


STANDARD ARTICLE

Biomarkers of oxidative stress as an assessment of the redox status of the liver in dogs

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Background: Oxidative stress is associated with a diverse group of liver disorders across species.

Objectives: Determine whether glutathione (GSH) concentration in plasma and red blood cells correlates with liver GSH concentration in dogs and evaluate whether other markers of systemic oxidative stress, plasma vitamin E and urine 8-isoprostanes/creatinine (F₂-IsoPs/Cr) concentrations, correlate with liver GSH.

Animals: Thirty-four client-owned dogs undergoing clinically indicated liver biopsy and 15 healthy control dogs.

Methods: Prospective, observational cross-sectional study. Urine and blood were collected before liver biopsy. Plasma, erythrocyte, and liver GSH were measured using high performance liquid chromatography (HPLC); vitamin E was measured by HPLC, and F₂-IsoPs/Cr was measured by gas chromatography/mass spectrometry.

Results: All dogs were treated at the discretion of the attending clinician (24/34 received antioxidants; 4/34 fed therapeutic liver diet), which included dogs with primary or secondary liver disease (inflammatory (n = 21), metabolic (n = 9), vascular (n = 2), and neoplastic (n = 2)). Median GSH concentrations in plasma, erythrocyte, and liver were 0.18 mg/dL (range 0.14 to 0.56 mg/dL), 56.7 mg/dL (18.3 to 79.2 mg/dL), and 181 mg/dL (39.9 to 527 mg/dL), respectively. No significant correlations were found between liver GSH and erythrocyte GSH, plasma GSH, vitamin E, or F₂-IsoPs/Cr. Dogs undergoing clinically indicated liver biopsy had significantly higher urine F₂-IsoPs/Cr than did healthy controls (5.89 vs 2.98 ng/mg; P < .0001).

Conclusions and Clinical Importance: Erythrocyte and plasma GSH are not indicative of liver GSH concentration in dogs. In addition, dogs undergoing clinically indicated liver biopsy have evidence of increased systemic oxidative stress compared to healthy controls.

KEYWORDS

glutathione, hepatopathy, isoprostane, vitamin E

Abbreviations: DTT, dithiothreitol; F₂-IsoPs/Cr, urine isoprostanes/creatinine; GSH, reduced glutathione; GSSG, oxidized glutathione; HPLC, high performance liquid chromatography; NAC, N-acetylcysteine; PBS, phosphate-buffered saline; ROS, reactive oxygen species; SAmE, S-adenosyl methionine; SSA, sulfosalicylic acid.

1 | INTRODUCTION

Reactive oxygen species (ROS) are produced as a consequence of oxidative metabolism, and oxidative stress occurs when the concentration of ROS exceeds the capability of the body's antioxidant defense mechanisms. Both acute and chronic liver disorders result in an

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oxidative imbalance contributing to the pathogenesis of these disorders.¹ The liver plays a vital role in regulating the body's endogenous antioxidant status, as it is the primary site for glutathione synthesis, the major intracellular antioxidant.²

In people with naturally occurring liver disease and in animal models of induced hepatopathies liver glutathione concentration is decreased.²⁻⁶ Similar to people, both dogs and cats with liver disease have decreased liver glutathione concentration.^{7,8} People with autoimmune liver disease have decreased glutathione concentration in peripheral whole blood, and decreases in plasma and erythrocyte glutathione can be directly correlated to the severity of the liver disease.^{3,9} In addition, plasma vitamin E concentration is negatively correlated with hepatocyte apoptosis in human patients with chronic hepatitis.⁹ Urinary isoprostanes variably correlate with oxidative stress in dogs, and urinary isoprostane concentration increases with severity of autoimmune liver disease in human patients.^{9,10} Taken together, these data indicate that increased oxidative stress is associated with progression of liver disease.

