THE EFFECTS OF RHEUM OFFICINALE ON THE PROGRESSION OF FELINE CHRONIC KIDNEY DISEASE

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Abstract

Chronic kidney disease (CKD) is a common cause of morbidity and mortality in cats. The purpose of this study was to investigate the effects of Chinese rhubarb (*Rheum officinale*) supplementation on the progression of feline CKD.

Cats with stable IRIS stage II or III CKD and without certain comorbidities were included in the study. Cats were randomly divided into 3 treatment groups and administered Chinese rhubarb extract (Group 1, Rubenal®, Vetoquinol, Forth Worth, TX; 75 mg tablet by mouth every 12 h), benazepril as a positive control (Group 2, 0.5 mg/kg by mouth every 24 h), or both (Group 3). Cats were fed a commercial renal specific diet and enteric phosphate binder as appropriate. Body weight, laboratory data, and blood pressure were recorded every 3 months.

Variables between groups at enrollment and within groups over visits were compared with ANOVA and repeated measures ANOVA, respectively. A treatment by visit interaction term was included in all repeated measures models. Significance was set at $p \leq 0.05$.

Except for body weight there was no significant differences between treatment groups at enrollment. There was no significant change in body weight, hematocrit (Hct), UPC, serum creatinine, or systemic blood pressure over time as compared to baseline within any group. There was no significant difference between groups over time in regards to change in body weight, Hct, UPC, serum creatinine, or systemic blood pressure. The treatment by time interaction was non-significant in all models.

Based on easily measured clinical parameters, this study failed to detect a significant difference in cats administered a Chinese rhubarb supplement, benazepril, or both.

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List of Abbreviations

CKD	Chronic kidney disease
IRIS	International Renal Interest Society
Hct	Hematocrit
ACEi	Angiotensin converting enzyme inhibitor
RAAS	Renin angiotensin aldosterone system
ACVIM	American College of Veterinary Internal Medicine
GFR	Glomerular filtration rate
H_2	Histamine 2 (receptor)
GI	Gastrointestinal
r-HuEPO	Recombinant human erythropoietin
ECM	Extracellular matrix
MMP	Matrix metalloproteinase
TIMP	Tissue inhibitor of matrix metalloproteinase
PAI-1	Plasminogen activator inhibitor-1
HIF	Hypoxia inducible factor
DC	Dendritic cell
CD8+	Cluster differentiation 8
PDGF	Platelet derived growth factor
CCN2	Connective tissue growth factor
EMT	Epithelial to mesenchymal transition
TGF-β	Transforming growth factors β
PKC-δ	Protein kinase C
c-Abl	Tyrosine kinase cellular Ableson
LAP	Latency associated peptide
SLC	Small latency complex
LTBP	Latent TGF-β binding protein
LLC	Large latency complex
ROS	Reactive oxygen species
ERK	Extracellular signal-regulated protein kinase

ELISA	Enzyme linked immunoassay
rt-PCR	Reverse transcriptase polymerase chain reaction
IgA	Immunoglobulin A
mRNA	Messenger ribonucleic acid
CFN	Cell surface fibronectin
MCP-1	Monocyte chemotactic factor-1
TNF-α	Tumor necrosis factor α
NGAL	Neutrophil gelatinase-associated protein-1
KIM-1	Kidney injury molecule-1
FABP-1	Fatty acid binding protein-1
HGF	Hepatocyte growth factor
LOX	Lipoxygenase
IL-6	Interleukin 6
TT4	Total thyroxine
RD	Renal-related death
NRD	Non-renal related death
AD	All cause death

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Chapter 1 - Review of Feline Chronic Kidney Disease

Clinical Importance

Chronic kidney disease (CKD) is one of the most common killers of cats and prevalence increases with age.[1,2] It has been estimated that over 30% of cats over the age of 15 are afflicted with CKD.[2] In a large epidemiologic study of feline patients presented to a primary care veterinarian, CKD was diagnosed in 1.9% of all cats.[3] It is important to note that the median age of cats in the aforementioned study was 4.3 years. Feline CKD has worldwide importance as the most common cause of all deaths in insured cats in Sweden are due to renal and ureteral disease (16%).[1] In the UK, 30% of non-azotemic, geriatric cats followed for a minimum of 12 months became azotemic.[4]

Clinical Stage

In order to facilitate communication between veterinarians concerning the appropriate treatment of feline CKD and to standardize classifications for scientific studies, a staging scheme has been developed by the International Renal Interest Society (IRIS) (Appendix A).[5] Staging is based on fasted serum/plasma creatinine concentration in an adequately hydrated patient. In addition the presence or lack of proteinuria and systemic hypertension are noted as sub-stages (Appendix A).[5]

Current Treatment

Treatment of feline CKD can be medical or in the form of renal replacement therapy (i.e. hemodialysis or renal transplant). The goals of medical management are to slow the progression of kidney disease and ideally prevent or at least detect and treat the consequences of declining renal function that lead to a decreased quality of life. Current medical treatment of CKD includes dietary modification and long term monitoring and treatment as appropriate of proteinuria, systemic hypertension, urinary tract infections, acid-base and electrolyte abnormalities, anemia, gastrointestinal ulcer and erosion, malnutrition, and dehydration.

