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Effect of *N*-Acetylcysteine Supplementation on Intracellular Glutathione, Urine Isoprostanes, Clinical Score, and Survival in Hospitalized Ill Dogs

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Abstract

Background

Antioxidant depletion and lipid peroxidation have been correlated with disease severity and associated with poor outcomes.

Hypothesis/Objectives

Supplementing dogs with *N*-acetylcysteine (NAC) during the first 48 hours of hospitalization will increase cysteine, normalize glutathione concentrations, and decrease the degree of lipid peroxidation associated with illness.

Animals

Sixty systemically ill hospitalized client-owned dogs and 14 healthy control dogs.

Methods

Randomized investigator-blinded, placebo-controlled prospective study. Dogs were randomized to treatment with NAC (n = 30) versus placebo (n = 30). Antioxidants, urine 8-isoprostane/creatinine (IP/Cr), and clinical score were determined before

and after treatment with NAC. Glutathione, cysteine, and vitamin E concentrations were quantified using high-performance liquid chromatography. Atomic absorption spectroscopy and enzyme-linked immunosorbent assays were used to quantify selenium and isoprostane concentrations, respectively.

Results

Ill dogs had significantly lower vitamin E concentrations (27 versus 55 μg/mL; *P* = .0005) as well as elevated IP/Cr ratios (872 versus 399 pg/mg; *P* = .0007) versus healthy dogs. NAC supplementation significantly increased plasma cysteine (8.67 versus 15.1 μM; *P* < .0001) while maintaining glutathione concentrations. Dogs in the placebo group experienced a statistically significant decrease in glutathione concentrations (1.49 versus 1.44 mM; *P* = .0463). Illness severity and survival were unchanged after short duration NAC supplementation.

Conclusions

Ill dogs experience systemic oxidative stress. Supplementation with NAC during the first 48 hours of hospitalization stabilized erythrocyte glutathione concentrations. The clinical impact of this supplementation and glutathione concentration stabilization was undetermined.

Abbreviations

CYS
: cysteine

GSH
: glutathione

IP/Cr
: urine 8-isoprostane/creatinine

NAC
: *N*-acetylcysteine

SPI2
: survival prediction index score

Reactive oxygen species (ROS) are a by-product of oxidative metabolism and are highly reactive molecules that damage lipids, proteins, carbohydrates, and DNA. When ROS are produced in excess of the capacity of the endogenous antioxidant network, increased cellular oxidative products are produced, resulting in a state of oxidative stress.[1], [2] Increasing evidence supports the role of oxidative stress in the pathogenesis of many systemic diseases in humans.[2], [3]

Multiple biomarkers are used to assess systemic oxidative stress, in part, owing to the transient nature of the ROS and the lack of consensus about which biomarker is the most specific, sensitive, and selective.[4] Biomarkers of oxidative stress include endogenous antioxidants (enzymatic, nonenzymatic, or micronutrients) and oxidation products of cellular components including lipids, proteins, or DNA.

By-products of lipid peroxidation are used as biomarkers of in vivo oxidative stress. Isoprostanes (or 8-isoprostanes) are produced by ROS-catalyzed peroxidation of arachidonic acid and are considered a specific marker of lipid peroxidation.[5] Urine or plasma 8-isoprostane concentrations correlate with disease severity in humans,[5, 6] are increased in animal models of oxidative injury,[5] and are modulated by antioxidants.[7]

By comparison, little is known about oxidative stress in veterinary patients. In dogs, GSH and selenium depletion are thought to play a role in aging.[8, 9] Decreased GSH levels are reported in dogs with liver disease,[10] acute acetaminophen toxicity,[11] and cardiac disease.[12] There are limited reports about vitamin E and selenium levels in ill dogs. Significantly, lower blood (plasma or serum) vitamin E concentrations have been documented in ill dogs with IMHA,[13] lymphoma,[14] and cardiac disease.[12] The use of isoprostanes is emerging in veterinary medicine as a biomarker of lipid peroxidation and oxidative stress.[4] Increased urinary 8-isoprostane concentrations are reported in dogs with intervertebral disk disease,[15] dogs in congestive heart failure,[12] and sled dogs after exercise.[16]

Critically ill dogs have decreased RBC GSH concentrations.[17] Erythrocyte glutathione (RBC GSH) concentrations correlated with illness severity and mortality, suggesting a population that would benefit from antioxidant therapy. However, little is published or known about the use of antioxidant supplementation during illness in dogs.[11, 18] In humans, therapeutic antioxidant supplementation has been used with varying degrees of success.[19, 20] While most antioxidants do not directly replenish a sulfhydryl deficiency, NAC is converted to L-cysteine, an essential amino acid for intracellular GSH synthesis.[21] Critically ill human patients treated with NAC have shown an increase in intracellular GSH levels, with many studies indicating a clinical benefit.[21-24]

The purpose of this study is to objectively evaluate if supplementation with NAC in a heterogeneous population of ill dogs with low RBC GSH concentrations affects (1) antioxidant status (ie, RBC GSH, plasma cysteine [CYS], plasma vitamin E, and whole blood selenium [Se]); (2) degree of oxidative stress (ie, urine isoprostane/creatinine ratio [IP/Cr]); (3) illness severity scores (ie, SPI2); and (4) outcome (ie, survival to discharge).

Materials and Methods

Dog Selection

Hospitalized ill dogs were recruited from the referral population admitted to the University of Wisconsin-Madison, Veterinary Medical Teaching Hospital from September 2008 through October 2009 by a single investigator (KV). In addition, blood from the 14 clinically healthy dogs was collected to establish a normal reference interval for plasma vitamin E, whole blood selenium, and urine 8-isoprostane concentrations. In our laboratory, we have previously established RBC GSH and plasma CYS in healthy dogs (n = 33).[17] An a priori power calculation was used to determine the target enrollment of healthy dogs to provide >90% power to detect a difference in urinary isoprostane concentrations in healthy versus ill dogs.[15] The healthy dogs were recruited from the VMTH primary care population and from pets belonging to VMTH employees, residents, and students.

All healthy and ill dogs with the following characteristics were considered eligible for the study: dogs of adequate size to safely remove 4 mL of blood, no clinical or biochemical evidence of significant anemia (no pallor, referral PCV > 20%), and a complete medical history including previous treatment history for the 2 weeks before sampling. Dogs previously receiving S-adenosylmethionine, NAC, total parenteral nutrition, or fluids containing vitamin supplementation during the 2 weeks before sampling, or having received a blood transfusion within 3 months of presentation to the VMTH were excluded. All study protocols were reviewed, approved, and conducted in accordance with the University of Wisconsin Animal Care and Use Committee. Informed consent was provided by all dog owners before enrollment into the study.