What these human and canine studies have not yet investigated is whether markers of oxidative stress in blood or urine reflect liver concentrations. In a recent review, blood reduced glutathione (GSH) and vitamin E concentrations trend with tissue concentrations in experimental animal models used to induce oxidative stress.¹¹ Glutathione concentration in blood accurately reflects the redox status of several tissues (ie, liver, heart, and skeletal muscle) in rats.⁴ Blood glutathione concentration is significantly and directly correlated with liver glutathione concentration in healthy rats, and rats experimentally subjected to an oxidative challenge. To our knowledge, similar studies in patients with naturally occurring liver disease have not been reported in either people or dogs.

The medical management of liver disease often includes antioxidant supplementation, most commonly with glutathione precursors to replenish glutathione concentrations. Measuring liver glutathione concentration requires biopsy specimen collection and is invasive. If peripheral antioxidant concentrations were found to correlate with liver antioxidant concentration, these peripheral markers would provide a clinically useful tool for assessing the progression of liver disease and response to treatment.

The purpose of this study was to determine liver and blood antioxidant concentrations, as well as a urinary marker of systemic oxidative stress prospectively in a population of dogs undergoing a clinically indicated liver biopsy. The primary aim of this study was to determine whether plasma or erythrocyte glutathione concentration would be an accurate reflection of liver glutathione concentration in dogs. The secondary aim was to evaluate whether other markers of systemic oxidative stress, specifically plasma vitamin E concentration and urine 8-isoprostanes concentration, would also correlate with liver glutathione concentration in these dogs. We hypothesized that erythrocyte and plasma glutathione concentrations, as well as plasma vitamin E concentration, would directly correlate with liver glutathione concentration in dogs. In addition, we hypothesized that liver glutathione concentration would be inversely correlated with urine 8-isoprostane concentration.

2 | MATERIALS AND METHODS

2.1 | Dog enrollment

Client-owned dogs of any age and sex that presented to UW Veterinary Care from July 1, 2014 through June 30, 2016 for a clinically indicated liver biopsy were enrolled in this prospective, observational, cross-sectional study. In addition, a group of 15 healthy control dogs was recruited for comparison of urinary isoprostanes. These dogs were presented to the hospital for routine wellness evaluations.

Dogs with clinical evidence of liver disease in which liver biopsy was deemed necessary by the attending clinician as part of the dogs' workup were eligible for study inclusion. Dogs were excluded if at the time of their work up if they were identified with other active or newly diagnosed systemic disease(s) (eg, diabetes mellitus, hyperadrenocorticism, and heart failure) that might impact the canine liver. However, dogs with stable chronic disease (eg, inflammatory bowel disease, hypothyroidism, and idiopathic epilepsy) were not excluded. Additional exclusion criteria included dogs with a history of a blood or plasma transfusion 3 months before or during the study period, if treatments administered before sample collection could not be determined, or if an adequately sized sample of liver tissue for study analysis could not be obtained. To ensure that 6-8 mL of whole blood could be safely removed, dogs with clinical or biochemical evidence of a clinically relevant anemia (pallor, packed cell volume, 20%) and dogs weighing less than 2 kg were excluded. All study protocols were reviewed, approved, and conducted in accordance with the University of Wisconsin, School of Veterinary Medicine Animal Care and Use Committee. Informed consent was obtained from owners of all dogs before enrollment into the study.

All dogs were cared for routinely and were treated at the discretion of the attending clinician. Because of the frequent prescription of antioxidant medications by veterinarians for dogs with suspected liver disease and our target being within dog correlations between serum or urine and liver antioxidant/oxidative biomarkers, such treatments were not exclusion criteria. However, to minimize the impact antioxidant supplementation could have in finding a correlation between serum and liver any antioxidant supplementation must have been taken by each dog long enough to achieve steady state: 7 days for a glutathione source (S-adenosyl methionine (SAME), N-acetylcysteine (NAC), or milk thistle), and 21 days for vitamin E.¹²⁻¹⁷

Data collected for each dog at the time of recruitment included age, breed, sex, neuter status, body weight, current medications and supplements, clinical signs, duration of clinically noted liver disease, and available clinical pathology data (ie, liver enzymatic concentrations, liver functional variables, coagulation panel, blood ammonia, post-prandial bile acids).

