Induction of thioredoxin-1 in response to oxidative stress in dogs

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OBJECTIVE
To determine whether thioredoxin (TRX)-1 can be used as a valid biomarker for oxidative stress in dogs.

ANIMALS AND SAMPLES
10 Beagles and Madin-Darby canine kidney cells.

PROCEDURES
Madin-Darby canine kidney cells were used to verify antigen cross-reactivity between human and canine anti–TRX-antibodies. Dogs were assigned to receive 21% or 100% O_2 (5 dogs/group) via an artificial respirator during a 3-hour period of isoflurane anesthesia (starting at 0 hours). Blood and urine samples were collected before (baseline) and at 6, 12, 24, and 48 hours after commencement of inhalation anesthesia. Concentrations of TRX-1 and 8-hydroxy-2'-deoxyguanosine (8-OHdG) in plasma and urine samples were analyzed; urine concentrations were reported as ratios against urine creatinine concentration.

RESULTS
Canine TRX-1 was recognized by monoclonal human anti-TRX-1 antibodies (clones of adult T-cell leukemia-derived factor [ADF]-11 and ADF21) by western blot analysis. Results of an ELISA indicated that plasma TRX-1 concentration and urine TRX-1-to-creatinine concentration ratio increased rapidly after the 3-hour period of hyperoxia with maximal peaks at 12 and 6 hours, respectively. Urine 8-OHdG-to-creatinine concentration ratio also increased significantly after hyperoxia induction. However, unlike the rapid increase in urine TRX-1-to-creatinine concentration ratio, maximal urine 8-OHdG-to-creatinine concentration ratio was attained at 48 hours after hyperoxia induction. These variables remained unchanged from baseline in the control group.

CONCLUSIONS AND CLINICAL RELEVANCE
Results indicated that human anti-TRX monoclonal antibodies cross-reacted with canine TRX, and plasma TRX-1 concentrations were rapidly increased in dogs following an oxidative stress challenge. Thus, TRX may be a valuable clinical biomarker for detecting oxidative stress more rapidly than 8-OHdG in dogs. (*Am J Vet Res* 2015;76:554–560)

Oxidative stress is caused by ROS produced within an organism by endogenous or exogenous sources and is a major cause of stress to cells or tissues. In human medicine, oxidative stress is known to be harmful to the body. Therefore, the human body has developed a redox (oxidation and reduction) regulating system, which acts defensively against oxidative stress. Within this system, TRX has an important role in maintaining homeostasis in response to oxidative stress.

Thioredoxin is now known to be a class of small redox proteins. Thioredoxin-1 is a small redox-active protein (12 kDa) consisting of 105 amino acids,[1]

which is ubiquitously expressed in tissues of humans and other animals. Thioredoxin was originally identified in *Escherichia coli* as an electron donor for ribonucleotide reductase, an essential enzyme for DNA synthesis.[2] Human TRX was cloned independently as an ADF in 1989.[3] In humans, TRX is a defensive protein induced by various stresses and has antioxidative, antiapoptotic, and anti-inflammatory effects.[4] Thioredoxin is present in all cells, including prokaryotes and fungi,[1] and is induced as a defensive protein by various stimuli such as UV light, radiation, ischemia and reperfusion injury, chemotherapy, infection, and inflammation.[4-6] In human medicine, TRX has an important role in inflammation during viral infection by HIV or hepatitis C virus as well as autoimmune and cardiovascular disease.[7-11] Thioredoxin expression is expected to influence the diagnosis of these disorders and their prognosis as well as be useful for treatment evaluation. Additionally, TRX might be useful for treatment of

ABBREVIATIONS
8-OHdG	8-hydroxy-2'-deoxyguanosine
ADF	Adult T-cell leukemia-derived factor
ROS	Reactive oxygen species
TRX	Thioredoxin

acute respiratory distress syndrome, acute lung injury, and contact dermatitis.[12,13]

Thus, in human medicine, TRX is generally recognized as an oxidative stress marker with clinical applicability.[14,15] In veterinary medicine, similar to human medicine, oxidative stress is likely involved in various diseases of animals, and TRX is expected to be a clinically applicable biostress marker and useful therapeutic agent for disorders associated with oxidative stress. However, to our knowledge, there have been no studies evaluating TRX in veterinary medicine. Measurements of plasma TRX-1 concentrations in companion animals such as dogs and cats have not been investigated.