Study Design

A randomized, investigator-blinded, placebo-controlled study design was used to allocate recruited dogs into one of 2 treatment groups to receive either parenteral NAC or placebo. A computerized random number generator was used to construct a treatment allocation table. Each randomized dog was dispensed the appropriate treatment directly by the pharmacy to the critical care unit technician for administration. The NAC dosing protocol was chosen based on reported supplementation protocols used in sepsis (animal models[25] and human clinical trials[26]), which reflect the administration protocol used in humans and animals for the treatment of paracetamol poisoning.[27] NAC was administered as an initial 140 mg/kg loading dose (a 5% solution diluted with 5% dextrose) given over 60 minutes, with

subsequent doses of 70 mg/kg every 6 hours for a total of 7 additional doses. NAC was given through an intravenous (IV) catheter using an inline 0.2 μm Millipore filter. The placebo group was treated similarly with an identical volume of 5% dextrose. Treatment was started within 24 hours of admission to the VMTH. Additional therapies as determined by the primary clinician were administered and recorded. Dogs were censored from after treatment data analysis if they received a transfusion (whole blood, packed RBC, or plasma) during the observation period.

Canine data collected at the time of recruitment included age, breed, sex, neutered/spayed status, body weight, current therapies, and duration of illness. Additional data recorded included final diagnosis, treatments administered, days of hospitalization, and clinical outcome (survival to discharge, death, or euthanasia because of deteriorating condition).

The survival prediction index clinical score (SPI2) was determined for each dog at the time of recruitment into the study and after antioxidant therapy by 1 clinician (KV).[28] Parameters assessed in generation of the SPI2 score included age, packed cell volume, respiratory rate, mean arterial pressure, plasma creatinine and albumin, and medical versus surgical case. Plasma creatinine and albumin concentrations were determined through the VMTH clinical pathology laboratory and blood pressure measurements were attained using noninvasive oscillometry.[29]

Sample Collection and Analysis

Blood and urine samples were collected from all ill dogs before and after the 48-hour treatment period. Before treatment samples were collected after a minimum of 12-hour fast and before any treatment at the VMTH. After treatment samples were collected 2 hours after the final infusion. This after treatment sampling time was chosen based on peak plasma concentrations and time for intracellular distribution of GSH observed in humans after IV NAC administration.[23, 30] This may not represent peak antioxidant concentrations in dogs, but the sampling time was uniform for group comparisons.

RBC GSH, and plasma CYS were measured in all dogs before and after treatment. Two milliliters of venous blood was immediately added to a heparinized blood tube. Monobromobimane was added to the heparinized blood within 1 minute of venipuncture and placed on ice. Within 30 minutes of phlebotomy plasma and packed RBC were separated, harvested, and frozen at −80°C. All assays were completed within 7 days of sample collection.[31] RBC GSH and plasma CYS were measured using high-

performance liquid chromatography as previously reported.[17], [31]

Additional antioxidants measured before and after the 48-hour treatment period included plasma vitamin E and whole blood selenium concentrations. Two milliliters of venous blood was immediately aliquoted into an EDTA blood tube and protected from light. Commercially available analytical methods were used to quantify blood vitamin E and selenium concentrations. A high-performance liquid chromatography method was used to quantify plasma vitamin E. Graphite furnace atomic absorption spectrophotometry was used to quantify whole blood selenium. Samples were stored frozen before analysis and both assays were completed within 7 days of sample collection.

Urine 8-isoprostane concentrations were measured in all dogs before and after treatment. Urine samples were collected via midstream free catch, aseptic catheterization, or cystocentesis and immediately centrifuged and frozen at −80°C until analysis. Urine 8-isoprostane concentrations were quantified using a commercially available competitive enzyme immunoassay validated in dogs.[a] Each urine sample was initially passed through an isoprostane affinity column before quantification. Urine 8-isoprostane concentrations were normalized to urine creatinine concentrations to account for variation in glomerular filtration rate and urine concentration. Urine creatinine concentrations were determined through the VMTH clinical pathology laboratory.[b]

Statistical Analysis

An a priori power calculation based on previously reported data was used to determine our target enrollment of 30 dogs recruited into each group (treatment and placebo). The enrollment of 60 dogs provided >90% power to show normalization of RBC GSH, [17] a 50% decrease in urine isoprostane concentrations,[15] and a 10% increase in SPI2 score in the NAC treated group.[32] For survival analysis, our power calculations were based on the mortality rate of the severely ill dogs in our clinically ill versus healthy dog study[17] to give 70% power to show a reduction in death rate by 30%.

At the time of enrollment, continuous variables (median age, body weight, RBC GSH, plasma CYS, plasma vitamin E, whole blood selenium, urine 8-isoprostane, and SPI2 scores) were compared between the NAC and placebo groups using a Mann–Whitney *U*-test. Categorical data (breed and sex) were compared at enrollment between NAC and placebo groups using a Fisher's exact test. Plasma vitamin E, whole blood selenium, and urine 8-isoprotane concentrations were compared between the clinically

healthy dogs and ill dogs using a Mann–Whitney *U*-test. Antioxidant concentrations (RBC GSH, plasma CYS, plasma vitamin E, and whole blood selenium), urine 8-isoprostane concentrations, and SPI2 scores were compared before and after treatment using a Wilcoxon signed-rank test. RBC GSH concentrations were evaluated for correlation with urine 8-isoprostane concentrations and SPI2 score before or after treatment using a Spearman correlation. Ill dogs that died or were euthanized because of declining clinical status after antioxidant infusion were compared between the NAC and placebo groups to those that survived to discharge using a Fisher's exact test. Dogs discharged from the hospital against medical advice, euthanized for financial reasons, or given a transfusion (blood or plasma) after initial sampling but before completion of antioxidant infusion were censored from analyses. Statistical calculations were performed with a commercial software package.[c] All values are reported as median and ranges. The level for determination of significance was .05.

Results

A total of 72 dogs were enrolled into the study. Twelve of the 72 dogs did not complete the study owing to discharge from the hospital before completion of antioxidant therapy. Sixty of the 72 ill dogs that were randomized to either NAC (n = 30) or placebo (n = 30) successfully completed the study. The baseline descriptive characteristics of the ill dogs recruited into the NAC versus placebo groups and healthy dog groups are summarized in Table 1. Disease categories or primary organ dysfunction making up the ill dog population included neoplasia (6), systemic infection (6), kidney (5), gastrointestinal (5), respiratory (2), endocrine (2), trauma (2), neurological (1), and liver/biliary/pancreas (1) in the NAC treatment group, and neoplasia (10), systemic infection (3), kidney (4), gastrointestinal (2), endocrine (1), neurological (7), and liver/biliary/pancreas (3) in the placebo group.

