Concentrations of 15F$_{2t}$ isoprostane in urine of dogs with intervertebral disk disease

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Objective—To measure 15F$_{2t}$ isoprostane concentrations in the urine of dogs undergoing ovariohysterectomy (OHE) and dogs undergoing surgery because of intervertebral disk disease (IVDD) and to assess relationships between urinary concentrations of 15F$_{2t}$ isoprostanes and neurologic score in dogs with IVDD.

Animals—11 dogs undergoing OHE and 32 dogs with IVDD undergoing hemilaminectomy.

Procedures—Paired urine samples were obtained at induction of anesthesia and approximately 1 hour after OHE (controls) and were collected from dogs with IVDD at induction of anesthesia (28 samples) and approximately 1 hour after hemilaminectomy (31 samples); 26 paired urine samples were obtained from dogs with IVDD. Urinary isoprostane concentrations were measured by use of a commercial ELISA, and results were adjusted on the basis of urinary creatinine concentrations. Differences in the mean isoprostane-to-creatinine ratio were analyzed. Neurologic score was determined in dogs with IVDD by use of the modified Frankel scoring system.

Results—Urinary isoprostane-to-creatinine ratios were significantly higher in dogs with IVDD than in control dogs before and after surgery. There was no significant difference between values before and after surgery for either group. There was a significant correlation of neurologic score and urinary isoprostane-to-creatinine ratio because dogs that had higher neurologic scores (ie, less severely affected) generally had higher isoprostane-to-creatinine ratios.

Conclusions and Clinical Relevance—Urinary isoprostane-to-creatinine ratios were higher in dogs with IVDD before and after surgery. Analysis of these data suggests that dogs with IVDD are in a state of oxidative stress and that preemptive treatment with antioxidants warrants further investigation. (Am J Vet Res 2006;67:1226–1231)

Abbreviations

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<tr>
<th>Term</th>
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<tr>
<td>IVDD</td>
<td>Intervertebral disk disease</td>
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<td>ROS</td>
<td>Reactive oxygen species</td>
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<td>OHE</td>
<td>Ovariohysterectomy</td>
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Intervertebral disk disease is characterized by intervertebral disk degeneration and protrusion or extrusion of disk material into the vertebral canal. It is the most common cause of paresis and paralysis in dogs.\(^1\) It has been estimated\(^1\) that IVDD is the reason for admission for 2% of dogs admitted to veterinary teaching hospitals. Damage to the spinal cord is dependent on the duration and location of the lesion, volume of the extruded disk material, dynamic considerations (eg, peracute massive extrusion vs chronic progressive protrusion), and secondary injury. Biochemical and vascular events that start at the time of injury and peak 4 days later result in local hypoxia, demyelination, axonal degeneration, and malacia.\(^1\) Severe injury or delayed surgical intervention may result in permanent neurologic deficits and, in many cases, result in the owner’s decision to euthanize a paralyzed dog. Although clinicians can do little to control the severity of the primary insult to the spinal cord, treatments aimed at minimizing secondary injury may improve outcome.\(^4\)

Neuronal intracellular concentrations of ATP are reduced during severe ischemia resulting from spinal cord injury, which leads to release of the excitatory neurotransmitter glutamate.\(^5\) Glutamate initiates a pathogenic cascade that results in cellular overload of calcium and increased production of ROS.\(^5\) In addition, an inflammatory response is initiated, with recruitment of neutrophils and macrophages and upregulation of cellular adhesion molecules.\(^6\) This inflammatory response is more pronounced in the spinal cord than in the brain during CNS injury in mice.\(^5\) Recruitment of inflammatory cells leads to the release of inflammatory mediators, including ROS. Release of ROS can lead to a number of secondary effects, which includes recruitment of additional inflammatory cells and thus perpetuates the cycle of ROS-mediated tissue damage. The ROS play a critical role in pathogenesis of several processes, including cardiac disease, ischemia-reperfusion injury, and neurologic disease.\(^7\)

Isoprostanes are considered an accurate marker of ROS activity in vivo in humans and several other animal species. Urinary or plasma concentration of isoprostanes correlate with disease severity in humans with asthma, heart failure, and pulmonary disease.\(^8,9\) Isoprostanes are produced when ROS react with arachidonic acid in cell membranes. Formation of iso-
Materials and Methods