2.2 | Sample collection

Dogs were enrolled in the study at the time of liver biopsy. Blood was collected via lateral saphenous, cephalic, or jugular venipuncture for the determination of circulating erythrocyte and plasma GSH concentrations and plasma vitamin E concentration after a minimum 12 hour fast, and urine collected for the determination of urine

8-isoprostane/creatinine (F₂-IsoPs/Cr) concentrations, within 24 hours before liver biopsy collection.

Liver biopsies were obtained from each dog under general anesthesia by 1 of 3 methods: ultrasound-guided 14 gauge Tru-Cut needle biopsy (Merit Medical, South Jordan, Utah), 5 mm laparoscopic biopsy forceps (33200 and 33121, Storz Medical, Southbridge, Massachusetts), or an exploratory laparotomy wedge or punch biopsy. After obtaining the necessary liver biopsy specimens as clinically indicated and determined at the discretion of the attending clinician (ie, histopathology, heavy metal analysis, culture, or any combination thereof), an additional single liver biopsy specimen was collected for the determination of liver GSH. Liver tissue was snap frozen in liquid nitrogen and stored at -80°C.

Liver biopsies were submitted for routine histopathology for clinical case management. All cases were reviewed by a single anatomic pathologist (Marie E. Pinkerton) and classified using WSAVA guidelines.¹⁸ Additional clinical diagnostics were pursued on per case basis at the discretion of the attending clinician to include heavy metal analysis (liver tissue) and bacterial culture (liver or bile), with results recorded when available.

2.3 | Erythrocyte, plasma, and liver glutathione analysis

Blood was added to heparinized tubes and monobromobimane added within 1 minute of venipuncture for evaluation of erythrocyte and plasma GSH. Within 30 minutes of heparinized blood collection, plasma and packed RBCs were separated, harvested, and frozen at -80°C before analysis. Plasma, erythrocyte, and liver GSH were determined within 7 days of blood collection. When possible, all samples were run in duplicate. Glutathione concentrations were determined using high performance liquid chromatography (HPLC). Plasma GSH concentration was measured by a method similar to a plasma cysteine determination used previously.¹⁹ Briefly, 7.5 µL 180 mM monobromobimane and 62.5 µL 1X phosphate-buffered saline (PBS) was added to 437 µL monobromobimane-treated plasma. Proteins were precipitated by addition of 40 µL 50% sulfosalicylic acid (SSA) in 500 µM dithiothreitol (DTT). After centrifugation, 400 µL was transferred to a fresh amber tube and 4.5 µL 50% SSA in 500 µM DTT, 10.2 µL 1 M N-Ethylmorpholine, and 16 µL acetonitrile was added. After incubation at 37°C in the dark, 15 µL trichloroacetic acid was added and the sample was filtered using a 0.2-µm centrifugal filter. The limit of quantitation of plasma GSH was 0.5 µM with an intra-assay CV of 0.37%-8.75%.

Erythrocyte GSH was determined as previously reported with the following exception.¹⁹ Packed erythrocytes (62.5 µL) were added to 62.5 µL 1X PBS for a total volume of 142.5 µL. Liver GSH was quantified using the same method as for erythrocytes with the following alteration. For liver tissue GSH determination, 0.1 g of tissue was combined with 900 µL of ice cold 2.7 mM monobromobimane in 1XPBS and homogenized for 1-2 minutes with 0.5 µm zirconium beads in a Bullet Blender (Next Advance, Averill, New York). The liver sample was centrifuged at 3000 g for 15 minutes at 4°C and the supernatant removed to a fresh amber tube before assay. The limit of quantitation for erythrocyte and liver GSH was 30 µM with an intra-assay CV of 0.25-2.59%. Samples were analyzed by HPLC using a

refrigerated autosampler unit (SIL-20 AC, Shimadzu Corp., Kyoto, Kyoto Prefecture, Japan), a C-18 5 µM ODS 2 Spherisorb column (4.6 mm × 25 cm; Waters Corp., Milford, Massachusetts), and fluorescence detection (ex. 394 nm, em. 480 nm; RF-20A, Shimadzu Corp., Kyoto, Kyoto Prefecture, Japan).