Table 1. Signalment, breed distribution, and weights of *N*-acetylcysteine (NAC), placebo, and healthy dog groups. All values reported as median and ranges

Number	Ill 60	Ill (Placebo) 30	Ill (NAC) 30	Healthy 14
Age (years)	8 (0.5–15)	5 (0.5–13)[a]	9 (2–15)[a]	6 (1–12)
Male (N/I)	35 (29/6)	18 (14/4)	17 (15/2)	4 (4/0)
Female (S/I)	25 (19/6)	12 (9/3)	13 (10/3)	10 (7/3)

Number	Ill	Ill (Placebo)	Ill (NAC)	Healthy
	60	30	30	14
Weight (kg)	26 (4.8–48)	27 (4.8–48)	23 (5.7–48)	28 (19–43)
Purebred/Mix	58/2 [b]	29/1	29/1	11/3 [b]
Breeds (top 3)	LR (12)	LR (8)	LR (4)	RR (4)
	GR (7)	GR (4)	GR (3)	LR (3)
	SS (4)	SS (4)	Sheltie (3)	GR (2)

NAC, *N*-acetylcysteine; N, neutered; S, spayed; RR, Rhodesian Ridgeback; LR, Labrador Retriever; GR, Golden Retriever; SS, Springer Spaniel.

[a] The ill dogs randomized to placebo group were significantly younger than the ill dogs in NAC group ($P = .0162$).

[b] The ill dog population was made up of significantly more purebred dogs compared with the healthy groups of dogs ($P = .0438$).

The dogs randomized to the NAC treatment group (median age 9 years, range 2–15 years) were significantly older than the dogs randomized to the placebo group (median age 5 years, range 6 months to 13 years), $P = .0162$. However, sex, breed distribution, and body weight of each population of ill dogs were similar.

Table 2 compares the biomarkers of oxidative stress (RBC GSH, plasma CYS, plasma vitamin E, whole blood selenium, and urine IP/Cr ratios) and illness severity scores (SPI2) at enrollment for the ill dogs randomized to treatment with NAC versus placebo. With the exception of whole blood selenium concentrations, no significant differences in illness severity or biomarkers of oxidative stress were reported between these 2 groups of ill dogs. The whole blood selenium concentrations were significantly lower in the dogs randomized to the placebo group versus those treated with NAC ($P = .0307$).

Table 2. Comparison of biomarkers of oxidative stress (RBC GSH, plasma CYS, plasma vitamin E, whole blood selenium, and urine IP/Cr ratio) and illness severity scores (SPI2) at enrollment for the ill dogs randomized to treatment with NAC treatment versus placebo. All values reported as median and ranges

Biomarker	Ill (Placebo)	Ill (NAC)	*P*-Value
RBC GSH (mM)	1.49 (0.36–2.09)	1.55 (1.13–1.99)	.0927
Plasma CYS (μM)	8.88 (3.68–16.6)	9.07 (3.25–13.9)	.3994
Plasma Vitamin E (μg/mL)	26 (5.4–61)	30 (13–120)	.2392
Whole blood Se (μg/mL)	0.34 (0.14–1.00)	0.40 (0.25–1.20)	.0307
Urine IP/Cr (pg/mg)	975 (147–4152)	804 (183–4581)	.7800
SPI2	0.76 (0.41–0.92)	0.73 (0.17–0.93)	.5336

NAC, *N*-acetylcysteine; RBC GSH, red blood cell glutathione; CYS, cysteine; Se, selenium; IP/Cr, isoprostanes/creatinine ratio; SPI2, survival prediction index 2.

Biomarkers of Oxidative Stress (Healthy versus Ill)

The healthy dog populations consisted of more mixed-breed dogs than purebred dogs relative to the group of clinically ill dogs (Table 1). There was no significant difference in age, sex, or body weight between the healthy and the ill dog populations.

Ill dogs had significantly lower plasma vitamin E concentrations (median 27 μg/mL, range 5.4–120 μg/mL versus median 55 μg/mL, range 18–88 μg/mL; $P = .0005$; Fig 1) and elevated urine IP/Cr ratios (median 872 pg/mg, range 147–4581 pg/mg versus median 399 pg/mg, range 184–1267 pg/mg; $P = .0007$; Fig 2) in comparison with healthy dogs. No significant differences in whole blood selenium (median 0.51 μg/mL, range 0.25–1.0 μg/mL in healthy dogs versus 0.37 μg/mL, range 0.14–1.2 μg/mL in ill dogs; $P = .0830$) were found when ill dogs were compared with healthy dogs.

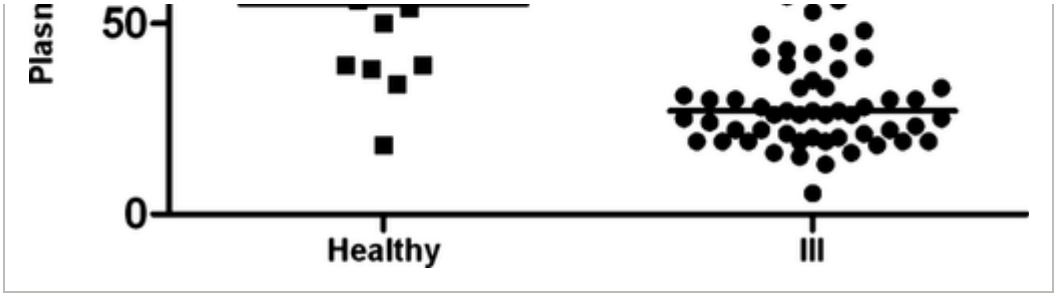

Figure 1

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Plasma vitamin E (Vit E) concentrations in healthy versus ill dogs; vitamin E concentrations in the ill dogs were significantly lower, median 27 μg/mL (n = 60), compared with vitamin E concentrations in healthy dogs, median 55 μg/mL (n = 14) with a *P*-value of .0005.