We previously reported immune cross-reactivity between humans and dogs by demonstrating that a rabbit antibody directed against human TRX (ADF) could also detect canine ADF by immunohistostaining.[14] Furthermore, antibodies directed against canine ADF successfully reacted with human ADF in a sensitive ELISA.[15]

The purpose of the study reported here was to determine whether TRX can be used as a valid biomarker for oxidative stress in dogs. Our intent was to measure plasma and urine TRX-1 concentrations in dogs to evaluate the clinical diagnostic application of an ELISA with cross-reactive anti-human TRX antibodies for use in clinical veterinary practice. Because TRX is an oxidative stress marker in humans, we hypothesized that 100% O_2–induced hyperoxia would induce oxidative stress in dogs and that increases in TRX-1 concentrations in dog urine and plasma samples could be measured by ELISA with human anti-TRX antibodies that are cross-reactive with canine TRX.

Materials and Methods

Western blot analysis

Immunohistochemical staining does not definitively demonstrate the cross-reactivity of antibodies for specific antigens. Therefore, to verify the antigen cross-reactivity between human and canine antibodies against TRX protein, we performed western blot analysis using monoclonal mouse anti-human TRX antibodies (ADF11 and ADF21). These antibodies have been successfully used for detecting human TRX in a sensitive ELISA by Kogaki et al.[15] Madin-Darby canine kidney[a] cells were cultured in Dulbecco modified Eagle medium containing 10% fetal bovine serum in a humidified incubator at 37°C and 5% CO_2. The Madin-Darby canine kidney cells were lysed in cell lysis buffer (10mM Tris-HCl [pH, 7.8], 1mM EDTA, 1% NP-40, and 0.15M NaCl) containing a protease inhibitor mixture.[b] The cell lysates were resolved by electrophoresis on 15% to 20% Tris-HCl gels,[c] and the proteins were subsequently transferred onto polyvinylidene fluoride membranes.[d] Nonspecific protein binding was blocked with 5% nonfat dry milk and 1% bovine serum albumin dissolved in Tris-buffered saline solution and Tween 20 (100mM NaCl, 10mM Tris-HCl

[pH, 7.5], and 0.1% Tween-20) for 1 hour, and then, the membranes were incubated with monoclonal mouse anti-human TRX antibodies (clones ADF11 or ADF21) for 1 hour at room temperature (approx 25°C). The final concentration of each antibody used was 0.5 μg/mL. Antigen signals were visualized with horseradish peroxidase–conjugated anti-mouse IgG[e] followed by an enhanced chemiluminescence substrate[f] according to the manufacturer's protocol.

Dogs

Ten healthy female Beagles[g] were used in the study. The mean ± SEM body weight and age of the dogs were 10.33 ± 0.41 kg and 12.4 ± 1.1 months, respectively. Dogs were considered healthy after general clinical observation and blood analyses. Food was withheld from the dogs for 12 hours and water was withheld for 6 hours prior to the experiment. This study was performed after approval from the Experimental Animal Committee of Nippon Veterinary and Life Science University, Tokyo. All animal experiments were performed within National Institutes of Health guidelines for the care and use of laboratory animals.

Anesthesia and induction of oxidative stress by inhalation of 100% oxygen

The 10 dogs were randomly assigned to receive inhalation of 21% O_2 (control group; n = 5) or 100% O_2 (experimental group; 5) during isoflurane-induced anesthesia. Regulated respiration management was performed by positive pressure ventilation with an artificial respirator.[h]

Each dog was administered droperidol (0.25 mg/kg, SC) for sedation and then received propofol (7 mg/kg) by injection into a cephalic vein via a venous catheter.[i] Induction of anesthesia was designated as 0 hours. An endotracheal tube was inserted when the dog was sufficiently anesthetized. For the maintenance of anesthesia, each dog inhaled isoflurane (2%) with the predetermined oxygen concentration for 3 hours. The flow of O_2 was set at 2 L/min. During anesthesia, lactated Ringer's solution (10 mL/kg/h) was administered into a cephalic vein via the venous catheter. In the artificial respirator, the rate of air circulation was set to 8 times/min with an airway pressure of 1.2 kPa.