Animals—Two groups of client-owned dogs were used in this study. Eleven healthy client-owned dogs undergoing OHE served as a control group, and there were 32 client-owned dogs with newly diagnosed IVDD. Inclusion criteria for dogs with IVDD included an intervertebral disk rupture in the region of T3 to L3 that required surgical decompression; was characterized by a decrease in neurologic score (increased severity of neurologic deficits), occurred within the preceding 7 days before our initial examination, and was accompanied by a lack of other documented inflammatory disease (eg, aspiration pneumonia). Exclusion criteria included extradural compressive lesions caused by something other than IVDD (eg, tumors, fractures, or abscesses), intradural or intramedullary lesions, or chronic neurologic disease with little change during the preceding 7 days. The study was conducted in a manner consistent with principles established by the US National Institutes of Health and the Animal Welfare Act.

Neurologic assessment—For each dog with IVDD, a neurologic score was assigned at the time of admission. Neurologic scores were determined on the basis of the modified Frankel scoring system and assigned on a scale of 0 to 2 (0, no deep pain sensation evident; 1, deep pain evident but no motor function; and 2, deep pain and motor function are evident). Sample collection—In all dogs, urine samples were obtained by use of midstream catch or aseptic catheterization of the bladder. Expression of urine from the bladder, as is clinically indicated and in accordance with the standard of care for our facility, was used on dogs before surgery. When necessary to obtain a urine sample, an indwelling urinary catheter was aseptically inserted into the bladder, and urine was collected from the closed urinary collection system by use of aseptic techniques. Urine samples were frozen immediately and stored frozen at −80°C until analyzed.

Samples were obtained from all dogs before surgery for use in urinalysis and after surgery for use in assessing hydration status. A urine sample was obtained from each of the 11 healthy dogs undergoing OHE before induction of anesthesia and again approximately 1 hour after surgery. Similarly, a urine sample was obtained at induction of anesthesia from 28 dogs with IVDD and approximately 1 hour after hemilaminectomy from 31 dogs with IVDD.

Analytic methods—Urinary concentrations of isoprostane were determined by use of a commercially available competitive enzyme immunoassay kit. Samples were analyzed in duplicate and in batches to reduce interassay variation. This assay has undergone extensive analytic validation by the manufacturers, as indicated in results provided in the package insert. Because 15F₂ isoprostane is not a species-specific compound, our in-house validation of this assay was intended to confirm the optimal use of this assay for urine samples obtained from dogs. In-house validation was performed with urine samples previously collected from healthy dogs as part of an unrelated project. Our validation of the assay included assessment of dilutional parallelism of canine urine samples (5 samples at 3 dilutions in which we determined the correct dilution for samples such that they would be within the working range of the assay), assessment of the correlation between direct analysis of canine urine samples and analysis after affinity purification of isoprostane (by use of commercially available isoprostane affinity purification columns) from canine urine samples (9 samples in which we assessed evidence of cross-reactive substances in canine urine), and determination of interassay variability (5 samples assayed on 5 separate days). Urinary creatinine concentrations were measured in all samples by personnel at another laboratory by use of an automated dry chemistry system. To account for variation in glomerular filtration rate and urinary concentrating capacity among dogs, urinary isoprostane concentrations were adjusted on the basis of the urinary creatinine concentration, and the isoprostane-to-creatinine ratio was calculated.

Statistical analysis—All statistical analyses were performed by use of a commercial software package. Mean ± SE of untransformed data was reported. Transformed data were analyzed for an effect of group (IVDD or control group) and time (before or after surgery) by use of a mixed linear model that accounted for the random variance of dog nested within each group and repeated measurements on each dog. When the group-by-time interaction was significant, predetermined comparisons were made within and between groups by use of least squares means with a Scheffé adjustment to maintain the type I error rate at 0.05.