Generally, commercial renal specific diets contain less protein, phosphorous, and sodium and more omega-3 fatty acids and potassium as compared to conventional diets.[6-8] Feeding a renal specific diet has been shown to increase survival and decrease uremic episodes in feline CKD.[9-11] Elliott et al. found that cats fed a renal diet had a mean survival of 633 days as compared to cats not fed a renal diet that survived an average of 264 days.[10] Ross et al. showed that cats fed a renal diet had fewer uremic episodes (clinically ill with an increase in creatinine and the lack of other detectable disease) (0%) as compared to cats not fed a renal diet (26%).[9] In the same study, renal-related death was also significantly lower in the cats fed a renal diet (0%) as compared to cats not fed a renal diet (21.7%) over a 12 to 24 month period.[9] In a third study, Plantinga et al. showed that cats fed a renal diet had a median survival of 16 months as compared to cats that ate a conventional diet who survived a median of 7 months.[11] Interestingly the diet that led to the longest median survival (23 months) contained the highest level of omega-3 fatty acids.[11]

As GFR decreases renal excretion of phosphorus also declines. CKD is the most common cause of hyperphosphatemia in the cat.[12] In a group of cats with various stages of CKD plasma phosphate concentrations were increased in 20%, 49%, and 100% of cats with mean plasma creatinine concentrations of 2.6, 3.6, and 10.3 mg/dL, respectively.[13] The deleterious effects of hyperphosphatemia include secondary hyperparathyroidism and soft tissue mineralization. Soft tissue mineralization could possibly contribute to the progression of CKD. Barber and Elliott found that 47% of cats with compensated CKD and 100% of cats with end stage CKD had increased parathyroid hormone concentrations.[13] Cats with partial renal ablation fed a phosphorous restricted diet had less severe renal mineralization, fibrosis, and inflammation as compared to cats fed a maintenance diet.[14] King et al. found a significant association between plasma phosphate concentration and shorter survival times.[15] In the aforementioned study plasma phosphate also correlated with plasma creatinine concentrations making it a dependent risk factor for survival time.[15] In another study with each 1 unit increment (mg/dL) increase in phosphorous at the time of diagnosis there was an 11.8% increase in the risk of death.[16] The most effective way to prevent hyperphosphatemia and hyperparathyroidism includes dietary phosphate restriction with or without an enteric phosphate binder. Barber et al. demonstrated that feeding a diet with reduced phosphate content and in some cases in

combination with an enteric phosphate binder could normalize serum phosphorous and parathyroid hormone concentrations in cats with CKD.[17] Interestingly a more recent study demonstrated the ability of an enteric phosphate binder to decrease serum phosphorous and parathyroid hormone concentrations in cats with reduced renal function fed a maintenance diet.[18] There are currently multiple enteric phosphate binders available, which have been recently reviewed.[12] Administering calcitriol, in the absence of hypercalcemia, makes physiologic sense and has been advocated by some. Unfortunately prospective studies investigating calcitriol supplementation have failed to show any significant effects on parathyroid hormone concentrations.[19]

There is a small amount of evidence to suggest that administering an angiotensin converting enzyme inhibitor (ACEi) may help slow the progression of feline CKD.[20,21] Cats that received 0.5-1 mg/kg/day of benazepril over a 6 month period were less likely to progress from stage II and III to stage IV CKD as compared to cats administered a placebo.[20] In one study cats receiving benazepril at 1.0 mg/kg/day had a significant decrease in serum creatinine, which was not seen in the control group.[21] A separate study failed to show a significant difference in survival (death or euthanasia due to to causes other than CKD are not considered) when benazepril was administered to cats with CKD although the drug was well tolerated and cats with a UPC $\geq$1 had a significantly better appetite.[22] Although prospective studies demonstrating the ability of an ACEi to prolong survival are lacking, there is strong evidence in humans and other species to support the use of an ACEi.[23-26] Apparently, the beneficial effects of RAAS inhibition go beyond simply decreasing proteinuria and normalizing systemic blood pressure.[27,28] Benazepril has been shown to significantly decrease proteinuria in cats.[22] The presence and degree of proteinuria has consistently been shown to have prognostic importance in feline CKD.[4,15,29,30] Syme et al. showed that cats with CKD and a UPC of 0.2-0.4 or >0.4 had a 2.9 and 4.0 hazard ratio for death or euthanasia as compared to cats with CKD and a UPC <0.2, respectively.[29] In agreement King et al. found that as the UPC increased above 0.2, survival time decreased.[15] In addition in a third group of cats with CKD, an increased UPC was associated with death within one month.[31] In non-azotemic geriatric cats that became azotemic over the subsequent 12 months, serum creatinine and either urine albumin or urine protein concentrations remained in the final statistical model as predictors of impending azotemia.[4] The clinical

importance of proteinuria paired with the fact that ACEi decreases proteinuria[21] may be the strongest argument for the use of an ACEi in feline CKD.

The UPC is considered the gold standard in quantification of urine protein content as it correlates well with 24 hour urine protein excretion in normal and experimentally induced renal disease.[32] It should be noted that the urine dipstick and sulfasalicylic acid assay have relatively low specificity and sensitivity, for detecting albuminuria, when interpreted in parallel or in series, respectively.[33] Increasing the cutoff for a positive test result increases specificity but makes the sensitivity unacceptably low.[33] Low grade proteinuria known as "microalbuminuria" will not be detected by the current methods of determining the UPC. The clinical importance of microalbuminuria remains unknown. It is yet to be determined if proteinuria is a marker for a more rapidly progressive renal pathology or if proteinuria itself contributes to declining renal function. In other species there is much evidence to support the latter, which has been reviewed elsewhere.[34,35]

At least 19% of cats with CKD have systemic hypertension.[36] Systemic hypertension can lead to further renal damage in addition to other end organ damage. Control of systemic hypertension has been shown to decrease proteinuria in cats with CKD.[30] In the same study it was found that the control of proteinuria and not systemic hypertension *per se* was significantly related to survival.[30] The ACVIM Consensus Statement recommends antihypertensive therapy when the systolic blood pressure is >160 mmHg and/or the diastolic blood pressure is >100 mmHg.[37] Activation of the RAAS is one contributing factor to systemic hypertension in cats with CKD.[38] In one study both enalapril and benazepril failed to completely inhibit the activation of RAAS and failed to normalize systolic blood pressure in 14/16 cats.[39] Since an ACEi is commonly not effective at controlling systemic hypertension alone, an additional therapy such as a calcium channel blocker may be required.