Blood and urine sample collection

For each dog in each group, blood and urine samples were collected before sedation (baseline) and at 6, 12, 24, and 48 hours after induction of anesthesia (ie, 3, 9, 21, and 45 hours after cessation of inhalation anesthesia). At each sample collection, 5 mL of blood was collected from a jugular vein to minimize the possibility of hemolysis. The blood sample was centrifuged at 3,000 X g for 15 minutes, and the plasma was collected aseptically and stored at −80°C until required for analysis. Urine samples were collected with a 10-mL disposable syringe and a catheter[j] previously inserted in the urinary bladder. The urinary bladder was empty after the urine sample was collected; any urine

volume in excess of 10 mL was discarded. After collection, each urine sample was centrifuged at 3,000 X g for 5 minutes; the supernatant was collected aseptically and stored at $-80°C$ until required for analysis.

Assessment of plasma TRX-1 concentration

Plasma TRX-1 concentrations were measured with monoclonal mouse anti-human TRX antibodies (ADF11 and ADF21) in a sandwich ELISA.[8,9,k] Absorption at a wavelength of 450 nm was measured with an ELISA reader.[l] Each sample was analyzed in duplicate; the mean value was calculated for data analysis.

Assessment of urine TRX-1-to-creatinine concentration ratio

Urine creatinine concentration was assessed by an enzymatic method with a clinical biochemistry analyzer.[m] Urine TRX-1 concentrations were measured with monoclonal mouse anti-human TRX antibodies (ADF11 and ADF21) in a sandwich ELISA.[8,9,k] Absorption at a wavelength of 450 nm was measured with an ELISA reader.[l] Each sample was analyzed in duplicate; the mean value was calculated for data analysis. To normalize for urine volume, the urine TRX-1-to-creatinine concentration ratio (ng/mg) was determined.

Assessment of urine 8-OHdG-to-creatinine concentration ratio

Urine 8-OHdG concentrations were measured in samples by immunoabsorption chromatography of ICR-001,[m] an oxidative stress marker in urine.[16] Each sample was analyzed in duplicate; the mean value was calculated for data analysis. To normalize for urine volume, the urine 8-OHdG-to-creatinine concentration ratio (ng/mg) was determined.

Statistical analysis

All results are reported as mean ± SD. For each time point, the value for the experimental group was compared with that for the control group. Differences between groups were analyzed by use of a Student t test. To compare obtained data with the baseline data within each group, an ANOVA followed by a Tukey honestly significant difference post hoc test was used. Values of $P \leq 0.05$ were considered significantly different.

Results

Detection of canine TRX protein with monoclonal mouse anti-human TRX antibodies (ADF11 and ADF21)

To confirm cross-reactivity between human and canine TRX, we determined whether monoclonal mouse anti-human TRX antibodies (ADF11 and ADF21) could recognize TRX from Madin-Darby canine kidney cells, a canine cell line, by western blotting. Both the ADF11 and ADF21 antibodies recognized canine TRX protein (12 kDa) from these cells,

demonstrating those antibodies were cross-reactive (data not shown).

Plasma TRX-1 concentration

Plasma TRX-1 concentration was determined at each time point for all dogs in each group (Figure 1). Baseline values in the experimental and control groups were similar. There were no significant changes from baseline in plasma TRX-1 concentration in the control group during the experiment. By contrast, plasma TRX-1 concentrations were significantly higher in the experimental group at 6 and 12 hours ($P = 0.023$ and $P = 0.043$, respectively) after induction of anesthesia; at 12 hours, the value was significantly greater than baseline. At 24 and 48 hours, plasma TRX-1 concentration was decreased, compared with the value at 12 hours; however, plasma TRX-1 concentrations remained high, compared with the control group, at those later time points although the differences were not significant.