The association between neurologic score and isoprostane-to-creatinine ratio was explored by use of Cochran-Mantel-Haenszel methods, with stratification and control for time (before or after surgery). The isoprostane-to-creatinine ratio was categorized on the basis of obvious breaks in the distribution observed on scatterplots and through quartile analysis. Exploration was performed by use of categorization of the isoprostane-to-creatinine ratio (< 10, 10 to < 25, 25 to < 40, and ≥ 40; with a simple dichotomized categorization of < 25 or ≥ 25). Because this was an exploratory investigation, associations were evaluated by use of the χ² statistic and considered significant at a conservative type I error rate of 0.1.

For all statistical analyses, the null hypothesis was that there was no difference between groups. This null hypothesis was rejected and statistical significance assigned for values of P < 0.05.

Results

Animals—Mean ± SE age of the OHE dogs was 2.63 ± 1.26 years. The group comprised 3 Labrador Retrievers, 2 Dachshunds, 1 Boxer, 1 Australian Shepherd, and 2 mixed-breed dogs; breed was not known for 2 dogs. There were 1136 urine samples collected from this group. Age of the dogs with IVDD was not normally distributed. Median age of the 32 dogs with IVDD was 5.0
years. This group comprised 24 Dachshunds, 5 Pekingese, 1 Golden Retriever, 1 Cocker Spaniel, and 1 mixed-breed dog. There were 16 females (13 spayed) and 16 males (11 castrated). Urine samples were collected from 28 dogs before surgery and 31 dogs after surgery. However, there were only 26 paired samples collected from these dogs. For the dogs with IVDD, 6 had an initial neurologic score of 0, 12 had a neurologic score of 1, and 14 had a neurologic score of 2.

**Assay validation**—Mean variation between concentrations determined by direct dilution of urine samples in enzyme immunoassay buffer and concentrations determined after affinity chromatography to remove competing substances from the urine was 43.0% for 9 samples. As per the manufacturer’s instructions for the enzyme immunoassay kit, all subsequent analyses were performed on affinity-purified samples.

Dilutional parallelism evaluations revealed acceptable linearity for 3 dilutions. The optimum dilution for initial analysis of a sample was determined to be 1:10 (urine:buffer). Samples with concentrations outside the linear range of the assay on initial analysis were reanalyzed at higher or lower dilutions (as necessary) to obtain valid results.

Interassay variability was good; mean interassay coefficient of variation for 5 samples analyzed in 5 separate assays was 9.3%. Other investigators who used this kit have reported interassay variability of 7.5% for analysis of exhaled breath condensates.

**Analysis of urine concentrations of isoprostane and creatinine**—The isoprostane-to-creatinine ratio was considered to be a continuous variable. Data were transformed (ie, 1 divided by the isoprostane-to-creatinine ratio) which resulted in a normal distribution (Kolmogorov-Smirnov statistic).

Mean ± SE urinary concentration of 15F_2t isoprostane for OHE dogs was 7.14 ± 0.94 pg/mL before OHE and 7.56 ± 1.18 pg/mL after OHE (Figure 1).

Mean urinary concentration of 15F_2t isoprostane for dogs with IVDD was 23.65 ± 5.87 pg/mL before hemilaminectomy and 30.47 ± 5.41 pg/mL after hemilaminectomy.

We detected a significant effect for the group-by-time interaction. There was not a significant difference between values before and after surgery for either group. However, the isoprostane-to-creatinine ratio for dogs with IVDD differed significantly from that of the control dogs both before (P = 0.002) and after (P = 0.003) surgery.

We detected a significant (P = 0.01) association between neurologic score and isoprostane-to-creatinine ratio. In that analysis, we controlled for the effect of time. The distribution among neurologic scores and isoprostane-to-creatinine ratios was not homogeneous because higher neurologic scores tended to have higher (≥ 25) isoprostane-to-creatinine ratios.

**Discussion**

Analysis of results from dilutional parallelism and interassay variation evaluations revealed that the commercial immunoassay kit used for the study reported here was stable and appropriate for the measurement of 15F_2t isoprostane in canine urine samples. The weak correlation between results obtained after solid-phase purification of 15F_2t isoprostane and values obtained by direct dilution of the urine samples in enzyme immunoassay buffer indicated that there may be substances in canine urine that cross-react with the anti-isoprostane antibodies used in the kit; thus, solid-phase purification of 15F_2t isoprostane from canine urine samples is necessary before analysis with this kit.