Urinary tract infections were identified in 16.9% and 22% of cats with CKD in two studies.[40,41] Being female, Persian, and increasing age were associated with a positive urine culture.[40] It was not uncommon for cats to lack clinical signs and urinalysis findings typically

associated with bacterial lower urinary tract infection.[41] The only reliable means of detecting a bacterial infection in this subset of cats is a urine culture.

Cats with CKD are at increased risk of developing hypokalemia (OR=14.4) as compared to cats with normal renal function.[42] In the same study cats with severe hypokalemia (<3.0 mEq/L) were 3.5 times more likely to have CKD than cats with moderate hypokalemia (3.1 – 4.1 mEq/L).[42] In a separate study cats with CKD excreted 2.5 times the amount of potassium in the urine as compared to normal cats.[43] In this group of cats GFR and tubular reabsorption of potassium were decreased and the quantity of urine produced was increased as compared to normal cats.[43] Hypokalemia due to increased urinary loss may be exacerbated by the fact that many cats with CKD are anorexic or hyporexic leading to decreased intake of dietary potassium.[15,44] As kidney disease progresses the occurrence of hypokalemia decreases and the frequency of hyperkalemia increases.[15] This is thought to be due to decreased urine production in end stage renal disease.[42] Deleterious effects of hypokalemia include muscular weakness, functional ileus, cardiac arrhythmias, renal vasoconstriction leading to decreased GFR, and exacerbation of polyuria and polydipsia due to decreased responsiveness to anti-diuretic hormone. In the case of severe hypokalemia intravenous potassium supplementation in the form of potassium chloride is recommended.[45] For long term maintenance therapy oral potassium gluconate may be administered as this is the most palatable potassium salt available.

Anemia is a common hematologic finding in cats with CKD and increases in occurrence and severity as kidney function declines.[15,16,44] The cause of anemia is multifactorial and includes the relative lack of erythropoietin, gastrointestinal blood loss, anemia of chronic disease, and shortened red blood cell lifespan.[46] Gastrointestinal blood loss may be due to gastroduodenal ulceration or erosion, which is secondary to hypergastrinemia.[47] In one study the prevalence of anemia ranged from 0% in cats without clinical signs (mostly IRIS Stage II) to 65% in cats with end-stage CKD (IRIS Stage IV).[44] Anemia in some studies has been shown to be a prognostic factor with cats that died within 1 month having a significantly lower hematocrit.[31] Boyd et al. showed that the median survival of cats that became anemic (Hct <25%) and that underwent intervention for their anemia was 100 days and 25 days, respectively.[16] Antacids in the form of H_2-receptor antagonists or proton pump inhibitors are

indicated in the case of anemia or GI upset. The lack of erythropoietin can be treated with recombinant human erythropoietin (r-HuEPO) or darbopoetin which will consistently increase RBC mass.[48] Because both erythropoietin products are non-feline proteins, both may evoke anti-erythropoietin antibodies.[48] In one small study, 5 of 7 cats receiving r-HuEPO developed refractory anemia and 4 of those 5 cats had detectable anti-r-HuEPO antibodies at the onset of the refractory anemia.[48] Erythropoietin therapy is generally instituted when the patient shows clinical signs of anemia. The benefits of treating anemia must be greater than the risks associated with inducing anti-EPO antibodies. According to the chronic hypoxia theory (see below) more aggressive treatment of anemia may be warranted.[49]

Clinical dehydration was apparent in 10% of uremic cats and 73% of cats with end-stage CKD in one study.[44] The prevalence of sub-clinical dehydration would be expected to be even higher. Dehydration occurs when water consumption does not keep pace with polyuria. Dehydration causes pre-renal azotemia, which in addition to renal azotemia exacerbates the deleterious effects of uremia. Some cats benefit from parenteral fluid supplementation and sub-cutaneous fluid administration is commonly a feasible at-home treatment option. An alternative option to sub-cutaneous fluid administration requires placing a feeding tube. Esophagostomy or gastrostomy (e.g., percutaneous endoscopically guided) tubes are safe and simple to place and facilitate the administration of water and nutritional supplementation if needed. In addition many medications can be administered via a feeding tube, which may improve owner compliance.

Cats with an appropriate temperament and the absence of concurrent disease including cardiac, endocrine, and infectious disease(s) may be candidates for renal transplant.[50] Following renal transplant, ongoing medical management including life-long immunosuppression to prevent allograft rejection is required. Due to stringent pre-operative patient screening and the relatively high monetary and time commitments required of the owner renal transplant is often not a feasible option.

Intermittent hemodialysis is an alternative renal replacement therapy in acute and chronic kidney disease and has been described in 14 cats with CKD.[51] Hemodialysis does improve

biochemical abnormalities but does not address the underlying cause of renal dysfunction. For this reason except in some cases of "acute on chronic" renal failure, life-long treatment would be required. Due to the small size of the domestic cat, performing hemodialysis is technically challenging with a substantial risk of various complications including; pulmonary edema, hypotension, disequilibrium, cardiopulmonary arrest, and seizures to name a few.[51] Because of the need for ongoing therapy and associated cost, technical challenges and risks, and the limited access to centers providing intermittent hemodialysis to feline patients the use of this treatment modality in feline CKD is limited.

Prognosis

A unique clinical aspect of feline CKD is the relatively long period of time (sometimes years) without apparent progression of renal dysfunction. Taking this into consideration, prolonged survival is expected in cats with mild to moderate disease (IRIS stage I, II, & early III). Prognostic indicators have been variable and have included the presence of anemia, degree of azotemia (plasma creatinine and urea nitrogen), hyperphosphatemia, increased blood leukocyte count, and proteinuria.[15,16,29,31,44] In a recent study cats with stage IIb (creatinine, 2.3-2.8 mg/dL), III, and IV CKD had median survivals of 1151 days, 679 days, and 35 days, respectively.[16] It is important to note that in this study there was considerable overlap of survival times between cats in different IRIS stages.[16] As such care must be taken when providing a prognosis for an individual patient. At one institution median survival following renal transplant was 613 days, with 6 month and 3 year survival rates of 65% and 40%, respectively.[52] There has been no study concerning survival times in cats treated with medical management alone vs. renal transplant, thus precluding direct comparisons of these two treatment modalities.

