics, maximum serum concentration (Figure 5) and area under the serum concentration-time curve (Figure 6) were plotted against increases in dose.

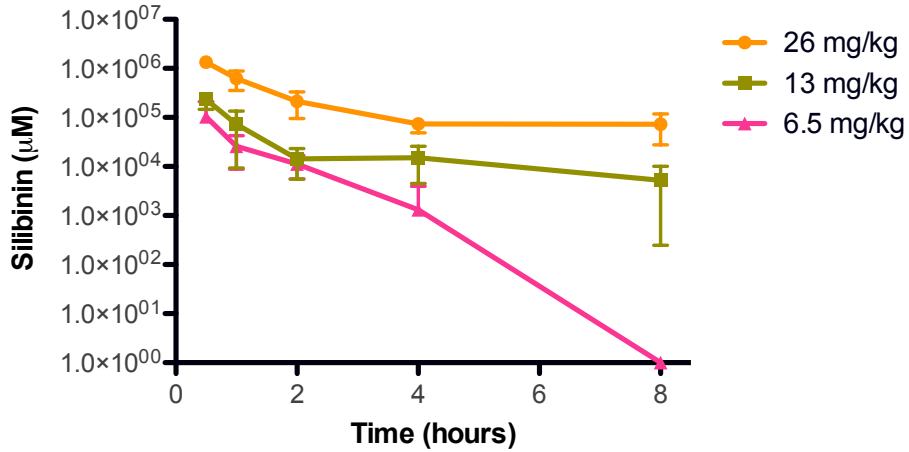


Figure 4: Cumulative mean serum drug elimination of single dose silybin phospholipid fed orally in healthy horses. Dosages listed in the figure legend reflect silybin content. Measured silybinin concentration (y axis) in μM is reported in log scale. Error bars represent standard deviation.

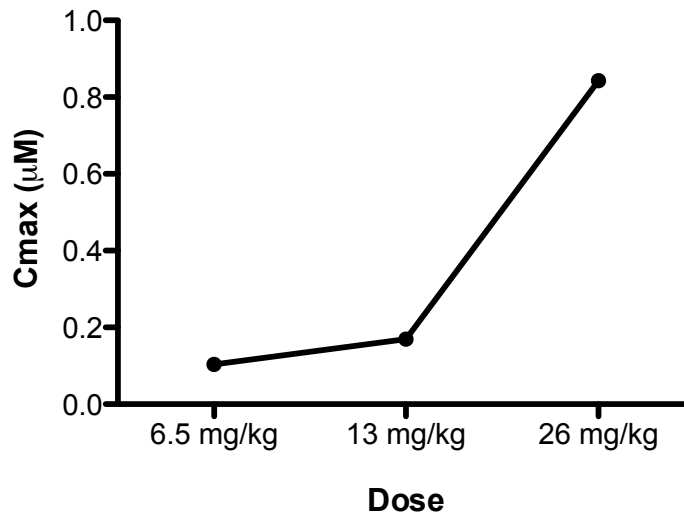


Figure 5: Cumulative mean maximum serum silybinin concentration plotted by dose. Dosages listed on the x axis reflect silybin content. Maximum silybinin concentration (y axis) in μM is reported in log scale.

Maximum serum concentration was found to increase disproportionately with increases in dose. The relationship between silybinin dose and maximum serum concentration was

nonlinear (Figure 5). Further, the relationship between silibinin area under the serum concentration-time curve was also found to be nonlinear (Figure 6).

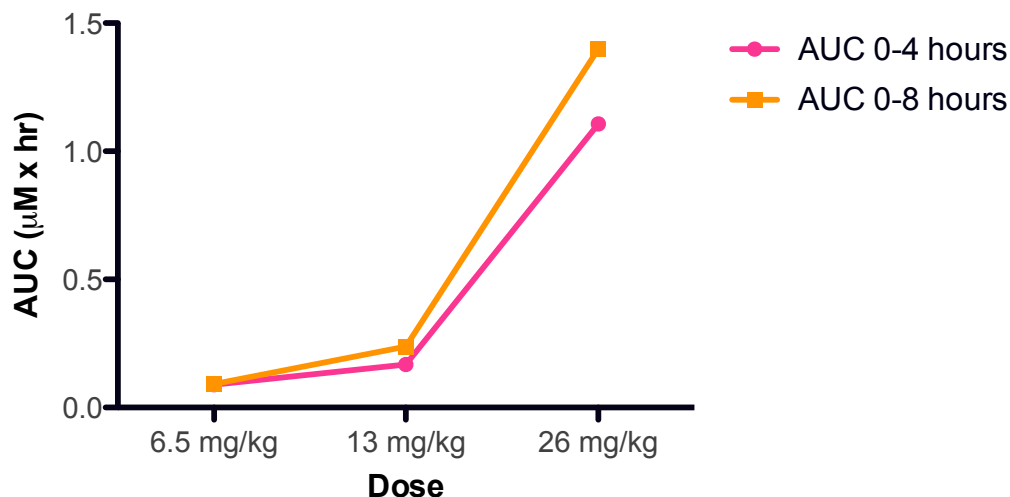


Figure 6: Cumulative mean silibinin exposure plotted by dose. Dosages listed on the x axis reflect silibinin content. Silibinin area under the serum concentration-time curve (AUC) (y axis) in $\mu\text{M} \times \text{hr}$ is reported in log scale.

The relationship between maximum serum concentration, area under the serum concentration-time curve, and dose indicated a nonlinear pharmacokinetic relationship.⁷⁵

On day 7 of the multi-dose escalation trial, trough concentrations of silibinin were found to be below the lower limit of quantitation of the assay. Concentration of silibinin measured 1 hour following oral administration did not differ between day 1 and day 7 for horses receiving 6.5 mg/kg ($p=0.3557$) and 13 mg/kg silibinin ($p=0.4135$). One hour post administration samples were lower on day 7 than on day 1 for horses receiving 26 mg/kg silibinin when the mean of all horses were considered ($p=0.0527$). If the horses that either incompletely consumed the dose (1 horse) or consumed the dose slowly (1 horse) were censored, 1 hour post administration serum silibinin levels did not differ.

Discussion

This is the first reported study of bioavailability and pharmacokinetic disposition of milk thistle extracts in horses. As expected, bioavailability of orally administered silybin phospholipid was below 2% and elimination half-life was short, with a mean of approximately 2 hours. Silibinin did not accumulate when given twice daily for 7 days. An unexpected finding was the observation of nonlinear pharmacokinetic behavior of silibinin in horses, as doses as high as approximately 80 mg/kg of silibinin evaluated in people with normal hepatic function undergo linear kinetics.¹⁵ This apparent anomaly in the kinetics of milk thistle extracts, when administered to healthy horses, could have more widespread ramifications in the study of effects when used to treat clinical hepatic disease. Investigation of milk thistle extract pharmacokinetics in normal horses is an important element of discovery and a necessary first step prior to study in horses with clinical hepatic disease.

Oral silibinin bioavailability was tested by administering silibinin complexed to phospholipids both by nasogastric tube and fed in typical feedstuffs. As reported in the literature, when silibinin alone is administered, absorption from the gastrointestinal tract is so low that levels are undetectable in the serum.⁶⁶ Therefore most investigators, as in the present study, use commercially available silibinin complexed with phosphatidylcholine forming a phytosome to enhance bioavailability. The present study confirms that silibinin compound bioavailability is low in horses, as it is in other species. For example, oral bioavailability of silibinin is estimated to be 0.73% in rats⁷⁴ and 6-7% in cats.⁷³ In the present study, silybin phospholipid was readily consumed by the horses at lower doses. Even though the data indicate that relative bioavailability is improved with

nasogastric administration when compared to feeding, this method of dosing is impractical for horse owners, especially those faced with chronic administration regimens. Though drug exposure is greater when feed is supplemented with 80 mg/kg silybin phospholipid, compliance may be problematic when feeding higher doses over time.

Nonlinear pharmacokinetics of silibinin observed in this study may be due to saturation of conjugation or enterohepatic circulation, as seen at higher doses in people with hepatitis.⁵ Enterohepatic circulation would contribute to increases in both plasma silibinin and silibinin glucuronide levels if metabolism capacity was not exceeded. Higher plasma levels could result from the greater dose exposure overwhelming hepatic metabolism. This could be tested through simultaneous measurement of plasma silibinin glucuronide conjugates and expression of relative amounts of measured silibinin glucuronide to silibinin parent compound. Though the relationship was not investigated in this study, it may be expected that portal blood levels would be significantly greater than peripheral serum levels, along with concurrent delivery to target tissues including Kupffer cells, stellate cells, and inflammatory infiltrate.⁵

Due to hepatobiliary excretion, silibinin maximum concentration within the bile is greater than twice plasma levels following intravenous administration in rats⁷⁴ and approximately 100 times higher than serum levels following oral administration in humans.⁶ High biliary concentrations and hepatobiliary recycling of silibinin and related compounds may be responsible for the hepatoprotective effects seen following ingestion, despite poor bioavailability. In addition, the pharmacokinetics of silibinin has been shown to be altered in hepatic disease.¹⁴ For example, human patients with hepatic

cirrhosis had nearly 5 times greater exposure of silymarin flavolignans following ingestion than healthy volunteers.¹⁴ This suggests that further study is needed to investigate if silybin phospholipid pharmacokinetics may be altered in horses with hepatic disease.

The concentration of silibinin required to elicit a biological effect is varied, with *in vitro* inhibition of xanthine oxidase between 10 and 50 μM .⁷⁶ Studies of silybin anti-inflammatory and anti-fibrinogenic effects on human hepatic stellate cells evaluated *in vitro* demonstrated dose dependent inhibition when concentrations between 5 and 50 μM were evaluated.³² Tissue concentrations of silibinin are known to be higher for a more prolonged period than peripheral blood levels following ingestion, and this phenomenon is reflected in the concentration of silibinin used in *in vitro* studies.⁷⁷ In the healthy horses in the present study, an oral dose of 20 mg/kg of silybin phospholipid resulted in peak serum silibinin concentrations of $0.10 \pm 0.11 \mu\text{M}$. This compares to plasma levels clinically effective in people that range from 0.03 to 0.14 μM .¹⁴ Evaluation of silybin phospholipid single dose oral pharmacokinetics in horses suggests that a dosing recommendation of 20 mg/kg may be effective, though confirmation in horses with hepatic disease would be required prior to suggesting a recommended dose to treat this population.

CHAPTER 3: SILIBININ PHARMACODYNAMICS IN HORSES; EFFECT ON BLOOD ENZYMOLOGY

Antioxidant properties of silibinin have been shown to be dose dependent in mice.⁷⁷ However, the effects of silibinin administration on antioxidant status in horses are unknown. Multiple assays, enzymes and substrates can be measured to evaluate antioxidant status *in vivo*. Estimation of oxygen radical absorbance capacity of plasma provides information on resistance to peroxy radical injury.⁷⁸ The glutathione peroxidase enzyme is a potent detoxifier of superoxide anions and hydrogen peroxide, and in this manner limits oxidative injury.⁷⁹ Another critical enzyme in oxidant protection, NAD(P)H:quinone oxidoreductase I, functions to catalyze the two-electron reduction of quinones to hydroquinones within cells, limiting one-electron reduction which results in reactive oxygen species formation.⁸⁰ Glutathione is the major intracellular reducing agent, functioning to protect against oxidative stress.⁸¹ Shifting of relative quantities of oxidized to reduced glutathione indicates a shift of redox status toward oxidative stress.⁸²

The objective of this study was to evaluate alterations in antioxidant potential of healthy horses consuming escalating doses of silibinin. Antioxidant potential was assessed by measuring (1) total plasma antioxidant content and (2) glutathione and antioxidant enzymes in peripheral blood cells. It was hypothesized that oral silibinin

administration may promote increased antioxidant potential in the peripheral blood of healthy horses and that these effects may be dose dependent.

Methods

This study was performed concurrently with the phase II pharmacokinetic study described in Chapter 2. Five horses consumed escalating oral doses of silybin phospholipid during 4 non-consecutive weeks resulting in consumption of 0 mg/kg, 6.5 mg/kg, 13 mg/kg, and 26 mg/kg silibinin twice daily for 7 days.

Sample collection

Blood samples were taken from horses on day 1, prior to administration of blank diet and silibinin, and on day 7, 1 hour following the final meal of the study diet. Blood was collected directly into EDTA tubes. Plasma was immediately separated via centrifugation at 2500 x g for 10 minutes. Plasma samples were then transferred to clean cryovials, submerged in liquid nitrogen until frozen, and stored at -80° until analysis. Following plasma removal, the middle buffy coat layer was transferred to a clean 15 mL tube, and the pelleted red blood cells (RBC) were transferred to a clean cryovial, snap frozen as above, and stored at -80° until analysis. The buffy coat sample was brought to a final 6 mL sample volume with phosphate buffered saline (PBS), layered onto 4 mL of a mixture of ficoll and sodium diatrizoate (Sigma Histopaque 1077), and centrifuged at 800 x g for 30 minutes at room temperature. Following centrifugation, the fraction containing peripheral blood mononuclear cells (PBMC) was collected and washed twice with PBS. PBMC's were counted using a hemacytometer and resuspended in PBS prior to freezing at -80° until analysis.

BCA

Protein measurement in plasma, RBC and PBMC samples was necessary to report protein corrected values of glutathione and antioxidant enzymes. Cell based samples required additional processing prior to analysis. PBMC and RBC samples were thawed on ice and diluted in 25 mM Tris (pH 7.4). Samples were then disrupted by sonication (Fisher Scientific Sonic Dismembrator 60) in three 2 second bursts at 30% power on ice, centrifuged at 15,000 x g, and supernatant collected for further analysis. Colorimetric measurement of protein was performed using bicinchoninic acid and standard curves of bovine serum albumin (Thermo Scientific, 23225 - BCA Protein Assay Kit). Sample or unknowns were added a 96-well microplate with 200 μ L of working reagent containing bicinchoninic acid, and incubated for 30 minutes at 37°C. Following incubation, absorbance was measured at 562nm using a microplate reader (Bio-tek Instruments Inc, Winooski, VT; Synergy HT). Net absorbance was calculated by subtracting blank from bovine albumin standards and unknown sample replicates. Standard curves were graphed by plotting average blank corrected albumin standards vs. concentration and unknowns estimated via linear regression. Protein for samples was reported in mg/mL.

ORAC

Oxygen radical absorbance capacity (ORAC) of plasma was measured using a commercially available assay (Cell Biolabs Inc., San Diego, CA) as described by Ungvari et al.⁸³ This assay evaluated the ability of unknown plasma samples to delay oxidation of a fluorescent probe by peroxy radicals relative to known concentrations of a water soluble vitamin E analog.⁸⁴ Plasma samples were thawed on ice, vortexed, and diluted

1:100. Fluorescein solution (Cell Biolabs 234502) 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 then read immediately following addition of 2,2'-azobis(2-methylpropionamide) hydrochloride (AAPH, Cell Biolabs 234503). Fluorescence was recorded every 84 seconds for one hour using a fluorescent microplate reader (Bio-tek Instruments Inc, Winooski, VT; Synergy HT) with an excitation wavelength of 485nm and an emission wavelength of 528nm. Net area under the fluorescence decay curve (AUC) for plasma samples was calculated by subtracting blank AUC from sample AUC, and then compared to an antioxidant standard curve of 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox, Cell Biolabs 234504). Linear regression was used to estimate the μM Trolox equivalents (TE) of plasma samples based on the Trolox standard curve. Plasma ORAC was reported as $\mu\text{mol TE/L}$.

NQO1

NAD(P)H:quinone oxidoreductase I (NQO1) of cell-based samples was measured using the method described by Gustafson et al.⁸⁵ 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 2,6-dichlorophenolindophenol (Sigma D1878) (40 μM final solution) and nicotinamide adenine dinucleotide (NADH, Sigma N4505) (200 μM final solution). Samples were evaluated in duplicate, mixing immediately prior to measuring absorbance at 600nm for 120 seconds by spectrophotometer (Beckman Coulter DU 800 UV/Vis), with and without the addition of dicumarol (20 μM final solution). NQO1 activity was defined as the dicumarol (DIC) inhibited decrease in

absorbance at 600nm, or the difference in the change in optical density per minute between DIC- and DIC+ samples. NQO1 activity was converted to nmol of DCPIP reduced per minute through calculations utilizing the extinction coefficient of DCPIP $21\text{mM}^{-1}\text{cm}^{-1}$. NQO1 was then normalized for protein content and expressed as nmol DCPIP/min/mg.

GPOX

Glutathione peroxidase (GPOX) of cell-based samples was measured using the method described by Gustafson et al.⁸⁶ Reaction mixture consisted of 2.59 mL of 50mM potassium phosphate buffer pH 7.0 with 1mM EDTA, 10 μ L of 5mM sodium azide, 100 μ L of 150mM reduced glutathione, 100 μ L of 2.2 mM hydrogen peroxide, 5 μ L glutathione reductase, and 100 μ L of 8.4mM nicotinamide adenine dinucleotide phosphate (NADPH) within a methacrylate cuvette. Unknown samples were added after a linear rate was established at 340nm absorbance for 240 total seconds measured by spectrophotometer (Beckman Coulter DU 800 UV/Vis). 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. GPOX activity was converted to pmol of NADPH reduced per minute through calculations utilizing the extinction coefficient of NADPH $6.2\text{ mM}^{-1}\text{cm}^{-1}$. GPOX was then normalized for protein content and expressed as pmol NADPH/min/g.

