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Review Of The Pathogenesis And Treatment Of Acute Spinal Cord Injury And Investigation Into The Use Of Urine 3-Hpma As A Novel Biomarker Of Secondary Injury After Acute Spinal Cord Injury In The Dog

Andrea Sangster
Purdue University

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By Andrea Sangster

Entitled REVIEW OF THE PATHOGENESIS AND TREATMENT OF ACUTE SPINAL CORD INJURY AND INVESTIGATION INTO THE USE OF URINE 3-HPMA AS A NOVEL BIOMARKER OF SECONDARY INJURY AFTER ACUTE SPINAL CORD INJURY IN THE DOG

For the degree of Master of Science

Is approved by the final examining committee:

T. Bentley

Riysi Shi

Rebecca A. Packer

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T. Bentley

Approved by Major Professor(s):

Approved by: Laurie A. Jaeger 10/13/2014

Head of the Department Graduate Program Date
REVIEW OF THE PATHOGENESIS AND TREATMENT OF ACUTE SPINAL CORD INJURY AND INVESTIGATION INTO THE USE OF URINE 3-HPMA AS A NOVEL BIOMARKER OF SECONDARY INJURY AFTER ACUTE SPINAL CORD INJURY IN THE DOG

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ABSTRACT

Sangster, Andrea DVM, MS, Purdue University, December 2014. Review of the pathophysiology of acute spinal cord injury and investigation into the use of urine 3-HPMA as a novel biomarker of secondary injury after naturally occurring acute spinal cord injury in the dog. Major Professors: Robin T. Bentley and Riyi Shi.

Acute spinal cord injury (ASCI) has two pathophysiological stages of injury: the primary injury and the secondary injury cascade. Primary injury includes the initial or mechanical insult to the spinal cord. Secondary injury is a cascade of biochemical events that propagates damage of adjacent, healthy tissue increasing the overall volume of spinal cord tissue that is affected. Acrolein is a toxic byproduct of lipid peroxidation produced during secondary injury. A metabolite of acrolein-glutathione adduct found in urine, 3-HPMA, has recently been shown to increase after spinal cord injury in a rat model. The aim of our study was to apply this urine 3-HPMA assay to dogs to indirectly quantify acrolein levels resulting from ASCI due to intervertebral disc herniation (IVDH). Urine was collected from ten client-owned dogs with ASCI upon presentation to PUVTH and analyzed for the acrolein metabolite, 3-HPMA per gram of creatinine. These concentrations were compared with urinary 3-HPMA levels from 10 healthy, control dogs. Mean urine 3-HPMA of ASCI dogs with IVDH (8.52 μmol 3-HPMA/g Cre) was significantly higher than the mean from control dogs (3.28 μmol 3-HPMA/g Cre). Therefore, urine 3-HPMA is higher in dogs after ASCI due to IVDH therefore supporting an important role of lipid peroxidation in canine ASCI. This study supports the use of
urine 3-HPMA in future clinical trials to measure the effect of therapeutic intervention targeted at reducing acrolein concentration after ASCI.
CHAPTER 1: INTRODUCTION

Acute spinal cord injury (ASCI) has debilitating and permanent consequences for companion animals and humans. Intervertebral disc herniation, fracture and luxation are a few of the pathologic mechanisms for spinal cord injury seen most commonly in companion animals. These can lead to severe functional deficits of paralysis, urinary and fecal incontinence and may ultimately result in euthanasia. In order of decreasing frequency, acute intervertebral disc herniation, fibrocartilagenous emboli and trauma are the most common causes for ASCI in dogs.\(^1\) ASCI accounts for the approximately 2% of veterinary patients seen in teaching hospitals across the United States and severe SCI is the cause of death for one in one hundred dogs.\(^2\) These figures are worse for certain breeds because of breed predilection. For example, the lifetime prevalence of intervertebral disc herniation in miniature dachshunds is approximately 20% of the breed population.\(^3\)

Approximately 2.5 million people are living with the devastating and permanent effects of ASCI worldwide with 130,000 new injuries occurring each year.\(^4\) Young adults are especially affected by complete SCI secondary to motor vehicle accidents, recreational sporting accidents or violent attacks. With only limited recovery possible after complete spinal cord injury in people, and a life long cost of care estimate reaching upwards of three million dollars per individual, novel and successful treatment options
for these severe injuries would dramatically benefit many dogs and people as well as our community as a whole.\textsuperscript{5}

Tremendous advances in the understanding of the pathophysiology of ASCI have been made in the last few decades. However, translating that knowledge into effective therapeutic remedies that prevent or reverse lifelong paralysis remains a goal for which researchers and clinicians continue to strive. Novel approaches continue to be attempted but have not yet consistently or irrefutably changed the expected clinical outcome for patients.\textsuperscript{6-9} This may be in part due to the complexity of the pathophysiology of SCI.\textsuperscript{10}

A non-invasive biomarker of acute spinal cord injury would provide researchers and clinicians alike an objective method of measurement to better evaluate the efficacy of treatments that target the biochemical effects of spinal cord injury. The purpose of this thesis is to introduce the pathophysiology of ASCI to readers with an emphasis on oxidative injury and acrolein toxicity, discuss a study performed at Purdue University Veterinary Teaching Hospital that investigated the novel use of a urine assay of a metabolite of acrolein and its use as a biomarker of ASCI in dogs, and present possible treatments that could be used in future experiments to combat secondary injury in dogs.
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CHAPTER 2: LITERATURE REVIEW

Review of the Pathophysiology of Acute Spinal Cord Injury

Within minutes of severe spinal cord trauma, a progressive and self-destructive series of pathological events occurs. Hemorrhagic necrosis leading to eventual cavitation of the central grey matter after contusive injury has been described as early as 7 days but may continue to worsen and form larger cystic cavities by 2 weeks.\textsuperscript{1} Degeneration of white matter at the epicenter as well as adjacent white matter tissue degeneration rostrally and caudally may be seen within one day of injury.\textsuperscript{2,3} An expanding volume of tissue destruction has been shown to continue to occur for days to even months following injury in rats and primates.\textsuperscript{3} In a rodent compressive injury model, 44% of epicenter neurons were lost 1 day following injury, 73% of neurons were lost by 3 days after injury and 81% loss was noted by 30 days post injury.\textsuperscript{4} The author of this study suggested that compressive injury leads to a more protracted timeline and extent of neuronal loss than contusive injury despite both types of forces leading to progressive injury. Fehlings found that the degree of axonal loss and recovery of neurological function after injury are directly related to the severity of the force of injury.\textsuperscript{5} Because astrocytes are more resistant to injury, they remain present to form scar like tissue around the defect while oligodendrocytes are more susceptible to injury and undergo apoptosis. Ultimately what remains after severe spinal cord injury is a large centrally located fluid-filled cyst with
surrounding astrocytosis and thin layer of subpial white matter tracts\textsuperscript{6}, although species variation does exist. An animal’s neurological function with such an injury may be determined by the percentage of white matter tracts left intact. It has been suggested that 10-15\% of the original tissue mass is necessary for functional recovery although this estimate varies somewhat in experimental studies.\textsuperscript{5}

It is now well recognized that two main phases occur after ASCI: primary and secondary injury. Primary injury to the spinal cord is characterized by the mechanical insult of the initial traumatic injury. The most common type of ASCI in dogs and humans is a combination of concussive, contusive and compressive trauma secondary to displacement of some component of the vertebral column causing both immediate injury and persistent compression.\textsuperscript{7,8} Other common forces in primary injury include shearing, laceration, stretching and acceleration-deceleration injuries.\textsuperscript{8,9} Direct trauma to neurons and glia occur as well as vascular compromise. This incites secondary injury, characterized by a cascade of biochemical events that lead to an expanding area of tissue damage beyond the borders of the initial lesion.\textsuperscript{10,11} The secondary injury phase involves further vascular compromise, excitotoxicity, ion dysregulation, reactive oxygen species production, and inflammation. These processes result in apoptosis and necrosis of adjacent neurons and glial cells.

ASCI may also be temporally differentiated into immediate, acute, subacute and chronic processes with secondary injury events predominating the acute phase and beyond.\textsuperscript{11,12} The immediate phase occurs within 2 hours of injury, acute phase has been described between 2 and 48 hours of injury, subacute denotes processes that occur between 48 hours and 14 days of injury and chronic events manifest after 2 weeks of the
initial insult and may continue on for many months.\textsuperscript{12} It is of great importance to consider the pathophysiological timeline of SCI as well as the effective time and duration of application for a given therapeutic intervention when investigating potential drugs that may be translated into clinical trials.\textsuperscript{13} Many experimental studies that have shown benefit with therapeutics have failed to translate those successful outcomes to clinical trials of naturally occurring injury.\textsuperscript{13-15} Although a multitude of variables exist that could account for this loss of efficacy in translation, temporal intervention is one of them. As a result, secondary injury has been the focus of much research. A temporal understanding of the course of secondary injury events will hopefully allow for therapeutics that target the biochemical processes that correspond with the time that patients are actually presented for medical care to be the focus of further investigations.

\textit{Primary Injury}

Primary injury does not usually completely transect all axons in the transverse plane but can lead to variable levels of necrosis of neurons. Based on animal studies, some axons, possibly those that are smaller in diameter and are near the periphery of the cord, are likely to be spared in many injuries.\textsuperscript{16} As a result, therapies aimed at remyelination of spared axons to improve conduction and therefore functional outcome for individual patients is an area of promise.