Chapter 2 - Pathophysiology of Chronic Kidney Disease

Fibrosis and the Progression of Kidney Disease

Fibrosis is an exaggerated deposition of extracellular matrix (ECM). ECM is vital to normal organ function facilitating structural support, cellular adhesion and growth, and the movement of fluid and macromolecules.[53] The specific components of ECM is unique to each

organ but includes proteins such as collagen I, collagen IV, fibronectin, laminin, elastin, and proteoglycans, amongst others.[53] In health there exists a balance between matrix deposition and degradation. Fibrosis occurs when matrix deposition exceeds degradation. ECM is produced by multiple cell types including fibrocytes, fibroblasts, and myofibroblasts. Degradation is catalyzed by proteases more specifically plasmin and matrix metalloproteinases (MMP).[53] Protease activity can be altered by enzyme inhibitors for example tissue inhibitors of metalloproteinases (TIMP) and plasminogen activator inhibitor-1 (PAI-1).

Renal fibrosis is a common end point to essentially all progressive kidney disease, including feline CKD. Renal fibrosis is ECM accumulation in the tubular interstitial compartment (tubulointerstitial fibrosis) and glomerulus (glomerulosclerosis). Histology studies have shown that the primary lesion in the majority of feline CKD is located in the tubular interstitium.[54] It is not surprising that feline plasma creatinine concentrations correlate with interstitial fibrosis but not with interstitial inflammation, glomerulosclerosis, or renal corpuscular diameter.[55] The fact that the interstitium is primarily affected does not mean that the glomerulus escapes undamaged. Any disease that alters the tubular interstitium also affects the glomerulus and *vice versa*. Interestingly in people, even when glomerular lesions are considered primary, progressive loss of renal function is more closely linked to tubulointerstitial disease.[56] It is important to note that when a portion of the nephron is irreversibly damaged the entire nephron is permanently lost.

The list of possible renal insults is long. In the case of feline CKD, at the time of diagnosis a specific underlying cause is rarely identified. Without an apparent underlying cause in the presence of progressive kidney dysfunction a therapeutic dilemma arises. As discussed previously, renal replacement therapy for multiple reasons is uncommonly a feasible option. Medical treatment is primarily supportive identifying and treating consequence(s) of renal dysfunction as they occur. As with any disease the identification and treatment of an underlying cause is desirable. Regardless of the underlying etiology tubulointerstitial fibrosis is considered by many to be central in the progression of CKD.[57] There is a paucity of research concerning the pathophysiology underlying the progressive decline in renal function in feline CKD. Partial nephrectomy models (one complete nephrectomy and partial infarction of the remaining kidney)

in cats have shown morphologic changes but have failed to demonstrate a progressive decline in GFR.[58] The lack of decline in GFR is consistent with what is seen in naturally occurring feline CKD, where cats may have stable kidney function for a relatively long period of time.[16,44] With a reduction in the number of functional nephrons compensatory changes occur. In cats with partial renal ablation there was an increase in single nephron GFR due to glomerular hypertension and hypertrophy.[59] In this study single nephron GFR increased not only due to increased glomerular filtration pressure but also because of an increased filtration coefficient.[59] As an illustration in the ability of the feline kidney to hypertrophy, 4-6 weeks after partial nephrectomy the remnant kidney weighed 36% less than the normal kidney despite a 70% reduction in nephron number.[59] One theory, that remains unproven in cats, is that even in the absence of the initial underlying insult progressive kidney dysfunction becomes "self-perpetuating".[58] It has been hypothesized that in mild or moderate CKD, GFR-raising effects dominate maintaining stable kidney function but in severe CKD, GFR-lowering effects prevail.[58] It is clinically apparent that once azotemia is present, feline CKD is progressive which indicates that in the majority of cases at least one of the following scenarios is occurring; (1) the initial underlying renal insult is present yet remains unrecognized and/or (2) renal dysfunction progresses even in the absence of the initial underlying cause.

One possible scenario to describe the progressive loss of kidney function is that fibrosis is not only the result but also the cause of functional nephron loss. Fibrosis may lead to further nephron loss by contributing to; (1) chronic renal hypoxia, (2) alterations in the glomerular filtration barrier, and (3) decreased renal cell survival.[57,60-65]

The chronic hypoxia theory first proposed by Fine et al. describes chronic hypoxia as the main driving force behind progressive renal disease.[49] In this theory proteinuria and systemic hypertension are "aggravating factors" to progressive renal disease but not causes of progression *per se*.[49] In this model fibrosis is one of many causes of hypoxia and hypoxia in turns leads to further renal parenchymal damage and fibrosis. Renal fibrosis leads to peritubular capillary damage (rarefaction and obliteration) leading to compromised blood flow and renal parenchymal hypoxia.[57,64,65] In response to hypoxia, the renal parenchymal cells alter genotypic expression. Most notably, hypoxia inducible factor (HIF), a transcription factor that is known to facilitate the

expression of over 100 genes, increases in concentration.[65] Although protective in acute hypoxia, in experimental models of chronic renal hypoxia HIF induces fibrosis.[64] Numerous other causes of renal parenchymal hypoxia have been postulated and include renal anemia, altered vascular tone, increased oxygen consumption due to hypertrophy, increased single nephron GFR, and oxidative stress.[65]