2.4 | Plasma vitamin E analysis

Two milliliters of venous blood were added to an EDTA tube within 1 minute of collection, protected from light with foil, and kept on ice for evaluation of vitamin E. Samples were stored frozen before analysis and assays were completed within 7 days of blood collection through the Wisconsin Veterinary Diagnostic Laboratory (Madison, Wisconsin). Quantification of plasma vitamin E was performed using HPLC, as previously described.

2.5 | Urine 8-isoprostane analysis

Urine samples were collected via midstream free catch, catheterization, or cystocentesis and immediately treated with 0.005% butylated hydroxytoluene, centrifuged, and frozen at -80°C until analysis. Urine 8-isoprostane concentration was quantified using gas chromatography/negative ion chemical ionization mass spectrometry assayed through the Eicosanoid Core Laboratory, Vanderbilt University (Nashville, Tennessee).^{20,21} Urine 8-isoprostane concentration was normalized to urine creatinine concentration (F₂-IsoPs/Cr) determined using the Jaffe reaction.

2.6 | Statistical analysis

Before study commencement, a power calculation was performed based on the previous work in rodents.⁴ Based on this calculation, the target enrollment was 34 dogs to provide 90% power to detect a modest correlation of 0.5.

Continuous variables (age, body weight, and F₂-IsoPs/Cr) were compared between the dogs with liver disease and healthy controls using the Mann-Whitney *U*-test. Categorical data (breed and sex) were compared using Fisher's exact test. Circulating markers of oxidative stress (ie, erythrocyte and plasma GSH, plasma vitamin E, and F₂-IsoPs/Cr) were compared to liver GSH using a Spearman's rank correlation (ρ). Statistical calculations were performed with a commercial software package (GraphPad Prism 7.04), with *P* < .05 considered significant.

3 | RESULTS

3.1 | Study population

A total of 49 dogs were enrolled into the study, 34 dogs with liver disease and 15 healthy control dogs for F₂-IsoPs/Cr analysis only. The baseline descriptive characteristics of the dogs recruited are summarized in Table 1. Represented purebred dogs included Havanese (2), Maltese (2), Border collie (2), French bulldog (2), English springer spaniel (2), Labrador retriever (2), and a single dog of each of the following breeds: shih tzu, miniature pinscher, Siberian husky, cocker spaniel,

TABLE 1 Enrollment characteristics of the dogs presenting for liver biopsy as part of their clinical evaluation, and healthy control dogs used for urine 8-isoprostane analysis. Age and weight are presented as median (range)

	Liver disease (n = 34)	Controls (n = 15)	P-value
Age	8 (0.9-14)	5 (1-13)	.27
Weight (kg)	19.8 (3.8-49.2)	30.6 (7.6-43.6)	.038
Male (N/I)	16 (15/1)	8 (4/4)	.76
Female (S/I)	18 (17/1)	7 (5/2)	
Purebred/mix	26/8	11/4	1

Abbreviations: I, intact; N, neutered; S, spayed.

west highland white terrier, Belgian malinois, Great Dane, papillon, bichon frise, Italian spinone, doberman pinscher, Australian cattle dog, greyhound, and pomeranian. In addition, the canine population included 8 mixed breed dogs.