GSH/GSSG

Reduced glutathione (GSH) and oxidized glutathione (GSSG) were measured according to instructions from a commercially available assay (Sigma-Aldrich, St. Louis,

MO). PBMC and RBC lysates were thawed on ice and acid precipitated with 5% sulfosalicylic acid (Sigma S2130). PBMC samples were diluted 2-fold or did not require dilution for analysis. RBC samples were diluted 10- to 100-fold for analysis. Samples were added to a 96-well microtiter plate with a working solution containing 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB, Sigma D8130) and glutathione reductase (Sigma G3664). Following 5 minutes of incubation at room temperature, NADPH solution (Sigma N6505) was added and the plate was read immediately. A microplate reader (Bio-tek Instruments Inc, Winooski, VT; Synergy HT) recorded absorption of samples every 60 seconds for 5 minutes at 412nm. The resulting slope was plotted from the change in absorbance at 412nm per minute. Standard curves of GSH (Sigma G4544) and GSSG (Sigma G4376) were analyzed and linear regression of the change in absorbance at 412nm per minute was used to estimate the quantity of GSH and GSSG in unknown samples. Estimated quantity of reduced and oxidized glutathione was normalized for protein content and expressed in nmol/mg.

Statistical analysis

Change in total plasma antioxidant content, and glutathione and antioxidant enzymes in peripheral blood cells, relative to silibinin dose, was analyzed using repeated measures analysis of variance, with Bonferroni's multiple comparisons for post hoc pairwise comparisons.

Results

All five horses completed the study and seven days of twice daily feeding of silybin phospholipid in carrier diet was well tolerated.

ORAC

Plasma ORAC values were obtained in horses for all time points. Plasma samples delayed oxidation of the fluorescent probe to a greater extent than blank samples, as illustrated in Figure 7.

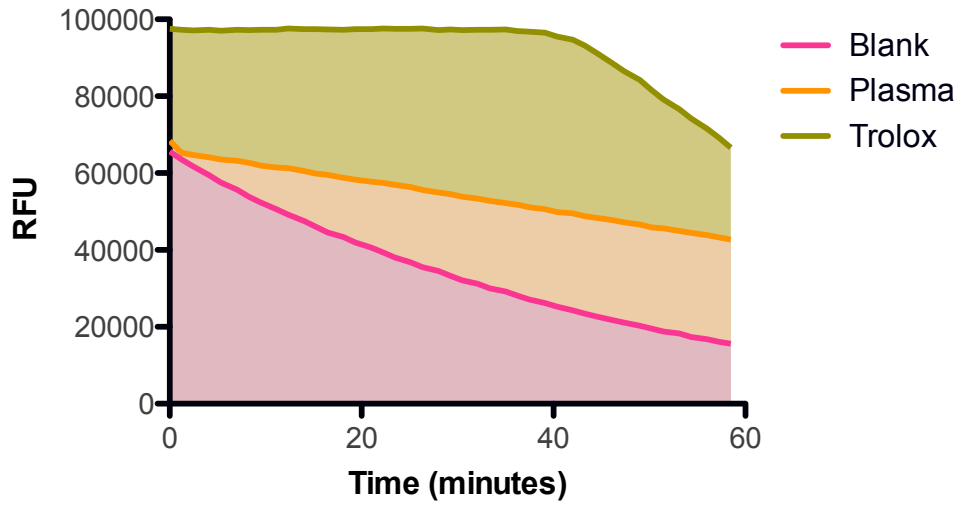


Figure 7: ORAC activity assay results for a blank sample, a 40 μ M standard sample of 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), and a plasma sample from a horse following one week of twice daily 26 mg/kg silibinin consumption. Relative area under the curve for each sample type is indicated by shading. Fluorescence in relative fluorescence units (RFU) is located on the y axis and time in minutes on the x axis.

A standard curve was constructed by graphing the net AUC of trolox samples of known concentrations. See Figure 8 for an example trolox standard curve.

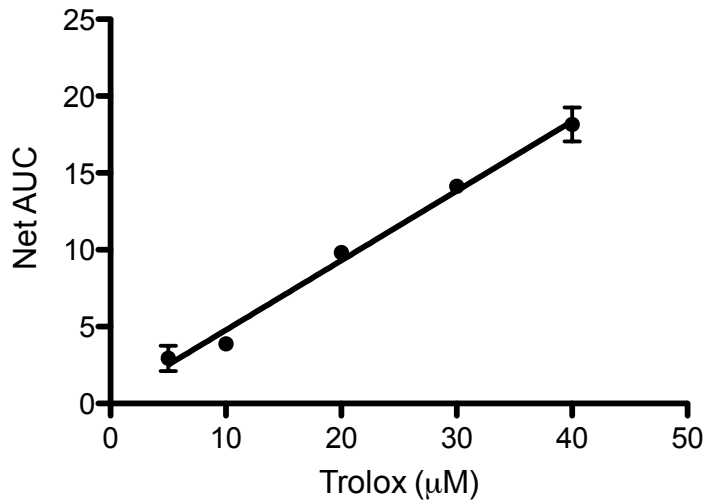


Figure 8: ORAC standard curve, used for estimation of ORAC values in plasma samples. Net area under the fluorescence decay curve (AUC) is located on the y axis in relative fluorescence units per minute and concentration of 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) in μM on the x axis. Error bars indicate standard deviation.

Based on the standard curve, plasma net AUC was converted to μmol trolox equivalents per liter and compared between horses receiving escalating doses of silibinin. Plasma ORAC values increased with silibinin administration in healthy horses, as illustrated in Figure 9.

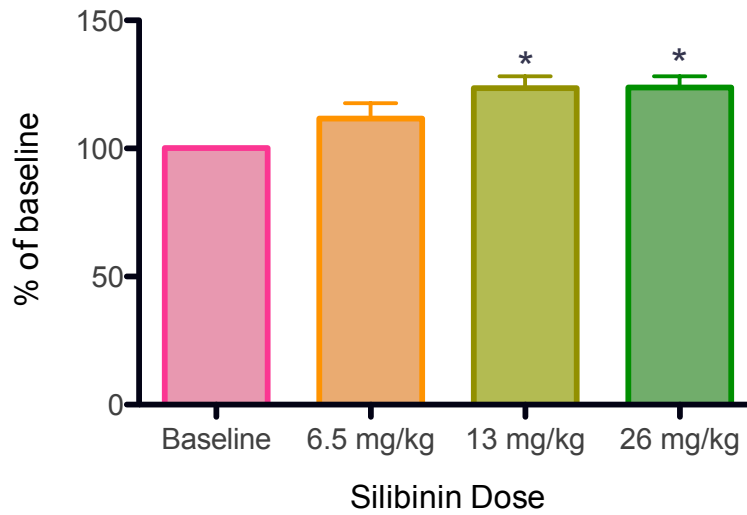


Figure 9: Silibinin administration results in a dose dependent increase in plasma oxygen radical antioxidant capacity in horses. Percent of the initial day 1 timepoint is located on the y axis. Dosages listed on the x axis reflect silibinin content. Significant differences from baseline with $p < 0.05$ denoted by ‘*’.

NQO1

NQO1 activity of PBMC samples did not differ significantly secondary to escalating doses of silibinin in horses, $p = 0.0868$. Alterations in NQO1 activity in PBMC samples secondary to silibinin dose escalation are illustrated in Figure 10.

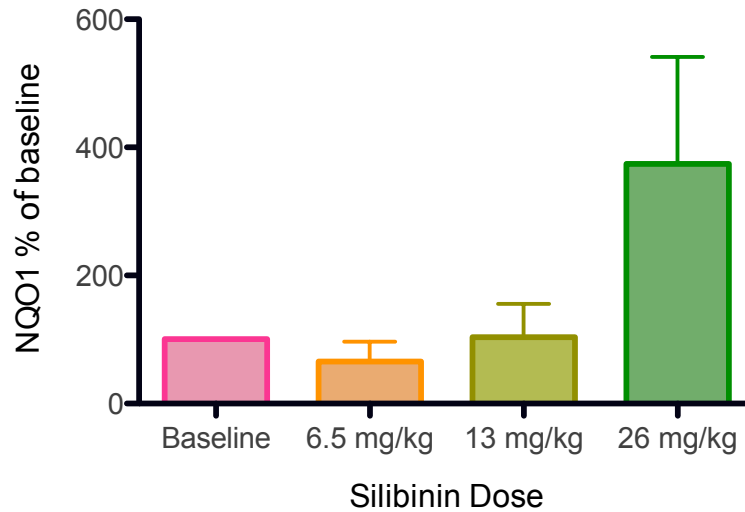


Figure 10: Alterations in PBMC NQO1 activity with increasing doses of silibinin in healthy horses. PBMC NQO1 activity did not differ significantly between groups. Percent of the initial day 1 time point is located on the y axis. Dosages listed on the x axis reflect silibinin content. Error bars indicate standard deviation.

In RBC samples, NQO1 activity decreased significantly with increasing doses of silibinin ($p=0.0166$). Alterations in NQO1 activity in RBC samples secondary to silibinin dose escalation are illustrated in Figure 11.

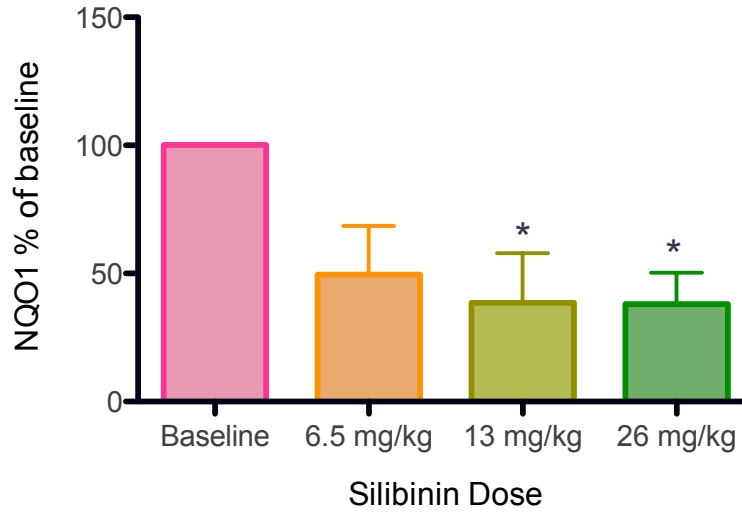


Figure 11: Alterations in RBC NQO1 activity with increasing doses of silibinin in healthy horses. Percent of the initial day 1 time point is located on the y axis. Dosages listed on the x axis reflect silibinin content. Error bars indicate standard deviation. Significant differences from baseline with $p < 0.05$ denoted by ‘*’.

GPOX

GPOX activity of PBMC samples did not differ significantly secondary to escalating doses of silibinin in horses, $p=0.3574$. Alterations in GPOX activity in PBMC samples secondary to silibinin dose escalation are illustrated in Figure 12.

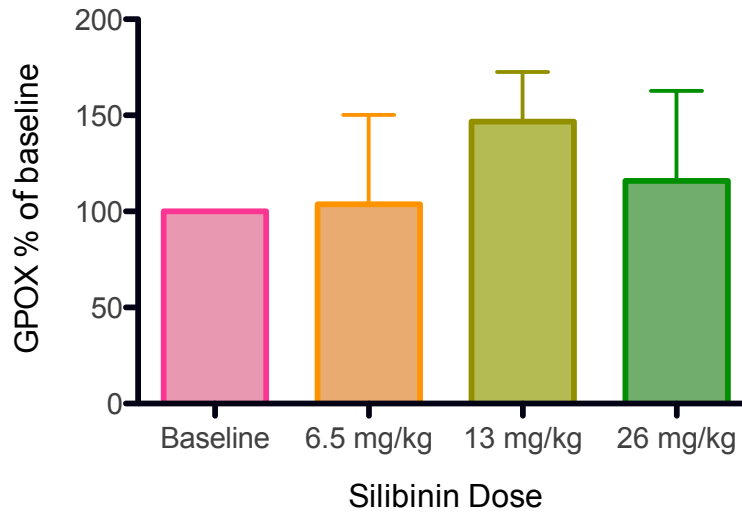


Figure 12: Alterations in PBMC glutathione peroxidase (GPOX) activity with increasing doses of silibinin in healthy horses. PBMC GPOX activity did not differ significantly between groups. Percent of the initial day 1 time point is located on the y axis. Dosages listed on the x axis reflect silibinin content. Error bars indicate standard deviation.

In RBC samples, GPOX activity did not differ significantly secondary to escalating doses of silibinin in horses ($p=0.2478$). Alterations in GPOX activity in RBC samples secondary to silibinin dose escalation are illustrated in Figure 13.

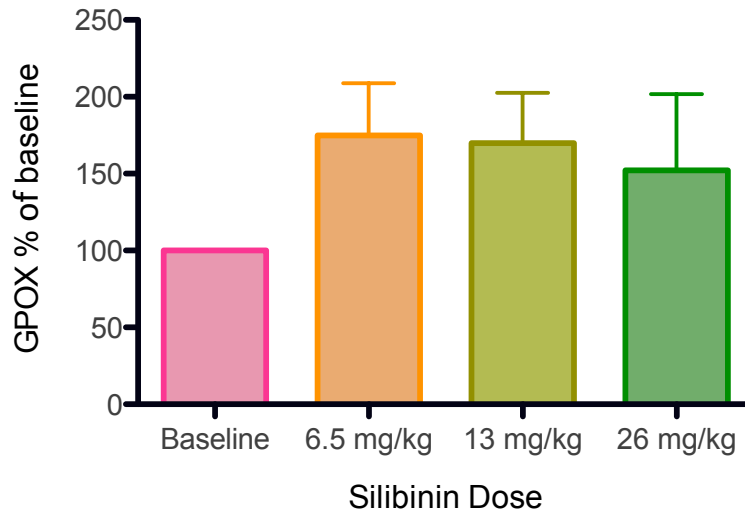


Figure 13: Alterations in RBC glutathione peroxidase (GPOX) activity with increasing doses of silibinin in healthy horses. RBC GPOX activity did not differ significantly between groups. Percent of the initial day 1 time point is located on the y axis. Dosages listed on the x axis reflect silibinin content. Error bars indicate standard deviation.

GSH/GSSG

Standard curves were constructed by graphing the change in absorbance at 412nm per minute with GSH or GSSG samples of known concentrations. See Figure 14 for an example of a GSH standard curve and Figure 15 for an example of a GSSG standard curve.

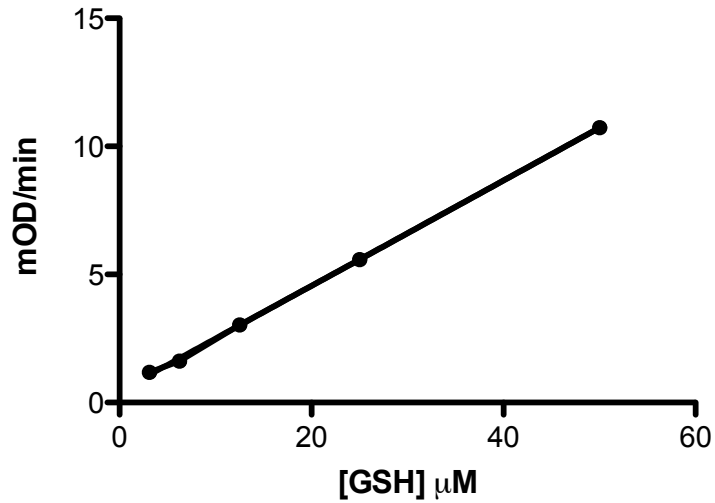


Figure 14: Reduced glutathione (GSH) standard curve, used for estimation of GSH content in cellular samples. Change in absorbance at 412nm per minute (mOD/min) (y axis) and concentration of GSH in μM (x axis).

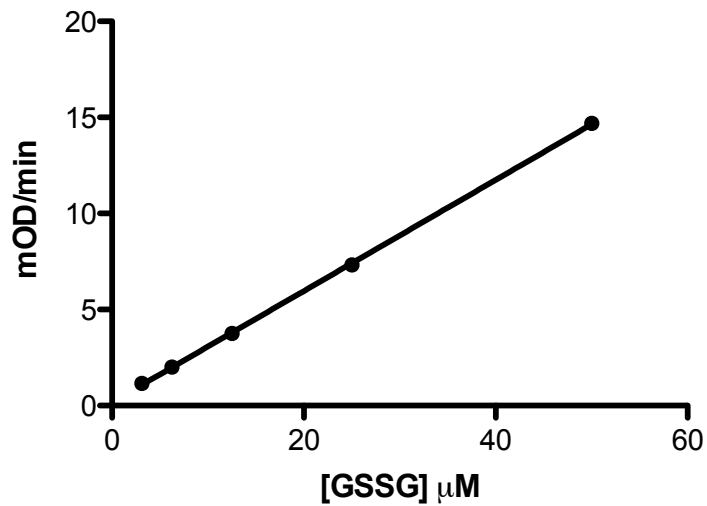


Figure 15: Oxidized glutathione (GSSG) standard curve, used for estimation of GSSG content in cellular samples. Change in absorbance at 412nm per minute (mOD/min) (y axis) and concentration of GSSG in μM (x axis).

Based on these standard curves, GSH and GSSG content was estimated for cellular samples and compared between horses receiving escalating doses of silibinin. GSH and GSSG content of PBMC and RBC samples did not increase in horses with increasing consumption of silibinin, as shown in Figures 16-19.

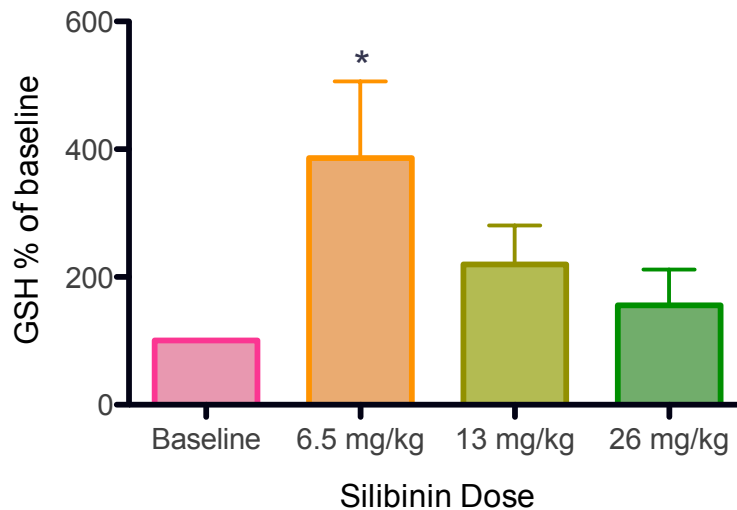


Figure 16: Reduced glutathione (GSH) content in PBMC samples was significantly elevated in horses consuming 6.5 mg/kg of silibinin (0.0349), but not with any other dose. Percent of the initial day 1 time point is located on the y axis. Dosages listed on the x axis reflect silibinin content. Error bars indicate standard deviation. Significant differences from baseline with $p < 0.05$ denoted by '*'.