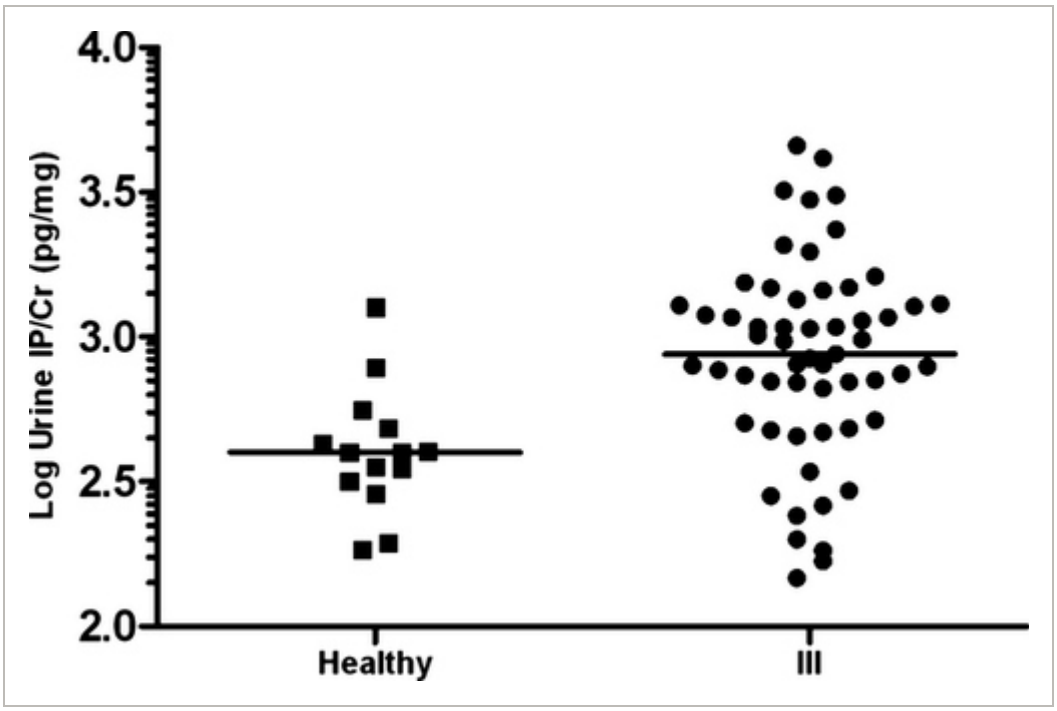

Figure 2

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Urine 8-isoprostane/creatinine ratios (IP/Cr) in healthy versus ill dogs; the IP/Cr ratio in the ill dogs were significantly higher, median 872 pg/mg (n = 57), compared with the IP/Cr ratio in healthy dogs, median 399 pg/mg (n = 14) with a *P*-value of .0007.

Biomarkers of Oxidative Stress and Illness Severity (before and

after NAC)

Dogs in the placebo group showed a statistically significant decrease in RBC GSH concentrations over the 48-hour observation period (median before 1.49 mM, range 0.36–1.86 mM versus median after 1.44 mM, range 0.30–1.94; P = .0463; Table 3) with no significant change in plasma CYS concentrations (median before 9.13 μM, range 3.68–16.69 μM versus median after 8.39 μM, range 4.80–13.16 μM; P = .5905; Table 3). NAC supplementation significantly increased plasma CYS levels (median before 8.67 μM, range 3.25–13.95 μM to median after 15.1 μM, range 7.31–59.01; P < .0001; Fig 3) with no significant change in RBC GSH concentrations (median before 1.55 mM, range 1.23–1.99 mM to median after 1.60 mM, range 1.01–1.99 mM; P = .2478; Table 3). However, plasma vitamin E, whole blood selenium, urine IP/Cr ratios, and illness severity (SPI2) were not significantly different before and after treatment for either the NAC or placebo treatment groups; Table 3. RBC GSH concentrations were not correlated with urine 8-isoprostane concentrations or illness severity (SPI2).

Table 3. Oxidative stress biomarkers (RBC GSH, plasma CYS, plasma vitamin E, whole blood selenium, and urine IP/Cr) and illness severity score (SPI2) before and after treatment in the ill dogs randomized to placebo versus NAC treatment groups. All before and after treatment data reported as median and ranges

Biomarker	Placebo			NAC		
	Before	After	*P*-Value	Before	After	*P*-Value
RBC GSH (mM)	1.49 (0.36–1.86)	1.44 (0.30–1.94)	.0463	1.55 (1.23–1.99)	1.60 (1.01–1.99)	.2478
Plasma CYS (μM)	9.13 (3.68–16.7)	8.39 (4.80–13.2)	.5905	8.67 (3.25–14.0)	15.1 (7.31–59.0)	<.0001
Plasma Vitamin E (μg/mL)	25 (16–61)	26 (0.66–60)	.5809	30 (13–120)	30 (11–64)	.1745
Whole blood Se (μg/mL)	0.32 (0.14–0.77)	0.35 (0.15–0.85)	.2470	0.40 (0.25–1.20)	0.39 (0.26–0.96)	.5772
Urine IP/Cr (pg/mg)	975 (147–4152)	777 (270–4833)	.6171	804 (183–4581)	946 (265–2837)	.3380

Biomarker	Placebo			NAC		
	Before	After	*P*-Value	Before	After	*P*-Value
SPI2	0.76 (0.41–0.92)	0.78 (0.47–0.93)	.6473	0.73 (0.17–0.93)	0.77 (0.17–0.90)	.8077

NAC, *N*-acetylcysteine; RBC GSH, red blood cell glutathione; CYS, cysteine; Se, selenium; IP/Cr, isoprostanes/creatinine ratio; SPI2, survival prediction index 2.

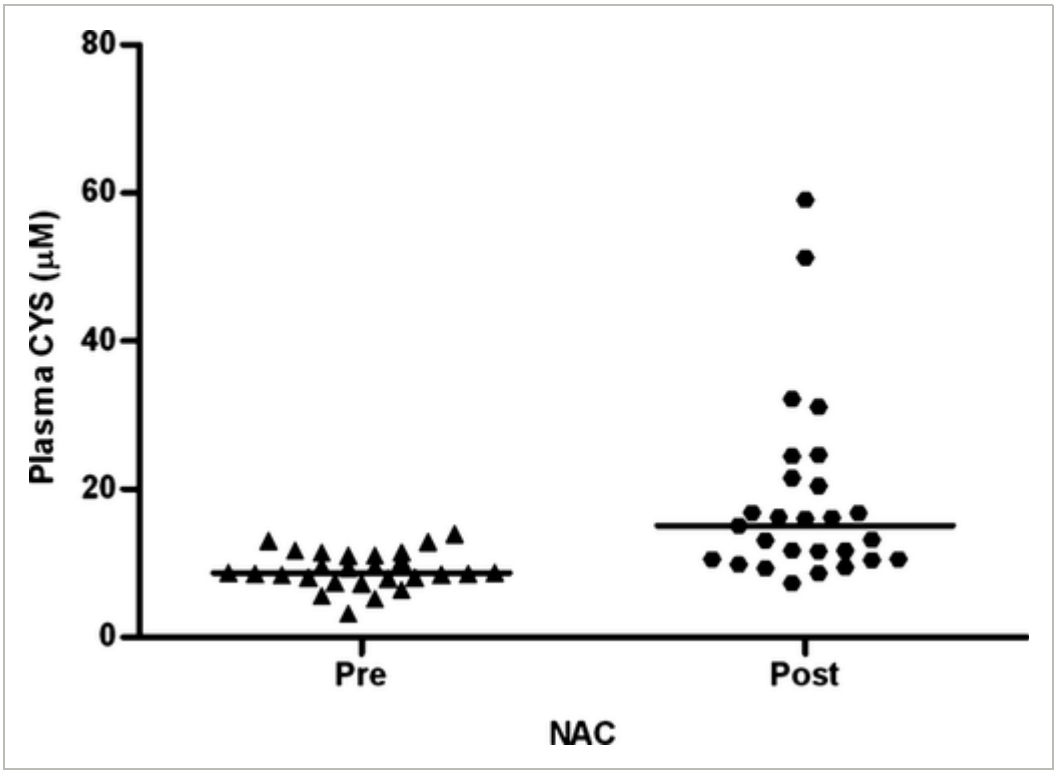

Figure 3

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Plasma cysteine (CYS) concentrations in ill dogs treated with *N*-acetylcysteine (NAC). Ill dogs treated with NAC had a significant increase in CYS concentrations from a median of 8.67 μM before treatment (n = 27) to a median of 15.1 μM after treatment (n = 27); $P < .0001$.