Intervertebral disc herniation is the most common type of ASCI seen in dogs and leads to the aforementioned concussive, contusive and compressive types of lesion.\textsuperscript{10,17,18} The initial impact of the disc herniation may cause hemorrhage and ischemia as well as a concussive injury to the spinal cord. Subsequent compression of the spinal cord by disc
material causes direct damage to neurons and glia by disrupting cell membranes and causing further vascular disruption/occlusion leading to ischemia. Edema and swelling ensue and perpetuate poor blood flow to the injured tissue. Immediate activation of microglial cells initiates the up-regulation of proinflammatory cytokines TNFα and IL-β that enhance vascular permeability. Extracellular glutamate and other amino acid concentrations increase within minutes of trauma, the degree of which is directly related to the severity of injury.

Current goals of treatment for primary injury include maintaining adequate perfusion to the injured spinal cord tissue as well as surgically relieving any persistent compression and correcting any vertebral instability that could lead to continued trauma. Whether the timing of surgical intervention relative to the injury makes a difference in neurological outcome has long been somewhat controversial. However, findings from a large human meta-analysis supported very early intervention leading to better neurological outcomes. This led to a recent human, multicenter, prospective cohort study known as STASCIS that found surgical decompression prior to 24 hours after SCI was associated with a significantly improved functional outcome.

Beyond early surgical decompression, there are not many effective therapeutic options available to lessen the damage that occurs to the cord during the primary injury phase. Once a patient is presented to the hospital for treatment, the earliest possible therapeutic intervention would likely be implemented during the secondary injury cascade. As a result, secondary injury has been implicated as the more important target of the two phases of injury in research for novel therapeutic interventions.
Secondary Injury

Hemorrhage, edema, and inflammation that begin during primary injury, and continue to proceed during the acute phase of secondary injury. In addition, other processes arise including ion dysregulation, glutamate accumulation resulting in excitotoxicity, lipid peroxidation with acrolein production and reactive oxygen species (ROS) production. Apoptosis of oligodendrocytes results leading to demyelination of axons and conduction failure. Necrosis of neurons and glial cells may also result in an enlarged zone of destruction as well as the propagation of inflammation, as neuronal and glial cell lysis releases pro-inflammatory agents. Astrocytes are also affected but appear more resistant to contusive injury than neurons and oligodendroglia.

Disruption of perfusion to the site of tissue damage is a central theme to primary and secondary injury. It has been shown that loss of perfusion to cord tissue is directly related to the severity of injury and that perfusion worsens with time after injury. Immediately after injury, hemorrhage is detected in the central zone that ultimately leads to an area of neuronal necrosis due to infarction. Not only does direct trauma compromise the microvasculature at this site but progressive edema leads to increased interstitial pressure and reduced perfusion to the spinal cord via small blood vessels.

Other microcirculatory defects that contribute to diminished perfusion include vasospasm, thrombosis, neurogenic shock and loss of autoregulation. Neurogenic shock has been described in experimental rat models of acute spinal cord injury evidenced by decreased cardiac output and systemic hypotension thought to result from reduced sympathetic tone and possibly direct myocardial injury. The central nervous system normally benefits from an autoregulatory system that is able to maintain perfusion to
spinal cord tissue despite variations in systemic blood pressure. Loss of this autoregulation becomes a concern after spinal cord injury, leaving the spinal cord tissue that is already compromised at risk to suffer reduced perfusion due to systemic hypotension. Systemic hypotension in the face of loss of autoregulation would greatly compound the level of tissue ischemia at the site of injury. Worsening ischemia leads to further cytotoxic edema and the cycle continues. Many patients that have experienced traumatic injuries experience systemic hypotension therefore patients with CNS lesions must be treated with this in mind and normotension should be maintained.\textsuperscript{18,25}

In this thesis, the basic components of the secondary injury biochemical cascade will be discussed, with subsequent discussion of oxidative stress and acrolein cytotoxicity as the main focus of the clinical study.

\textit{Cytokines and Cellular Inflammatory Response}

The effects of the inflammatory response on the spinal cord after injury are not yet as well understood or agreed upon as some of the other secondary injury processes. The inflammatory reaction is complex with many of the inflammatory constituents, including microglia, cytokines, non-resident macrophages, lymphocytes and neutrophils having multiple functions. Some of these processes have been interpreted as detrimental due to worsening secondary injury and others have been found to be beneficial for healing by removing cellular debris and promoting regeneration.\textsuperscript{23,27} In addition, the timing of activity and relative importance of these various cell types and compounds differ among species and even among strains within species of rodent models.\textsuperscript{19} Unfortunately, there are substantially fewer data regarding immune responses to naturally
occurring SCI in companion animals and humans compared with rodent models.\textsuperscript{28} As a result, although immune manipulation to improve neurologic functional outcome after injury is a goal of many experimental studies, it is not likely to be the subject of clinical trials in the immediate future. However, as the inflammatory response becomes better elucidated in each species, it may become an important area for intervention.

The complexity and multiplicity of functions of individual components of the immune response make a comprehensive understanding of its effects on the spinal cord after injury daunting. Within the neuroinflammatory response, neutrophils are thought to contribute to tissue damage and rodent studies have shown that blocking neutrophil infiltration lessens the extent of secondary injury.\textsuperscript{29-32}

Microglia are resident CNS macrophages that become activated after injury and are therefore a predominant cell seen in all species after ASCI. Microglia may be destructive to adjacent tissue early in the inflammatory response but may also have a beneficial effect later in the course of inflammation. Both neutrophils and macrophages have the potential to cause further damage to SC tissue through the generation of reactive oxygen species that lead to lipid peroxidation and its toxic byproducts.\textsuperscript{33} In fact, microglial production of reactive oxygen species seemed to be directly related to severity of clinical signs in one study of naturally occurring canine SCI.\textsuperscript{34} One study found that markers for oxidative injury produced by neutrophils and macrophages were increased 1-3 days after human spinal cord injury, suggesting that these inflammatory cells may directly damage adjacent cells via lipid peroxidation.\textsuperscript{35} Microglia can also perpetuate inflammation due to cytokine release. However, they are also thought to promote healing and repair by secreting neurotrophic and axonal growth factors and carrying out
the phagocytic task of cleaning up necrotic cells and debris at the epicenter of the lesion.\textsuperscript{36,37} Removing debris helps to promote healing as it creates a path for axonal budding to occur and removes myelin and myelin inhibitory proteins that can suppress axonal regrowth. In addition, a circular argument is made that removal of lipid membranes from the injury site by phagocytosis may reduce the secondary damage caused by lipid peroxidation.\textsuperscript{33}

The overall effect of lymphocytes in secondary injury remains controversial. Some studies have suggested a beneficial effect and others have supported improved outcome when T-lymphocyte infiltration is suppressed.\textsuperscript{38}

The timing and type of immune response varies among species. Neutrophil infiltration immediately after injury is common to rats, mice and humans however variation in the timing and duration of peak concentration of cells exists between these species. Neutrophil numbers are maximally expressed 1-3 days after injury and remain increased for up to 10 days in humans.\textsuperscript{35} Neutrophil numbers are similarly high for 1 to 2 days in rats following SCI, however in mice, they peak 3-14 days post injury and may remain increased for several weeks.\textsuperscript{29,35,39} Interestingly, in a histopathological study of canine spinal cords after naturally occurring injury, neutrophil infiltration was not appreciated.\textsuperscript{40} However, no immunostaining was performed in that study, which may have detected neutrophils better than morphological evaluation alone. Immunostaining was thought to explain the increased number of neutrophils found in a more recent study compared with two other human studies of SCI that did not use similar techniques.\textsuperscript{35,41,42}

Common to all species discussed is the microglial/macrophage response. Microglial activation occurs immediately after injury and macrophage numbers peak at 5-10 days in
dogs, rodents and humans. Lymphocyte infiltration appears to be a prominent component of the neuroinflammatory response in mice but does not occur as significantly in dogs, rats or humans. T cell infiltration into the injured spinal cord has been shown to peak 3-7 days after injury in rat models but is delayed until 7-14 days in mice. This is in contrast to findings in humans, which seem to exclude a major role by T and B cells after SCI. There are also differences in cell predominance and time of peak numbers of cells after injury within species among strains of mice suggesting that genetic variation may play a role in the neuroinflammatory response. These differences among and within species present an additional challenge when it comes to unraveling and understanding the inflammatory response to SCI and applying therapeutic intervention studied in experimental rodent models to canine and human inflammation.

Pro-inflammatory cytokines including IL-6, IL-8 and possibly TNFα are immediately increased after spinal cord injury in dogs. These cytokines are thought to be produced by microglia. Interleukin-6 promotes the activation of inflammatory macrophages, M1 phenotype. The anti-inflammatory cytokine TGF-β is not upregulated in dogs until three to four days after injury. This is similar to humans who share peak levels of the pro-inflammatory cytokines IL-6 and IL-8 in CSF after injury and a delayed increase in IL-10 and TGF-β. The anti-inflammatory cytokines have been shown to possess neuroprotective qualities as IL-10 knockout mice suffered more severe inflammation and injury compared to control animals after artificially induced ASCI. As a result, a therapeutic intervention aimed at altering the balance of cytokine levels toward the anti-inflammatory cytokines, IL-10 and TGF-β, to peak earlier in the course of the neuroinflammatory response is an interesting consideration.