Urine TRX-1-to-creatinine concentration ratio

Urine TRX-1-to-creatinine concentration ratio was determined at each time point for all dogs in each group (Figure 2). Baseline values in the experimental and control groups were similar. Although no sig-
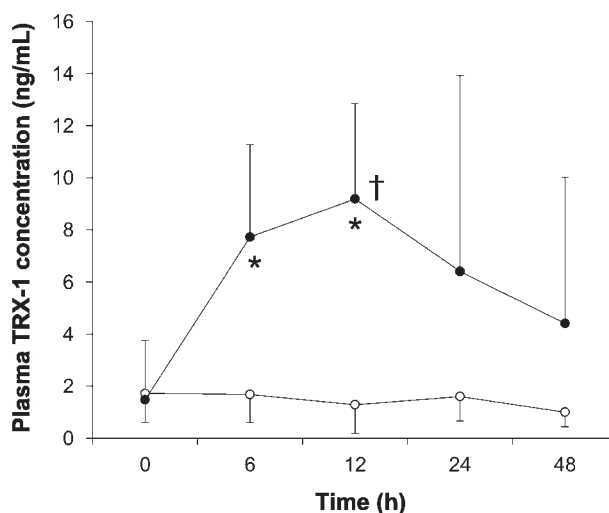

Figure 1—Mean ± SD plasma TRX-1 concentration in dogs anesthetized via inhalation of isoflurane in 21% O_2 (control conditions; white circles [n = 5]) or 100% O_2 (hyperoxic conditions; black circles [5]) for 3 hours. Induction of anesthesia was designated as 0 hours. Blood samples were collected before sedation (baseline) and at 6, 12, 24, and 48 hours after induction of anesthesia (ie, 3, 9, 21, and 45 hours after cessation of inhalation anesthesia). Regulated respiration management was performed by positive pressure ventilation with an artificial respirator; the rate of air circulation was set to 8 times/min with an airway pressure of 1.2 kPa. The flow of O_2 was set at 2 L/min. Plasma TRX-1 concentration was assessed by use of monoclonal mouse anti-human TRX antibodies (clones ADF11 or ADF21) in a sandwich ELISA. *At this time point, the experimental group value is significantly ($P < 0.05$; paired t test) different from the control group value. †At this time point within a group, the value is significantly ($P < 0.05$; Tukey honestly significant difference post hoc test) different from the baseline.
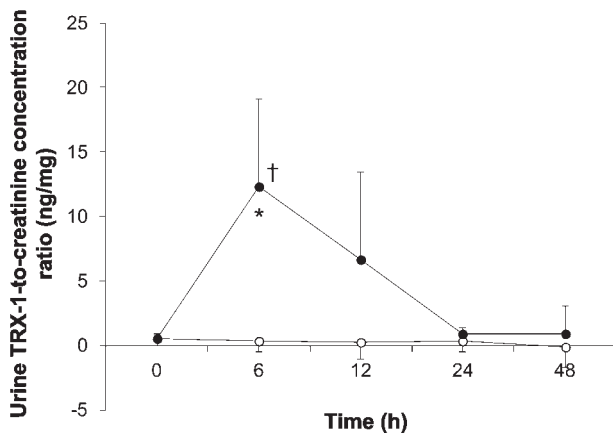
Figure 2—Mean ± SD urine TRX-1-to-creatinine concentration ratio in dogs anesthetized via inhalation of isoflurane in 21% O_2 (control conditions; white circles [n = 5]) or 100% O_2 (hyperoxic conditions; black circles [5]) for 3 hours. Induction of anesthesia was designated as 0 hours. Urine samples were collected before sedation (baseline) and at 6, 12, 24, and 48 hours after induction of anesthesia (ie, 3, 9, 21, and 45 hours after cessation of inhalation anesthesia). Urine TRX-1 concentration was assessed by use of monoclonal mouse anti-human TRX antibodies (clones ADF11 or ADF21) in a sandwich ELISA. Urine creatinine concentration was assessed by an enzymatic method with a clinical biochemistry analyzer. *See* Figure 1 for remainder of key.