Outcome

Twenty-three of the 30 dogs in the placebo group (77%) and 25 of the 30 dogs treated with NAC (83%) survived to discharge. There was no significant differences in outcome (survival to discharge) between the NAC and placebo groups after treatment (P = .740).

Discussion

This study evaluated the impact of illness and treatment on endogenous antioxidant concentrations, lipid peroxidation, clinical score, and outcome in ill dogs supplemented with either NAC versus placebo within the first 48 hours of hospitalization. The results of this study further support that hospitalized ill dogs experience systemic oxidative stress including depletion of endogenous antioxidants and increased lipid peroxidation. Illness in dogs is associated with decreased RBC GSH concentrations (median RBC GSH ill dogs 1.22 mM, range 0.55–3.61 versus healthy dogs 1.91 mM, range 0.87–3.51; P = .0004).[17] In addition, compared with healthy dogs, ill dogs have lower plasma vitamin E plasma concentrations and experience excessive production of urine isoprostanes, a biomarker for systemic lipid peroxidation. Antioxidant supplementation with NAC within the first 48 hours of hospitalization significantly increased plasma CYS concentrations and modulated the degree of oxidative stress by preventing further RBC GSH depletion in ill dogs. Plasma vitamin E, urinary 8-isoprostanes, illness severity, and survival to discharge were unchanged after short duration NAC supplementation.

Illness in humans is associated with increased cellular oxidative injury and lipid peroxidation in part owing to a reduction in antioxidant potential. Decreased antioxidants including RBC GSH,[33, 34] vitamin E,[35, 36] and selenium[19, 37] have been reported in association with illness including critically ill human patients. Factors influencing circulating antioxidant concentrations during illness include inflammatory-mediated antioxidant redistribution, increased antioxidant utilization, loss, or both, decreased vitamin intake, or decreased antioxidant synthesis, redox recycling or both.[38]

Increasing evidence supports that oxidative stress plays a role the pathogenesis of many systemic diseases. The interplay between ROS and antioxidants maintains cellular redox balance. For example, endogenous antioxidants function independently and synergistically within a highly interconnected cellular antioxidant network to modulate or quench endogenous ROS produced as a by-product of aerobic metabolism.[39] Glutathione, the main intracellular antioxidant, is intimately linked to other antioxidants including cysteine, vitamin E, and selenium. Glutathione functions as a direct antioxidant but also an essential cofactor for enzymes like glutathione

peroxidase and functions synergistically with vitamins E and C as well as selenium to maintain cellular redox homeostasis. Vitamin E is a lipid soluble vitamin that functions as a free radical scavenger that prevents lipid peroxidation and is recycled in part by glutathione and vitamin C.[40] Selenium is an essential cofactor for the function of the antioxidant selenoenzyme, glutathione peroxidase, which is necessary for the reduction of lipid peroxides to hydroxyl acids.[41] The GSH peroxidase-Se enzyme complex plays a significant role in the detoxification of lipid peroxides especially in absence of vitamin E.[42] Selenium via GSH peroxidase has a sparing effect on vitamin E.

Similar to hospitalized human patients, we previously reported decreased low RBC GSH concentrations during illness in dogs[17] and decreased glutathione concentrations has been associated with canine liver disease[10] and acetaminophen toxicity.[11] The results of this study support that illness in dogs is associated with a cellular redox imbalance. Ill dogs have decreased RBC GSH and plasma vitamin E concentrations without a concurrent deficiency in whole blood Se. Others have reported decreased blood (plasma or serum) vitamin E concentrations in association with both pathological conditions in dogs, including immune-mediate hemolytic anemia,[13] cardiac disease,[12] and physiological conditions including pregnancy[43] and endurance exercise.[16, 44] During illness, decreased dietary intake may contribute to the decreased circulating vitamin E concentrations in ill dogs, as most mammals cannot synthesize vitamin E.[45] Much less is known about selenium concentrations during illness in dogs. Decreased selenium levels have been reported in association with canine aging.[8]

The results of this study do not provide a mechanistic understanding of the complex interplay between endogenous antioxidants during disease-induced oxidative stress, but support that illness in dogs is associated with oxidative stress. Excessive lipid peroxidation occurs concurrently with decreases in RBC GSH and plasma vitamin E. Interestingly, in dogs, whole blood selenium levels were not decreased during illness. These findings may suggest that ill dogs have sufficient Se for continued GSH peroxidase activity but are limited in available GSH and vitamin E for adequate lipid peroxide detoxification.

Systemic oxidative stress triggers a series of progressive cellular adaptive responses involving not only alterations in endogenous antioxidants but also the production of lipid peroxidation products. Urinary isoprostanes are nonenzymatic products of lipid peroxidation, more specifically stable eicosanoids used as an in vivo biomarker of oxidative damage.[46-48] In humans, urinary or plasma 8-isoprostanes concentrations or both have been correlated with illness severity and modulated after antioxidant supplementation.[5-7] Animal models of oxidative injury are associated with increased

isoprostanes concentrations.[49] There is increased urinary 8-isoprostane concentrations in healthy dogs after exercise[16] and in dogs in congestive heart failure[12] and acute intervertebral disk disease.[15] In our heterogeneous group of systemically ill dogs, urinary isoprostanes concentrations were significantly increased relative to healthy dogs. Increased urinary isoprostanes occurred in association with decreased endogenous antioxidant concentrations. The clinical utility of urinary isoprostane concentrations during illness in dogs needs further investigation. However, its use as a biomarker of lipid peroxidation further supports a state of redox imbalance during illness in dogs.

A variety of antioxidants for the modulation of disease-associated oxidative stress have been studied in humans. Mixed results are reported, limited by the lack of standardization of methodologies, doses, and routes of administration.[50, 51] Many clinical studies in critically ill humans have evaluated antioxidant supplementation during illness to target blocking the formation of ROS, scavenging ROS, and augmenting endogenous antioxidants. A recent meta-analysis of clinical studies evaluating antioxidant supplementation during illness concluded that trace element and vitamin supplementation in critically ill humans not only supports antioxidant function but may reduce mortality.[20] The combination of antioxidants and doses used vary between studies, making it difficult to standardize the combination, doses, or both of antioxidants or micronutrients needed during illness.