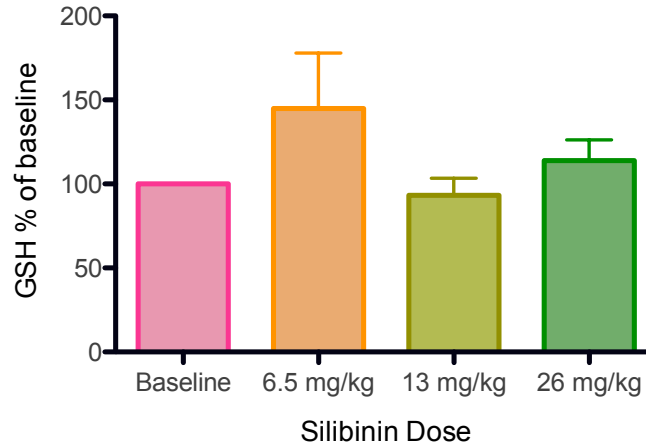


Figure 17: Alterations in RBC reduced glutathione (GSH) content with increasing doses of silibinin in healthy horses. GSH content did not differ significantly between groups ($p=0.2231$). Percent of the initial day 1 time point (y axis). Dosages (x axis) reflect silibinin content. Error bars indicate standard deviation.

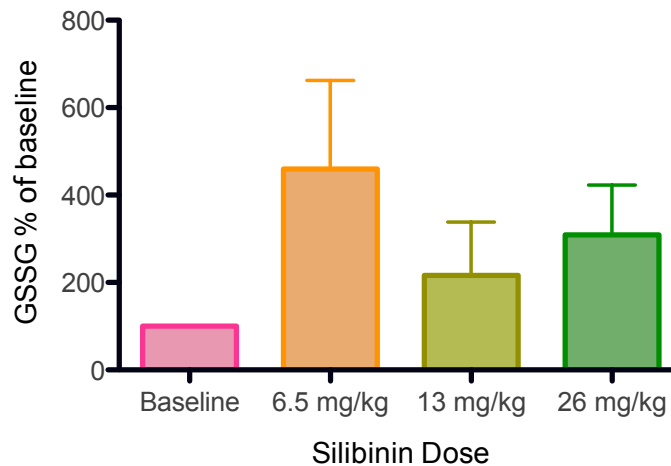


Figure 18: Alterations in PBMC oxidized glutathione (GSSG) content with increasing doses of silibinin in healthy horses. GSSG content did not differ significantly between groups ($p=0.1550$). Percent of the initial day 1 time point (y axis). Dosages listed (x axis) reflect silibinin content. Error bars indicate standard deviation.

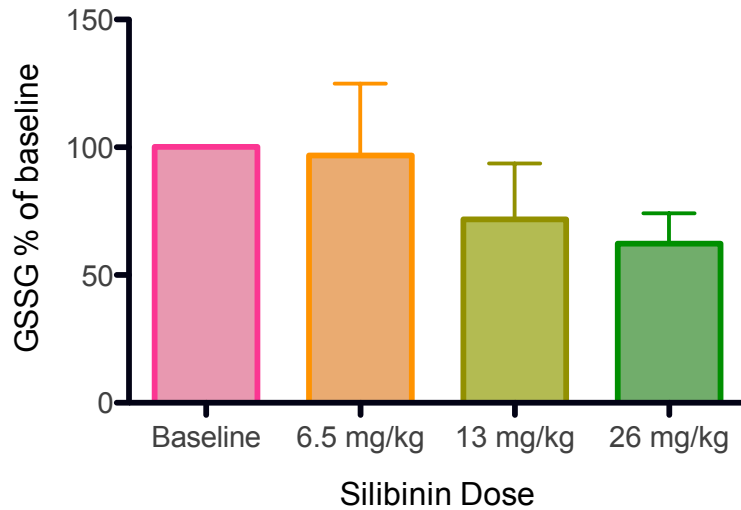


Figure 19: Alterations in RBC oxidized glutathione (GSSG) content with increasing doses of silibinin in healthy horses. GSG content did not differ significantly between groups ($p=0.4332$). Percent of the initial day 1 time point is located on the y axis. Dosages listed on the x axis reflect silibinin content. Error bars indicate standard deviation.

GSSG to GSH ratio was unchanged with silibinin dose in PBMC samples ($p=0.4527$).

GSSG to GSH ratio decreased with silibinin dose in RBC samples (see Figure 20), but this difference was not significant ($p=0.4710$).

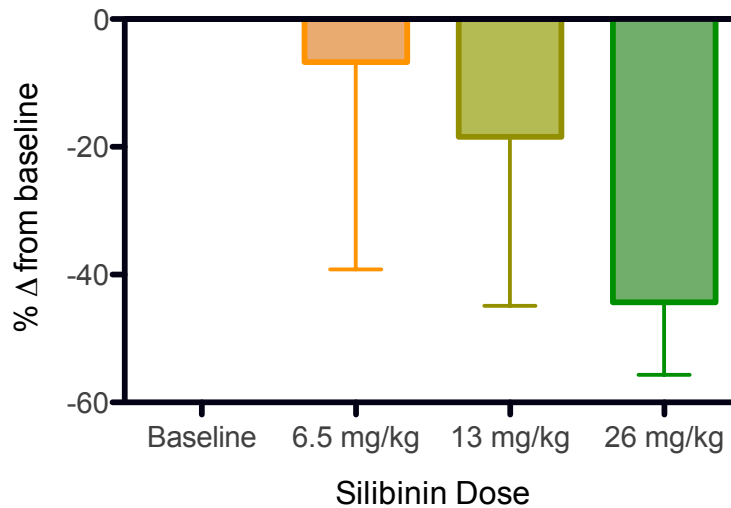


Figure 20: Alterations in oxidized to reduced glutathione ratio (GSSG:GSH) with increasing doses of silibinin in healthy horses. The decrease in GSSG:GSH with increasing dose did not reach statistical significance. Percent difference from the initial day 1 time point is located on the y axis. Dosages listed on the x axis reflect silibinin content. Error bars indicate standard deviation.

Discussion

Multiple antioxidant treatment effects were identified in horses fed diets supplemented with silibinin. Silibinin administration in horses significantly increased plasma radical absorbance capacity in a dose dependent manner. Antioxidant enzyme GPOX was elevated with silibinin administration in peripheral blood cells. Peripheral blood cell glutathione levels were also elevated with silibinin treatment. The change seen in the GSSG:GSH ratio in RBC samples was a cumulative effect of both increased GSH content and decreased GSSG content.

Much is known about the antioxidant protective effects of silibinin administration. Silibinin has positive effects on antioxidant reserve within peripheral blood cells and plasma, including restoring antioxidant defenses in pathologic disease states.

Maintenance of GPOX activity is a primary mechanism of silibinin protection against RBC lipid peroxidation and damage secondary to oxidation.⁸⁷ Silibinin administration significantly increased RBC GPOX levels in people undergoing hemodialysis⁸⁸ and ameliorated serum GPOX decreases secondary to renal ischemia and reperfusion in a rat model.⁸⁹ Silibinin also has been found to be protective against decreases in GPOX and GSH in human RBC secondary to peroxide exposure *in vitro*.⁸⁷ Silibinin restored PBMC GSH levels in people with β -thalassemia major, as well as improving the PBMC proliferative responses.⁸¹ Silibinin also improved the PBMC GSSG:GSH ratio *in vitro* following oxidant insult.⁸² In healthy cats, silibinin increased PBMC GSH content, but did not alter the GSSG:GSH ratio.⁹⁰ Though ORAC has not been specifically measured following silibinin administration, protective effects on serum and plasma total antioxidant capacity (TAC) have been shown.^{89,91} Information regarding silibinin effects on NQO1 activity was not available, although dose dependent increases in NADPH2:quinone reductase have been reported in mice *in vivo*.⁷⁷

In a study of rats receiving intraperitoneal silibinin, increases in GSH were tissue specific, focused primarily in the liver and intestines.²⁶ The authors attributed the tissue specific effects to the basic pharmacokinetics of silibinin, undergoing predominantly biliary excretion and 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 peripheral blood of horses consuming silibinin will be magnified in the liver, which is the target organ in horses with liver disease. Hepatic antioxidant effects of silibinin are well documented.^{51,62,92,93} Less is known regarding the relative

antioxidant effect on peripheral blood vs. hepatic tissues. In a model of carbon tetrachloride induced hepatitis, hepatic and RBC GSH were both measured.⁹⁴ Silibinin administration improved GSH content in both tissues, but to a greater degree in hepatic tissue.⁹⁴

In the present study, modest increases were observed in antioxidant reserves in healthy horses consuming silibinin. This outcome was predicted based on previous observations in healthy animal populations.⁹⁰ Though not tested, it is presumed that a larger effect could be expected with diseases in which oxidant injury and oxidative stress are prominent. In addition, due to the pharmacokinetic behavior of silibinin, the majority of antioxidant effects will likely concentrate in the liver and intestinal tract of horses.

CHAPTER 4: SILIBININ PHARMACODYNAMICS IN HORSES; EFFECT ON PERIPHERAL CYTOKINES

Amplification of inflammation due to potent cytokines released by myeloid cells, such as tumor necrosis factor alpha (TNF α) and interleukin-1 (IL-1), promotes a cascade of events that results in sequestration of polymorphonuclear leukocytes within tissues and parenchymal dysfunction.⁹¹ Inflammatory responses are beneficial in recognition of foreign antigens and clearing infection. However, prolonged or over-exuberant expression of inflammatory cytokines can be detrimental and has been linked to pathologic changes associated with disease.⁹⁵ Hepatocellular injury may be due to a combination of the primary insult and the secondary inflammatory response attracted by stressed hepatocytes.³³ TNF α is directly toxic to hepatocytes and induces apoptosis.⁹⁶ IL-1 β and interleukin-6 (IL-6) reduce hepatocyte protein synthesis, carbohydrate metabolism, and cytochrome P450-dependent detoxification.⁹⁷ Peripheral blood chemokine concentration has been linked to severity of hepatic disease in people.⁹⁸ Derangement in balance of pro- and anti-inflammatory serum cytokines is characteristic of alcoholic cirrhosis and is predictive of prognosis and mortality.⁹⁹ Inhibiting cytokine release and subsequent inflammatory cell recruitment may limit organ damage. Silibinin has been shown to protect against inflammation by limiting oxidative injury, inhibiting neutrophil migration, and regulating inflammatory mediators.⁹¹ Silibinin inhibits

expression and synthesis of inflammatory cytokines TNF α , IL-1, and interleukin-2 (IL-2).^{100,101}

The objective of this study was to evaluate if escalating doses of silibinin consumed by healthy horses produce an anti-inflammatory action. Inflammation was assessed by evaluating alterations in inflammatory cytokines in peripheral blood. It was hypothesized that oral silibinin administration would not increase inflammatory cytokines in the peripheral blood of healthy horses

Methods

This study was performed concurrently with the phase II pharmacokinetic study described in Chapter 2. Five horses consumed escalating oral doses of silybin phospholipid during 4 non-consecutive weeks resulting in consumption of 0 mg/kg, 6.5 mg/kg, 13 mg/kg, and 26 mg/kg silibinin twice daily.

Sample collection

Blood samples were taken from horses on day 1, prior to administration of blank diet and silibinin, and on day 7, 1 hour following the final meal of study diet. Blood was collected directly into commercially available evacuated tubes containing a proprietary additive to stabilize the *in vivo* gene transcription profile by reduction *in vitro* RNA degradation (Qiagen PreAnalytix PAXgene blood RNA tube, Valencia, CA). Blood tubes were frozen at -20°C and shipped on ice to the Gluck Equine Research Center at the University of Kentucky for further analysis.

RT-PCR

Real-time PCR (RT-PCR) was used to quantitate cytokine gene expression within blood samples. Samples were thawed and total RNA was extracted using a commercial kit (Qiagen PreAnalytix PAXgene blood RNA extraction kit, Valencia, CA) and manufacturers instructions. Samples were converted to cDNA via reverse transcription using 1.0 µg of RNA sample and reverse transcription master mix (Promega, Madison, WI), and incubating at 42°C for 15 minutes and 95°C for 5 minutes.¹⁰² Equine specific intron-spanning primer/probe sets were used.¹⁰³ Cytokines evaluated included TNFα, IL-1, and IL-2. Reaction mixtures composed of 5 µL cDNA, 6.25 µL nuclease-free water, 1.25 µL 20X assay mix for the primer/probe set, and 12.5 µL TaqMan[®] Universal PCR Master Mix (Applied Biosystems, Foster City, CA) were incubated at 95°C for 10 minutes and underwent 40 cycles within a sequence detection system (Applied Biosystems 7500, Foster City, CA). Beta-glucuronidase (β-GUS) was used as the housekeeping gene. Changes in gene expression were calculated using $\Delta\Delta C_T = ((\text{Cytokine threshold cycle} - \beta\text{-GUS threshold cycle})_{\text{SILIBININ DOSE GROUP}} - (\text{mean cytokine threshold cycle} - \text{mean } \beta\text{-GUS threshold cycle})_{\text{DAY 1 SAMPLE}})$.¹⁰⁴ Results were reported as relative cytokine gene expression (RQ) calculated by $2^{-\Delta\Delta C_T}$, calibrated to samples from day 1 prior to administration of the blank diet for each individual gene.

Statistical analysis

Change in quantity of cytokine mRNA relative to silibinin dose was analyzed using repeated measures analysis of variance, with Bonferroni's multiple comparisons for post hoc pairwise comparisons.

Results

All five horses completed the study and seven days of twice daily feeding of silybin phospholipid in carrier diet was well tolerated. In one horse, the RT-PCR housekeeping gene amplified too late, invalidating the cytokine data. Therefore, this horse was excluded and only 4 horses were included in the final analysis.

Levels of TNF α did not differ significantly secondary to escalating doses of silybin in horses. Relative change in TNF α with differing doses of silybin are illustrated in Figure 21.

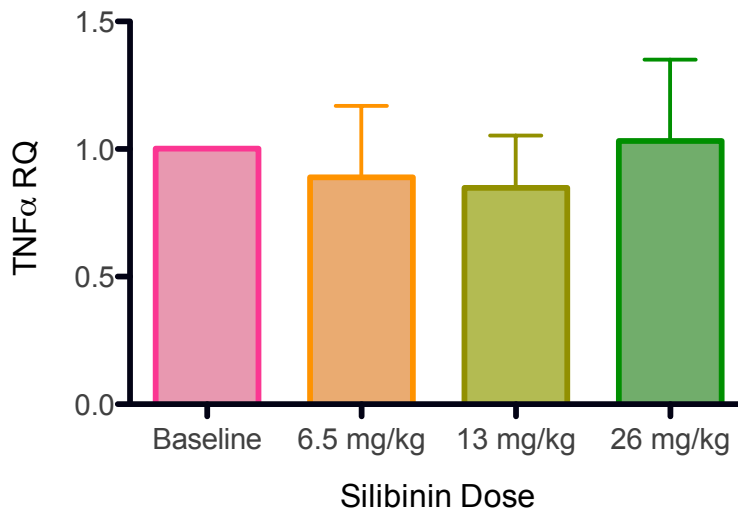


Figure 21: Alterations in TNF α with increasing doses of silybin in healthy horses. TNF α did not differ significantly between groups ($p=0.8644$). The relative TNF α cytokine gene expression of (RQ), normalized to baseline levels, is located on the y axis. Dosages listed on the x axis reflect silibinin content. Error bars indicate standard deviation.

Levels of IL-1 did not differ significantly secondary to escalating doses of silybin in horses. Relative change in IL-1 with differing doses of silybin are illustrated in Figure 22.

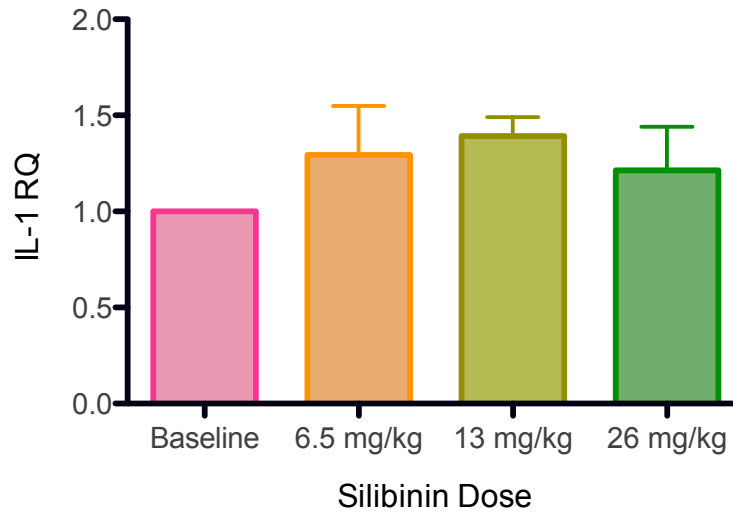


Figure 22: Alterations in IL-1 with increasing doses of silibinin in healthy horses. IL-1 did not differ significantly between groups ($p=0.5016$). The relative IL-1 cytokine gene expression (RQ), normalized to baseline levels, is located on the y axis. Dosages listed on the x axis reflect silibinin content. Error bars indicate standard deviation.

Levels of IL-2 did not differ significantly secondary to escalating doses of silibinin in horses. Relative change in IL-2 with differing doses of silibinin are illustrated in Figure 23.