**Excitotoxicity**

Excitotoxicity refers to neuronal cell death secondary to excessive exposure to glutamate.\(^{6,46}\) Within the central nervous system, glutamate is the main neurotransmitter responsible for synaptic transmission of excitatory impulses between neurons.\(^{46,47}\) After transmission, glutamate is removed from the synaptic cleft and extracellular space via active transport by adjacent astrocytes and neurons.\(^{48}\) This allows for the recycling of glutamate into glutamine and back to glutamate by glial cells and neurons respectively. Glutamate uptake also stimulates glycolysis which results in lactate release from astrocytes that may be used as an energy substrate by neurons.\(^{49}\) Most importantly, glutamate uptake maintains a high intracellular to extracellular concentration gradient, as glutamate is toxic to neurons at high extracellular levels. Maintenance of this gradient is dependent upon energy supply.\(^{47}\) During pathological states such as brain or spinal cord injury and other neurological diseases, this homeostasis is no longer maintained and excitotoxic levels of glutamate may accumulate.

Ischemia, hypoxia and hypoglycemia are conditions that lead to increased release of glutamate or failure of glutamate active transporters and can result in excitotoxic levels of the neurotransmitter in the extracellular space.\(^{48}\) Neurotoxic levels of glutamate accumulate within the extracellular space within minutes of SCI.\(^{6,50}\) Glutamate, administered at quantities consistent with experimental spinal cord injury, has been shown to result in significant neuronal cell death.\(^{50}\) Oligodendrocytes are also vulnerable to excitotoxic levels of glutamate via AMPA receptors.\(^{6,10,47}\) Extensive demyelination is a concerning sequel because a single oligodendrocyte is responsible for myelinating several axons.
Glutamate was found to be significantly increased in the CSF of dogs obtained via lumbar collection following acute and chronic spinal cord injury compared with controls. Additionally in this study, increases in glutamate were directly associated with the severity of clinical signs.\textsuperscript{10} It is interesting to note that increased glutamate concentrations compared to controls were still detected despite injury occurring up to months prior to CSF sampling. Experimental studies suggest that glutamate levels decline to 25\% of their peak values within 30 minutes of injury.\textsuperscript{51} Olby theorized that persistent parenchymal compression perpetuated the presence of increased levels of glutamate in the case of naturally occurring disc herniation. The levels detected in the CSF of dogs following naturally occurring IVDH were much lower (0.9-20.33 μM)\textsuperscript{10} than the extracellular concentrations found at the site of experimental injury (0.53+/−0.14 mM)\textsuperscript{50,51} and those used to mimic excitotoxic concentrations, capable of inducing neuronal cell death. However, depending upon the type of injury, these findings may support excitotoxicity as a potential target of secondary injury worth directing interventional therapy towards in future clinical trials.

\textit{Ion Dysregulation}

Studies have shown alterations in Na\textsuperscript{+}, K\textsuperscript{+} and Ca\textsuperscript{2+} ions in the area of the lesion after experimental SCI.\textsuperscript{6} Not only are ion concentration gradients affected by direct damage to the cellular membrane but Na\textsuperscript{+}/K\textsuperscript{+} ATPase failure may also occur. This may lead to Na\textsuperscript{+} influx and cell swelling or cytotoxic edema that not only results directly in cell death but also tissue swelling that compromises microvascular perfusion of adjacent cells. Calcium ion influx causes cell death via lipid peroxidation and ROS production.
Dysregulation of calcium also causes mitochondrial dysfunction that leads to cell death via second messengers such as cytochrome c resulting in apoptosis.

**Oxidative Stress and Acrolein Cytotoxicity**

Several of the secondary injury mechanisms previously mentioned including calcium influx, excitotoxicity, inflammation and ischemia lead to the production of reactive oxygen species (ROS) that induce cellular damage or oxidative stress. Excessive oxidative injury then begins a chain reaction leading to further ROS accumulation that can overwhelm the endogenous stores of antioxidants in the tissue. These unchecked oxidative reactions such as lipid peroxidation can directly and indirectly cause neuronal and glial cell death.

ROS are free radicals derived from oxygen. Secondary injury phenomena such as calcium influx can lead to the loss of an electron from oxygen, producing superoxide radical (O$_2$•⁻). Superoxide dismutase (SOD) normally squelches this free radical by catalyzing its reaction into H$_2$O$_2$ and O$_2$. In fact, the administration of SOD has been shown to provide some benefit after spinal cord ischemia in dogs and rabbits. The proper storage of iron and other catalyzing metal ions is a key factor in reducing the production of free radicals. If they are not stored and transported properly then they are free to catalyze the reactions that produce free radicals including lipid peroxidation. In the ischemic and therefore more acidic environment of injured tissue, iron is freed from transferrin (iron transport protein) and ferritin (storage protein) to catalyze ROS production reactions. The CNS may be especially sensitive to this phenomenon compared with other tissues because CNS tissue is rich in iron and cerebral spinal fluid is
not able to buffer free iron released after trauma.\textsuperscript{53} Iron reacts with hydrogen peroxide to create hydroxyl radical. Hydroxyl radical reacts with carbohydrates, DNA and membrane phospholipids and disrupts their functions. The reaction of ROS with membrane phospholipids also results in lipid peroxidation or oxidative degeneration of lipids. It is caused by ROS, such as the hydroxyl radical, removing an electron from polyunsaturated fatty acids in the cell membrane and leaving behind a carbon-centered radical. These carbon-centered radicals react with oxygen to form peroxyl radicals. Peroxyl radicals in turn may destroy membrane proteins including receptors and enzymes necessary for cell function or may react with adjacent membrane lipids and continue on to self-propagatate a series of these chain reactions. The cell membrane becomes less fluid and therefore leaky to ions. Not only are the cell membranes directly damaged from lipid peroxidation but lipid peroxidation also produces cytotoxic aldehydes such as acrolein, malondialdehyde, 4-hydroxynonenal (HNE). This propagation stops with the reaction of two peroxyl radicals with one another or with the help of antioxidants such as vitamin E, superoxide dismutase and glutathione reductase.\textsuperscript{11,53,54}

The α,β-unsaturated aldehyde products of lipid peroxidation including acrolein, malondialdehyde, and 4-HNE are toxic to adjacent cells because they create bonds with proteins and amino acids in DNA, altering their structure and cellular function.\textsuperscript{55,56} Acrolein may also be produced from polyamines such as spermine and spermidine, released after RNA injury by hydroxyl radical, making it more abundant than 4-HNE in CNS tissue.\textsuperscript{57,58} Acrolein and 4-HNE have been well documented to be associated with the pathological effects of secondary injury in ASCI in experimental studies.\textsuperscript{59,60} 4-HNE was found within grey matter immediately after contusive injury and found in white
matter tracts later; it was also found at the site of injury immediately but diffused to adjacent spinal cord segments 24 hours later. The author surmised that the cytotoxic aldehyde spread to adjacent tissue in time. Acrolein has also been shown to diffuse to adjacent healthy tissue at pathological concentrations after incubation with injured spinal cord tissue in an *ex vivo* experiment. Luo et al found that acrolein levels increased at the injury site 4 hours after the insult, peaked at 24 hours and remained increased for at least 7 days. Additionally, adjacent segments of cord within 10 mm of the epicenter contained increased acrolein levels starting at 24 hours post injury. Acrolein is estimated to have a longer half-life of 7-10 days than free radicals and is a more volatile aldehyde than 4-HNE having been estimated as having 100 times greater intracellular reactivity toward nucleophiles. In toxicity studies, acrolein has been compared with hydroxyl radical and hydrogen peroxide and was found to be more toxic than both. It readily reacts with glutathione thereby exacerbating oxidative stress once glutathione tissue stores are depleted. Due to its ability to damage tissue that was not compromised by the primary injury, its longer half-life and high reactivity, it stands to reason that scavenging acrolein may be a beneficial therapeutic target of the secondary injury cascade.

Acrolein’s cytotoxicity may be due to its ability to cause ROS production, damage DNA, disrupt the function of cellular proteins and deplete endogenous glutathione stores. Although the specific mechanisms of action that cause acrolein’s toxicity are still being investigated, its cytotoxicity is likely to be partially due to its reactivity with nucleic acid residues within proteins and DNA such as cysteine, lysine, histidine, and arginine therefore altering or disrupting the transcription or function
of proteins. Nakamura recently showed that conjugation of acrolein with glyceraldehyde-3-phosphate dehydrogenase (GAPDH), by binding with a cysteine active site, may be an important mechanism of cytotoxicity. Using mouse mammary carcinoma cells and exposing them to 8μM acrolein, cellular ATP was reduced and the acrolein-conjugated GAPDH moved from the cytoplasm into the nucleus possibly inducing apoptosis. Interestingly, an earlier study using acrolein-bathed PC12 cells showed that the majority of cell death was due to necrosis rather than apoptosis. This toxicity was also concentration dependent with 92% of cells dying after being exposed to 100 μM of acrolein for 24 hours; 58 and 25% cell death was seen after incubation with 10 μM and 1 μM respectively. Pre-incubation with antioxidant therapy significantly reduced ROS production and cell death in this study. The authors similarly demonstrated that antioxidants protected spinal cord neurons from oxidative injury after acrolein administration. One study quantified ROS production secondary to the administration of 25 μM of acrolein to increase by a factor of 1.8 in murine hippocampal cells. Excessive ROS production, as can occur in ASCI, overwhelms the endogenous antioxidant capacity of the CNS resulting in oxidative injury of lipids, proteins and DNA.