The rationale for the use and success of NAC in patients with low GSH concentrations is that it provides a source of L-cysteine needed for GSH synthesis and replenishment of intracellular GSH. The majority of circulating GSH is synthesized in the liver and subsequently undergoes secretion into circulation.[52] NAC also functions as a free radical scavenger of hydroxyl radicals and hypochlorous acid[53] as well as having anti-inflammatory properties via attenuation of neutrophil and macrophage activation, cytokine release, leukocyte-endothelial cell adhesion, and capillary leakage.[54, 55] In animal models, treatment with NAC was considered protective against experimentally induced endotoxemia,[25] toxic gas inhalation,[56] and ischemia-reperfusion injury.[57] In humans, many studies indicate a clinical benefit associated with NAC therapy.[22, 23] For example, NAC attenuated ischemia reperfusion associated with cardiac catheterization and has demonstrated benefits in treating sepsis,[23] acute respiratory distress[24], and contrast-induced nephropathy.[58] However, in at least 1 human study, early NAC supplementation in critically ill septic patients is reported to not affect cytokine concentrations, gastric pH, patient hemodynamics, or outcome.[59]

In this study, supplementing dogs with NAC within 48 hours of hospitalization resulted

in a significant increase in plasma CYS concentrations and the maintenance of RBC GSH concentrations. Plasma vitamin E, urinary 8-isoprostanes, illness severity, and survival to discharge were unchanged after short duration supplementation. These results support the maintenance of intracellular GSH in ill dogs with short-term NAC supplementation. However, the resolution of systemic oxidative stress or repletion of RBC GSH was incomplete during the first 48 hours of hospitalization.

Reasons for the lack in resolution of systemic oxidative stress after supplementation with NAC may include the time NAC was supplemented during the course of illness, the relatively short duration of therapy, the NAC dosage administered, or the need for combination antioxidant therapy. Experimental and clinical studies evaluating the use of NAC in sepsis or septic shock report early supplementation (during the first hours of disease) to be beneficial[23, 60] versus the lack of benefit reported in studies in which supplementation was delayed.[61, 62] The NAC dosage used in this study was extrapolated from protocols used to treat acetaminophen toxicosis. Prospective trials support the safety and efficacy of this dosage protocol,[27, 30] but no randomized controlled trials are available to support optimal dose and duration of NAC supplementation. The pharmacokinetics and pharmacodynamics of NAC have not been adequately studied to support an alternative dosing regimen in dogs.[30] The use of a different NAC dosage, supplementation beyond 48 hours, or NAC supplementation in combination with vitamin E may have improved the outcome of these ill hospitalized dogs. Further studies are necessary in clinically ill dogs to investigate if longer duration or combination antioxidant supplementation is needed to normalize endogenous antioxidant levels, resolve systemic oxidative stress, and impact long-term outcome.

The strength of this study is the randomized, investigator-blinded, placebo-controlled study design to reduce treatment bias. However, this study is not without limitations. A major limitation of this study was the heterogeneity of this population of ill dogs. Dogs in this study were diagnosed with a variety of underlying diseases with varying degrees of illness severities and durations (median 7 days, range 0.5–90 days) at the time of presentation. This heterogeneity may have confounded the degree of improvement to NAC supplementation, limited our ability to provide statistical comparisons of endogenous antioxidants, the degree of oxidative stress between specific disease states, or identify disease states that maybe more or less responsive to NAC supplementation.

In spite of these limitations, this study used multiple biomarkers to further characterize oxidative stress during illness in hospitalized dogs and provided an initial assessment of targeted single agent antioxidant supplementation in GSH depleted dogs. In

summary, clinically ill dogs have decreased endogenous antioxidant concentrations (ie, RBC GSH and plasma vitamin E) and increased systemic lipid peroxidation as determined by urinary 8-isoprostane concentrations. Supplementation with NAC within the first 48 hours of hospitalization significantly increased plasma CYS concentrations and stabilized RBC GSH concentrations. Plasma vitamin E, urinary 8-isoprostanes, illness severity, and survival to discharge were unchanged after short-duration NAC supplementation. Further studies are necessary to investigate whether longer duration or combined antioxidant supplementation in clinically ill dogs normalizes their redox state and impacts long-term outcome.

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Conflict of Interest: Authors disclose no conflict of interest.

Footnotes

1 Cayman Chemical Co, Ann Arbor, MI
2 Vitros 5,1 FS Chemistry analyzer by Ortho Clinical Diagnostics, Rochester, NY
3 Graphpad Prism 5, La Jolla, CA

References

1 Cowley HC, Bacon PJ, Goode HF, et al. Plasma antioxidant potential in severe sepsis: A comparison of survivors and nonsurvivors. *Crit Care Med* 1996; **24**: 1179–1183.

CAS | PubMed | Web of Science® | Google Scholar

2 Roth E, Manhart N, Wessner B. Assessing the antioxidative status in critically ill patients. *Curr Opin Clin Nutr Metab Care* 2004; **7**: 161–168.

CAS | PubMed | Web of Science® | Google Scholar

3 Nathens AB, Neff MJ, Jurkovich GJ, et al. Randomized, prospective trial of antioxidant supplementation in critically ill surgical patients. *Ann Surg* 2002; **236**: 814–822.

PubMed | Web of Science® | Google Scholar

4 McMichael MA. Oxidative stress, antioxidants, and assessment of oxidative stress in dogs and cats. *J Am Vet Med Assoc* 2007; **231**: 714–720.

CAS | PubMed | Web of Science® | Google Scholar

5 Morrow JD, Roberts LJ. The isoprostanes: Unique bioactive products of lipid peroxidation. *Prog Lipid Res* 1997; **36**: 1–21.

CAS | PubMed | Web of Science® | Google Scholar

6 Basu S. Isoprostanes: Novel bioactive products of lipid peroxidation. *Free Radic Res* 2004; **38**: 105–122.

CAS | PubMed | Web of Science® | Google Scholar

7 Fischer UM, Cox CS Jr, Allen SJ, et al. The antioxidant N-acetylcysteine preserves myocardial function and diminishes oxidative stress after cardioplegic arrest. *J Thorac Cardiovasc Surg* 2003; **126**: 1483–1488.

CAS | PubMed | Web of Science® | Google Scholar

8 Stowe HD, Lawler DF, Kealy RD. Antioxidant status of pair-fed Labrador Retrievers is affected by diet restriction and aging. *J Nutr* 2006; **136**: 1844–1848.