The hallmark of glomerular disease is proteinuria. When the glomerular filtration barrier is compromised increased concentrations of albumin and other macromolecules enter the glomerular ultrafiltrate. Brenner et al. first described the hyperdynamic theory, which postulates that the glomerular hypertrophy and hyperfiltration leads to progressive interstitial disease.[66] It has since been shown that proteinuria and hydrodynamic changes *in vitro* stimulates the production of profibrotic and inflammatory mediators by renal tubular cells.[67-73] In addition human clinical studies have shown that proteinuria is correlated with disease progression and that the control of proteinuria slows the decline of kidney function.[23,24,26,74] Recent work by Heymann et al. and Macconi et al. provides the basis for a model that links glomerular disease to progressive interstitial disease by means of autoimmunity.[75-77] In health, albumin and other macromolecules that enter the tubule undergo endocytosis by renal tubular epithelial cells that then present antigen to dendritic cells (DCs).[77] Alternatively antigen may be taken up directly from the tubular lumen by DCs.[77] In either case DCs then travel to renal lymph nodes where in the healthy kidney cross-tolerance to the presented antigen develops and activated CD8+ T cells undergo apoptosis without stimulating an immune response.[78] In the diseased kidney constituents from the damaged renal cells in addition to albumin enter the filtrate and an inflammatory response to self antigen ensues leading to inflammation or tubulointerstitial nephritis.[75-77] In this scenario continuous antigenic stimulation leads to perpetual inflammation and progressive renal fibrosis and parenchymal damage. It is noteworthy that as peritubular capillaries arise from the glomerular efferent arterioles, glomerular disease may also contribute to renal parenchymal hypoxia by decreasing peritubular capillary blood flow.[79,80]

Fibrosis alters ECM composition and three dimensional structure. ECM is a vital component of the structure and function of all parenchymal organs and this fact is most obvious in renal fibrosis. Not only does ECM facilitate three dimensional cellular orientation but ECM is

also important for the spatial distribution of cytokines/chemokines, growth factors, and cell signaling required for cell survival.[62] *In vitro* studies have shown that the specific composition of ECM is important for renal cell survival.[60,61] For example rat mesangial cells, exposed to starvation and DNA damage, were protected by collagen IV and laminin whereas collagen I, fibronectin, or osteonectin did not improve cell survival.[61] ECM binding and cell survival appears to be β_1 integrin mediated.[61] In addition to composition, three dimensional structure and tissue architecture are also crucial to renal cell survival and the maintenance of a differentiated phenotype.[63]

Whether progression of CKD results from hypoxia, proteinuria (autoimmunity), or a decreased stimulus for cell survival − the common histologic denominator is fibrosis. Understanding what precedes fibrosis has therapeutic, diagnostic, and prognostic importance. The deposition of fibrous tissue requires a well-coordinated chain of events including: (1) an initiating event, (2) release of profibrotic cytokines and growth factors, (3) associated secondary sub-cellular signaling, and (4) production of ECM primarily by fibroblasts and myofibroblasts.

The number of people with chronic kidney disease continues to climb with as many as 6.3% of the population now affected.[81] In 2008 within the Medicare population the yearly cost to treat CKD was $19,752 US per person, which increased with common comorbidities such as diabetes and congestive heart failure.[81] This accounted for 14.2% of all Medicare expenditures.[81] For this reason an immense amount of resources is currently being directed toward the investigation of novel therapies to slow, stop, or reverse renal fibrosis. Recognized key mediators in renal fibrosis include transforming growth factor β (TGF-β), platelet derived growth factor (PDGF), connective tissue growth factor (CCN2), HIF, and RAAS directing key cellular events such as: inflammation, autoimmunity, epithelial to mesenchymal transition (EMT), hypoxia, and growth arrest and apoptosis. Therapy intended to slow or reverse tissue fibrosis could potentially be directed at any of the aforementioned cellular events and/or mediators. As the fibrotic pathway is complex there are a large number of possible therapeutic targets with the caveat being that blocking a single signaling cascade or inhibiting a single fibrotic mediator may not substantially alter fibrous tissue deposition.

Transforming Growth Factors β

Transforming growth factors β (TGF-β) are a family of profibrotic cytokines with anti-inflammatory and immunoregulatory properties that are instrumental in wound healing and organ development and function.[82] TGF-β expression is upregulated in essentially all naturally occurring and induced states of fibrosis. There are three TGF-β isoforms (1, 2, and 3) all of which are expressed in the kidney.[83] TGF-β1 is the primary isoform produced by mammalian cells and is most important in tissue fibrosis. TGF-β exerts its cellular effects by binding to cell surface, transmembrane TGF-β receptors (RI, II, and III).[84] Type III receptor, betaglycan, has no ability to transduce signal, but rather binds TGF-β and presents it to type II receptors.[84] Once bound, type II receptors bind type I receptors which are then phosphorylated by type II receptor kinases. TGF-β signals act primarily through the smad pathway. Smad-independent signaling occurs via protein kinase C-δ (PKC-δ) and nonreceptor tyrosine kinase cellular Ableson (c-Abl) amongst others. Eight Smad proteins have been identified in vertebrates.[85] Smad 2 and 3 are phosphorylated in response to TGF-β bound receptors. Once phosphorylated, smad 2 and 3 bind to smad 4. The resulting smad complex translocates to the nucleus, where it interacts with multiple transcription factors, regulating the transcription of pro-fibrotic genes. Smad 6 and 7 inhibit phosphorylation of smad 2 and 3 and thereby interfere with transcellular signaling. In addition, internalization in caveolin positive lipid rafts leads to TGF-β receptor degradation and is another important regulatory mechanism of TGF-β activity.[86,87] Finally the activation of latent TGF-β, in response to cell damage or alterations in the cellular micro-environment provides the most important means of altering TGF-β function.[88]