Membrane damage may lead to mitochondrial and plasmalemma dysfunction, resulting in cell death. ROS produced during the secondary injury cascade contribute to this dysfunction. This is supported by the protection that anti-oxidant therapy has provided against membrane damage from acrolein. As a result, acrolein’s ability to perpetually produce ROS that damage the integrity of the mitochondrial and cell membranes has been suggested as a mechanism of its cytotoxicity.
mitochondria specifically, an increased ROS production of 50% was shown after exposure to 10 uM of acrolein.\textsuperscript{73} Furthermore, oxidative injury is likely exacerbated because the concentration of mitochondrial endogenous antioxidant, glutathione, is reduced upon exposure to acrolein and its resultant increased ROS production.\textsuperscript{73} Acrolein has been shown to increase the permeability of neuronal cell membranes in a time and dose dependent manner more severely than 4-HNE.\textsuperscript{74} Additionally, acrolein can diffuse to tissue adjacent to the epicenter of a traumatic lesion. Using a guinea pig SCI model, researchers found cell membrane damage 10 mm from the primary injury as well as increased levels of acrolein.\textsuperscript{74,77}

Mitochondria are targets of cytotoxic aldehydes and have been proposed to be an important factor in several neurodegenerative diseases.\textsuperscript{67} The brain and spinal cord have little energy reserve therefore under circumstances of increased energy requirements, cells become more dependent upon the function of mitochondrial respiration. Mitochondrial respiration and energy production are reduced in the presence of acrolein and ROS production with spinal cord mitochondria being more sensitive to the toxic effects of acrolein than brain mitochondria.\textsuperscript{67,73,74,78,79} Several mechanisms of action have been discussed including the ability of acrolein to bind to a cysteine residue within adenine nucleotide translocase (ANT) as ANT activity was significantly inhibited by acrolein in guinea pig brain mitochondria.\textsuperscript{76,78} ANT is thought to be necessary for normal mitochondrial electron transport function. This inhibition of function not only leads to decreased energy production for the cell but also results in even more ROS production that potentiates the cycle of oxidative stress.\textsuperscript{62,73} Another mechanism of action attributed to acrolein is its effect on the mitochondrial membrane potential necessary for normal
function. Acrolein has been shown to reduce mitochondrial membrane potential in a dose-dependent manner. Pretreatment with either glutathione or N-acetylcysteine have been shown to significantly reduce the effects of acrolein on mitochondrial respiration in vitro. Several of these mechanisms of mitochondrial dysfunction ultimately lead to the initiation of apoptosis of the cell.

Acrolein has been shown to cause hepatocyte destruction not only due to direct toxicity of protein adduction but also likely due to its adduction of liver glutathione. This may cause an indirect toxic effect due to the depletion of available antioxidant protection as lipid peroxidation has been described to occur secondary to excessive loss of glutathione in one study. Glutathione is composed of three amino acids including glutamate, glycine, and cysteine. It acts as an antioxidant transporting free radicals out of the cell. Glutathione depletion alone without the influence of acrolein has in fact been proposed to lead to apoptosis. It is well known that glutathione reserves can be severely depleted by increased acrolein levels while concurrently causing further ROS production. It has been shown that pretreatment with a low or sublethal level of acrolein not only upregulates the endogenous production of glutathione but this increased glutathione level becomes protective against subsequent acrolein-mediated injury from an additional high dose of acrolein. Therefore, it is likely that severe glutathione depletion after ASCI plays a key mechanistic role in the cytotoxicity of acrolein.

It has also been suggested that the macromolecular adducts of acrolein may maintain some cytotoxicity. Each acrolein molecule has two reactive electrophilic centers available therefore after addition of an amino acid at one carbonyl center, the other remains available to undergo further addition creating crosslinking of
Higher cellular glutathione levels before administration of acrolein led to reduced protein polymerization via acrolein crosslinking.\(^\text{70}\)

As previously discussed, the toxicity of acrolein appears to be concentration and time dependent and may also depend upon the exposed cell type. Functionally, acrolein has been shown to induce axolemmal injury and reduce axonal conduction in a time and concentration dependent manner.\(^\text{59}\) The level of acrolein administered to cause axonal damage was found to be between 50-200 \(\mu\)M.\(^\text{59}\) It has also been shown to inhibit mitochondrial function, lower cellular ATP and induce neuronal cell death in a concentration dependent manner.\(^\text{75,78,80}\). In cell culture, neuronal membrane damage has been detected after exposure to as little as 1 \(\mu\)M of acrolein with the severity of injury worsening with rising concentrations.\(^\text{74}\) Pulmonary arterial endothelial cell death has been described to occur with the administration of 25-100 \(\mu\)M of acrolein.\(^\text{66}\) In respiratory epithelium, glutathione was able to trap acrolein and prevent cytotoxicity at levels \(\leq \) 3 \(\mu\)M. When the level of acrolein was further increased, glutathione levels decreased and cytotoxicity was detected between 3-10 \(\mu\)M of acrolein. Acrolein levels \(\geq 10 \mu\)M overwhelmed the endogenous protection of glutathione and 80\% of the administered acrolein was recovered unbound to glutathione.\(^\text{86}\) The amount of acrolein found in normal spinal cord tissue versus the amount endogenously produced after ASCI has not yet been determined therefore whether it reaches pathological levels after naturally occurring injury remains to be seen.
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CHAPTER 3: CLINICAL STUDY

Urine 3-HPMA Is Increased in Acute Spinal Cord Injury Due to Intervertebral Disc Herniation in Dogs

1.1 Introduction

Acute spinal cord injury (ASCI) occurs in dogs secondary to intervertebral disc herniation, fibrocartilagenous emboli and less commonly due to spinal fracture or subluxation. Depending upon the severity of the injury, debilitating neurological deficits can persist and may ultimately result in euthanasia. Surgical decompression of intervertebral disc herniation (IVDH) can provide a good to excellent prognosis for functional recovery in many of these patients, except for dogs that suffer from loss of nociception. Functional outcome for patients that present with loss of nociception, the most severe clinical manifestation of ASCI, carries a more guarded prognosis and has been variably reported to be between 25% and 78%.\textsuperscript{1-5}

It has been well established that ASCI has two pathophysiological stages of injury.\textsuperscript{6-14} These include the primary injury and the secondary injury cascade. The primary injury includes the initial or mechanical insult to spinal cord parenchyma and vasculature. If it is due to a compressive lesion that persists, such as in the case of a herniated disc, then the primary injury phase can be prolonged. The secondary injury is a cascade of biochemical events that propagates the damage of adjacent, healthy tissue
increasing the overall volume of spinal cord tissue that is affected. Secondary injury may be more detrimental to the spinal cord parenchyma than the primary injury itself. This cascade includes ischemia, inflammation, ion dysregulation, excitotoxicity, reactive oxygen species production and lipid peroxidation. These processes ultimately lead to tissue necrosis and oligodendrocyte apoptosis.

The effectiveness of medical treatments that could be used to compliment surgical intervention to improve functional outcome after ASCI has been limited to date. However, extensive research into novel therapies to target specific pathways within the injury cascade has been performed over the last few decades and promising results using experimental models have paved the way for clinical trials in veterinary patients with naturally occurring injury.

Various biomarkers of ASCI in dogs have been proposed. Glutamate, a neurotransmitter that accumulates and causes excitotoxicity during the secondary injury cascade, has been shown to be increased in the cerebral spinal fluid of dogs with ASCI after disc herniation and the degree of change appeared to be directly related to the severity of the injury. Oxidative stress has been shown to be an important component of secondary injury in rodent models of ASCI. Urinary isoprostane, which is a biomarker of oxidative stress, was found to be higher in dogs with intervertebral disc disease (IVDD) compared to dogs undergoing ovariohysterectomy. However, further aspects of oxidative stress after ASCI have not been investigated extensively in the dog.

Acrolein is a reactive aldehyde byproduct of lipid peroxidation, an oxidative injury reaction that has been shown to begin soon after injury and continue for approximately 7 days in a mouse model of ASCI. The same study showed that acrolein remained
increased beyond the duration of lipid peroxidation before it decreased back to baseline levels at approximately 14 days after injury.\textsuperscript{6} It is extremely cytotoxic and thought to be a major cause of tissue damage in the secondary injury cascade.\textsuperscript{24-26} In fact, acrolein scavenging or neutralization has been shown to be neuroprotective in rodent models of both ASCI and traumatic brain injury.\textsuperscript{24,25} Because acrolein is a highly volatile substance and forms adducts with cellular compounds, an assay for its direct measurement from urine is not currently available. However, a metabolite of acrolein-glutathione adduct found in urine, 3-hydroxypropylmercapturic acid (3-HPMA), has been previously quantified for use in human toxicology studies and used as an indirect measurement of free acrolein and has recently been shown to increase after spinal cord injury in a rat model.\textsuperscript{18-22,35} The intention of our study was to apply this urine 3-HPMA assay to dogs to indirectly quantify acrolein levels resulting from ASCI. This biomarker could then be used to quantify response to treatment in future clinical trials of novel therapeutic interventions focused on quenching lipid peroxidation or acrolein trapping. If a correlation between biomarkers of acrolein and clinical outcome can be made, then acrolein reduction could become an important target for therapeutic intervention in ASCI. As a result, our goal was to evaluate the use of 3-HPMA as a biomarker of naturally occurring ASCI in dogs. Our hypothesis was that the concentration of 3-HPMA found in urine of dogs with ASCI due to IVDH would be higher than that of control dogs, and would be directly related to the severity of neurological grade. We also hypothesized that the concentration of 3-HPMA would decline over time after spinal cord decompression.
1.2 Materials and Methods

This was a prospective, blinded, controlled study. Urine from ten client-owned dogs with ASCI was collected and analyzed for the acrolein metabolite, 3-HPMA upon presentation to Purdue University Veterinary Teaching Hospital and for three consecutive days. Urine from ten dogs with no evidence of disease was analyzed and used as controls. This study was approved by the Purdue University Animal Care and Use Committee (#1309000935). Informed consent was obtained from all owners before enrollment in the study.