CAS | PubMed | Web of Science® | Google Scholar

9 Vajdovich P, Gaal T, Szilagyi A, et al. Changes in some red blood cell and clinical laboratory parameters in young and old Beagle dogs. *Vet Res Commun* 1997; **21**: 463–470.

CAS | PubMed | Web of Science® | Google Scholar

10 Center SA, Warner KL, Erb HN. Liver glutathione concentrations in dogs and cats with naturally occurring liver disease. *Am J Vet Res* 2002; **63**: 1187–1197.

CAS | PubMed | Web of Science® | Google Scholar

11 Wallace KP, Center SA, Hickford FH, et al. S-adenosyl-L-methionine (SAMe) for the treatment of acetaminophen toxicity in a dog. *J Am Anim Hosp Assoc* 2002; **38**: 246–254.

PubMed | Web of Science® | Google Scholar

12 Freeman LM, Rush JE, Milbury PE, et al. Antioxidant status and biomarkers of oxidative stress in dogs with congestive heart failure. *J Vet Intern Med* 2005; **19**: 537–541.

PubMed | Web of Science® | Google Scholar

13 Pesillo SA, Freeman LM, Rush JE. Assessment of lipid peroxidation and serum vitamin E concentration in dogs with immune-mediated hemolytic anemia. *Am J Vet Res* 2004; **65**: 1621–1624.

CAS | PubMed | Web of Science® | Google Scholar

14 Winter JL, Barber LG, Freeman L, et al. Antioxidant status and biomarkers of oxidative stress in dogs with lymphoma. *J Vet Intern Med* 2009; **23**: 311–316.

CAS | PubMed | Web of Science® | Google Scholar

15 McMichael MA, Ruaux CG, Baltzer WI, et al. Concentrations of 15F2t isoprostane in urine of dogs with intervertebral disk disease. *Am J Vet Res* 2006; **67**: 1226–1231.

CAS | PubMed | Web of Science® | Google Scholar

16 Hinchcliff KW, Reinhart GA, DiSilvestro R, et al. Oxidant stress in sled dogs subjected to repetitive endurance exercise. *Am J Vet Res* 2000; **61**: 512–517.

CAS | PubMed | Web of Science® | Google Scholar

17 Viviano KR, Lavergne SN, Goodman L, et al. Glutathione, cysteine, and ascorbate concentrations in clinically ill dogs and cats. *J Vet Intern Med* 2009; **23**: 250–257.

CAS | PubMed | Web of Science® | Google Scholar

18 Villar D, Buck WB, Gonzalez JM. Ibuprofen, aspirin and acetaminophen toxicosis and treatment in dogs and cats. *Vet Hum Toxicol* 1998; **40**: 156–162.

CAS | PubMed | Web of Science® | Google Scholar

19 Bulger EM, Maier RV. Antioxidants in critical illness. *Arch Surg* 2001; **136**: 1201–1207.

CAS | PubMed | Web of Science® | Google Scholar

20 Heyland DK, Dhaliwal R, Suchner U, et al. Antioxidant nutrients: A systematic review of trace elements and vitamins in the critically ill patient. *Intensive Care Med* 2005; **31**: 327–337.

PubMed | Web of Science® | Google Scholar

21 De Rosa SC, Zaretsky MD, Dubs JG, et al. N-acetylcysteine replenishes glutathione in HIV infection. *Eur J Clin Invest* 2000; **30**: 915–929.

CAS | PubMed | Web of Science® | Google Scholar

22 Bernard GR, Wheeler AP, Arons MM, et al. A trial of antioxidants N-acetylcysteine and procysteine in ARDS. The Antioxidant in ARDS Study Group. *Chest* 1997; **112**: 164–172.

CAS | PubMed | Web of Science® | Google Scholar

23 Ortolani O, Conti A, De Gaudio AR, et al. The effect of glutathione and N-acetylcysteine on lipoperoxidative damage in patients with early septic shock. *Am J Respir Crit Care Med* 2000; **161**: 1907–1911.

CAS | PubMed | Web of Science® | Google Scholar

24 Soltan-Sharifi MS, Mojtahedzadeh M, Najafi A, et al. Improvement by N-acetylcysteine of acute respiratory distress syndrome through increasing intracellular glutathione, and extracellular thiol molecules and anti-oxidant power: Evidence for underlying toxicological mechanisms. *Hum Exp Toxicol* 2007; **26**: 697–703.

CAS | PubMed | Web of Science® | Google Scholar

25 Vassilev D, Hauser B, Bracht H, et al. Systemic, pulmonary, and hepatosplanchnic effects of N-acetylcysteine during long-term porcine endotoxemia. *Crit Care Med* 2004; **32**: 525–532.

CAS | PubMed | Web of Science® | Google Scholar

26 Henderson A, Hayes P. Acetylcysteine as a cytoprotective antioxidant in patients with severe sepsis: Potential new use for an old drug. *Ann Pharmacother* 1994; **28**: 1086–1088.

CAS | PubMed | Web of Science® | Google Scholar

27 Yip L, Dart RC, Hurlbut KM. Intravenous administration of oral N-acetylcysteine. *Crit Care Med* 1998; **26**: 40–43.

CAS | PubMed | Web of Science® | Google Scholar

28 King LG, Wohl JS, Manning AM, et al. Evaluation of the survival prediction index as a model of risk stratification for clinical research in dogs admitted to intensive care units at four locations. *Am J Vet Res* 2001; **62**: 948–954.

CAS | PubMed | Web of Science® | Google Scholar

29 Stepien RL. Blood pressure measurement in dogs and cats. *In Pract* 2000; **22**: 136–145.

Web of Science® | Google Scholar

30 Prescott LF, Donovan JW, Jarvie DR, et al. The disposition and kinetics of intravenous N-acetylcysteine in patients with paracetamol overdosage. *Eur J Clin Pharmacol* 1989; **37**: 501–506.

CAS | PubMed | Web of Science® | Google Scholar

31 Trepanier LA, Yoder AR, Bajad S, et al. Plasma ascorbate deficiency is associated with impaired reduction of sulfamethoxazole-nitroso in HIV infection. *J Acquir Immune Defic Syndr* 2004; **36**: 1041–1050.

CAS | PubMed | Web of Science® | Google Scholar

32 Prittie JE, Barton LJ, Peterson ME, et al. Pituitary ACTH and adrenocortical secretion in critically ill dogs. *J Am Vet Med Assoc* 2002; **220**: 615–619.

CAS | PubMed | Web of Science® | Google Scholar

33 Lyons J, Rauh-Pfeiffer A, Ming-Yu Y, et al. Cysteine metabolism and whole blood glutathione synthesis in septic pediatric patients. *Crit Care Med* 2001; **29**: 870–877.

CAS | PubMed | Web of Science® | Google Scholar

34 Wu G, Fang YZ, Yang S, et al. Glutathione metabolism and its implications for health. *J Nutr* 2004; **134**: 489–492.