Twenty-four dogs were receiving supplementation with a glutathione precursor. No dogs were receiving supplemental vitamin E. Dogs received a variety of supportive medications and antimicrobials, summarized in Table 2. A subset of enrolled dogs (n = 9) had concurrent stable chronic disease (some with more than 1 condition) including separation anxiety (3/9, treated with fluoxetine, trazodone, or both), keratoconjunctivitis sicca (1/9, treated with topical cyclosporine), inflammatory bowel disease (1/9, treated with budesonide, tylosin, folic acid, and cyanocobalamin), idiopathic epilepsy (1/9 treated with potassium bromide), hypothyroidism (1/9, treated with levothyroxine), atypical hypoadrenocorticism (1/9, treated with physiologic prednisone), urinary sphincter mechanism incompetence (1/9, treated with diethylstilbestrol), and osteoarthritis (3/9, treated with combinations of meloxicam, tramadol, gabapentin, glucosamine, and fish oil). Four of the dogs were eating a prescription liver diet (L/D, Hill's Pet Nutrition, Inc., Topeka, Kansas, n = 3; Hepatic, Royal Canin, St. Charles, Missouri, n = 1). Thirteen dogs were not fed a prescription liver diet, and in 17 dogs, diet was not specified.

TABLE 2 Medical treatments received by the enrolled dogs at the time of liver biopsy. The number of dogs receiving each medication is reported in parentheses

Category	Medical treatments of the enrolled dogs at the time of liver biopsy
Treatments for liver disease	Antioxidants: SAME + silymarin (20), NAC (3), SAME (1) Antibiotics: potentiated penicillin (8), fluoroquinolone (5), metronidazole (4) Choleric: ursodiol (8) Diuretic: spironolactone/hydrochlorothiazide (2) Treatment for hepatic encephalopathy: lactulose (2) Vitamin supplementation: phytonadione (1)
Symptomatic treatments	Gastric acid suppression: proton pump inhibitor (4), histamine H2-receptor antagonist (1) Antiemetic/anti-nausea: maropitant (4), ondansetron (2), metoclopramide (1) Prokinetic: cisapride (1) Appetite stimulant: mirtazapine (1)
Chronic treatments unrelated to current visit	Behavior modification: fluoxetine (3), trazodone (2) Osteoarthritis: glucosamine (2), meloxicam (1), gabapentin (1), tramadol (1), fish oil (1) Steroids: budesonide plus tylosin (1, previously biopsy diagnosed stable IBD), physiologic prednisone (1, previously diagnosed stable atypical hypoadrenocorticism) Vitamin supplementation: folic acid (1), cyanocobalamin (1) Hypothyroidism: levothyroxine (1) Idiopathic epilepsy: potassium bromide (1) Hormone responsive incontinence: diethylstilbestrol (1) Keratoconjunctivitis: topical ophthalmic cyclosporine (1)

Abbreviations: IBD, inflammatory bowel disease; NAC, N-acetylcysteine; SAME, S-adenosyl methionine.

3.2 | Liver histopathology

Primary or secondary liver disease was confirmed in all dogs on the basis of liver histopathology and classified as: (1) Inflammatory (eg, chronic hepatitis, nonspecific reactive hepatitis); (2) Metabolic (eg, steroid-induced hepatopathy, vacuolar degeneration); (3) Vascular (portal vein hypoplasia); and (4) Neoplastic (hepatocellular carcinoma). Table 3 summarizes these dogs categorized based on histopathology along with the results of associated liver values, synthetic markers, and liver function testing.

3.3 | Biomarkers of oxidative stress

Median GSH concentrations in plasma, erythrocyte, and liver were 0.18 mg/dL (5.76 μM; range 0.14-0.56 mg/dL, n = 31), 56.7 mg/dL (1.85 mM; range 18.3-79.2 mg/dL, n = 30), and 181 mg/dL (5.90 mM, range 39.9-527 mg/dL, n = 31), respectively. Median plasma vitamin E was 29 μg/mL (0-77, n = 31). Median F₂-IsoPs/Cr was 5.89 ng/mg (2.29 to 16.31, n = 26) in the dogs with liver disease compared to 2.98 ng/mg (0.89-4.48, n = 15) in the control group; P < .0001 (Figure 1).