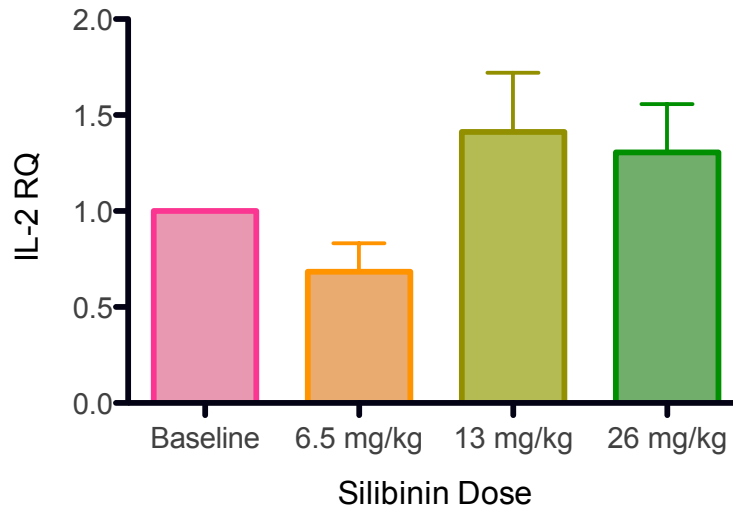


Figure 23: Alterations in IL-2 with increasing doses of silibinin in healthy horses. IL-2 did not differ significantly between groups ($p=0.0963$). The relative IL-2 cytokine gene expression (RQ), normalized to baseline levels, is located on the y axis. Dosages listed on the x axis reflect silibinin content. Error bars indicate standard deviation.

Overall, silibinin administration in healthy horses did not impact the levels of inflammatory cytokines measured.

Discussion

Levels of inflammatory cytokines $TNF\alpha$, IL-1, and IL-2 did not change in healthy horses fed diets supplemented with silibinin. In the absence of active inflammatory stimulus, it is unlikely that the established antioxidant and other anti-inflammatory effects of silibinin would result in measureable changes in levels of inflammatory cytokines. The present study provides baseline data on the effects of silibinin in health, and excludes pro-inflammatory effects of silibinin and the carrier diet. Results of the present study have application as a basis for future investigations of silibinin in disease.

Much has been learned from *in vitro* and *in vivo* models of disease with respect to the peripheral anti-inflammatory effects of silibinin administration. It has been found that silibinin reversed elevations in serum TNF α , IL-1 β , and IL-6 in a rat model of sepsis induced by cecal ligation and perforation.⁹¹ A decrease in acute lung and brain injury accompanied this anti-inflammatory effect.⁹¹ In a rat model of non-alcoholic fatty liver disease, silibinin decreased plasma levels of TNF α concurrent with improvements in liver inflammation and fatty infiltration evident on histologic examination.¹⁰⁵ In rats undergoing partial hepatectomy and resultant inflammatory response, silibinin significantly reduced serum TNF α and IL-1 activity.¹⁰⁶ TNF α release and cytotoxicity secondary to toxic insult was decreased with silibinin in perfused livers and isolated Kupffer cells of rats.¹⁰⁷ In canine hepatocytes, silibinin ameliorated the pro-inflammatory influence of IL-1 β , including production of chemotactic cytokines, and reduced hepatocyte damage.³³ Silibinin also diminished the pro-inflammatory influence of IL-1 in human hepatic stellate cells.³²

Anti-inflammatory effects of silibinin also have been documented in the peripheral blood of patients with naturally occurring disease, especially those diseases in which oxidative injury is prominent. In chronic hepatitis C infection, silibinin administration inhibits TNF α production by PBMCs.⁹⁵ Production of TNF α from peripheral blood lymphocytes in people with end-stage diabetic nephropathy was significantly reduced following silibinin supplementation.¹⁰⁸ Silibinin also reduces inflammatory cytokines and disease severity in people with non-alcoholic fatty liver disease.⁶³⁻⁶⁵

The anti-inflammatory effect silibinin in horses warrants further investigation. In a recent study of rats, *in vitro* effects of silibinin were of variable length, with prevention of cytotoxicity ochratoxin A lasting >24 hours and prevention of cytotoxicity by lipopolysaccharide (LPS) prominent at 4 hours, but not persisting to 24 hours.¹⁰⁷ The concentration of silibinin required to reduce the pro-inflammatory effects of IL-1 β in canine hepatocytes was approximately equal to the maximum serum concentration of the lowest dose tested in horses in the present study, equivalent to 6.5 mg/kg of silibinin. Relative to what is currently known about silibinin pharmacokinetics and pharmacodynamics in horses and other animals, a dose of 6.5 mg/kg at 12 hour intervals may be effective in reducing deleterious hepatic inflammatory responses in disease.

CHAPTER 5: SAFETY OF SILIBININ ADMINISTRATION IN HORSES

Silibinin toxicity and adverse events are reported to be low in humans and animals.⁴⁵ National Cancer Institute Common Terminology Criteria for Adverse Events version 3.0 (CTCAE v3.0) published August 9th, 2006 was designed for evaluation of new cancer therapies and has been used for adverse event reporting in human clinical studies evaluating high dose silibinin administration.¹⁶ Standardized criteria for adverse events are not available for veterinary species. However, adverse events guidelines as defined by the National Cancer Institute have been adapted by the Veterinary Cooperative Oncology Group for use in studies of dogs and cats undergoing chemotherapy or biological antineoplastic therapy.¹⁰⁹ Though complete, these guidelines are not universally applicable to all mammalian species, and specifically to horses. The American Society for Veterinary Clinical Pathology, in a joint task force with the American Association for Clinical Chemistry's Division of Animal Clinical Chemistry, published consensus recommendations for routine clinical pathology testing for animals in nonclinical toxicity and safety studies.¹¹⁰ In this document, the importance of pre-study health screen clinical pathology testing and recognition of patterns of effects caused by treatments of increasing doses were emphasized.¹¹⁰ The United States Food and Drug Administration's Division of Gastroenterology Products Guidance for Industry on Drug-Induced Liver Injury has outlined criteria to identify drugs with the potential for drug-induced liver injury.¹¹¹ Such drugs cause hepatocellular injury, identified by elevations

in aminotransferase enzymes, with concurrent alterations in liver function.¹¹¹

Specifically, injury is defined as an increase in aminotransferase enzymes greater than 3 times the upper limits of normal, accompanied by an unexplained increase in serum total bilirubin greater than twice the upper limits of normal.¹¹¹ Drug-induced liver injury can be idiosyncratic, that is, based on individual susceptibility rather than predictable or clearly dose-related. Thus, criteria for identification becomes more critical to discover the drug's potential for hepatotoxic injury.

The objective of this study was to determine if escalating doses of silibinin consumed by healthy horses results in adverse events. Adverse events were assessed by evaluating clinical and clinical laboratory measurements. It was hypothesized that oral silibinin administration would not result in alterations in clinical examination and plasma chemistry profiles in healthy horses, regardless of dose administered.

Methods

This study was performed concurrently with the phase II pharmacokinetic study described in Chapter 2. Five horses consumed escalating oral doses of silybin phospholipid during 4 non-consecutive weeks resulting in consumption of 0 mg/kg, 6.5 mg/kg, 13 mg/kg, and 26 mg/kg silibinin twice daily. Blood samples were taken from horses on day 1, prior to administration of blank diet and silibinin, and on day 7, 1 hour following the final meal of study diet. Blood was collected directly into evacuated tubes containing lithium heparin anticoagulant. A plasma chemistry panel was analyzed by the Colorado State University Veterinary Diagnostic Laboratory (CSUVDL), which included hepatic enzyme evaluation. The plasma chemistry panel included all relevant core

chemistry test recommended for nonclinical toxicity and safety studies, including those specifically recommended to evaluate hepatocellular and hepatobiliary health.¹¹⁰ Reference intervals previously generated by the CSUVDL and based on confidence intervals of plasma chemistry results in healthy horses, were used to interpret clinical laboratory measurements. Horses were monitored daily for abnormal clinical signs and weekly for serum chemistry abnormalities. Dose limiting toxicity was defined as: (1) an increase in either γ -glutamyl transferase greater than 3 times the upper normal limits (>60 U/L) or sorbitol dehydrogenase greater than 3 times the upper normal limits (>64 U/L), with concurrent increase in plasma bilirubin above the upper normal limits (>1.7 mg/dL), or (2) any health abnormality evident upon clinical examination. Clinical laboratory measure criteria used current CTCAE 4.03 guidelines (corresponding to Grade 2 hepatic enzyme elevation) and FDA recommendations.^{111,112} Specific criteria for clinical adverse events included gastrointestinal signs (diarrhea, colic pain, anorexia), neurologic signs (ataxia, seizures, abnormal mentation), respiratory signs (stridor, tachypnea), and vascular signs (venous thrombosis, hemorrhage, petechiation). Dose level of silibinin was increased only if no signs of toxicity were observed at the previous dose. As a precondition, if signs of toxicity occurred, individual horses were to be withdrawn from the study.

Change in clinical laboratory measurements relative to silibinin dose was analyzed using repeated measures analysis of variance, followed by Bonferroni's multiple comparisons for post hoc pairwise comparisons.

Results

All horses received escalating doses of silibinin, including the highest dose administered of 26 mg/kg silibinin, with no horses withdrawn. No dose limiting toxicity, as previously defined by critical hepatic enzyme elevation or clinical adverse events, was observed. Median γ -glutamyl transferase increased above the upper limit of the normal reference range (>20 U/L) in 3 horses following 7 days of twice daily treatment with 13 mg/kg and 26 mg/kg silibinin. Maximal elevation was observed at the 13 mg/kg dose, where median concentration was 1.4 times the upper limit of normal. Median values of other enzymes and clinical laboratory measurements related to hepatic function were within the normal laboratory reference range for horses consuming all tested doses of silibinin. The ranges and median values are given in Figure 24.

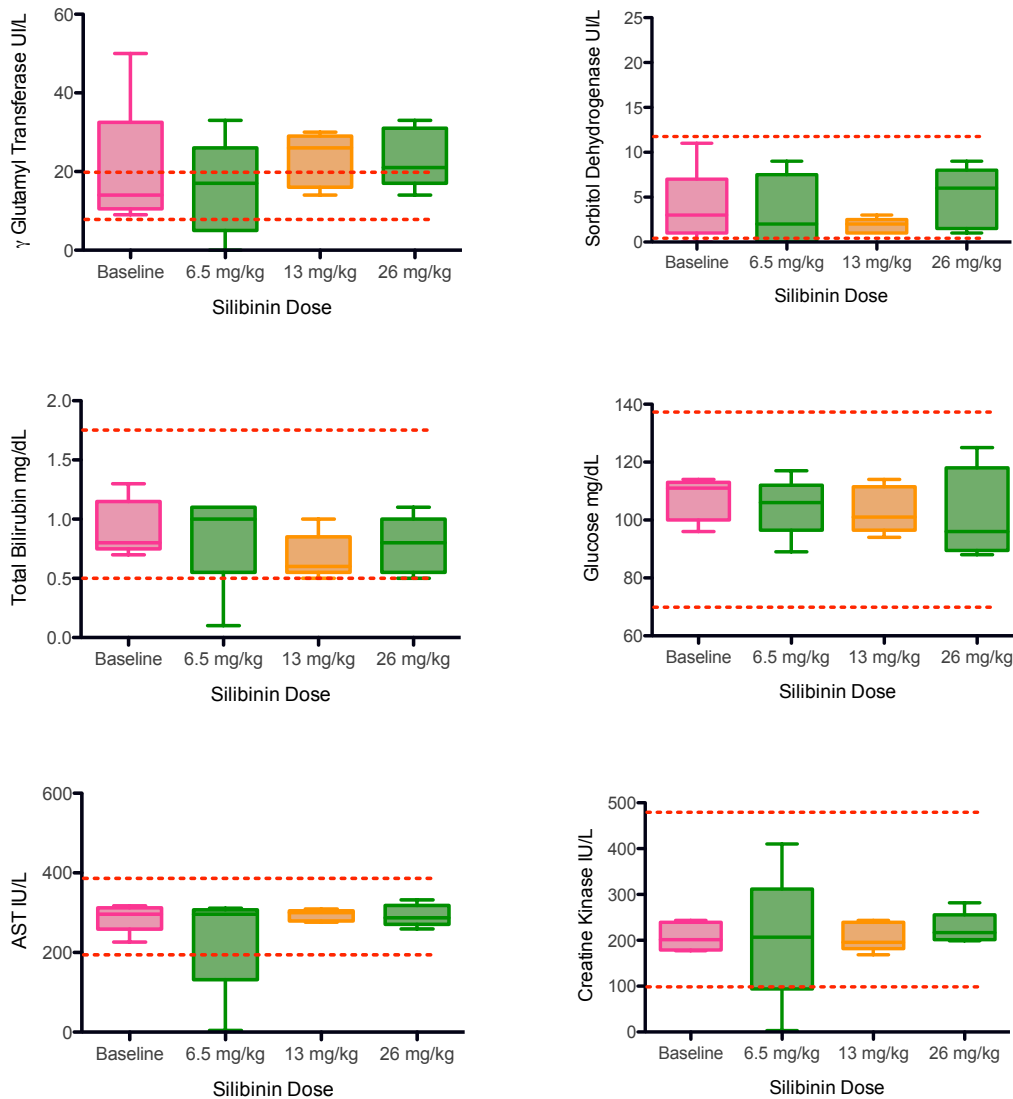


Figure 24: Clinical laboratory measurements related to hepatic function in horses consuming escalating doses of silibinin. The box-and-whisker plots divide the data into four parts using median and quartiles. Concentrations of γ -glutamyl transferase (U/L), sorbitol dehydrogenase (U/L), total bilirubin (mg/dL), aspartate aminotransferase (IU/L), and creatine kinase (IU/L) are located on the y axis. Dosages listed on the x axis reflect silibinin content. Horizontal red dashed lines indicate the boundaries of the laboratory normal reference range.

Median values of clinical laboratory measurements related to plasma proteins were within the normal laboratory reference range for horses consuming all tested doses of silibinin. The ranges and median values are given in Figure 25.

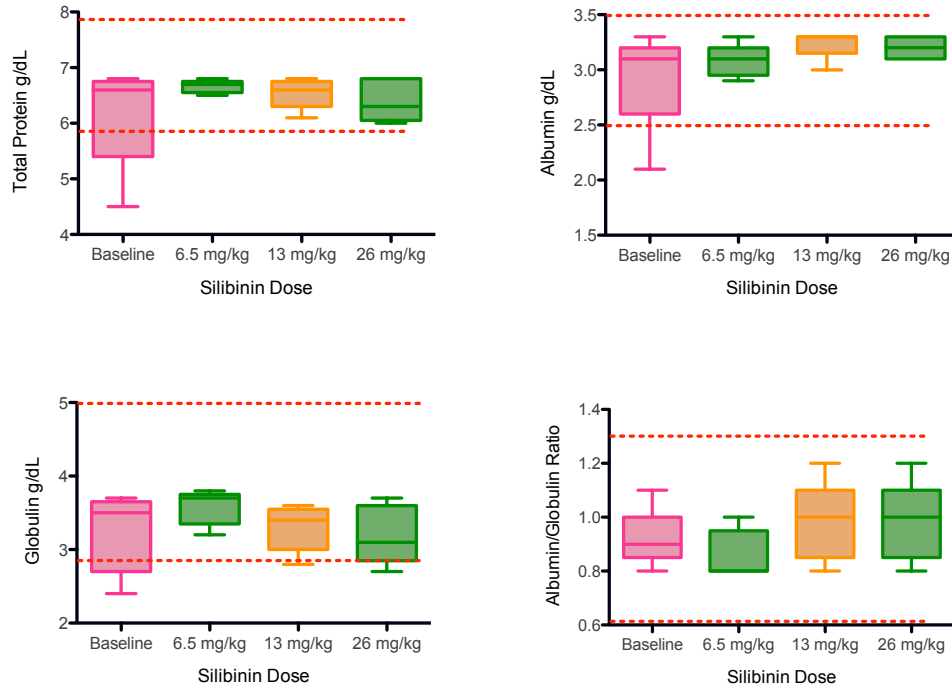


Figure 25: Clinical laboratory measurements related to plasma proteins in horses consuming escalating doses of silibinin. The box-and-whisker plots divide the data into four parts using median and quartiles. Concentrations of total protein (g/dL), albumin (g/dL), globulin (g/dL), and albumin to globulin ratio are located on the y axis. Dosages listed on the x axis reflect silibinin content. Horizontal red dashed lines indicate the boundaries of the laboratory normal reference range.

Plasma creatinine concentration was within the normal reference range for all horses throughout the study, though a significant decrease was observed in horses between baseline and consumption of both 13 mg/kg and 26 mg/kg doses of silibinin. Median plasma bicarbonate was 1.02 times the upper limits of normal in horses consuming 6.5 mg/kg silibinin. Other clinical laboratory measurements related to renal function were

within the normal laboratory reference range for horses consuming all tested doses of silibinin. The ranges and median values are given in Figure 26.