Figure 3—Mean ± SD urine 8-OHdG-to-creatinine concentration ratio in dogs anesthetized via inhalation of isoflurane in 21% O_2 (control conditions; white circles [n = 5]) or 100% O_2 (hyperoxic conditions; black circles [5]) for 3 hours. Induction of anesthesia was designated as 0 hours. Urine samples were collected before sedation (baseline) and at 6, 12, 24, and 48 hours after induction of anesthesia (ie, 3, 9, 21, and 45 hours after cessation of inhalation anesthesia). Urine 8-OHdG concentration was assessed by immunoabsorption chromatography of ICR-100. Urine creatinine concentration was assessed by an enzymatic method with a clinical biochemistry analyzer. *See* Figure 1 for remainder of key.

nificant changes from baseline were observed in the control group, an increase in urine TRX-1-to-creatinine concentration ratio was evident in the experimental group. At 6 hours after induction of anesthesia, urine TRX-1-to-creatinine concentration ratio in the experimental group was increased significantly from baseline (P = 0.003) and significantly greater than the value in the control group (P = 0.023). At 12 hours, urine TRX-1-to-creatinine concentration ratio in the experimental group had decreased; although it remained higher than baseline and the value in the control group, the differences were not significant. After 24 hours, the urine TRX-1-to-creatinine concentration ratio in the experimental group reached baseline concentrations.

Urine 8-OHdG-to-creatinine concentration ratio

Urine 8-OHdG-to-creatinine concentration ratio was determined at each time point for all dogs in each group **(Figure 3)**. Baseline values in the experimental and control groups were similar. In contrast to the control group, which did not have any significant changes from baseline, the urine 8-OHdG-to-creatinine concentration ratio in the experimental group was significantly greater at 24 (P = 0.039) and 48 (P = 0.022) hours after induction of anesthesia. At 48 hours in the experimental group, the urine 8-OHdG-to-creatinine concentration ratio was significantly (P = 0.005) greater than baseline.

Discussion

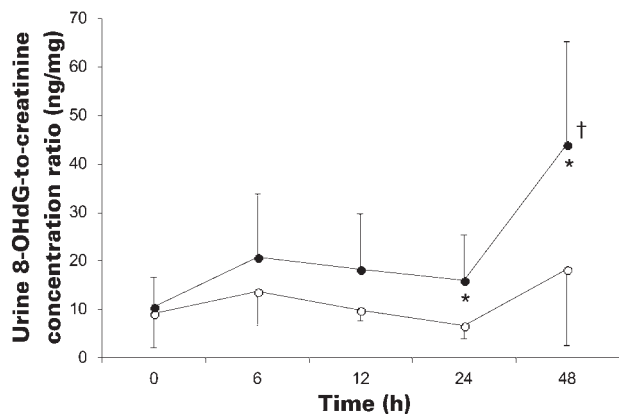Thioredoxin is an oxidative stress marker in humans.[17,18] We hypothesized that exposure of dogs to hyperoxic conditions via inhalation of 100% O_2 during anesthesia for 3 hours would induce oxidative stress and that subsequent increases of TRX-1 concentration in urine and plasma samples could be measured by an ELISA with monoclonal mouse anti-human TRX antibodies that were cross-reactive with canine TRX. In the present study, monoclonal mouse anti-human TRX antibodies (ADF11 and ADF21) were found to be highly immunologically cross-reactive with canine TRX. Therefore, these antibodies were used to measure canine plasma and urine TRX-1 concentrations by use of a sandwich ELISA. It was observed that TRX-1 was induced in dogs under in vivo conditions of oxidative stress and could be measured in plasma and urine up to 48 hours after hyperoxia induction.

The *N*-terminal regions in human and canine TRX proteins are conserved according to protein analysis data in the Ensembl database. Western blot analysis revealed that canine TRX protein (predicted molecular weight of 12 kDa [ie, TRX-1]) was recognized by monoclonal antibodies (clones ADF21 and ADF11) against human TRX. Both antibodies were used for human TRX ELISA systems[15] in the present study.