Liver GSH did not correlate significantly with any of the biomarkers evaluated. Correlation between liver and plasma GSH yielded a rho value of -0.05 (P = .80). Correlation between liver and erythrocyte GSH yielded a rho value of 0.06 (P = .76, Figure 2). Correlation between liver GSH and plasma vitamin E yielded a rho value of 0.01 (P = .96). Correlation between liver GSH and F₂-IsoPs/Cr yielded a rho value of 0.02 (P = .92).

Liver GSH did not differ significantly between dogs receiving antioxidant supplementation and dogs not receiving supplementation (P = .50), nor did plasma or erythrocyte GSH differ between these groups (P = .94 and P = .41 respectively). Likewise, plasma vitamin E and F₂-IsoPs/Cr did not differ between supplemented and nonsupplemented groups (P = .45 and P = .15 respectively). No correlations were found between liver GSH and any of the peripheral markers studied when groups were divided into those receiving antioxidant

TABLE 3 Histopathology and biochemical characteristics of dogs with primary or secondary liver disease. Values are reported as median (range) represented by the number of dogs (n) listed at the top of each of the respective columns unless otherwise noted

Chem./function (Ref. interval)	Histopathological diagnosis			
	Inflammatory (n = 21)	Metabolic (n = 9)	Vascular (n = 2)	Neoplastic (n = 2)
ALT (U/L) (14-87)	516 (145-2000)	517 (42-2000)	274 (89/458)	1191 (381/2000)
ALP (U/L) (20-157)	402 (63-3000)	159 (73-961)	69 (62/75)	1612 (824/2400)
GGT (U/L) (5-16)	29 (10-413)	26 (10-45)	10 (10/10)	23 (10/36)
Bilirubin (mg/dL) (0.1-0.8)	0.4 (0.2-20.9)	0.3 (0.1-0.7)	0.2 (0.2/0.2)	0.45 (0.4/0.5)
Albumin (g/dL) (2.3-3.9)	3.3 (1.9-3.9)	3.5 (3.3-4.4)	2.8 (2.5/3.1)	4.3 (4.1/4.5)
Cholesterol (mg/dL) (149-319)	212 (128-650)	237 (179-432)	173 (155/190)	244 (175/312)
BUN (mg/dL) (7-32)	11 (3-17)	13 (10-22)	14 (11/16)	33 (10/56)
Glucose (mg/dL) (67-132)	92 (77-111)	99 (80-120)	90 (87/92)	105 (99/111)
PT (sec) (7-9.4)	8.5 (7.4-11)	7.4 (6.4-9.6)	7.5 (7.3/7.7)	7.1 (7-7.1)
PTT (sec) (8.5-13.8)	12.2 (9.7-23)	11 (9.5-13.3)	10.2 (9.9/10.6)	20 (14.5/26)
Ammonia (μmol/L) (1-46)	9.5 (9-113)	9 (9-26)	9 (9/9)	NA
Post bile acids (μmol/L) (0-28)	104 ^a (23-206)	26 (12-70)	28.3 (9.6/46.9)	NA
Bile cultures (NG/G)	19/1 ^b	7/0	2/0	NA
Copper (μg/g dry) (137-400)	708 (173-2268)	348 (143-595)	183 (161-204)	NA

Abbreviations: ALT, alanine aminotransferase; ALP, alkaline phosphatase; BUN, blood urea nitrogen; GGT, gamma-glutamyl transferase; NA, not applicable; PT, prothrombin time; PTT, partial thromboplastin time; NG, no bacterial growth; G, bacterial growth.

^a Number of dogs (n = 5).

^b Bacterial isolates from liver tissue included heavy growth of *Escherichia coli*, *Pseudomonas aeruginosa*, *Enterococcus* spp.