In the kidney TGF-β1 is expressed by mesangial cells, visceral epithelial cells, parietal epithelium of the glomerulus, and renal tubular cells.[83] In addition TGF-β is produced by invading cell types including macrophages, monocytes, and platelets.[89] Initially TGF-β is produced in a latent form bound to latency-associated peptide (LAP) which forms the small latency complex (SLC).[90,91] This complex, when bound to latent TGF-β-binding protein (LTBP 1-4), is known as the large latency complex (LLC).[90,91] The LLC via LTBP-1 is covalently bound to the ECM.[92] For TGF-β to be biologically active it must dissociate from the LAP. The LLC has been likened to a "molecular sensor" responding to perturbations in the ECM and releasing biologically active TGF-β.[88] Primarily through interactions with LAP, latent TGF-β

can be activated by proteases (plasmin, MMP 2 and 9), thrombospondin-1, integrins ($\alpha_v\beta_6$ and $\alpha_v\beta_8$), reactive oxygen species (ROS), nitric oxide, and low pH.[93-101] In addition mechanical strain and exposure to elevated glucose concentration or advanced glycation-end products leads to increased production of TGF-β.[73,102-104] Finally an important stimulator of TGF-β production in kidney disease is the RAAS.[105,106]

TGF-β contributes to renal fibrosis by increasing ECM synthesis, decreasing ECM degradation, and facilitating cell matrix interactions.[107-110] TGF-β contributes to ECM in multiple ways one of which is the induction of epithelial to mesenchymal transition (EMT).[111,112] EMT is the phenotypic transformation of tubular epithelial cells (cytokeratin positive) to myofibroblasts expressing smooth muscle actin and/or vimentin. The phenotypic transformation is accompanied by cellular translocation into the interstitium and the production of ECM.[113] This phenotypic transition has been demonstrated *in vitro* and in the dog and rodent.[111,114,115] There is evidence that the tubular basement membrane is important for maintaining the epithelial phenotype and damage to the basement membrane leads to increased TGF-β and EMT.[116] Although there is a substantial amount of evidence indicating that tubular epithelial cells contribute to interstitial fibrosis[112], recent research has also shown that interstitial myofibroblasts may also originate from interstitial perivascular fibroblasts.[117] In addition to increasing ECM production, TGF-β reduces ECM degradation by inhibiting proteases and increasing TIMPs.[102,109]

CCN2 (Formerly - Connective Tissue Growth Factor)

There is a multitude of cytokines, growth factors, and other signaling molecules that work downstream or in concert with TGF-β to promote fibrosis. CCN2 is a matricellular protein that is important in development, tissue healing, and fibrosis.[118] Once incorrectly referred to as a growth factor, it is now known that CCN2 primarily works by modifying the signaling of other molecules and effects of such can vary widely.[118] In people CCN2 expression is upregulated in various kidney diseases and correlates with the degree of tubulointerstitial damage.[119] The production of CCN2 is induced by TGF-β and CCN2 modulates TGF-β activity by decreasing smad 7 and increasing smad 2.[120] In addition cellular stress such as hypoxia can induce the production of CCN2.[118] There is evidence that TGF-β may induce fibrosis but that CCN2 is

required to maintain the production of matrix in chronic disease.[121] *In vitro* CCN2 increases cellular proliferation and the production of fibronectin and type I collagen by renal myofibroblast-like cells.[122] These cellular effects were dependent upon activation of extracellular signal-regulated protein kinase (ERK)1/2 mitogen activation protein kinase pathway.[122] The aforementioned profibrotic effects may be partially offset by the fact that *in vitro* CCN2 augments the activity MMP-2 and inhibits TIMP-2.[123]

Chapter 3 - Assessment and Modulation of Renal Fibrosis

Clinical Assessment of Fibrosis

The gold standard of quantifying organ fibrosis involves histologic examination. Depending on the patient and the organ, there are times when obtaining an adequate tissue sample may not be clinically feasible. The desire to find non-invasive, reliable, and cost effective ways of assessing normal biology, pathologic states, and therapeutic response has led to much interest in "biomarkers". In regards to biomarkers the kidney is a unique organ in that it produces a bodily fluid (urine) that is in sufficient quantity and easily obtained. Methods of quantifying growth factor, cytokine/chemokine, signaling molecules and other proteins or their expression in urine has included urine supernatant ELISA and Western blot, urine sediment rt-PCR, and the analysis of entire urine proteomes via mass spectrometry. An ideal biomarker is one that is safely obtained, rapidly quantitated, and that provides important prognostic, diagnostic, or information concerning therapeutic monitoring.

In people urinary TGF-β has been shown to be elevated as compared to normal subjects in membranous glomerulonephropathy, mesangial glomerulonephritis, rapidly progressive glomerulonephritis, systemic lupus erythematosus, IgA nephropathy, glomerulosclerosis, crescentic nephritis, various pediatric renal diseases, nephritic patients, and following renal transplant. [124-130] In addition urinary TGF-β has been shown to be elevated in feline CKD and feline diabetes mellitus.[131] In this group of cats serum TGF-β concentrations were not increased indicating that the urinary TGF-β likely represented local production in the kidney.[131] Urinary TGF-β mRNA in urine sediment correlated to the amount of interstitial fibrosis and estimated GFR in a group of people with IgA nephropathy and glomerulosclerosis.[125] In addition, urinary

TGF-β was shown to be higher in patients with crescentic nephritis that did not respond to immunosuppressive therapy as compared to those who did.[127]

Urinary CCN2 concentrations are increased in rats and people with experimentally induced renal disease and diabetic nephropathy, respectively. In addition urinary CCN2 is significantly elevated in rodents and humans with histologically confirmed chronic allograft nephropathy (interstitial fibrosis and tubular atrophy) following renal transplant.[132,133] In fact urinary CCN2 concentrations increased weeks before serum creatinine and the presence of histologic evidence of renal allograft injury.[134] Although urinary CCN2 concentrations increased earlier, when both were present, urinary CCN2 concentrations directly correlated to the amount of interstitial fibrosis.[134] In rats with experimentally induced diabetic nephropathy CCN2 production originated primarily from the cortex, thus is likely related to glomerular ECM accumulation and glomerulosclerosis.[135]