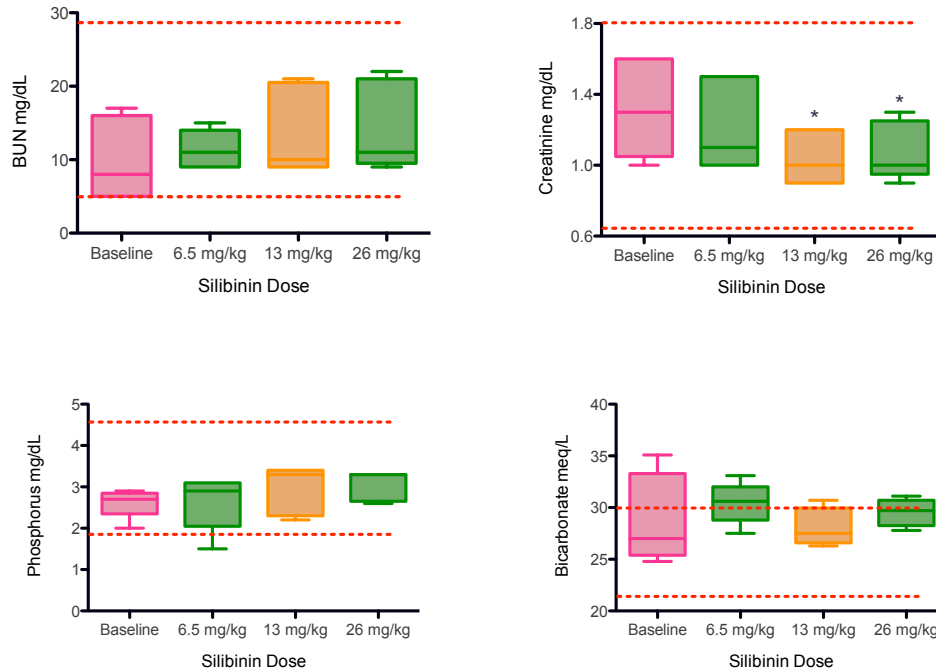


Figure 26: Clinical laboratory measurements related to renal function in horses consuming escalating doses of silibinin. The box-and-whisker plots divide the data into four parts using median and quartiles. Concentrations of blood urea nitrogen (BUN, mg/dL), creatinine (mg/dL), phosphorus (mg/dL), and bicarbonate (meq/L) are located on the y axis. Dosages listed on the x axis reflect silibinin content. Horizontal red dashed lines indicate the boundaries of the laboratory normal reference range. Significant differences from baseline with $p < 0.05$ denoted by ‘*’.

Median values of clinical laboratory measurements related to plasma electrolytes were within the normal laboratory reference range for horses consuming all tested doses of silibinin. The ranges and median values are given in Figure 27.

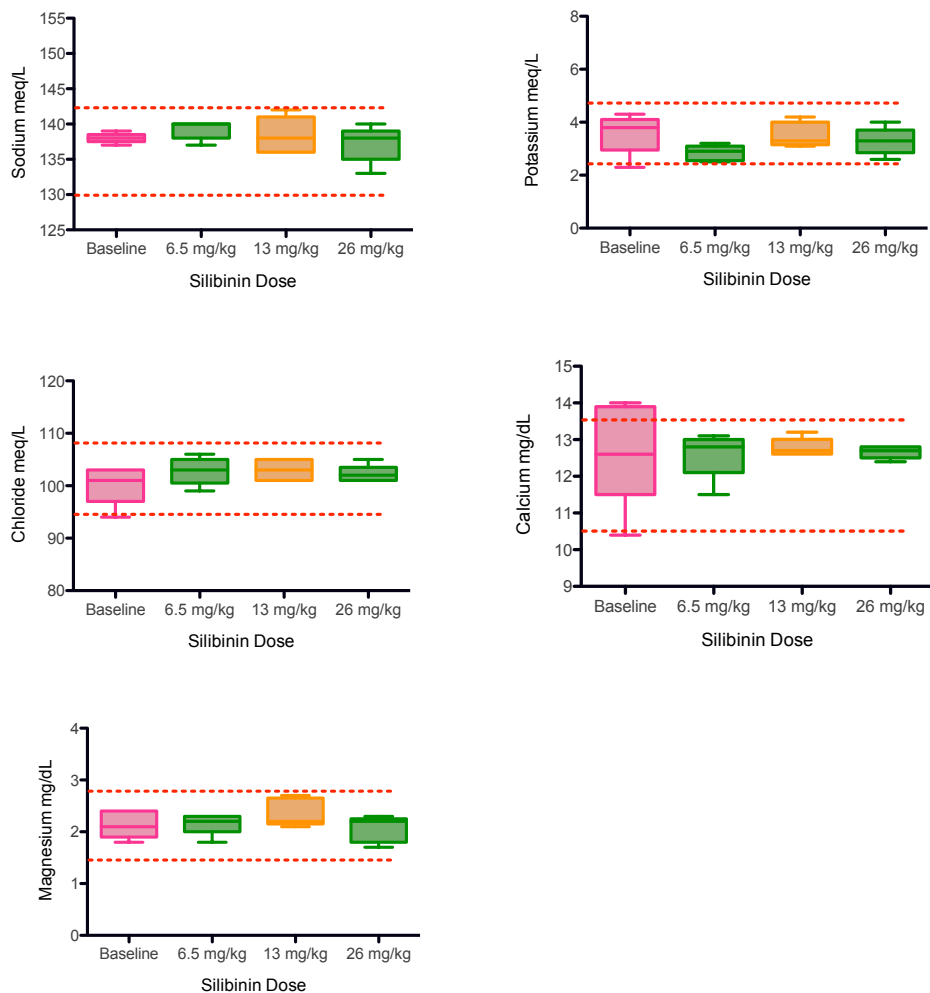


Figure 27: Clinical laboratory measurements related to electrolyte concentrations in horses consuming escalating doses of silibinin. The box-and-whisker plots divide the data into four parts using median and quartiles. Concentrations of sodium (meq/L), potassium (meq/L), chloride (meq/L), calcium (mg/dL) and magnesium (mg/dL) are located on the y axis. Dosages listed on the x axis reflect silibinin content. Horizontal red dashed lines denote the boundaries of the laboratory normal reference range.

All clinical laboratory measurements for horses consuming silibinin were within normal reference ranges or met the current definition of a Grade 1 adverse event proposed by CTCAE.¹¹²

Discussion

This is the first report of safety and toxicity of escalating doses of milk thistle extracts in horses. As expected, silibinin administration did not result in alterations in health evident upon clinical examination. However, multiple treatment effects were identified in clinical laboratory measurements of horses, including median γ -glutamyl transferase (13 mg/kg and 26 mg/kg silibinin dose) and bicarbonate (6.5 mg/kg silibinin dose) above the upper limit of normal. Neither γ -glutamyl transferase nor bicarbonate differed significantly from baseline values at any tested dose of silibinin. Plasma creatinine concentration was decreased significantly following consumption silibinin (13 mg/kg and 26 mg/kg doses), though values were within the normal reference range and not considered clinically significant. All elevations in clinical laboratory measurements met the current CTCAE definition for a Grade 1 adverse event. Despite minor alterations in clinical laboratory measurements, no dose limiting toxicity was observed.

Elevation in γ -glutamyl transferase (GGT) concentration was unexpected. Median GGT concentration was highest in horses consuming the intermediate dose of 13 mg/kg silibinin, therefore degree of elevation was not considered strictly dose related. Despite the rise in median GGT above the reference range, one horse had a GGT concentration above the upper limits of normal at the baseline measurement and concentration of GGT did not change significantly with silibinin treatment. GGT is associated with microsomal membranes in biliary epithelium, and production and release of GGT is elevated in cholestasis.¹¹³ A current reference describes typical values of GGT in normal horses as less than 30 U/L.¹¹³ Median values, and 5 of 6 individual horse values above the laboratory reference range at the higher silibinin doses tested (13 mg/kg

and 26 mg/kg), were less than 30 U/L. A rise in hepatocellular enzymes was not identified with concurrent alteration in liver function. Even when strict exclusion criteria that included a 'zero tolerance' for elevations in bilirubin were enforced, no horses were excluded based on dose limiting toxicity.

Silibinin consumption in horses was safe and did not result in clinical adverse events. Documentation of the effects of silibinin administration on clinical laboratory measurements in healthy horses may assist in interpretation of similar analysis performed in horses with liver disease undergoing silibinin treatment. Elevations in plasma GGT have not been reported in other species and identification of the mechanism of elevation may warrant further study in the horse.

CHAPTER 6: HEPATIC DISEASES OF HORSES IN THE WESTERN UNITED STATES

Hepatic disease in horses often has serious consequences, with severe illness and death of affected horses occurring commonly. Because signs of liver disease in horses are often nonspecific, hepatic biopsy and histologic evaluation is necessary to determine the presence and etiology of disease. Reported causes of liver disease in horses include serum hepatitis,¹¹⁴ aflatoxicosis,¹¹⁵ pyrrolizidine alkaloids,^{116,117} hepatic metastasis,¹¹⁸ and hyperlipemia.¹¹⁹ As the liver is the primary site of detoxification of ingested substances, horses are at risk for developing liver disease through ingestion of toxic plants while grazing.¹²⁰ For this and other reasons, type of hepatic diseases observed in horses may be regionally specific. Much of what is known about horses with liver disease is based on 2 retrospective reports of naturally occurring disease. These studies included groups of 40 or 50 horses and were both conducted in the United Kingdom.^{116,120} Histologic descriptions either were not available for all cases, lacked detail, or were not standardized. In these 2 retrospective reports, the mortality rate of liver disease in horses was reported to be 60%¹²⁰ and 72%.¹¹⁶

The objectives of this study were to describe clinical and histologic features of horses with hepatic disease admitted to the Colorado State University Veterinary Teaching Hospital and to determine outcomes and survival times of horses.

Methods

A computerized search of records was performed to identify horses older than 1 year of age that were admitted to the Colorado State University Veterinary Teaching Hospital between January 1, 2000 and December 31, 2010 that were diagnosed with liver disease, with histopathology of the liver confirmed either by surgical biopsy or necropsy examination. Slides were then retrieved from the Colorado State University Veterinary Diagnostic Laboratory repository. Slides of hepatic tissue were reviewed in conjunction with a single Board Certified Veterinary Pathologist (Patricia C. Schultheiss DVM, PhD, DACVP-AP). A standardized data collection sheet was used to record histologic observations. Observations were only recorded for the liver, even if other organ tissues had been evaluated.

Hepatic histologic specimens were observed for the presence or absence of neoplasia, bile stasis, congestion, and reversible disease. Reversible disease was defined as histologic change that did not result in permanent architectural changes, for example, inflammation and lipidosis without concurrent necrosis. Conversely, examples of irreversible disease included fibrosis and necrosis. Portal fibrosis, biliary hyperplasia, inflammation, lipidosis, hydropic degeneration and necrosis were graded within each specimen based on severity as: absent, mild, moderate, or severe. Severity scales were generated to promote consistency in observation among specimens. Specimens with portal fibrosis were further classified for the presence or absence of cirrhosis. A primary histologic diagnosis was assigned to each specimen based on relative severity of concurrent lesions. The histologic severity score for each specimen was defined as the

maximum severity of lesion between 0 and 3, recorded among all lesion categories evaluated.

Clinical data retrieved from the medical record included signalment, presenting complaint, state of residence, length of illness prior to admission, admission clinicopathologic values, and diagnosis. The clinicopathologic values recorded included bilirubin, gamma-glutamyltransferase (GGT), aspartate aminotransferase (AST), sorbitol dehydrogenase (SDH), total white blood cell count (WBC), segmented neutrophil count, band neutrophil count, albumin, globulin, packed cell volume (PCV), and plasma total protein (TP). Primary disease was further classified as hepatic, gastrointestinal, or other.

For horses that survived to hospital discharge, follow-up information was obtained through phone interview between August 14th and August 18th, 2011. Time and cause of death for each horse were recorded, if available. Survival was reported as number of days from hospital discharge until death or time of follow-up.

All continuous variables underwent Shapiro-Wilk analysis for normality, and as most variables were not normally distributed, results were presented as median and range. Analysis of Variance was used to test relationships among severity of portal fibrosis and biliary hyperplasia, severity of lipidosis and inflammation, and duration of illness and lipidosis. A Mann-Whitney test was used to compare continuous variables between horses that underwent necropsy versus surgical biopsy. Survival curves were constructed for horses discharged from the hospital. A cutpoint of 359 days was determined from the survival curve, and continuous and categorical data were compared to associate impact of

these variables on long-term survival. A log-rank Mantel-Cox test was used to investigate effect of reversible disease on survival.

Results

The computerized records search for the time period described identified 117 horses with liver disease and concurrent liver histologic examination. Slides were retrieved from the Colorado State University Veterinary Diagnostic Laboratory repository for 109 horses. Slides for 8 horses were not found. Four horses were excluded when a lesion was not identified from a review of the specimens. Five horses were excluded when the specimens were judged to be uninterpretable. Of these, specimens of 2 horses had undergone severe autolysis prior to fixation and specimens of 3 horses contained either no hepatic tissue (1 horse - skin, 1 horse - skeletal muscle) or did not contain enough hepatic tissue to evaluate the specimen completely (1 horse – no portal areas). For reasons outlined, a total of 17 horses were censored, leaving 100 horses in the sample for final analyses. Of these, histologic specimens were collected during necropsy examination from 75 horses, whereas surgical biopsy specimens were obtained from 25 horses.

Thirty-four horses had evidence of portal fibrosis on histology. Eight horses had mild fibrosis, 8 horses had moderate fibrosis, and 18 horses had severe fibrosis. Histologic images of equine hepatic tissue illustrating mild, moderate and severe fibrosis can be found in Figure 28.

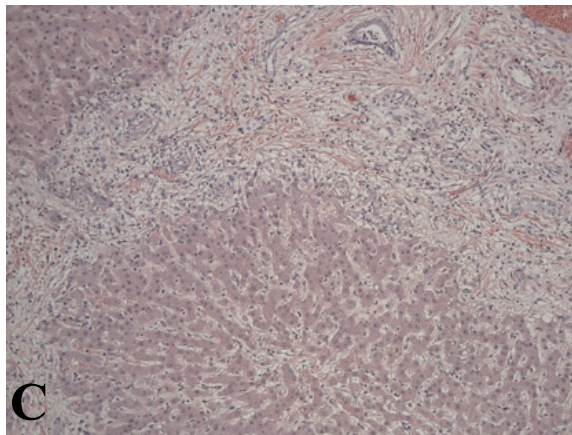
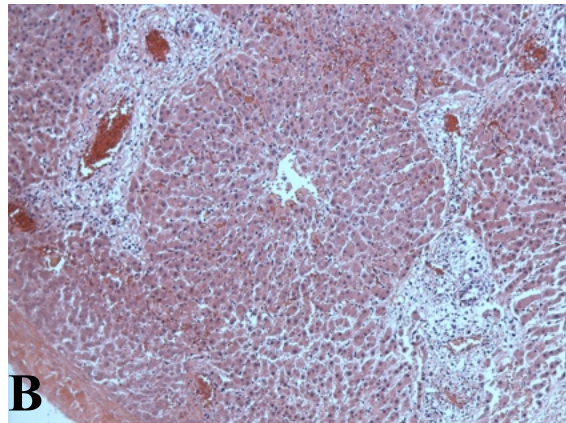
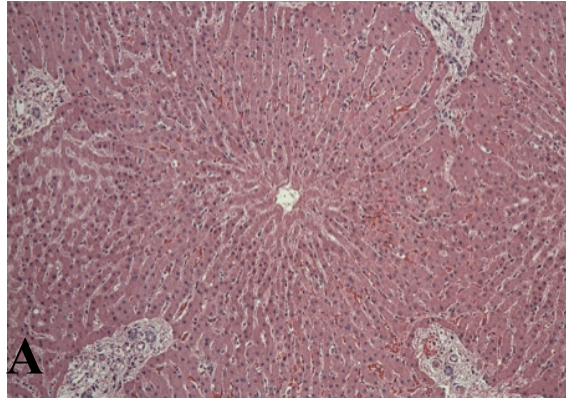


Figure 28: Images above illustrate examples of mild (A), moderate (B), and severe (C) portal fibrosis (100X magnification). Specimens were graded based on the amount of fibrous connective tissue present. Mild fibrous expansion of the portal tracts was characteristic of mild portal fibrosis. In moderate portal fibrosis, an increase in fibrous expansion was seen with rare portal-portal septa. Severe portal fibrosis was accompanied by septal fibrosis with multiple connective tissue bridges linking portal tracts.

Sixteen horses with severe fibrosis were further classified as cirrhotic. Histologic images of equine hepatic tissue illustrating cirrhosis can be found in Figure 29.

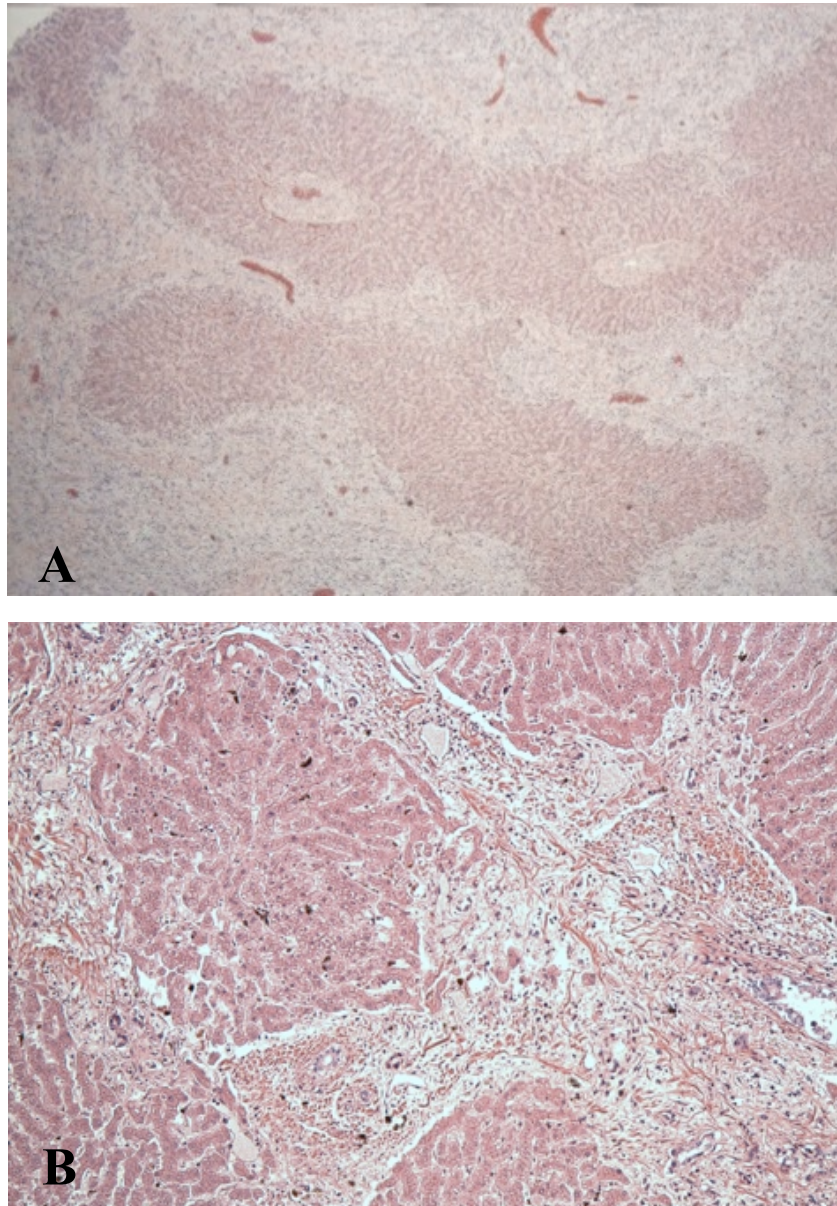


Figure 29: Images above illustrate examples of hepatic cirrhosis. Cirrhosis was defined as severe bridging portal fibrosis and architectural distortion (A - 400X magnification). Connective tissue accumulation was severe enough to result in hepatocyte isolation (B - 100X magnification).