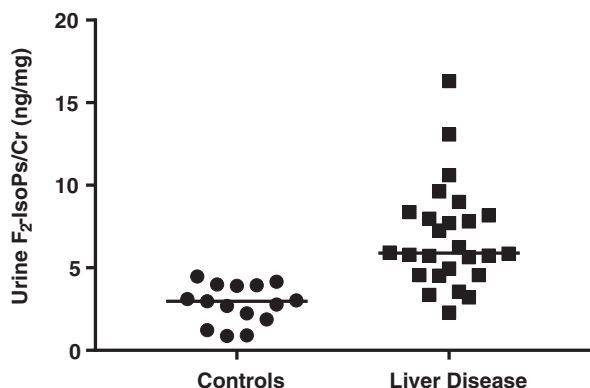


FIGURE 1 Urine 8-isoprostanes/creatinine ratio (F₂-IsoPs/Cr) in healthy controls (●) vs dogs with primary or secondary liver disease (■). F₂-IsoPs reported in nanograms normalized to Cr in milligrams. Controls were healthy dogs presenting for wellness examination (median: 2.98 ng/mg; range: 0.89-4.48). Dogs with primary or secondary liver disease underwent biopsy as routine evaluation for their disease, and were affected by a range of diseases (median: 5.89 ng/mg; range: 2.29-16.3). Groups differed significantly ($P < .0001$)

supplementation and those not receiving supplementation (data not shown).

4 | DISCUSSION

In this study, no significant correlations were identified between liver GSH and any of the other peripheral markers (plasma or erythrocyte GSH, plasma vitamin E, and F₂-IsoPs/Cr) of the body's redox status. Compared to healthy dogs, this population of dogs undergoing a clinically indicated liver biopsy had significantly increased F₂-IsoPs/Cr

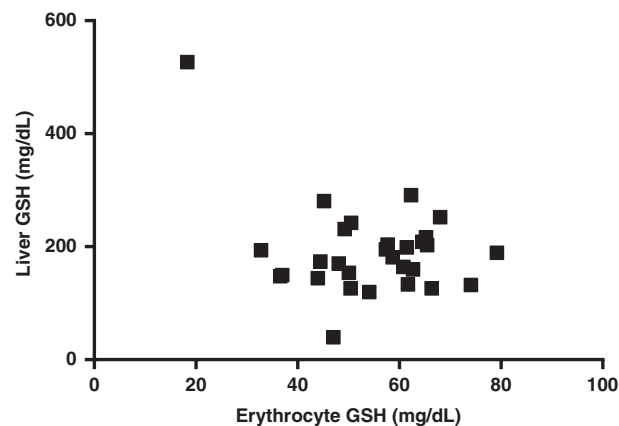


FIGURE 2 Reduced glutathione concentration (GSH (■); mg/dL) in erythrocytes vs liver (n = 29). Spearman correlation ($\rho = 0.06$, $P = .76$) between erythrocyte and liver GSH concentrations in a heterogeneous group of dogs with liver disease undergoing liver biopsy as part of their clinical evaluation

concentration, a marker of lipid peroxidation. This increased lipid peroxidation occurred despite many dogs (24/34) receiving antioxidant supplementation, indicating ongoing systemic oxidative damage.

Liver GSH concentration did not correlate with other systemic markers of oxidative stress in this group of dogs undergoing a clinically indicated liver biopsy. This is in contrast to what we hypothesized based on experimentally induced acute oxidative injury models in rodents.⁴ In rats, oxidized glutathione (GSSG) concentrations correlate between blood and liver within <12 hours of exhaustive exercise or allupurinol administration.⁴ In rats treated with L-buthionine-[S,R]-sulfoximine (BSO) to deplete body stores of glutathione, both GSH and GSSG in the plasma and in the liver decrease with exhaustive exercise, though correlation between plasma and liver has not been

directly assessed.²² Other than species or analytical method differences, possible reasons for the observed study differences from these studies and this clinical study could be in the oxidative markers studied (GSSG vs GSH) as well as systemic compartment measured (blood vs urine), the duration of oxidative injury (acute vs chronic), or the source of oxidative injury (experimentally induced vs spontaneous disease). Similarly, other studies of induced oxidative injury and systemic vs tissue concentrations of selected biomarkers of oxidative stress have reported mixed results. For example, rats fed high iron diets and treated with manganese to exacerbate oxidative damage and desferrioxamine to limit it have correlation only of serum lipid peroxides with tissue concentrations, not protein carbonyls or total antioxidant capacity.²³ In exercised rats with normal glutathione stores, plasma GSH does not change significantly with exhaustive exercise, while liver GSH decreases.²² The overall findings of the current study do not support the use of systemic markers of oxidative stress in blood or urine as an indicator of liver redox status in dogs, despite these compartments being more readily assessable for routine noninvasive sampling.