Fibronectin is a major component of ECM and is also found on cell surfaces (cFN) and as soluble fibronectin in circulation.[136] Because the accumulation of ECM accompanies all progressive kidney diseases, fibronectin is significantly increased in the renal tissue in essentially all human glomerulopathies.[137] Urinary fibronectin originates from local renal production and from systemic circulation (only if the glomerular filtration barrier is compromised).[136] In people with newly diagnosed glomerulonephritis urinary fibronectin concentrations were significantly higher in those who had progressive loss of renal function over the subsequent four years as compared to those who had non-progressive disease.[136] In contrast neither proteinuria nor systemic blood pressure was higher in this group of people. Interestingly urinary fibronectin was not associated with proteinuria thus indicating that fibronectin was likely produced locally in the kidney. Although in the previous study there was no correlation between urinary fibronectin and the severity of glomerular lesions, in a separate human study urinary fibronectin correlated with the relative area of interstitial fibrosis.[136,138] In addition to renal disease urinary fibronectin may also be elevated in bladder and urethral cancer and may be utilized to facilitate the initial detection and reoccurrence of certain urinary neoplasms.[139,140] Other constituents of ECM that have been quantified in urine include various collagen and collagen fragments. As measured by ELISA, urinary type III collagen was increased in people with diabetic nephropathy.[141]

Interestingly, in a separate study, when quantified via mass spectrometry various collagen fragments were decreased in the urine of people with diabetic nephropathy.[142] This could be explained by the decreased ECM degradation that occurs in all progressive kidney disease which is at least in part due to the influence of TGF-β. In this same study the urinary proteome could differentiate between diabetic nephropathy and other CKD with 81% sensitivity and 91% specificity.

Other Urinary Biomarkers

A significant decrease in nephron number deleteriously affects glomerular epithelial cells.[143] Podocytes are unique epithelial cells, which are attached to the glomerular basement membrane and are an important component of the glomerular filtration barrier. Podocyte loss contributes to glomerular disease including glomerulosclerosis. Quantifying podocyte damage may include urine sediment cytology detecting podocyturia or by the detection of urinary podocyte products.[144-147] Podocalyxin, a sialomucin, is a cell surface protein expressed by podocytes. In children with glomerular disease, urinary sediment podocalyxin concentration is reliable marker for the severity of active glomerular injury.[147] Other podocyte proteins that have been quantified in urine include podocin and nephrin amongst others.[148]

Various other cytokines/chemokines, growth factors, enzymes, and signaling molecules have been detected and quantified in urine and include; monocyte chemoattractant factor-1 (MCP-1), granzyme A, tumor necrosis factor-α (TNF-α), MMP, TIMP, neutrophil gelatinase-associated lipocalin (NGAL), IL-18, kidney injury molecule-1 (KIM-1), and urinary fatty acid binding protein-1 (FABP-1) amongst many others.[149-155]

Modulating Renal Fibrosis

The incidence of CKD is growing, as is the need for alternative treatments.[81] Targeting organ fibrosis and more specifically TFG-β and associated mediators of fibrosis has gained much attention. Experimental and clinical therapies that have been investigated with mixed success include; RAAS inhibition, anti-TGF-β antibodies, TGF-β anti-sense oligonucleotides, soluble

TGF-β co-receptor, smad 7 gene transfer, hepatocyte growth factor (HGF) gene transfer, PKC inhibition, tyrosine kinase inhibition, and statin therapy amongst many, many others.[156-162]

Chinese Rhubarb (*Rheum palmatum, Rheum officinale*), also known as Rheum, has been used for thousands of years in traditional Chinese medicine. Many substances have been isolated from rhubarb but the anthraquinones (emodin, rhein, and chrysophanol) and stilbenes (resveratrol) have gained the most attention.[163] *In vitro* rhein inhibits renal cellular hypertrophy, ECM production, and PAI-1 upregulation induced by TGF-β.[164,165] In mice with diabetic nephropathy rhein corrected dyslipidemias and significantly decreased glomerular hypertrophy and ECM expansion via TGF-β inhibition.[166] Emodin inhibits the effects of angiotensin II on vascular smooth muscle.[167] In addition emodin has anti-fibrotic properties via the inhibition of TGF-β induced collagen production.[168] Stilbene derivatives have shown remarkable inhibitory effects on lipoxygenase (LOX) and as free radical scavengers.[163] In rats, with partial nephrectomy and adenine induced renal failure, rhubarb extracts decreased glomerulosclerosis, renal cell necrosis, proteinuria, and renal values and increased survival.[169,170] In people with CKD, small uncontrolled studies have shown that oral rhubarb administration alleviated uremic symptoms and slowed the progression of disease.[171,172] The most compelling *in vivo* evidence of the anti-inflammatory and anti-fibrotic effects of rhubarb come from people with radiation induced lung injury.[173] In a double-blind, randomized, controlled study people that received rhubarb had significantly decreased systemic TGF-β and IL-6 concentrations and radiation induced lung injury and significantly improved pulmonary function as compared to placebo.[173]

Chapter 4 - Clinical Study

Introduction

Feline CKD is one of the most common killers of cats and prevalence increases with age.[1,2] Current medical treatment of feline CKD includes the prevention, detection, and treatment of the consequences of decreased renal excretory and endocrine function. Feline CKD, after the onset of azotemia, is invariably progressive. An underlying cause is rarely found at the time of diagnosis, which indicates that the cause simply remains undetected or more likely that progressive decline in kidney function is self-perpetuating. Interstitial fibrosis and mononuclear

inflammation are common findings in all progressive kidney diseases, including feline CKD. There is evidence to support the fact that advanced renal fibrosis is not only the result but may also be the cause of progressive CKD.[90,174,175] Fibrosis may contribute to progressive disease by causing renal hypoxia, altering growth signals and cell survival, and leading to a maladaptive hyperdynamic state and proteinuria .[49,60,62,65,176]