Age, sex, and breeds of the dogs with IVDD reported here are similar to those reported elsewhere. In our population of dogs, 15F_2t isoprostane concentrations were higher for the IVDD group, compared with concentrations for the control OHE dogs. There was no significant difference observed in the urinary isoprostane-to-creatinine concentrations before and after surgery for either of the 2 groups.

Analysis of the results of the study reported here suggests that IVDD is associated with substantial oxidative stress, most likely through ischemia of the spinal cord during disk extrusion. Resulting ischemia and inflammation likely contributes to the ROS-associated injury. The half-life of 15F_2t isoprostane reported-ly is 16 minutes in rats, and urinary concentrations of 15F_2t isoprostane peak 20 minutes after IV injection in rabbits. In several studies, isoprostane concentrations increased immediately after reperfusion, peaked at 1 hour after reperfusion, and then decreased to baseline values by 2 to 3 hours after reperfusion.

Dogs with higher neurologic scores (less affected clinically) tended to have higher isoprostane-to-creatinine ratios, whereas dogs with lower neurologic scores (more severely affected) tended to have lower isoprostane-to-creatinine ratios. It is intriguing that the more severely affected dogs had a lower isoprostane-to-creatinine ratio. One possible explanation could be that with greater spinal cord damage, there may be reduced perfusion of the damaged cord and an inability to mobilize isoprostane formed after partial reperfus-
sion. However, many factors may influence the interpretation of neurologic score, including variability among clinicians who perform the evaluation and administration of medications. Any of these factors could have contributed to these results. Additional studies with multiple urine samples, particularly after improvement in neurologic score, may be warranted.

Evidence points to a central role of ROS in the pathophysiologic cascade of secondary damage that results after initial spinal cord injury. In 1 study, investigators detected ROS formation within 4 hours after spinal cord injury and ROS continued to be formed up to 24 hours later. An increase in ROS concentrations has also been detected in the semen of men with spinal cord injury. Furthermore, increases in ROS concentrations can contribute to death of motor neuron cells in mice with experimentally induced spinal cord injuries.

Once formed, ROS may attack nucleic acids, proteins, and phospholipids that are critical to cellular function. Lipid peroxidation, which tends to be a self-propagating event, is one of the most damaging results that ROS has on spinal cord tissue, which results in the spread of deterioration to regions outside of the original zone of trauma and causes extensive tissue damage proximal and distal to the original trauma zone. Products of lipid peroxidation have been detected as early as 1 hour after spinal cord injury, which suggests that lipid peroxidation is one of the earliest biochemical changes after damage to the spinal cord.

The CNS is particularly vulnerable to injury from ROS. Although the CNS comprises only 2% of the body mass, it uses 20% of the available oxygen, and high oxygen consumption correlates positively with ROS formation. The CNS also has a relatively low amount of glutathione, a critical component of the endogenous antioxidant defense mechanisms that protect cells from ROS-induced damage, which makes the spinal cord extremely susceptible to unchecked ROS-induced damage resulting from primary trauma. Finally, the CNS contains a high concentration of polyunsaturated fatty acids in the membranes of neurons and oligodendrocytes, which make it exquisitely sensitive to lipid peroxidation.

It has been suggested clinically and in 1 study that improving the defenses of spinal cord cells against lipid peroxidation is a promising strategy for minimizing damage associated with spinal cord injury. Methylprednisolone, the most widely used drug for improving damage associated with spinal cord injury, is administered specifically because of its antioxidant activity. Unfortunately, the dose of methylprednisolone necessary to exert an antioxidant effect exceeds the dose necessary for activation of glucocorticoid receptors by a factor of approximately 1,000; thus, such a dose would contribute to the many adverse effects reported with use of this drug. Bleeding in the gastrointestinal tract, an increase in the number of wound infections, and pneumonia are the most commonly reported adverse effects associated with high doses of methylprednisolone in humans. In veterinary medicine, 36 of 40 (90%) dogs receiving a high dose of methylprednisolone in 1 study developed occult hemorrhage of the gastrointestinal tract, whereas in another study, dogs receiving high doses of methylprednisolone had a significant increase in the incidence of gastrointestinal complications, increased use of gastrointestinal tract protectants, and increased cost for each hospital stay, compared with results for dogs receiving other corticosteroids. Although reports revealed a slight benefit in neurologic score with the use of methylprednisolone, an evidence-based meta-analysis failed to detect benefits of methylprednisolone in people with acute spinal cord injury. The authors are unaware of similar studies in dogs.