Twenty-four horses with portal fibrosis had concurrent biliary hyperplasia. In addition, 28 of the horses with fibrosis had inflammatory changes and 9 had lipidosis. Bile stasis was a prominent feature in horses with cirrhosis, seen in 11 of 16 cases.

Twenty-eight horses had biliary hyperplasia on histology. Eight had mild biliary hyperplasia, 9 had moderate biliary hyperplasia, and 11 had severe biliary hyperplasia. Twenty-four horses with biliary hyperplasia had concurrent portal fibrosis. The relationship between severity of portal fibrosis and biliary hyperplasia was significant ($p=0.0037$), as illustrated in Figure 30. Histologic images of equine hepatic tissue illustrating mild, moderate and severe biliary hyperplasia can be found in Figure 31.

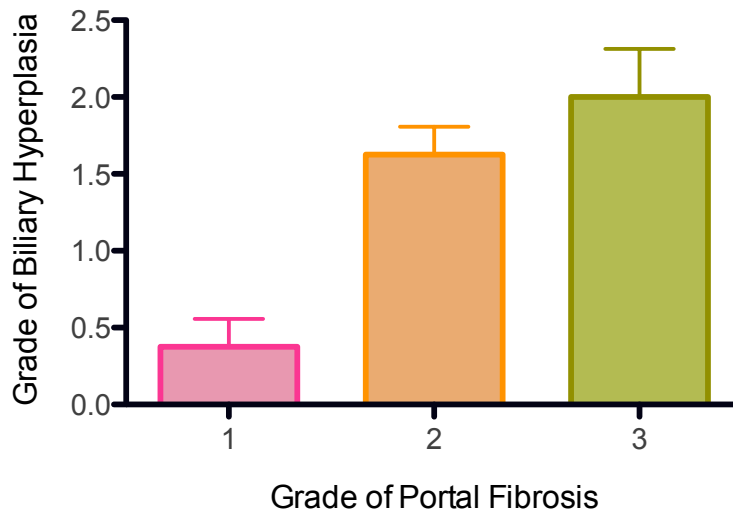


Figure 30: Relationship between severity of fibrosis and hyperplasia.

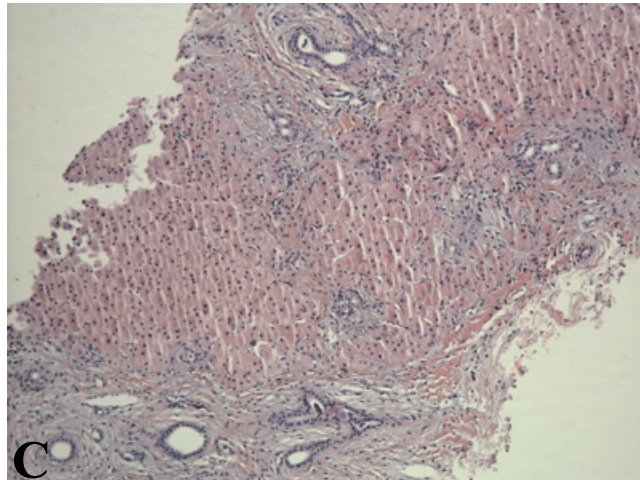
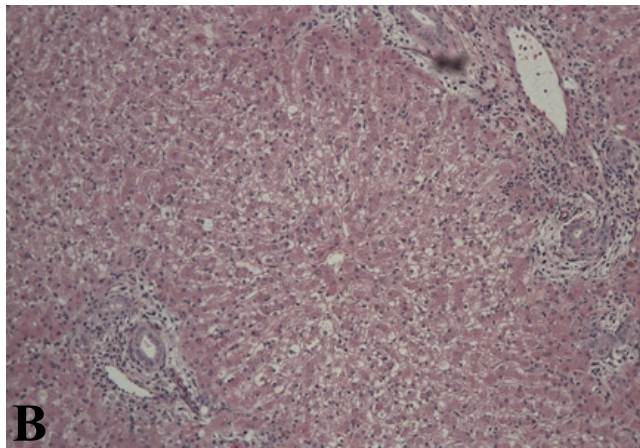
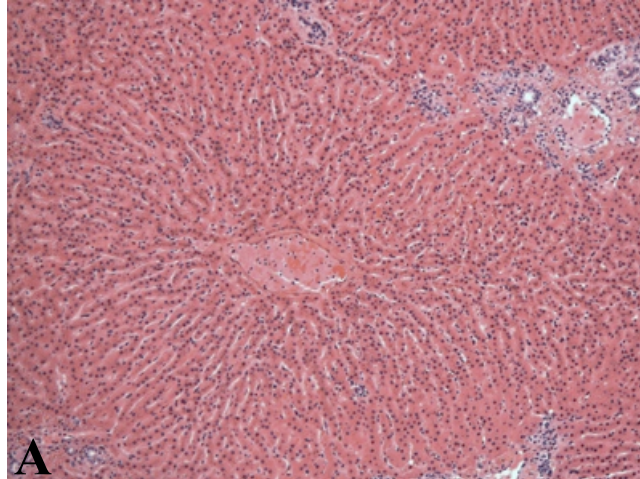


Figure 31: Images above (100X magnification) illustrate examples of mild (A), moderate (B), and severe biliary hyperplasia (C). Specimens were graded based on the relative quantity of biliary ducts present within portal areas.

Thirty-four horses had evidence of cellular necrosis on histology. Eleven horses had mild necrosis, 4 had moderate necrosis, and 19 had severe necrosis. Histologic images of equine hepatic tissue illustrating mild, moderate and severe biliary hyperplasia can be found in Figure 32. Thirty-three of 34 horses with necrosis had concurrent histologic changes. The most common accompanying lesion was inflammation (31), with eighteen showing evidence of severe inflammation.

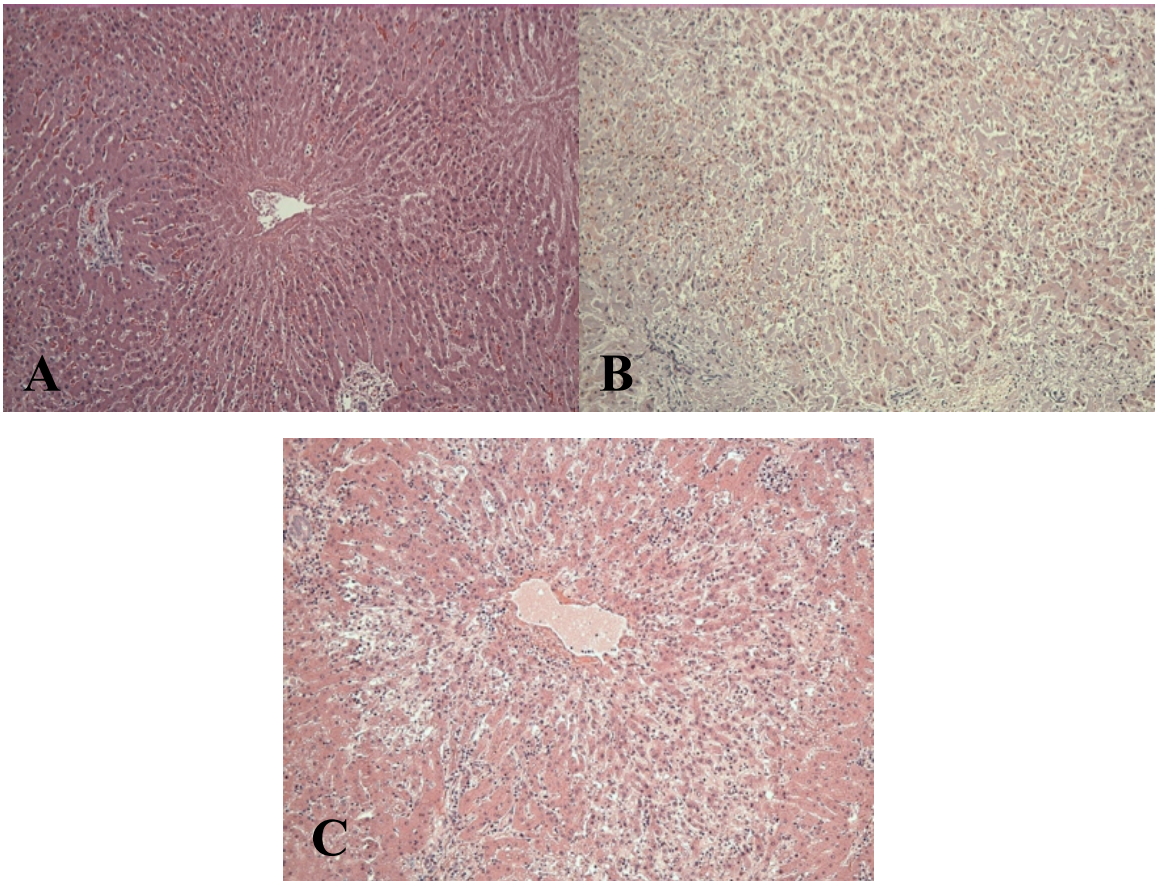


Figure 32: Images above illustrate examples of mild (A), moderate (B), and severe (C) necrosis (100X magnification). Specimens were graded based on quantity of cellular necrosis, independent of location. In specimen A, cellular necrosis is evident focally around the central vein. In specimen C, severe and extensive necrosis is evident throughout the lobule.

Thirty-eight horses had evidence of lipidosis on histology. Fifteen horses had mild lipidosis, 13 horses had moderate lipidosis, and 10 horses had severe lipidosis. Inflammatory changes were often seen concurrently in 26 of 38 horses with lipidosis. Severity of lipidosis did not seem to be related to severity of inflammatory changes observed ($p=0.2788$), as illustrated in Figure 33. However, when severity of inflammation was considered among all horses with lipidosis, the data suggested there was a link between severity of inflammation and duration of illness as depicted in Figure 34. The relationship between grade of inflammation in lipidosis and duration of illness did not reach statistical significance ($p=0.3278$).

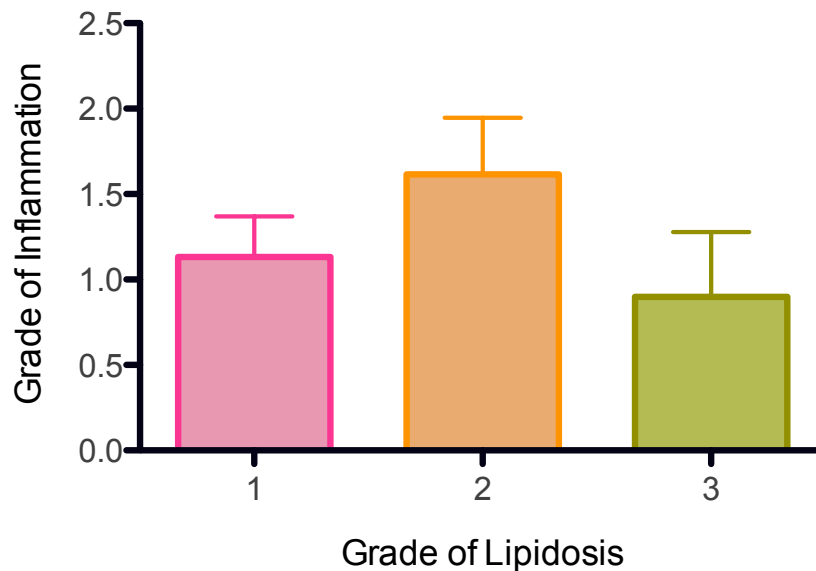


Figure 33: Relationship between severity of inflammation and lipidosis

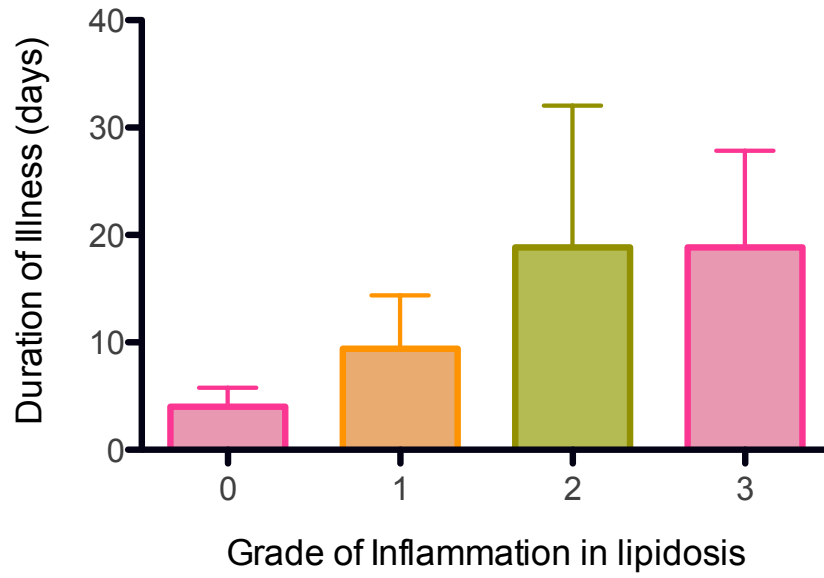


Figure 34: Relationship between steatohepatitis and duration of illness.

Histologic images of equine hepatic tissue illustrating mild, moderate and severe lipidosis can be found in Figure 35.

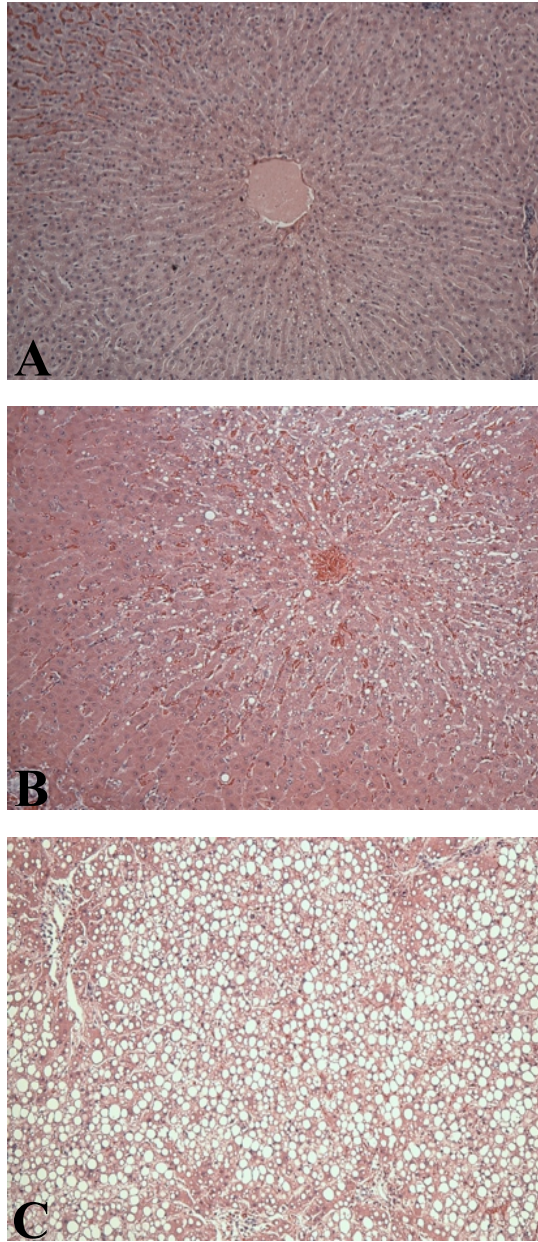


Figure 35: Images above illustrate examples of mild (A), moderate (B), and severe (C) lipidosis (100X magnification). Specimens were graded based on quantity and location of lipid infiltrate within hepatocytes. In specimens with mild lipidosis, microvesicular lipid infiltrate was restricted to the area surrounding the central vein. Moderate lipidosis was characterized by macro- and microvesicular lipid infiltrate extending from the central vein to portal tracts. Severe lipidosis was characterized by prominent hepatocellular ballooning throughout the lobule.

Hydropic degeneration was observed in 14 horses. Eight horses had mild hydropic degeneration, 5 horses had moderate hydropic degeneration, and 1 horse had severe hydropic degeneration. Hydropic degeneration was never identified without concurrent histologic lesions, and was most commonly associated with inflammatory change (13 of 14 horses). Histologic images of equine hepatic tissue illustrating mild, moderate and severe hydropic degeneration can be found in Figure 36.