Dogs were eligible for inclusion if they suffered an acute, thoracolumbar spinal cord injury within 72 hours prior to admission and were classified with grade three neurological deficits or higher. The preoperative neurological status was graded using a 5 point scale as follows: grade 1, spinal pain only; grade 2, ambulatory paraparesis; grade 3, non-ambulatory paraparesis; grade 4, paraplegic animals with nociception; and grade 5, paraplegia without nociception. Duration of paraparesis/plegia prior to presentation was classified as < 24 hours, 24-48 hours, and 48-72 hours. Dogs were excluded if they had a history of spinal cord disease greater than 3 days, concurrent illness or recently (< 7 days) sustained a traumatic injury affecting the body outside of the spinal cord.

MRI and/or CT was used to confirm a diagnosis of intervertebral disc herniation with spinal cord compression in all dogs but one. The study design included urine collection via expression or catheterization on day 1 (day of presentation), and days 2, 3 and 4 when possible.

Power analysis indicated that a sample size of 10 dogs per group would be
necessary to detect a difference of 50% between 3-HPMA levels in urine of dogs suffering from ASCI versus urine of control dogs, with an alpha level of significance of 0.05 and power of 90%.

3-HPMA and Creatinine Assay

Three-HPMA was analyzed in urine according to Eckert et al. Solid phase extraction with Isolute ENV+ cartridges (Biotage, Charlotte, NC) was used to prepare each sample before LC/MS/MS analysis. Each cartridge was conditioned with 1mL of methanol, followed by 1mL of water, then 1mL of 0.1% formic acid in water. A volume of 500 µL of urine was spiked with 200 ng of deuterated 3-HPMA (d3-3-HPMA)(Toronto Research Chemicals Inc., New York, Ontario) and mixed with 500 µL of 50 mM ammonium formate and 10 µL of undiluted formic acid. This mixture was immediately loaded onto the prepared ENV+ cartridges. Each cartridge was washed twice with 1 mL of 0.1% formic acid, followed by 1 mL of 10% methanol/90% 0.1% formic acid in water. The cartridges were dried with nitrogen gas and subsequently eluted with three volumes of 600 µL methanol + 2% formic acid. The eluates were combined and dried with a rotary evaporation device. Each sample was then reconstituted in 100 µL of 0.1% formic acid before LC/MS/MS analysis.

An Agilent 1200 Rapid Resolution liquid chromatography (LC) system coupled to an Agilent 6460 series QQQ mass spectrometer (MS) was used to analyze 3-HPMA in each sample. A Waters Atlantis T3 2.1mm x 150mm, 3 µm column was used for LC separation. The buffers were (A) water + 0.1 % formic acid and (B) acetonitrile + 0.1%
formic acid. The linear LC gradient was as follows: time 0 minutes, 0 % B; time 1 minute, 0 % B; time 9 minutes, 95 % B; time 10 minutes, 95 % B; time 11 minutes, 0 % B; time 15 minutes, 0 % B. Retention time of the 3-HPMA/d3-3-HPMA was 6.8 minutes. Multiple reaction monitoring was used for MS analysis. The 3-HPMA data were acquired in negative electrospray ionization (ESI) mode by monitoring the following transitions: 220.1⇒91 with collision energy of 5 V and 220.1⇒89 with collision energy of 15 V. The d3-3-HPMA data were acquired in negative electrospray ionization (ESI) mode by monitoring the following transitions: 223⇒91 with collision energy of 5 V and 223⇒89 with collision energy of 15 V. The jet stream ESI interface had a gas temperature of 325°C, gas flow rate of 8 L/minute, nebulizer pressure of 40 psi, sheath gas temperature of 250°C, sheath gas flow rate of 7 L/minute, capillary voltage of 4000 V, and nozzle voltage of 1000 V. Creatinine measurement was performed using creatinine (urinary) assay kit (Cayman Chemical Co.) according to the accompanied manual. Creatinine measurement was performed in order to correct for variations in urine concentration between samples. Urine samples were frozen at -80 °F immediately after collection. They were stored for up to 2 weeks prior to analysis.

**Statistical Analysis**

Urinary 3-HPMA levels were logarithmically transformed to correct the right-skewness. A Welch t-test was used to compare geometric means of urinary 3-HPMA levels between the affected group and the control group. A paired t-test was used to compare 3-HPMA levels from day 1 and day 2. Additionally, spearman rank correlation
(r₃) was used to investigate if a relationship existed between 3-HPMA levels and neurological grade, body weight, and duration of injury prior to urine sample collection. Mann-Whitney testing was used to compare urinary 3-HPMA of intact versus neutered dogs. Sample size calculations were performed using software available on Hedwig.mgh.harvard.edu. Statistical analysis was performed using software available on MedCalc® (Version 12.6.1.0, MedCalc software, Ostend, Belgium). P < 0.05 was considered significant.
1.3 Results

Of the dogs with ASCI, several breeds were represented, including four dachshunds and one of each of the following breeds: Pug-Beagle mix, Cocker spaniel, Pekingnese, Doberman pincer, Shih tzu and Beagle. Ages ranged from 1-10 years with a mean of 5.5 years. Three neutered males, 1 intact male, 4 spayed females and 2 intact females were included. Urine was collected on day 1 in all cases, and on day 2 (n=5) where possible. The neurological grade at admission was grade 3 (n=3), grade 4 (n=3) or grade 5 (n=4).

The geometric mean concentration of urinary 3-HPMA in ASCI dogs on day 1 of 8.52 μmol 3-HPMA/g Cre (95% confidence interval (CI) 3.76-19.32) was significantly higher than the mean concentration in control dogs of 3.28 μmol 3-HPMA/g Cre (95% CI 2.73-3.94; p = 0.028) (Fig. 1). The mean urine 3-HPMA from ASCI dogs was 2.6 (95% CI 1.1 – 6.0) times higher than controls. No significant difference in concentration was appreciated between geometric means of day 1 (12.36 μmol 3-HPMA/g Cre) and day 2 (7.04 μmol 3-HPMA/g Cre; p = 0.179).

A moderate correlation (r_s = -0.51, 95% CI -0.86 - 0.18) suggesting an inverse relationship between neurological grade and urine 3-HPMA was noted but was not statistically significant (p = 0.135) (Fig. 2). A correlation between the degree of urine 3-HPMA and the duration of time from initial injury to urine collection was not significant (r_s = 0.33, 95% CI -0.38 – 0.8; p = 0.349). Urinary 3-HPMA of intact dogs was significantly higher than neutered dogs (p = 0.017) (Fig. 3).

One patient with a neurological grade of 5 was euthanized prior to imaging and treatment per owner’s request (13.61 μmol 3-HPMA/g Cre at presentation). Seven of the
remaining 9 dogs recovered ambulatory function after surgical decompression. Two dogs failed to recover ambulatory function; both were classified with a neurological grade of 5 at presentation. One of these two dogs developed myelomalacia (initial, 2.75 μmol 3-HPMA/g Cre) and was euthanized soon after surgery. The other dog persistently lacked nociception on recheck exam, 1 year after surgical decompression (initial, 4.20 μmol 3-HPMA/g Cre). The median urine 3-HPMA of the 7 dogs that recovered ambulation post-operatively was 5.80 μmol 3-HPMA/g Cre.

No other body weight, gender or age correlation was found (p > 0.05).
Figure 1. Urine 3-HPMA in ASCI dogs (n=10) is significantly higher than in control dogs (n=10), p<0.03. The top, middle and bottom line of the box correspond to the 75th percentile, the 50th percentile (median) and the 25th percentile respectively. The asterisk indicates an outlier (157.36 μmol 3-HPMA/gram creatinine).
Figure 2. Urine concentrations of 3-HPMA versus neurological grade. Note the slight inverse relationship between severity of neurological grade and severity of urine 3-HPMA concentration. Horizontal line denotes the median and the asterisk corresponds with an outlier (157.36 μmol 3-HPMA/gram creatinine).
Figure 3. Urine 3-HPMA concentrations in intact dogs were significantly higher than neutered dogs, p<0.02. The horizontal line denotes the median and the asterisk corresponds with an outlier (157.36 μmol 3-HPMA/gram creatinine.)
1.4 Discussion

The current study showed a statistically significant difference in urine 3-HPMA from dogs affected with ASCI due to IVDH compared with control dogs, thereby providing evidence for using urine 3-HPMA as a biomarker of ASCI in dogs. This is consistent with the known role of lipid peroxidation in secondary injury. 3-HPMA is the first assay used in dogs to indirectly assess acrolein concentration by measuring an acrolein-glutathione adduct after naturally occurring ASCI. Considering that acrolein is cytotoxic, it is likely that the increased concentrations of acrolein play a clinically relevant role in secondary injury in dogs, and may warrant therapeutic intervention. It is also important to note that a previous study in rats found that urine 3-HPMA values were 2.6 times lower than concurrent spinal cord tissue protein-acrolein values in the same individuals at the same time point. The author theorized that a dilutional effect occurred in the systemic circulation from the area of injury. This would suggest that the concentration of acrolein in the spinal cord tissue is much higher than what the urine samples reflect. Studies in people have established plasma acrolein as a sensitive biomarker for cerebrovascular accidents and studies in animal models of brain and spinal cord injury have shown that sequestering aldehydes with drugs that reduce acrolein concentration in the central nervous system (CNS) is neuroprotective. Establishing a biomarker for acrolein concentration in dogs after ASCI may aid in the development of neuroprotective agents after naturally occurring ASCI.