Reactive oxygen species cause oxidative damage to lipids, proteins, and nucleic acids in vivo. In humans, 8-OHdG is a major form of DNA damage induced by ROS[19] and has received increasing attention as an oxidative stress marker in recent years.[20] In the present study, there was minimal (and nonsignificant) change in urine 8-OHdG-to-creatinine concentration ratio from baseline over time under control conditions (inhalation of 21% O_2). By contrast, urine 8-OHdG-to-creatinine concentration ratio changed over time in

dogs exposed to hyperoxic conditions (inhalation of 100% O_2) and reached a maximum (significant increase from baseline) at the 48-hour time point. These results indicated that inhalation of pure oxygen for 3 hours during isoflurane anesthesia was sufficient to induce oxidative stress in the study dogs. Similarly, inhalation of pure oxygen for 3 hours during isoflurane anesthesia also significantly increased urine TRX-1-to-creatinine concentration ratio at the 6-hour time point, compared with findings for the control dogs. These findings suggested that hyperoxia can alter plasma and urine TRX-1 concentrations in dogs as it can in humans.

Thioredoxin can be induced by several causes of oxidative stress in humans.[4] Thioredoxin eliminates single oxygen or hydroxyl radicals by removing intracellular ROS cooperatively with peroxiredoxin.[21] Therefore, it is likely that rapid increases in plasma and urine TRX-1 concentrations have a protective role against oxidative stress following 100% O_2 inhalation in dogs. In the present study, plasma TRX-1 concentration and urine TRX-1-to-creatinine concentration ratio increased in the dogs exposed to hyperoxic conditions and reached a maximum at 12 and 6 hours, respectively. Blood and urine samples were collected before sedation and anesthesia and at 6, 12, 24, and 48 hours after induction of anesthesia (ie, 3, 9, 21, and 45 hours after cessation of inhalation anesthesia). In preliminary experiments, plasma and urine TRX-1 concentrations were higher than baseline for a 48-hour period, at which point concentrations decreased. In the present study, plasma TRX-1 concentration and urine TRX-1-to-creatinine concentration ratio were not significantly different from baseline after attaining their maximum values but plasma TRX-1 concentration decreased slowly, whereas urine TRX-1-to-creatinine concentration ratio decreased much more rapidly. These findings suggested that the elevation and elimination of the urine TRX-1 concentration were more rapid than those of the plasma TRX-1 concentration. A slower elimination of TRX-1 from plasma may occur because TRX-1 is produced in blood in response to oxidative stress.

Plasma TRX-1 is likely eliminated by primitive urine, which is then reabsorbed in the kidney tubules. This process was identified in mice undergoing renal ischemia and reperfusion, in which IV injections of TRX were filtered in the glomeruli and then reabsorbed in the tubules of the kidneys.[7] That study[7] also revealed that medullary thick ascending limb–specific retention of TRX might be protective against renal ischemia and reperfusion injury. Furthermore, TRX tubular reabsorption likely decreased in those mice because of oxidative stress–induced renal tubular injury.[7] The increased elimination of TRX in urine through direct secretion or decreased reabsorption in the kidney tubules during the early stages of hyperoxia-induced oxidative stress might explain why the peak of urine TRX-1-to-creatinine concentration ratio occurred earlier than the peak of plasma TRX-1 concentration in the dogs exposed to hyperoxic conditions in the present study. Results of the pres-

ent study indicated that urine 8-OHdG-to-creatinine concentration ratio reached a maximum at 48 hours after hyperoxia induction, much later than the peak of urine TRX-1-to-creatinine concentration ratio. This was likely because 8-OHdG is eliminated after ROS damages DNA. Therefore, the peak of urine 8-OHdG-to-creatinine concentration ratio was shifted later than that of urine TRX-1-to-creatinine concentration ratio because the process from DNA damage to 8-OHdG generation and elimination may take longer, compared with urine TRX formation.

A previous study[22] on the clinical course of hyperoxia and TRX-1 concentrations in the brains of 6-day-old rats revealed that exposure to 80% O_2 for up to 48 hours induced significant upregulation of TRX-1 expression. Results of another study[23] indicated that lung TRX-1 mRNA expression at days 1, 6, and 10 in newborn baboons receiving 100% O_2 since birth were increased, compared with findings in controls breathing room air. However, other newborns receiving a lower percentage of O_2 since birth had increased TRX concentrations at days 1 and 6 but not at day 10, compared with findings in controls breathing room air, indicating a dose response effect of hyperoxia in the lungs of newborn primates.