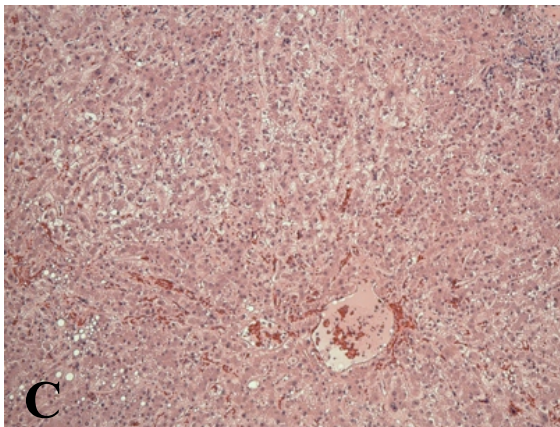
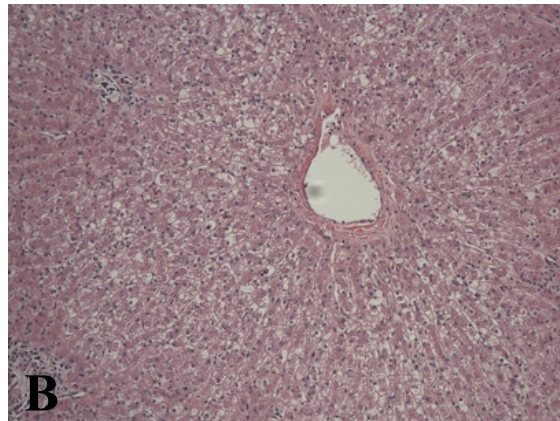
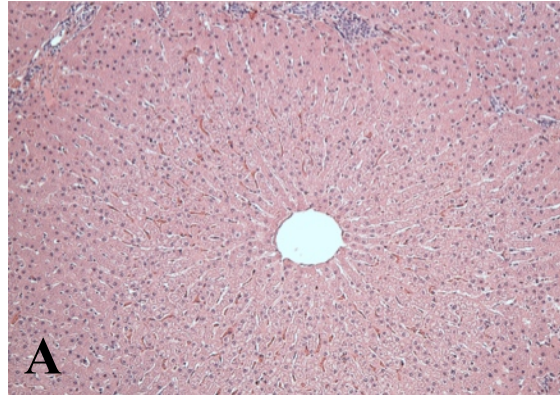


Figure 36: Images above illustrate examples of mild (A), moderate (B), and severe (C) hydropic degeneration (100X magnification). Specimens were graded based on quantity of hydropic change, independent of location. Hydropic degeneration was characterized by cell swelling and a cloudy cytoplasmic appearance secondary to intracellular water accumulation. This change was precipitated by cell membrane injury affecting transmembrane ion transfer.

Bile stasis was observed in 40 horses and was never observed without concurrent histologic lesions. A histologic image of equine hepatic tissue illustrating bile stasis can be found in Figure 37.

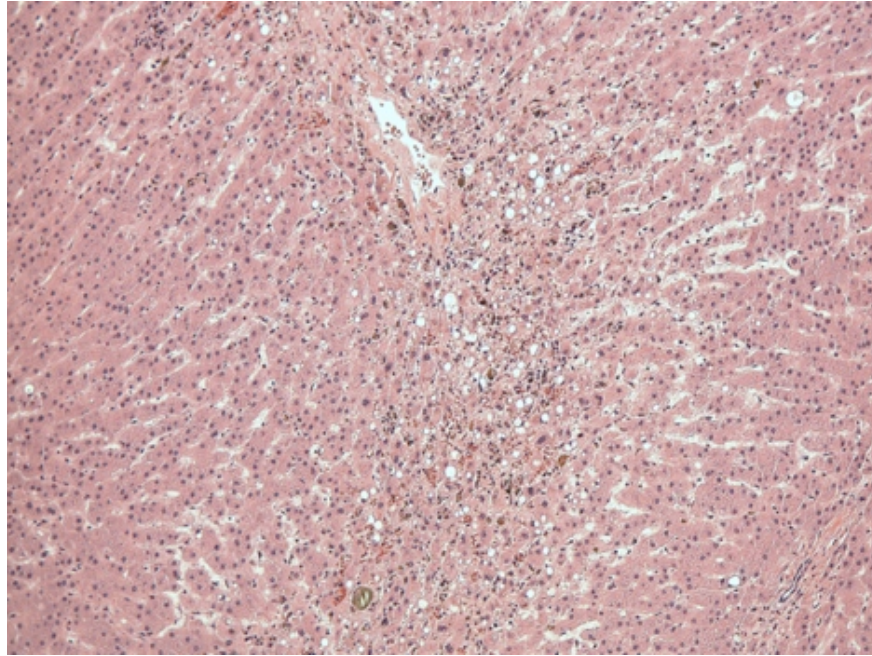


Figure 37: In this histologic image (100X magnification), bile stasis is a prominent finding. Bile lakes were variably located in canaliculi, hepatocytes, and sinusoids.

Bile stasis was observed in 34 horses with inflammatory changes and 22 horses with portal fibrosis, 11 of which were classified as cirrhotic. Bile stasis tended to be observed in horses with a higher overall histologic severity score. Horses with bile stasis had a median severity score of 3 and horses without bile stasis had a median severity score of 2. Difference in bile stasis relative to histologic severity score did not reach statistical significance ($p=0.2109$).

Congestion was observed in 18 horses and was never observed without concurrent histologic lesions. A histologic image of equine hepatic tissue illustrating congestion can be found in Figure 38.

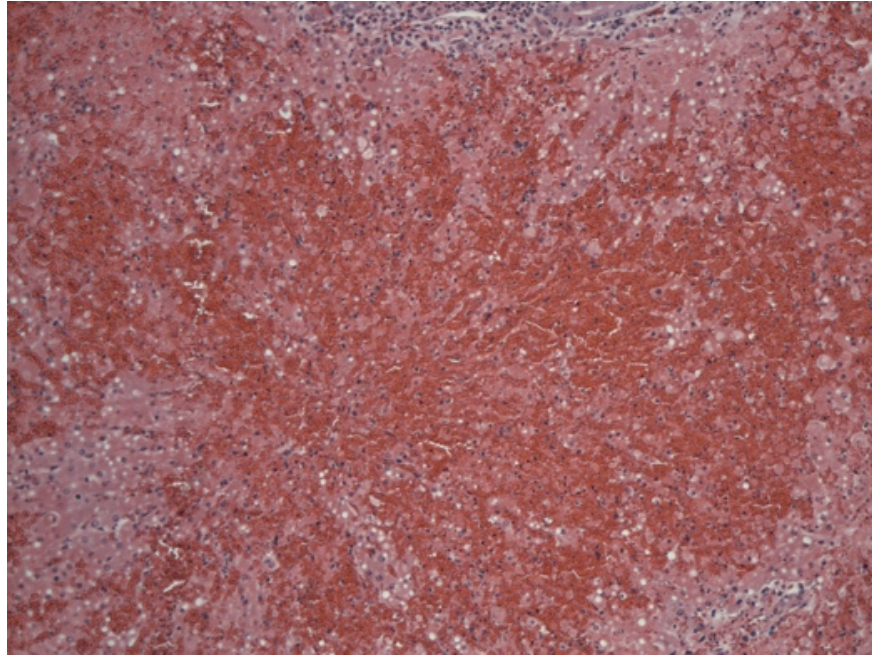


Figure 38: In this histologic image (100X magnification), congestion is a prominent finding. Sinusoidal dilation with erythrocytes is characteristic of this change.

Congestion was observed in 15 horses with inflammatory changes and 8 horses with portal fibrosis, 5 of which were classified as cirrhotic. Hepatic congestion did not appear to be linked to overall histologic severity ($p=0.7818$). Horses with congestion had the same median histologic severity score (2) as horses without evidence of congestion.

Eighty horses had histologic evidence of inflammation. Forty horses had mild inflammation, 20 horses had moderate inflammation, and 20 horses had severe inflammation. Histologic images of equine hepatic tissue illustrating mild, moderate and severe inflammation can be found in Figure 39.

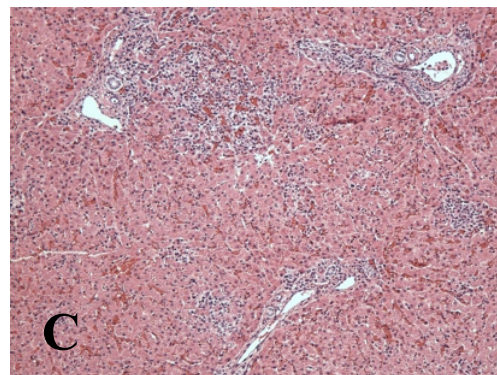
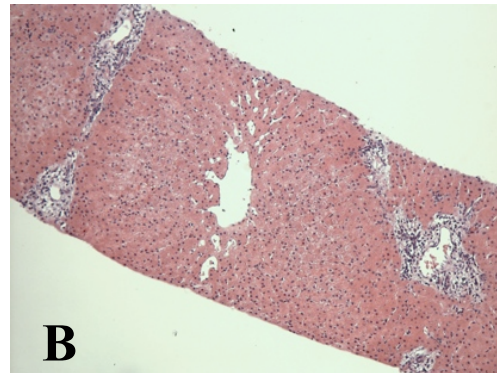
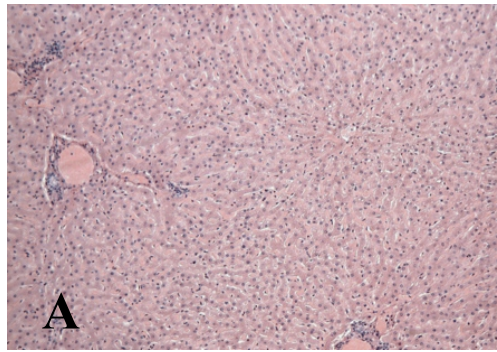


Figure 39: Images above illustrate examples of mild (A), moderate (B), and severe (C) inflammation (100X magnification). Specimens were graded based on quantity of inflammation, independent of pattern. Three patterns of inflammation were observed: periportal (A and B), central, and random multifocal (C). Mild inflammation was characterized by focal aggregates of inflammatory cells. In specimens with moderate inflammation, areas were moderately expanded with inflammatory cells. Severe accumulation of inflammatory cells was characteristic of specimens with severe inflammatory change.

Based on the predominant location observed, 53 horses had periportal inflammation, 22 horses had random multifocal inflammation, and 5 horses had central inflammation. Severe inflammation often accompanied cellular necrosis. Ten horses had hepatic inflammation without any other concurrent histologic changes. Of these ten horses, 9 had periportal inflammation and 1 had random multifocal inflammation.

Five horses had histologic evidence of neoplasia. Specific neoplasms included lymphosarcoma (2), metastatic glandular thyroid carcinoma (1), undifferentiated neuroendocrine carcinoma (1), and leukemia (1). Three of 5 horses with neoplasia had concurrent moderate to severe lipidosis. Other than a single horse with evidence of bile stasis in this group, no other concurrent histologic changes were observed. Histologic images of equine hepatic tissue illustrating three different types of neoplasia encountered, including lymphosarcoma, metastatic glandular thyroid carcinoma, and leukemia, can be found in Figure 40.

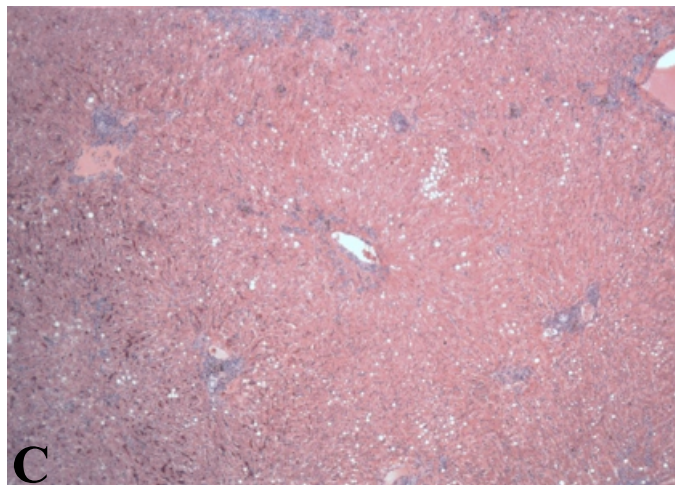
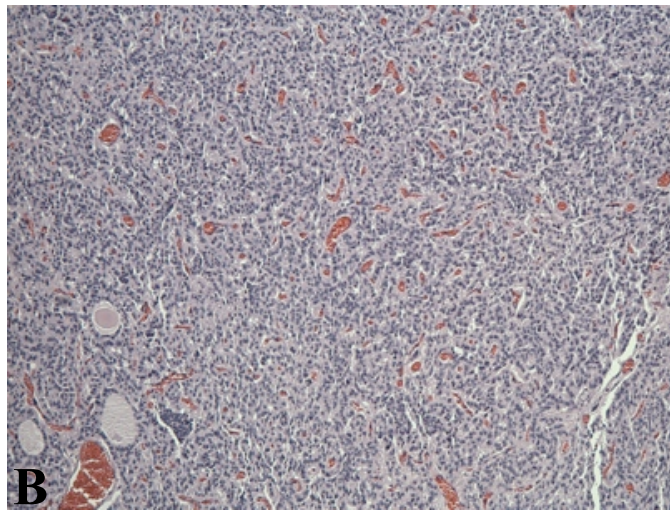
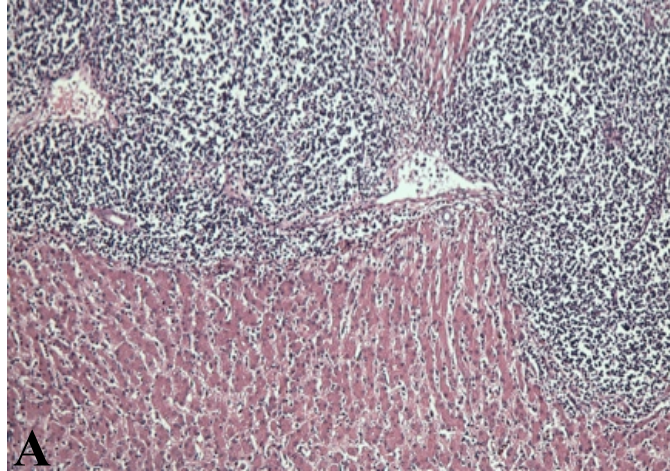


Figure 40: Images above were from horses with hepatic neoplasia (100X magnification). Lesions represented include lymphosarcoma (A), metastatic glandular thyroid carcinoma (B), and leukemia (C).

The primary histologic diagnosis for the 100 horses analyzed in this study were identified and are summarized in Table 4.

Table 4: Primary histologic diagnoses of horses with hepatic disease

Primary Histologic Diagnosis	Number of Horses
Portal inflammation	27
Portal fibrosis	24
Necrosis	15
Lipidosis	12
Abscess	8
Random multifocal inflammation	7
Neoplasia	5
Biliary Hyperplasia	2

Median histologic severity score was 2, with a range of 1 to 3. Twenty-eight horses had a histologic severity score of 1, 24 horses had a histologic severity score of 2, and 48 horses had a histologic severity score of 3. Thirty-five horses were classified as having reversible hepatic disease and 65 had irreversible disease.

Within the group of 100 horses, sex was evenly distributed to include 51 males (4 stallions, 47 geldings) and 49 females. Median age was 10 years (range 1-31 years). Multiple breeds were represented including 42 Quarter Horses, 12 American Paint Horses, 8 Arabians, 8 Thoroughbreds, 8 Donkeys, 4 Morgans. There were 2 horses of each of the following breeds: Appaloosa, Warmblood, American Miniature, Percheron, and Pony. There was 1 horse of each of the following breeds: Icelandic Pony, Spanish Mustang, Clydesdale, Friesian, Belgian, Shire, Paso Fino, and Tennessee Walking Horse. Presenting complaints were primarily colic (55), lethargy (27), anorexia (24), elevated liver enzymes (18), fever (13), abnormal neurologic behavior (14), weight loss (13),

diarrhea (7), icterus (5), and dermal lesions suspicious for photosensitivity (4). All horses currently resided in the Western United States, to include 80 from Colorado, 8 from Wyoming, 3 from Nebraska, 3 from Texas, 2 from New Mexico, and 1 each from Nevada, California, South Dakota, and Utah. Median duration of illness prior to admission was 3 days (range 1-730 days). Clinicopathologic variables were evaluated and are summarized in Table 5.

Table 5: Summary of clinicopathologic variables evaluated in horses with hepatic disease. Abbreviations: GGT = γ -glutamyl transferase, AST = aspartate aminotransferase, SDH = sorbitol dehydrogenase, WBC = white blood cell count, PCV = packed cell volume, TP = total protein.

Clinicopathologic variable	Median	Range	Number of Horses	Reference Range
Bilirubin	2.6	0.1-17.2	91	0.5-1.7 mg/dL
GGT	55	7-1623	91	7-20 IU/L
AST	695	164-5752	91	185-375 IU/L
SDH	20	1-650	91	0-12 IU/L
WBC	8000	1300-29200	91	5500-10500 c/ μ L
Segmented neutrophils	5600	0-25100	90	3000-7000 c/ μ L
Band neutrophils	0	0-5000	91	0-100 c/ μ L
Albumin	3	1.5-3.9	90	2.5-3.5 g/dL
Globulin	4.1	2.5-9.3	90	2.9-5.0 g/dL
PCV	37	11-72	91	30-45%
TP	7.2	4.7-11.5	90	5.8-7.8 g/dL

Median values of bilirubin, GGT, AST, and SDH were all above the hospital reference range. These values of liver function were elevated in 87 of 92 horses in which at least one was evaluated. Though all horses had evidence of hepatic disorders, the primary disease was classified as hepatic in 52 horses, gastrointestinal in 34 horses, or other in 14 horses.

Continuous variables were further evaluated for horses that underwent necropsy versus horses that underwent surgical biopsy, as all horses undergoing surgical biopsy were discharged from the hospital alive. A summary of these continuous variables and comparisons, with the corresponding p-value, is outlined in Table 6.

Table 6: A comparison of age, duration of illness and clinicopathologic variables of horses with hepatic disease identified either at necropsy or surgical biopsy. P-values listed in bold were considered significant with $p < 0.05$.