Treatment of patients with spinal cord injury with anti-inflammatory doses of corticosteroids in combination with an effective antioxidant may optimize healing while minimizing adverse effects. Unfortunately, a major limitation in the assessment of clinical efficacy of antioxidants is the difficulty of quantifying their effect. Testing for markers of oxidative stress is not standardized and is fraught with inaccuracies.

Results vary depending on the test used, the biological fluid or tissue tested, and the laboratory method used. Because testing is not standardized, results of treatment trials with antioxidants vary dramatically, and it is difficult to discern the role, if any, antioxidants play in treatment.

The F3 isoprostanes are a relatively recently recognized class of free-radical–catalyzed products of the arachidonic acid pathway. They are produced in vivo independent of cyclooxygenase enzymes by free-radical–catalyzed peroxidation of arachidonic acid. These chemically stable products of ROS activity are now used extensively in the quantification of lipid peroxidation in vivo and have a high sensitivity and specificity for assessing oxidative stress in several diseases in humans and laboratory animals. In addition to acting as a marker for oxidant-mediated damage, F3 isoprostanes possess potent vasoconstrictive activity and may be important mediators for the adverse effects of oxidant injury.

The F3 isoprostanes have been studied most extensively. There is disagreement as to the correct nomenclature of the isoprostanes, and 15F2t isoprostane may also be referred to as 8-isoprostaglandin F3t.

Measurement of urinary F3 isoprostanes as a biomarker of lipid peroxidation is now considered to be the criterion-referenced standard for assessment of oxidative damage in vivo. Approximately 90% of F3 isoprostanes are carried in plasma as lipid esters, with the remainder circulating in the free form. Free F3 isoprostanes are formed via oxidation of plasma or tissue lipids, and quantification of the free form is believed to provide an index of total body F3 isoprostane production.

Isoprostanes have been detected in all types of biological fluids and tissue. However, urine may be the ideal biological fluid for measurement of isoprostanes because of the lack of artifactual lipid peroxidation in most species and the noninvasive method of collection.

In pigs with experimentally induced spinal cord ischemia, investigators found that 15F2t isoprostane was significantly increased during ischemia and reper-
fusion, with plasma concentrations increasing almost instantaneously, which was followed by increased urinary concentrations within 60 minutes. An excellent correlation was found between plasma and urinary concentrations of isoprostane in that study. Increased urinary concentrations of isoprostane have also been found in humans with hyperreflexic neurogenic bladder resulting from spinal cord injury. In dogs used to evaluate ischemia-reperfusion injury, $^{13}$F$_2$ isoprostane concentrations were increased significantly after reperfusion, compared with baseline concentrations. In the study reported here, we found a significant increase in ROS-generated isoprostane concentrations in dogs with IVDD unrelated to general anesthesia or surgical trauma (surgical control dogs and dogs with IVDD both were subjected to general anesthesia and major surgical procedures), which suggests that oxidative stress is increased in dogs with spinal cord injury. Additional studies will be needed to determine whether increased urinary isoprostane-to-creatinine ratios affect the degree of function and time to return of function in dogs with IVDD undergoing decompressive surgery. Studies to determine whether oxidative damage to the spinal cord affects neurologic recovery and prognosis following spinal cord surgery are also warranted.

Several factors may have affected the results in the study reported here and prompt us to urge caution in interpretation of the results. This study was designed to detect a difference between control dogs and affected dogs. Therefore, the sample size may have been too small to find a difference between the measurements obtained before and after surgery in dogs with IVDD.

Major limitations of the study reported here include a lack of matching on the basis of age and breed for the OHE and IVDD dogs. Most studies do not support a lack of matching on the basis of age and breed for the samples obtained before and after surgery in dogs with IVDD. In the future, measurement of urinary concentrations of F$_2$ isoprostanes may be used as a noninvasive biomarker to determine the role of ROS in dogs with IVDD. It may also be used to guide dosing regimens and for use in evaluating effectiveness of antioxidants in clinical trials.

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