The etiologies of liver disease in people are often different from those that affect dogs, despite excessive lipid peroxidation being a common feature of liver injury across species.^{23,24} In people, increased markers of lipid peroxidation, both urine 8-isoprostanes and serum protein carbonyls, are associated with noncirrhotic and cirrhotic autoimmune cholestatic liver disease and cirrhotic autoimmune hepatitis.⁹ Other studies in dogs have demonstrated altered endogenous antioxidants in dogs with liver disease, with decreased GSH reported in both the liver and erythrocytes.^{5,7,8} Taken together, multiple markers of oxidative stress are altered in dogs undergoing a clinically indicated liver biopsy. Extrapolating from what is known about mechanisms of liver injury and studies in animals and humans with liver injury, systemic oxidative stress likely contributes to disease progression.^{24–27} In addition, this study further suggests that exogenous antioxidants, limited to supplementation with a glutathione source, do not resolve underlying systemic lipid peroxidation.

This study had several limitations. GSH was evaluated, but not oxidized or total glutathione, and correlations might have been found between these alternate forms of glutathione. Indeed, in rats subjected to oxidative stress, GSSG in blood and liver tissue, rather than GSH, correlates significantly.⁴ However, GSH represents the most biologically active form (capable of halting redox reactions), and thus seemingly the most clinically relevant; it has also previously been found to be decreased within the liver in dogs with spontaneous hepatopathies.⁷ This study also evaluated a heterogeneous group of dogs with primary or secondary liver disease, and it is possible that correlations might exist only in certain subsets of dogs, such as those with inflammatory disease or minimal fibrosis. The number of dogs in this study did not allow further analysis by category of disease. In addition, the overall number of dogs analyzed in this study was relatively small, which might have limited our power to observe statistical correlations. In addition, this study did not control antioxidant supplementation provided to dogs. As so few direct treatments for liver disease exist, antioxidant supplementation regardless of etiology is a mainstay of its treatment. Thus, most dogs are receiving antioxidant supplementation at the time of liver biopsy. Such supplementation

could have affected results (if, eg, supplementation increased glutathione concentration in the blood but not the liver). Lastly, the dogs in the control and study populations differed significantly in both weight and breed. While this difference could have contributed to the significant difference found in urine isoprostane concentrations between the 2 groups, the control group was heavier, and oxidative stress is greater in larger animals.²⁸

In conclusion, this study did not demonstrate significant correlations between liver GSH concentration and several peripheral markers of oxidative stress. This information is clinically relevant, demonstrating that measuring only blood or urine markers in dogs with liver disease is inadequate for assessing the degree of oxidative damage at the primary site of disease, or the therapeutic response. In addition, this study demonstrated significantly higher F₂-IsoPs/Cr concentration in dogs undergoing a clinically indicated liver biopsy compared to healthy controls. This increased concentration suggests that in this study population oxidative stress persisted despite prevalent antioxidant supplementation.

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CONFLICT OF INTEREST DECLARATION

Authors declare no conflict of interest.

OFF-LABEL ANTIMICROBIAL DECLARATION

Authors declare no off-label use of antimicrobials.

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE (IACUC) OR OTHER APPROVAL DECLARATION

This study was approved by the University of Wisconsin Institutional Animal Care and Use Committee.

HUMAN ETHICS APPROVAL DECLARATION

Authors declare human ethics approval was not needed for this study.

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