Continuous variable	Necropsy	Biopsy	p-value	Reference Range
Age (years)	12 (1-31)	9 (2-25)	0.3348	
Duration of illness (days)	2 (1-365)	26 (1-730)	0.0003	
Bilirubin	2.5 (0.3-17.2)	3.1 (0.1-12.8)	0.6746	0.5-1.7 mg/dL
GGT	45 (7-912)	141 (10-1623)	0.0206	7-20 IU/L
AST	606 (212-5752)	762 (164-2167)	0.6989	185-375 IU/L
SDH	18 (1-488)	26 (2-650)	0.7455	0-12 IU/L
WBC	8100 (1300-29200)	7600 (4000-23600)	0.8148	5500-10500 c/ μ L
Segmented neutrophils	5800 (0-25100)	4900 (2300-19600)	0.6076	3000-7000 c/ μ L
Band neutrophils	0 (0-5000)	0 (0-700)	0.0152	0-100 c/ μ L
Albumin	2.8 (1.6-3.9)	3.1 (1.5-3.8)	0.0317	2.5-3.5 g/dL
Globulin	3.9 (2.5-9.3)	4.3 (3.0-8.3)	0.392	2.9-5.0 g/dL
PCV	39 (11-72)	37 (26-60)	0.2219	30-45%
TP	7.1 (4.7-11.5)	7.4 (6.0-10.4)	0.1429	5.8-7.8 g/dL

Median values of bilirubin, GGT, AST, and SDH were again elevated above reference range in both groups. Horses in the surgical biopsy group had a longer duration of illness prior to presentation, a higher concentration of GGT, lower numbers of band neutrophils, and a greater albumin concentration.

Twenty-five horses were discharged from the hospital alive. Of these, follow-up information was available by telephone interview for 21 horses. Follow-up time ranged

from 2-3973 days (median 1065 days). Thirteen horses were alive at the time of follow-up and 8 had been euthanized. Four horses were euthanized for progression of primary liver disease (2, 10, 11, and 60 days), 3 horses were euthanized for colic (100, 276, and 359 days), and 1 was euthanized for progressive arthritis (3168 days). Definitive diagnosis by necropsy examination was not elected. Survival of horses following hospital discharge is illustrated in Figure 41. Thirty-three percent of horses died within 359 days of hospital discharge.

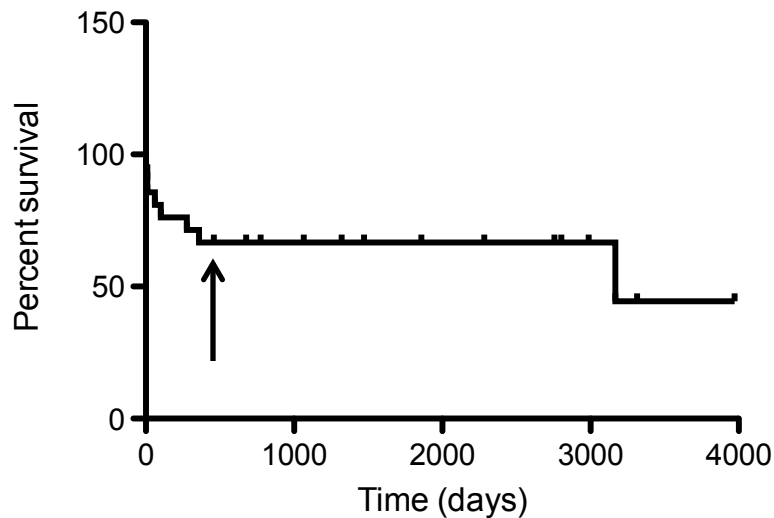


Figure 41: Survival of horses with liver disease on biopsy examination. Survival is plotted as a function of time. Percent survival is on the y axis and time in days on the x axis. An arrow indicates the point on the survival curve corresponding to 359 days. Censored horses, alive at the time of follow-up, are indicated by vertical marks located on the horizontal lines of the graphs.

Associations between survival of less than or equal to 359 days and multiple variables were investigated. All but one case in both groups had a primary diagnosis of hepatic disease. Histologic severity was similar between groups, though severity tended to be higher in the group with shorter survival times (Median severity 3 vs. 2, $p=0.4757$).

The majority of horses in both groups had a histologic diagnosis of either portal fibrosis and periportal inflammation; 7 of 7 in the ≤ 359 days survival and 10/14 in the >359 days survival. Three horses that survived longer than 359 days had a primary histologic diagnosis of severe hepatic lipidosis. Presence or absence of reversible hepatic disease differed between groups; 3 of 7 in the ≤ 359 days survival and 9/14 in the >359 days survival group had reversible disease. The difference in survival rate (percentage) relative to presence of reversible disease is illustrated in Figure 42 ($p=0.1251$).

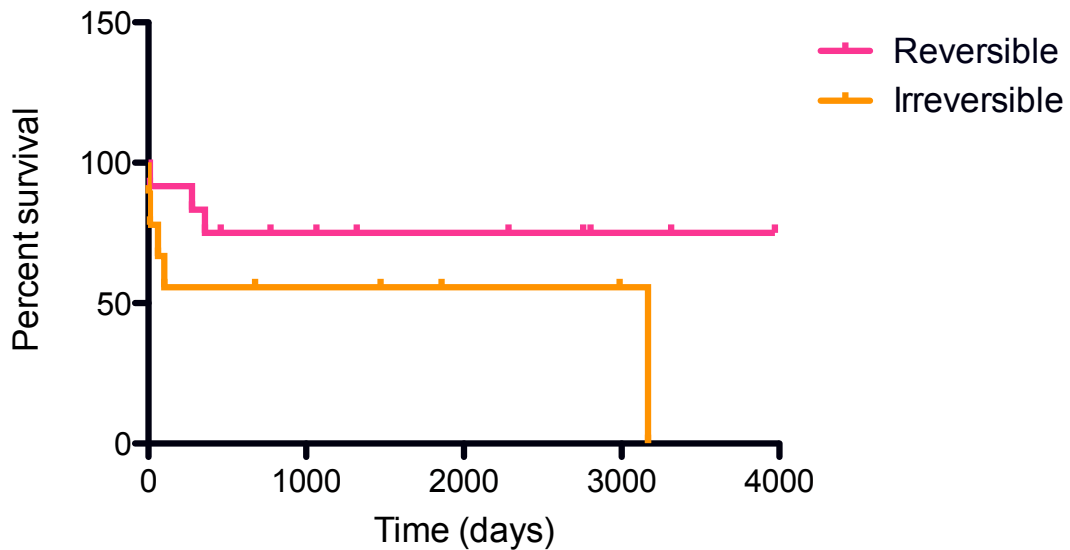


Figure 42: Survival proportions relative to histologic change. Horses were more likely to survive if reversible disease was detected on histologic examination. Survival is plotted as a function of time. Percent survival is on the y axis and time in days on the x axis. Censored horses, alive at the time of follow-up, are indicated by vertical marks located on the horizontal lines of the graphs.

Clinical data for horses that survived longer or less than 359 days are summarized in Table 7.

Table 7: A comparison of age, duration of illness and clinicopathologic variables of horses with hepatic disease that survived longer or less than 359 days.

Continuous variable	Death ≤ 359 days (n=6)	Survived > 359 days (n=14)	p-value	Reference Range
Age (years)	9 (4-25)	10 (2-22)	p=0.7489	
Duration of Illness (days)	2 (1-42)	30 (1-730)	p=0.2778	
Bilirubin	4.0 (2.6-8.8)	2.1 (0.1-12.8)	p=0.5908	0.5-1.7 mg/dL
GGT	166 (28-1036)	142 (10-1623)	p=0.8800	7-20 IU/L
AST	854 (387-1277)	589 (164-2167)	p=0.7251	185-375 IU/L
SDH	71 (36-251)	11 (2-232)	p=0.0531	0-12 IU/L
WBC	6200 (4400-6900)	8200 (4000-23600)	p=0.0628	5500-10500 c/μL
Segmented neutrophils	4600 (2800-5700)	5150 (2300-19600)	p=0.1923	3000-7000 c/μL
Band neutrophils	0 (0-100)	0 (0-700)	p=0.3632	0-100 c/μL
Albumin	3.3 (2.6-3.5)	3.0 (1.5-3.8)	p=0.4631	2.5-3.5 g/dL
Globulin	4.3 (3.5-6.7)	4.3 (3.0-8.3)	p=0.8087	2.9-5.0 g/dL
PCV	34 (31-43)	36 (26-60)	p=0.8452	30-45%
TP	7.5 (6.8-9.3)	7.4 (6.0-10.4)	p=0.9688	5.8-7.8 g/dL

Age, duration of illness, and clinicopathologic variables did not differ significantly between survival groups, though horses surviving less than 359 days tended to have a lower white blood cell count and a higher level of sorbitol dehydrogenase enzyme. These clinicopathologic derangements could indicate increased disease severity in horses with shorter survival times.

Discussion

This study is the first to report clinical findings and standardized histological examination of a large group of horses with liver disease residing within the United States. Histologic evidence of fibrosis, necrosis, lipidosis, and inflammation were common findings, whereas neoplasia was rarely observed. Median values of bilirubin, GGT, AST, and SDH were above the hospital reference range, and 87 of 92 horses had an elevation of at least one of these four values. This finding was not unexpected, as a laboratory diagnosis of liver disease is commonly made in horses based on elevations in GGT, AST, SDH, and bilirubin,¹²¹⁻¹²³ though enzymes may be normal in disease.

West reported a series of 40 horses with liver disease identified from a hospital population in Liverpool, UK.¹²⁰ Predominant clinical signs were similar to the present study and other reports, comprised of weight loss, anorexia, lethargy, and abdominal pain.^{116,120} Standardized histologic examination was not reported. Lipidosis, neoplasia, and necrosis were reported more frequently than in the present study and overall mortality rate was 60%.¹²⁰ In the present study, eighty-five percent of horses diagnosed with hepatic disease died during hospitalization or within 1 year of hospital discharge. The high rate of mortality may be related to severity of hepatic disease in this group of horses, as 48 out of 100 horses received the highest possible histologic severity score. The present study's mortality rate is higher than other studies, including that from McGorum et al.¹¹⁶ McGorum described a mortality rate of 72% in a group of 50 horses with suspected liver disease. There were several differences between the present study and that of McGorum. Differences included geographic location (UK), horses with lipidosis and disseminated neoplasia were excluded, and horses were primarily identified

with clinicopathologic data with only 40 of 50 undergoing hepatic histopathologic examination. Clinical studies of naturally occurring disease are inherently diverse. Regardless of study differences, mortality rates in horses with hepatic disease are consistently high.

In a recent abstract published by the Equine Colic Research Symposium, authors from 5 University hospitals reported on 97 horses older than 1 year of age with liver disease.¹²⁴ Histologic diagnoses were obtained from pathology reports and were not confirmed by review of a single pathologist. Outcomes were not reported. The focus of the abstract was to describe that erythrocytosis (PCV>45%) accompanied liver disease in 37% of cases, and that this clinicopathologic variable was linked with disease severity, with concurrent significant increases in bile acids and bilirubin. In the present study, only 20% of horses had erythrocytosis and this change did not seem to be linked significantly with histologic severity scores (p=0.2872).

Though previously recognized, the link between gastrointestinal and hepatic disease in horses is poorly defined. In the present study, 34 of 100 horses with liver pathology had a primary clinical diagnosis of gastrointestinal disease. A recent pilot study of 32 horses undergoing emergency gastrointestinal surgery focused on prospective evaluation of hepatic indices and identified a high incidence of hepatic dysfunction.¹²⁵ Furthermore, in this group of horses, the level of hepatic dysfunction impacted survival.¹²⁵ Elevations in hepatic enzymes have been documented retrospectively in horses with duodenitis-proximal jejunitis and right dorsal large colonic displacements.^{126,127} Potential etiologies of hepatic injury in horses with colic included hypoxia, ischemia, endotoxemia, extrahepatic biliary obstruction, and ascending infection

from the intestinal tract.¹²⁵ Horses undergoing colic surgery are also predisposed to metabolic disturbances in the post-operative period,¹²⁵ as are horses undergoing medical treatment of colic that is accompanied by anorexia or prolonged periods of fasting. Metabolic disturbances of sufficient severity can predispose to hepatic lipidosis. Colic, gastrointestinal disease affecting between 4% and 26% of horses per year, is the number 1 cause of death in horses, other than old age, and results in a high economic cost to horse owners.¹²⁸ Though primary hepatic disease is thought to be rare, hepatic disease secondary to primary gastrointestinal disorders may be as yet under-recognized.

All horses in the present study undergoing hepatic biopsy were discharged from the hospital alive. Horses discharged from the hospital had a longer duration of illness prior to admission, which may have been related to a lesser severity of disease. Significantly higher levels of albumin and lower levels of band neutrophils seen in this group support a lower disease severity. Seven horses died of progression of hepatic disease or colic within 359 days of hospital discharge. Though necropsy examination was not available for these 7 horses, the deaths are considered related to their primary disease. Horses surviving longer than 359 days were more likely to have reversible disease based on histologic examination. Overall, 9 of 35 horses with reversible disease and 5 of 65 horses with irreversible disease survived longer than 359 days. Diseases resulting in reversible anatomical hepatic pathology likely have a better long-term prognosis for survival than diseases resulting in irreversible change.

An important element of this study was the inclusion of prospective and consistent histologic evaluation. Quality and severity of histologic interpretation often differed from the pathological report included in the medical record. Evaluation of slides

as a group allowed generation of lesion severity scales, which improved consistency of observations. Inter-observer variation was avoided as all slides were evaluated by a single Board Certified Veterinary Pathologist. This pathologist was well versed in evaluation of equine liver histopathology and subtle species-specific differences, such as equine portal tracts that ordinarily have prominent fibrous elements under normal conditions.

CHAPTER 7: CONCLUSIONS OF DISSERTATION

Hepatitis is an important disease with high morbidity and mortality in horses. Investigation of new therapeutics for hepatitis in horses is needed. Based on information gained from analyzing horses admitted to Colorado State University with liver disease, metabolic disruptions and primary hepatic injury coexist, as do acute and chronic hepatic injuries. Horses appeared to be prone to injury from food and digestive system borne insults as well as toxins within the systemic circulation. Colic, a common disease in horses, may be an under-recognized etiology of hepatic injury. Primary histologic lesions of the liver in horses evaluated at Colorado State University were portal inflammation, portal fibrosis, hepatocyte necrosis, and lipidosis. Therefore, modes of action of an ideal drug for clinical application would be anti-inflammatory, anti-fibrotic, and favor hepatocyte regeneration. Anti-fibrotic and regenerative processes assume primary importance with regards to diseases resulting in irreversible histologic changes within the liver.

Silibinin has been used to promote liver health and manage liver disease of multiple etiologies. The multiple functions of silibinin make this extract especially promising in the treatment of hepatic disease in horses. A number of effects relevant equine liver disease can be attributed to silibinin. The anti-inflammatory actions of silibinin were initially thought to be due primarily to antioxidant effects.¹²⁹ However, direct anti-inflammatory effects also result from NF- κ B and inflammatory cytokine

suppression, as well as inhibition of nitric oxide synthesis and leukocyte migration.^{27,28,31,35} The anti-fibrotic effects of silibinin are due to decreases in stellate cell activation and collagen synthesis.^{32,36} The success of fatty liver treatment in humans is largely attributed to the antioxidant effects of silibinin, which are a key component in the prevention of progression from steatosis to steatohepatitis.⁶²

If silibinin is to be used as a treatment in equine liver disease, a basic pharmacokinetics evaluation is justified. In the present study, the horses used for silibinin evaluation were typical of Colorado, and age and breed were similar to those horses affected by liver disease evaluated at Colorado State University. From the outset, silibinin was considered to be safe and non-toxic in the horses evaluated. As expected, silibinin had low bioavailability, a short elimination half-life, and did not accumulate when administered twice daily for 7 days. There was an unexpected finding of nonlinear pharmacokinetic behavior at the highest dose evaluated (26 mg/kg silibinin), which may have been due to saturable enzymatic elimination. Thus, pharmacokinetic and safety evaluation in diseased populations is necessary to confirm the most appropriate dose and dose interval. In the future, therapeutic drug monitoring may assist in the identification of horses with liver disease that require dose modification secondary to nonlinear kinetics.

Evaluation of the effect of silibinin on antioxidant reserve in normal horses was an important element of discovery in the investigation of new drugs for liver disease in horses. Despite the use of healthy horses, improvement in multiple measures of antioxidant reserve was observed. Hepatic and oxidative injury are closely linked, and the positive effects of silibinin on antioxidant reserve, as well as its direct oxidant

scavenging ability, are implicated heavily in its hepatoprotection. Peripheral blood evaluation of antioxidant reserve has not yet been established in horses with hepatic disease and may mimic reductions seen in other species.⁶⁷

Inflammatory cytokine production is a common feature of equine diseases. Horses with colic have increased expression of TNF α and interleukin 6 (IL-6).¹³⁰⁻¹³² In addition, the degree of inflammatory cytokine expression is related to severity of disease and prognosis in horses. Higher levels are seen in those with severe gastrointestinal disease, such as colitis, peritonitis, acute perforation, and strangulating intestinal disease, and in nonsurvivors.¹³⁰⁻¹³² Horses with laminitis increase expression of inflammatory cytokines, such as IL-1 β and IL-6, following the onset of lameness.¹³³ Inflammatory cytokine profiles and gene expression in horses with hepatitis have yet to be established, though severity of hepatic disease in horses often is severe and may mimic high levels observed in severe hepatic diseases in humans.⁶⁴ Though silibinin administration did not significantly impact inflammatory cytokines in healthy horses, studies in animal models and human clinical disease indicate silibinin administration has significant anti-inflammatory effects, especially in diseases with prominent oxidant injury.⁴⁵ Concentration in hepatic and intestinal tissues improve the utility of silibinin in diseases affecting these organs.²⁶

Establishing silibinin multi-dose pharmacokinetics and pharmacodynamics in normal horses provides a basis for future study. One specific area of interest is the evaluation of the silibinin treatment effect on active hepatic disease in horses. A positive effect has been observed in periparturient cows with hepatic lipid infiltration, but dose response could not be evaluated due to lack of basic pharmacokinetic evaluation.^{69,70}

Horse owners often are given a poor prognosis for horses afflicted with liver disease, especially those with severe histologic lesions or irreversible histologic changes.

Silibinin is a plausible choice as a hepatoprotectant in horses, as positive treatment effects have been primarily established in severe toxic or end-stage hepatic diseases in people.

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