There was some degree of overlap between the 3-HPMA concentrations from the affected dogs and the control subjects. The purpose of this biomarker is not to establish a
diagnosis of spinal cord injury for dogs. Instead, the goal is to establish this as a companion analytical tool to identify and further characterize ASCI. Furthermore, we intend to utilize urine 3-HPMA in future studies as a measure of acrolein-adduct reduction with treatment using drugs that suppress lipid peroxidation and/or scavenge acrolein. Serial sampling may be required with the baseline being established for each individual as the first sample taken after injury. This acrolein biomarker may be of use in understanding the success or failure of any putative ASCI medical therapy, as well as its mechanism of action.

The concentration of 3-HPMA from exogenous sources has not yet been previously established and exogenous acrolein may partially contribute to the overlap seen between the control and affected groups. Acrolein is not only endogenously produced via lipid peroxidation but is present in the environment as a product of combustion.\textsuperscript{18} It is an air pollutant associated with diesel engine and industrial emissions. Acrolein is present in small amounts in various foods and quite high levels are found in cigarette smoke.\textsuperscript{18-20} It has been well established that the level of 3-HPMA in the urine of smokers is significantly higher than non-smokers.\textsuperscript{19,20} Individual variation of exogenous acrolein is likely due to variable environmental exposure. Data on diet and second hand smoke exposure were not available in this study, but would be warranted in future studies.

An increased urine 3-HPMA concentration was not directly correlated with a more severe neurological grade as was hypothesized. Instead, although an inverse correlation was not statistically significant, it is interesting to note that the highest 3-HPMA concentrations were seen in dogs that presented with the least severe neurological grade. The relatively lower concentrations of 3-HPMA in the present study seen in clinically
more severely affected dogs could be due to glutathione depletion rather than a relatively lesser degree of production of acrolein. In people with late stage Alzheimer’s disease, glutathione transferase activity was decreased in certain areas of the brain but unchanged elsewhere in the brain.\textsuperscript{23} This was postulated to be due to glutathione depletion in pathologic areas of the brain while spared tissue maintained its ability to detoxify.\textsuperscript{31} This may apply to clinically less affected ASCI patients having more functional tissue to produce glutathione to bind to acrolein. Similarly, another study found an inverse correlation with lesion severity of cerebral strokes in people. Urinary 3-HPMA levels were lower in stroke patients than in controls and concentrations were significantly lower if the lesions were ≥ 1cm compared with lesions < 1cm.\textsuperscript{32} It has been suggested that acrolein preferentially reacts with glutathione rather than the lysine residues in proteins and that glutathione plays a vital role in the endogenous detoxification of acrolein.\textsuperscript{28} It has also been shown that plasma acrolein-protein adducts increase after stroke, supporting acrolein production overwhelming the glutathione stores of the CNS so that as glutathione-acrolein adducts decline, protein-acrolein adducts climb.\textsuperscript{33} Concurrent measurement of the plasma protein-acrolein concentrations of the patients in our study would have assisted in determining if this relationship exists after ASCI in dogs.

Another possible cause for the relatively lower levels of urine 3-HPMA in more severely affected dogs in this study is that CNS glutathione may also be depleted by excessive glutamate release during secondary injury. Glutamate accumulates after ASCI and is known to be excitotoxic to adjacent neurons but it can also prevent the uptake of cysteine, which is the rate limiting amino acid necessary for the production of glutathione.\textsuperscript{34} Glutamate concentrations have been shown to increase in the lumbar CSF
of dogs after ASCI in direct relationship to the degree of clinical neurological grade. Further studies into the relationship between the severity of ASCI and urine 3-HPMA levels in dogs and possibly their relationship to glutamate concentrations are warranted.

It was also interesting to note that urine 3-HPMA concentrations from the dogs that did not recover neurological function post-operatively (median 3.48 μmol 3-HPMA/g Cre) were lower than concentrations from dogs that did recover function (median 5.80 μmol 3-HPMA/g Cre). In fact, the lowest level (2.75 umol 3-HPMA/g Cre) was from the only dog that developed myelomalacia post-surgical decompression. This finding poses the question, are dogs with lower inherent glutathione levels at greater risk for more severe sequela to spinal cord injury such as myelomalacia or is the injury and therefore acrolein production so severe that the glutathione level is more greatly depleted, therefore leading to a lower urine 3-HPMA value? This was not statistically analyzed due to the low number of dogs that failed to recover function (n=2), however it may be of value to investigate if the degree of urine 3-HPMA might serve as a prognostic indicator of outcome in future studies. Additionally, measuring whole blood glutathione in future studies of naturally occurring ASCI and comparing these findings with urine 3-HPMA may be beneficial in assessing the degree to which glutathione depletion plays in the ultimate severity of secondary injury.

The urine 3-HPMA of intact dogs (2 females, 1 male) was significantly higher than that of neutered males and females, which was not expected. This finding has not previously been reported. The urine 3-HPMA assay has not previously been evaluated in a sexually altered population as it has only been performed in humans and rat studies using intact specimens. Had our sample size been larger, multivariate analysis could
have been used to investigate whether urine 3-HPMA was most strongly associated with neutering status, or factors such as neurological grade. Future studies are required to confirm or reject an association with neutering status.

Limitations of this pilot study included the inability to analyze data within subgroups, such as degree of urine 3-HPMA versus neurological outcome due to sample size. Similarly, the lack of urine samples following presentation for several consecutive days affected our ability to find a statistical difference in urine 3-HPMA over time after surgical decompression due to inadequate power of analysis. Because our study design selected for dogs with more severe neurological deficits by including grades 3-5 only, we cannot apply the findings to dogs suffering from ASCI with a neurological grade of 1 or 2.
1.5 Conclusions

Urine 3-HPMA, a metabolite of acrolein, is a valid biomarker of ASCI in dogs; associations with factors such as duration and severity of injury and neutering status warrant further investigation. This assay allows for an indirect but quantitative measure of acrolein that can be used in future studies of pharmaceutical interventions that target lipid peroxidation and/or drugs that sequester acrolein. The ability to measure the effect of a targeted treatment in the secondary injury cascade could support a cause and effect relationship and isolate acrolein and its reduction as an effective interventional focus in the ongoing battle to reduce the severity of the lesion and long-term outcome after ASCI in dogs and humans. If a correlation between treatment and urine 3-HPMA is validated, then the assay could be used in an individual to better evaluate treatment and to alter treatment dosages based upon changes in the urine 3-HPMA level.
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CHAPTER 4: INTERVENTIONAL THERAPY

Antioxidant Therapy

As previously discussed, antioxidant therapy has been shown to reduce acrolein induced injury and cell death of neurons demonstrating that oxidative injury has a chief mechanistic role in the toxicity of acrolein.\(^1\)

Recently, rapamycin has been shown to reduce acrolein-induced apoptosis of male germ cells. Rapamycin reduced ROS production, increased the ratio of Bcl2/Bax mRNA and proteins and protected mitochondrial membranes. The authors suggested that one of the mechanisms of action responsible could be the known upregulation of expression of antioxidant genes that occurs with rapamycin therapy.\(^2\) Rapamycin, an mTOR inhibitor, has several other clinical effects in the body complicating its clinical use. It is currently used in human transplant patients as an immunosuppressant and it can also affect insulin sensitivity. Rapamycin is being investigated as a therapy in several other human diseases in canine models including osteosarcoma and glycogen storage disease type IIIa and some pharmacokinetic analyses have been performed.\(^3,4\) Further investigation into its efficacy and safety for use after ASCI may be warranted.

In another study, caffeic acid, a natural, food-derived antioxidant, protected hippocampal neurons from acrolein induced ROS production, glutathione depletion and
cell death. Only limited investigation into caffeic acid has been performed to date therefore further studies are needed.

Vitamin E is an important antioxidant that acts by reacting directly with ROS, donating a hydrogen ion and breaking the cycle of lipid peroxidation. Each \( \alpha \)-tocopherol molecule, located within the plasmalemma, traps two peroxyl radicals. It competes with lipid substrate and binds at a rapid rate making it an effective antioxidant.\(^{6,7}\) It has been well established that vitamin E is necessary to normal neurological function and animals and humans with vitamin E deficiency may develop a distal sensory axonopathy and secondary demyelination;\(^{8,9}\) however, it may take weeks for concentrations within the nervous system to increase with supplementation making its use for ASCI impractical and ineffective.\(^{8}\) It may however be beneficial to consider regular vitamin E supplementation for populations (e.g. chondrodystrophic dogs) who are at greater risk for IVDH.