CAS | PubMed | Web of Science® | Google Scholar

35 Goode HF, Cowley HC, Walker BE, et al. Decreased antioxidant status and increased lipid peroxidation in patients with septic shock and secondary organ dysfunction. *Crit Care Med* 1995; **23**: 646–651.

CAS | PubMed | Web of Science® | Google Scholar

36 Takeda K, Shimada Y, Amano M, et al. Plasma lipid peroxides and alpha-tocopherol in critically ill patients. *Crit Care Med* 1984; **12**: 957–959.

CAS | PubMed | Web of Science® | Google Scholar

37 Geoghegan M, McAuley D, Eaton S, et al. Selenium in critical illness. *Curr Opin Crit Care* 2006; **12**: 136–141.

PubMed | Web of Science® | Google Scholar

38 Berger MM, Chiolero RL. Antioxidant supplementation in sepsis and systemic inflammatory response syndrome. *Crit Care Med* 2007; **35**: S584–S590.

CAS | PubMed | Web of Science® | Google Scholar

39 Temple MD, Perrone GG, Dawes IW. Complex cellular responses to reactive oxygen species. *Trends Cell Biol* 2005; **15**: 319–326.

CAS | PubMed | Web of Science® | Google Scholar

40 Traber MG, Stevens JF. Vitamins C and E: Beneficial effects from a mechanistic perspective. *Free Radic Biol Med* 2011; **51**: 1000–1013.

CAS | PubMed | Web of Science® | Google Scholar

41 Christophersen BO. Reduction of linolenic acid hydroperoxide by a glutathione peroxidase. *Biochim Biophys Acta* 1969; **176**: 463–470.

CAS | PubMed | Web of Science® | Google Scholar

42 Tappel AL. Selenium-glutathione peroxidase and vitamin E. *Am J Clin Nutr* 1974; **27**: 960–965.

CAS | PubMed | Web of Science® | Google Scholar

43 Vannucchi CI, Jordao AA, Vannucchi H. Antioxidant compounds and oxidative stress in female dogs during pregnancy. *Res Vet Sci* 2007; **83**: 188–193.

CAS | PubMed | Web of Science® | Google Scholar

44 Scott KC, Hill RC, Lewis DD, et al. Effect of alpha-tocopheryl acetate supplementation on vitamin E concentrations in Greyhounds before and after a race. *Am J Vet Res* 2001; **62**: 1118–1120.

CAS | PubMed | Web of Science® | Google Scholar

45 Colombo ML. An update on vitamin E, tocopherol and tocotrienol-perspectives. *Molecules* 2010; **15**: 2103–2113.

CAS | PubMed | Web of Science® | Google Scholar

46 Lawson JA, Li H, Rokach J, et al. Identification of two major F2 isoprostanes, 8,12-iso- and 5-epi-8, 12-iso-isoprostane F2alpha-VI, in human urine. *J Biol Chem* 1998; **273**: 29295–29301.

CAS | PubMed | Web of Science® | Google Scholar

47 Moore K. Isoprostanes and the liver. *Chem Phys Lipids* 2004; **128**: 125–133.

CAS | PubMed | Web of Science® | Google Scholar

48 Navarro F, Navas P, Burgess JR, et al. Vitamin E and selenium deficiency induces expression of the ubiquinone-dependent antioxidant system at the plasma membrane. *FASEB J* 1998; **12**: 1665–1673.

CAS | PubMed | Web of Science® | Google Scholar

49 Morrow JD, Roberts LJ. The isoprostanes: Their role as an index of oxidant stress status in human pulmonary disease. *Am J Respir Crit Care Med* 2002; **166**: S25–S30.

PubMed | Web of Science® | Google Scholar

50 Halliwell B. Oxidative stress, nutrition and health. Experimental strategies for optimization of nutritional antioxidant intake in humans. *Free Radic Res* 1996; **25**: 57–74.

CAS | PubMed | Web of Science® | Google Scholar

51 Hermans N, Cos P, Maes L, et al. Challenges and pitfalls in antioxidant research. *Curr Med Chem* 2007; **14**: 417–430.

CAS | PubMed | Web of Science® | Google Scholar

52 Griffith OW, Meister A. Glutathione: Interorgan translocation, turnover, and metabolism. *Proc Natl Acad Sci U S A* 1979; **76**: 5606–5610.

CAS | PubMed | Web of Science® | Google Scholar

53 Scott BC, Aruoma OI, Evans PJ, et al. Lipoic and dihydrolipoic acids as antioxidants. *A critical evaluation. Free Radic Res* 1994; **20**: 119–133.

CAS | PubMed | Web of Science® | Google Scholar

54 Kharazmi A, Nielsen H, Schiotz PO. N-acetylcysteine inhibits human neutrophil and monocyte chemotaxis and oxidative metabolism. *Int J Immunopharmacol* 1988; **10**: 39–46.

CAS | PubMed | Web of Science® | Google Scholar

55 Schmidt H, Schmidt W, Muller T, et al. N-acetylcysteine attenuates endotoxin-induced leukocyte-endothelial cell adhesion and macromolecular leakage in vivo. *Crit Care Med* 1997; **25**: 858–863.

CAS | PubMed | Web of Science® | Google Scholar

56 Sciuto AM, Strickland PT, Kennedy TP, et al. Protective effects of N-acetylcysteine treatment after phosgene exposure in rabbits. *Am J Respir Crit Care Med* 1995; **151**: 768–772.

CAS | PubMed | Web of Science® | Google Scholar

57 Carroll JE, Howard EF, Hess DC, et al. Nuclear factor-kappa B activation during cerebral reperfusion: Effect of attenuation with N-acetylcysteine treatment. *Brain Res Mol Brain Res* 1998; **56**: 186–191.

CAS | PubMed | Web of Science® | Google Scholar

58 Mautone A, Brown JR. Contrast-induced nephropathy in patients undergoing elective and urgent procedures. *J Interv Cardiol* 2010; **23**: 78–85.

CAS | PubMed | Web of Science® | Google Scholar

59 Emet S, Memis D, Pamukcu Z. The influence of N-acetyl-L-cystein infusion on cytokine levels and gastric intramucosal pH during severe sepsis. *Crit Care* 2004; **8**: R172–R179.

PubMed | Web of Science® | Google Scholar

60 Rank N, Michel C, Haertel C, et al. N-acetylcysteine increases liver blood flow and improves liver function in septic shock patients: Results of a prospective, randomized, double-blind study. *Crit Care Med* 2000; **28**: 3799–3807.

CAS | PubMed | Web of Science® | Google Scholar

61 Agusti AG, Togores B, Ibanez J, et al. Effects of N-acetylcysteine on tissue oxygenation in patients with multiple organ failure and evidence of tissue hypoxia. *Eur Respir J* 1997; **10**: 1962–1966.