In humans, there is no significant difference in urinary excretion of 8-OHdG between persons who exercise regularly and those with a sedentary lifestyle.[24] However, other studies[25] detected significant increases in urine 8-OHdG concentration at 1, 2, 4, and 6 hours after exercise. Furthermore, the urinary excretion of 8-OHdG varied with age and sex.[26] Because of these differences in the excretion of 8-OHdG, its reliability as a biomarker for oxidative stress is unclear.

Thioredoxin is rapidly detectable in the plasma and urine because it is released by cells after oxidative stress–induced damage to the lipid membranes.[27] Results of the present study indicated that the induction in response to oxidative stress of TRX-1 is more rapid, compared with that of 8-OHdG, in dogs. Therefore, TRX-1 may be more useful than 8-OHdG as a biomarker for the rapid diagnosis of disorders associated with oxidative stress.

An objective of the present study was to evaluate plasma and urine TRX-1 concentrations in dogs by use of an ELISA with cross-reactive monoclonal mouse anti-human TRX antibodies to evaluate the test's clinical diagnostic application in veterinary practice. It is well-known that lung tissue TRX is upregulated by hyperoxia. Indeed, it is induced at birth by breathing O_2 and to a greater extent by hyperoxia.[23] A limitation of the present study was the use of plasma and urine samples rather than lung tissue samples for the measurement of TRX-1. However, because the present study investigated the use of cross-reactive monoclonal mouse anti-human TRX antibodies for diagnostic use, we did not wish to cause surgical stress to dogs or euthanize study animals to obtain lung tissue samples. In future studies, correlations among lung tissue, plasma, and urine TRX-1 concentrations should be investi-

gated. In addition, it is important for the clinical application of TRX-1 as a biomarker for oxidative stress to consider possible associations between TRX and other disease markers (eg, vascular epidermal growth factor, cytokines, and apoptotic molecules), which might link TRX-1 with clinical conditions. Future studies should determine the contribution of disease-associated molecules on plasma and urine TRX-1 concentrations in dogs.

Results of the present study indicated that monoclonal mouse anti-human TRX antibodies are highly immunologically cross-reactive with canine TRX. This is important because the human TRX ELISA kit can also be used for canine TRX measurement. We observed that canine TRX-1 was rapidly induced by oxidative stress, suggesting TRX might have a protective role against oxidative stress in dogs, as it does in humans. The present study used inhalation of 100% O_2 to induce hyperoxia in dogs. Although metabolic conditions other than hyperoxia, such as disease, can induce oxidative stress, these potential conditions were not investigated in this study. Nevertheless, findings of the present study indicated that the measurement of plasma concentration of TRX-1 and urine TRX-1-to-creatinine concentration ratio might be a useful as a biostress marker for diagnosing disorders associated with oxidative stress in dogs. Comparisons of plasma and urine TRX-1 concentrations in healthy dogs and those with metabolic disease are warranted. Future studies should develop reliable and affordable techniques for measurement of plasma and urine TRX-1 concentrations that can be used in dogs in veterinary practice.

Acknowledgments

Footnotes

a. Madin-Darby Canine Kidney cells, ATCC, Manassas, Va.
b. Protease inhibitor cocktail, Complete Mini, Roche Applied Science, Tokyo, Japan.
c. 15% to 20% Tris-HCl gels, Bio Craft, Tokyo, Japan.
d. Immobilon-P membranes, Millipore, Tokyo, Japan.
e   Horseradish peroxidase-conjugated anti-mouse IgG, Promega, Tokyo, Japan.
f. Enhanced chemiluminescence substrate, SuperSignal West Femto Maximum Sensitivity Substrate, Thermo Scientific, Tokyo, Japan.
g. Beagles, Kitayama Labs, Nagano, Japan.
h. Artificial respirator, Kimura Ika Kougyou, Tokyo, Japan.
i. Venous catheter, Terumo, Tokyo, Japan.
j. 6F Catheter, Atom Medical, Tokyo, Japan.
k. Sandwich TRX ELISA, Redox Bioscience Corp, Kyoto, Japan.
l. ELISA reader, BioRad Laboratories, Calif.
m. Hitachi 7180, Hitachi, Tokyo, Japan.
n. ICR-001, Techno Medica, Yokohama, Japan.

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