One of the only studies to address the oxidative stress of secondary injury from ASCI in dogs looked at the administration of N-acetylcysteine and its association with neurological outcome and \( 15F_2 \) isoprostane excretion.\(^{10}\) One IV dose of N-acetylcysteine, a precursor of glutathione, was administered to dogs before surgical decompression to dogs with intervertebral disc extrusion. There was no difference in \( 15F_2 \) isoprostane levels or neurological outcome between treated and control groups.\(^{10}\) The authors described several limitations that could have contributed to the lack of efficacy seen in the study: the dose, distribution into the central nervous system as well as the fact that only one dose of one type of antioxidant was administered. Although the half-life of ROS is relatively short, lipid peroxidation reactions are self-perpetuating once
a certain threshold has been reached and endogenous glutathione stores are depleted. ROS are therefore continually produced and oxidative stress continues to be a concern for up to a week likely secondary to the lingering aldehyde products of lipid peroxidation. Therefore, it is possible that subsequent doses of N-acetylcysteine would have been beneficial. Additionally, a study using mouse mammary carcinoma cells found that N-acetylcysteine fully protected cells against acrolein-mediated toxicity suggesting that the antioxidants benefit may be due to acrolein attenuation rather than removal of hydroxyl radical alone.\textsuperscript{11} The authors went on to cite their unpublished findings of decreased protein-conjugated acrolein and brain infarct lesion size reduction in mice after use of N-acetylcysteine. The distribution of N-acetylcysteine into CNS tissue is not definitively supported. One study showed that it is distributed into many tissues of mice except for the brain and spinal cord;\textsuperscript{12} however, another study in mice showed increased parenchymal concentrations of N-acetylcysteine after intravenous injection that supports its ability to cross the BBB.\textsuperscript{13} Due to the question of CNS distribution, N-acetylcysteine was not chosen as a therapy in our follow up study. However, further investigation into its ability to prevent acrolein-mediated injury along with its concurrent ability to scavenge ROS may be warranted.

Despite success in experimental studies, no antioxidant has yet been shown to change clinical outcome after ASCI in dogs. Interest in high dose methylprednisolone (MP) came from experimental cat models of SCI that supported MP as an inhibitor of lipid peroxidation.\textsuperscript{14} High dose MP has been investigated in several prospective placebo-controlled human trials (National Acute Spinal Cord Injury Study [NASCIS]) for its antioxidant properties and its improved functional outcome if administered within
hours of injury was confirmed. This benefit was dose and time dependent as no improvement was seen if MP was administered after 8 hours. Also, questions regarding the accuracy of the data analysis have been raised therefore the results of the studies remain controversial. Significant neurological improvement using high dose MP has not yet been shown in dogs after ASCI therefore it is less commonly used in veterinary medicine due to the concern that the risk to benefit ratio is too great.

Previous attempts of therapeutic intervention with anti-oxidants after ASCI in rodent models have not significantly improved outcome; however, acrolein scavenging and/or lipid peroxidation suppression may provide a more realistic target for dogs after ASCI due to their timeline of onset and referral. As previously stated, although both lipid peroxidation and acrolein concentration begin to climb within hours of injury, they remain increased for 7 and 14 days respectively. Because most dogs present for medical and surgical treatment within 1-5 days of injury, intervention targeted at reducing lipid peroxidation and this cytotoxic aldehyde could reduce the effects of secondary injury.

Acrolein Scavenging

Due to the well-established cytotoxicity of acrolein as well as the lack of clinical efficacy of ROS scavengers after ASCI thus far, acrolein trapping has become a focus of experimental research to reduce the severity of secondary spinal cord injury. A therapeutic acrolein scavenger that reacts with this toxic carbonyl more quickly than cell macromolecules could spare the function of cell proteins and nucleic acids and allow for the excretion of non-toxic drug-adducted carbonyl. It stands to reason that the
relatively longer half-life of acrolein when compared to ROS would provide a larger window of opportunity for therapeutic intervention to break the cycle of oxidative stress. Furthermore, scavenging acrolein and other aldehydes along with the use of direct antioxidants may provide a complimentary approach to reducing oxidative stress. Phthalazine drugs such as hydralazine and dihydralazine have been proven to scavenge acrolein and acrolein-protein adducts efficiently and rapidly.\textsuperscript{18,19} Several studies have demonstrated hydralazine’s ability to mitigate acrolein-mediated cell injury in PC12 cells and cultured hepatocytes.\textsuperscript{19-21} An \textit{ex-vivo} experiment using guinea pig spinal cords produced convincing results that hydralazine reduced secondary injury after spinal cord tissue compression compared with controls.\textsuperscript{22}

Although hydralazine is a drug that has been used in dogs for other purposes and its pharmacokinetics have been described, there are several limitations to its use as an acrolein scavenger in this species. Hydralazine has historically been used as an effective afterload reducer and anti-hypertensive agent in dogs with congestive heart failure but has mostly been replaced by safer more preferable options. The dose range used in dogs with congestive heart failure is 0.5-2.0 mg/kg, and it is recommended to start at the low end of the dose range with slow titration to effect as severe hypotension may occur even at recommended dosages.\textsuperscript{23} This adverse effect would be quite undesirable because maintaining normotension in the face of neurogenic shock and loss of autoregulatory mechanisms after ASCI is an important component of current standard therapy, and would also complicate anesthesia for surgical intervention. Reducing spinal cord perfusion even further after injury-induced vascular compromise should be avoided as a therapeutic side effect. Furthermore, the cardiovascular effects of hydralazine in dogs are
long lasting (11-13 hours) relative to the plasma half-life (20-50 minutes) due to either
the drug binding to smooth muscle in arterioles or possibly due to metabolite activity.\textsuperscript{24}
Therefore, attempting to maintain plasma concentrations high enough to scavenge
acrolein would likely result in prolonged risk.

As previously discussed, the concentration of acrolein that is attributed with
causing more tissue damage than the primary injury and other aspects of secondary injury
is currently unknown. However, Hamann has estimated that tissue levels of acrolein may
exceed the concentration of hydralazine that can be administered to scavenge it.\textsuperscript{25}
Hamann estimated that CNS tissue levels likely reach 1-5 nmol/mg of protein.\textsuperscript{22} This
would equate to 100-500 umol/L. It is known that hydralazine traps acrolein in a 1:1
ratio;\textsuperscript{21} however, antihypertensive doses of hydralazine reach estimated \textit{in vivo}
concentrations of 0.5-1.0 umol after IV administration.\textsuperscript{26} Furthermore, the drug is highly
protein bound making the plasma levels low at therapeutic doses and tissue to blood
ratios lowest in the brain due to poor lipid solubility.\textsuperscript{27}

Another limitation to the use of hydralazine is associated with the theory that
cross-linking of acrolein adducts in addition to free acrolein alone is partially responsible
for acrolein-induced toxicity. Using bovine pancreas ribonuclease A as a model protein,
hydralazine has been shown to inhibit cross-linking of acrolein adducts if administered
within 30 minutes of acrolein exposure but was not effective if administered after 90
minutes.\textsuperscript{28} Depending upon the degree of contribution that cross-linking makes to
acrolein toxicity, delayed therapy with a scavenger like hydralazine may or may not
effectively reduce acrolein-induced injury. Furthermore, hydralazine did not prevent
 glutathione depletion or increased extracellular acrolein concentration in mouse
hepatocytes. The author therefore suggested that free acrolein scavenging is not the primary mechanism of cell protection with hydralazine, but rather concluded that intracellular acrolein-protein adduct trapping is its main mechanism of action. Trapping free acrolein may not be clinically necessary to its efficacy against acrolein–induced cytotoxicity as MESNA (sodium 2-mercaptoethanesulfonate) is a better extracellular scavenger of free acrolein but did not protect cultured hepatocytes as well as hydralazine in one study when hydralazine and acrolein were given concurrently. Furthermore, acrolein-protein trapping may come with subsequent risks including the induction of an autoimmune reaction to the hydralazine bound acrolein-protein adduct acting as an antigen to B cells. Therefore, delayed hydralazine administration as would occur after naturally occurring ASCI, lack of glutathione sparing and delayed immune mediated sequela may be limitations to its clinical use.

Dihydralazine is structurally similar to hydralazine and could be a more efficient scavenger due to a second hydrazine group with which to bind with acrolein. However, the only pharmacokinetic study that has assessed the concentration of dihydralazine in the cerebrospinal fluid of dogs found no drug after IV administration of 5 mg/kg and 7.5 mg/kg. Because these acrolein scavengers are expected to work at the site of injury in the CNS, it would be prudent to ensure that hydralazine and/or dihydralazine cross the blood brain barrier in dogs and reach therapeutic tissue concentrations before their experimental use as acrolein scavengers in dogs is assessed. Based upon assessment of the current knowledge regarding the pharmacokinetics of hydralazine and its effects on the cardiovascular system, dihydralazine is also likely to cause severe hypotension before reaching adequate tissue concentrations in the CNS to scavenge acrolein. It is suspected
that after trauma, extensive inflammation would increase the permeability of the barrier and thus make entry of the drug into the CNS more likely but the extent of which is currently unknown.

Phenelzine is a monoamine oxidase inhibitor with a hydrazine group capable of scavenging reactive aldehydes. Furthermore, its functional inhibition of amine oxidases may provide another mechanism of neuroprotection by reducing the production of acrolein from polyamines such as spermine. Phenelzine at a dose of 15 mg/kg/day for 7 days provided significant neuroprotection in a gerbil model of ischemic brain infarction. Additionally, phenelzine sequestered acrolein and provided greater than 95% protection from LDH release in a retinal cell line. Phenelzine may hold some advantage over hydralazine in vivo. In people, the half-life of phenelzine is longer at 11.6 h (Nardil) than hydralazine (0.5-1 h). Also, it may be possible to reach a higher concentration of phenelzine in spinal cord tissue without the adverse effect of hypotension that hydralazine induces, however its pharmacokinetics are not yet well known in dogs. Side effects are described but the overall tolerability of phenelzine is not well characterized, as the drug is not regularly used in veterinary medicine. Subcutaneous injections of 2 mg/kg/day for 3 days led to hyperexcitability and agitation in beagles. Tremors and cerebellar ataxia are reported to occur at a single dose greater than 5.5 mg/kg and at doses of 12 mg/kg for 3 days respectively. Although a relatively short course of phenelzine would be required to scavenge acrolein, the dose required to effectively scavenge endogenously produced acrolein after ASCI is not known and one should be aware that an experimental model using dogs caused severe cerebellar nuclei necrosis, cortical demyelination and convulsions with high doses (12 mg/kg to 20 mg/kg) and with chronic
administration (11 weeks). Additionally, concurrent use of phenelzine and with local analgesics or systemic anesthetics may cause hepatic failure in people. Consuming foods with tyramine may also lead to hypertensive crisis therefore the diet should be evaluated before its use is considered.
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CHAPTER 5: SUMMARY AND FUTURE DIRECTIONS

1.1 Summary

In summary, the secondary injury cascade created after ASCI is exceptionally complicated and multifactorial making the use of only one therapeutic compound to lessen its deleterious effects less conceivable. However, if one could target a major component of this cascade that might reduce the propagation of other reactions then improved outcome may be achieved. For this reason, lipid peroxidation and acrolein production are two aspects of the cascade that may make for profitable targets.

We have shown that 3-HPMA, a metabolite of acrolein is increased after naturally occurring ACSI in dogs. Although our initial plan was to perform a clinical trial with an acrolein scavenger, after extensive investigation (described previously) the drug options and presumed doses required to lower acrolein levels were not considered to be adequately safe for our veterinary patients. Advances in targeted nanocarriers for phthalazine drugs or the creation of safer derivatives may allow for their use in the future, as proof of principal for their mechanism of action has already been established.¹ For the current study, a therapeutic intervention that suppresses lipid peroxidation and reduces acrolein production was instead used in the follow up study in lieu of phthalazines.
1.2 Future Directions

**UDCA Neuroprotection/A Novel Use for Ursodeoxycholic Acid**

Ursodeoxycholic acid (UDCA) is an endogenous bile acid that has been shown to protect hepatocytes in cholestatic liver disease in dogs and humans.\(^2\)\(^-\)\(^5\) UDCA is conjugated with taurine or glycine after oral ingestion to form tauroursodeoxycholic acid (TUDCA) and glycoursodeoxycholic acid (GUDCA) respectively. UDCA is currently used commonly in veterinary and human medicine for cholestatic disease, hepatic cirrhosis and other liver diseases and is considered to be safe and effective in this setting. However, to the author’s knowledge, it has not been used for acute CNS injury or neurodegenerative disease in dogs. Experimentally, it has been shown to be neuroprotective in Alzheimer disease and Huntington disease models as well as retinal degeneration and traumatic brain injury.\(^6\)\(^-\)\(^8\) UDCA has more recently been evaluated for safety and tolerability in 18 humans with amyotrophic lateral sclerosis (ALS). It was found to be very safe at doses higher than the standard dose range and was distributed into the CSF in a dose dependent manner.\(^8\) Rodrigues has similarly reported detecting significantly increased levels of TUDCA in the brain of rats and mice after IV administration.\(^9\) The concentration of UDCA required to cause a beneficial effect in various naturally occurring CNS diseases is not yet well established however, recently, a new formula of UDCA has been created that allows for UDCA to accumulate at a greater concentration in plasma and therefore the CNS.\(^10\) Although not significant, longer survival times and a slower rate of progression of neurological decline were noted in a recent randomized cross-over trial using the solubilized form of UDCA in ALS patients,
though a statistical conclusion could not be made regarding its efficacy due to a high attrition rate.\textsuperscript{11}

Additional support for further investigation into the use of UDCA in CNS disease has been established due to the neuroprotective effects seen in experimental ASCI and stroke models in rats. It has been shown that TUDCA protected rat cortical neurons from the effects of glutamate excitotoxicity when pretreated with a sufficient concentration of TUDCA.\textsuperscript{12} After experimentally induced SCI, treatment with 200 mg/kg of UDCA daily until sacrifice reduced neuronal necrosis and apoptosis and improved functional outcome in rats.\textsuperscript{13} TUDCA administered 1 hour after middle cerebral artery occlusion protected rat neurons from cell death and substantially decreased the size of the ischemic infarct.\textsuperscript{9} Treatment with 100 mg/kg of TUDCA up to 6 hours after acute hemorrhagic cerebral infarction reduced the lesion volume by up to 50\% in rats.\textsuperscript{9} In this study and in another study using an ischemic stroke model, neurological deficits were significantly improved in TUDCA treated rats compared to controls.\textsuperscript{9,14}

The neuroprotective mechanism of UDCA is not completely known however it is thought to be related to its ability to stabilize cell and mitochondrial membranes thereby preserving mitochondrial function and preventing apoptosis induction.\textsuperscript{9,14,15} UDCA prevents the production of ROS and inhibits lipid peroxidation and glutathione depletion in neuron cell culture.\textsuperscript{15} In addition to its ability to suppress lipid peroxidation and directly reduce ROS production, UDCA has also been shown to reduce the effects of oxidative stress by increasing glutathione production through the upregulation of gene transcription in cultured hepatocytes and retinal cells.\textsuperscript{4,16,17}
It is possible that UDCA may block the action of acrolein through its ability to stabilize mitochondrial membranes and directly prevent ROS production. Mitochondrial dysfunction is thought to be an important component in the induction of apoptosis. In a cell model of Huntington’s disease, Rodrigues showed disruption of the outer mitochondrial membrane and mitochondrial swelling can lead to cytochrome c efflux that induces caspase activation and apoptosis. TUDCA was able to significantly protect neuronal cells from apoptosis via stabilizing the mitochondrial membrane by reducing membrane oxidative injury/ROS production and membrane depolarization and, therefore, completely abolishing cytochrome c efflux from mitochondria.6 Maintaining mitochondrial integrity and function was also a component of the proposed mechanism of action responsible for neuroprotection seen with UDCA after SCI in rats.13 Several anti-apoptotic molecular mechanisms have been proposed including increased production of Bel-2, blocked translocation of Bax protein into the mitochondria and inhibited release of cytochrome c.8,9,13,14

Lipid peroxidation is a self-perpetuating reaction that leads to damaged cell and mitochondrial membranes, persistent ROS production and the production of acrolein and other toxic aldehydes. As a result, the ability of UDCA to suppress lipid peroxidation could be very beneficial in mitigating the effects of secondary injury after ASCI. UDCA has been shown to suppress lipid peroxidation in an experiment using a human bile model and in the liver of rats after common bile duct ligation.3,5 The component of lipid peroxidation that the author is most interested in is the reduction of acrolein production by UDCA. We are currently studying if the administration of UDCA at 5-15 mg/kg/day for 7 days will lower urine 3-HPMA levels of dogs with naturally occurring ACSI. This
is the standard therapeutic dosing currently used in veterinary medicine for cholangiohepatitis.\textsuperscript{18} Data collection is in progress and results are pending data analysis. Although much higher dosing may be required to reach adequate UDCA concentrations to cause an effect in CNS tissue, this dose was chosen in our baseline pilot study because it is extremely well tolerated in dogs. It is likely that higher doses will be well tolerated in dogs based on safety and tolerability studies in humans however further investigation is required to confirm this in dogs. It has been shown that administering a dose of 50 mg/kg to humans for over 3 weeks was safe and well tolerated.\textsuperscript{8} The adverse events reported were low grade, mild and mostly gastrointestinal in nature including constipation, loose stool, nausea and abdominal bloating.\textsuperscript{8} UDCA was also shown to cross the blood brain barrier in humans in a dose-dependent manner. Mean CSF concentration after oral dosing of 50 mg/kg were significantly higher than after 15 mg/kg, producing CSF concentrations of 191.11 nmol/L and 86.69 nmol/L respectively.\textsuperscript{8} Therefore, if no change in urine 3-HPMA is seen in our pilot study after administration of UDCA, it should be considered that the CNS tissue concentration may not be adequate and a dose increase could be considered. Additionally, use of the new solubilized form could be considered. Determination of UDCA concentration in CSF or CNS tissue would be ideal however may not be easily obtained due to invasiveness of testing in client-owned patients with naturally-occurring injury.

The toxicity of acrolein is also reduced by UDCA via its ability to up-regulate transcription of endogenous glutathione in several cell types. As previously discussed, a component of acrolein-induced cytotoxicity can be attributed to its ability to deplete glutathione. Evidence to this protective mechanism of action was demonstrated by an
experiment using cultured neurons whereby increased glutathione concentration provided protection against acrolein-induced neuronal cytotoxicity.\textsuperscript{19} Detoxification by UDCA was accomplished through conjugation of acrolein by glutathione that prevented acrolein from otherwise reacting with and disrupting cellular proteins and DNA.\textsuperscript{19} UDCA has also been shown to reduce the effects of oxidative stress by increasing glutathione production through the upregulation of gene transcription in cultured hepatocytes, retinal cells and cultured neurons.\textsuperscript{4,15-17,19} Therefore, even if the level of acrolein is not secondarily reduced by UDCA through suppression of lipid peroxidation then the toxicity of acrolein may still be mitigated by its use. An investigation into the neurological outcome after UDCA therapy is therefore warranted regardless of the results of change of urine 3-HPMA levels. Additionally, it would be beneficial to measure whole blood glutathione concentrations in these dogs before and after UDCA administration and compare these results with urine 3-HPMA results. If urine 3-HPMA levels do not decline after UDCA treatment, and if glutathione levels increase, then increased glutathione transcription secondary to treatment administration could be supported.
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