A treatment that can slow, stop, or reverse renal fibrosis is desirable. Transforming growth factors β are upregulated in essentially all experimentally induced and naturally occurring fibrosis, including renal fibrosis. For this reason TGF-β, associated signaling cascades, and downstream mediators of TGF-β are the focus of much research related to renal fibrosis. Chinese rhubarb, *Rheum palmatum* and *Rheum officinale*, has been used for thousands of years to treat various disease in traditional Chinese medicine. Chinese rhubarb and its constituents have been shown to have anti-fibrotic and anti-inflammatory effects *in vivo* and *in vitro*.[168,170,173,177] In addition, in rodent models rhubarb supplementation has been shown to decrease proteinuria and slow the progression of kidney disease.[170,177] These effects appear to be synergistic with concurrent RAAS blockade.[171,177] Since 2009 a rhubarb supplement marketed toward veterinarians for the treatment of feline and canine kidney disease has been commercially available.[178] The purpose of this study is to investigate the effects of a commercially available Chinese rhubarb supplement[a] on the progression of feline CKD. The tested hypothesis is that Chinese rhubarb extract will slow the progression of feline CKD as compared to benazepril and Chinese rhubarb extract and benazepril together will be more effective than either alone.

Materials and Methods

Cats

Feline patients were actively recruited from the Kansas State University Veterinary Medical Teaching Hospital patient population and from surrounding primary care veterinary hospitals. The study was advertised during continuing education seminars and flyers were mailed to referring veterinarians. Financial compensation was provided to referring veterinarians at initial examination and patient inclusion into the study. Financial compensation was provided to the owners at initial visit and after 1 year of participation in the study. The study in its entirety

was approved by the Institutional Animal Care and Use Committee of Kansas State University and owner written consent was obtained before patient entry into the study.

Inclusion criteria

Cats were required to have stable IRIS Stage II or Stage III CKD, as diagnosed by a serum creatinine of 1.6 – 3.9 mg/dL in a euhydrated state. Evidence of stable disease by demonstrating a less than 20% change in serum creatinine from initial visit to the first follow up visit 10-14 days later was required. In addition cats must not be affected with certain comorbidities or consequences of CKD including pyelonephritis, neoplasia, reno- or ureterolithiasis, uncontrolled hyperthyroidism, systemic hypertension requiring treatment (Doppler >170 mmHg on two occasions or >160 mmHg with evidence of end organ damage), or other unrelated disease leading to clinical illness. The cat was also required to be willing to eat a commercially available renal specific diet.[b,h,i] In addition the patient's temperament had to be such as the required diagnostic evaluation and at home drug administration would not cause undue risk to the clinician, student, technical staff, owner, or the cat. Neither the presence nor degree of proteinuria was considered an exclusion criterion.

Study design

A prospective, positive-controlled study was conducted. Cats that fulfilled the inclusion criteria were randomly placed into 1 of 3 treatment groups; rhubarb extract (Group 1), benazepril[b] (Group 2), or both (Group 3). To evaluate drug tolerance rhubarb extract was initially administered at 75 mg PO q 24 h. If well tolerated this dose was increased to 75 mg PO q 12 h at 1 month. Benazepril was administered at approximately 0.5 mg/kg (0.24 mg/lb) PO q 24 h, with the dose rounded to the nearest ¼ of a 5mg tablet.

Screening examination

Initial diagnostic investigation required to diagnose stable CKD and rule-out comorbid diseases included; CBC, chemistry panel, urinalysis with sediment, UPC, species-specific semiquantitative urine albumin ELISA[c], bacterial urine culture, total thyroxine concentration (TT4), thoracic radiographs, abdominal radiographs and ultrasound, and Doppler[d] blood pressure. In addition historical findings, full physical examination, and body weight were

recorded. To facilitate owner compliance all diagnostic examination and treatment was provided free of charge.

Subsequent examinations

A repeat examination was scheduled to be performed at 10-14 days at which time if stable disease was present the cat was included in the study. Further examinations were at 1 month after initial examination and then every 3 months thereafter. More frequent examinations could be scheduled at the discretion of the attending clinician, referring veterinarian, or owner. A CBC, chemistry panel, urinalysis with sediment, UPC, species-specific semi-quantitative urine albumin ELISA[c], and Doppler[d] blood pressure were performed at each examination, excluding the CBC which was not performed at the first recheck examination. In addition a bacterial urine culture and TT4 was performed every 6 months and abdominal radiographs and/or ultrasound every 12 months. Finally, historical findings, full physical examination, and body weight were recorded at each examination.

Blood sample collection and analysis

Owners were instructed to withhold food but not water for at least 12 hours prior to presentation for examinations. Blood samples were collected from the jugular or medial saphenous vein. Serum biochemical panel, CBC, and TT4 were analyzed on the same day as the visit.

Urine sample collection and analysis

Urine samples were collected via cystocentesis. Urinalysis was performed with a refractometer for urine specific gravity, commercially available reagent strip[e] for chemical evaluation, and standard microscopic sediment examination. A few drops of urine were saved from each urine sample and refrigerated until submitted, on the same day, for aerobic culture and sensitivity. Urine protein content was quantified via the benzethonium reaction method and urine creatinine was quantified via the buffered Jaffe reaction, both with an automated chemistry analyzer[f]. A UPC ratio was derived from this data for each urine sample. For individual visits, a UPC result was not included in the statistical analysis if any of the following were detected: (1) pyuria or >10 WBC/hpf, (2) a positive bacterial urine culture, (3) gross hematuria, (4) semen, or (5) bacteriuria, except for the presence of cocci in the absence of pyuria or a positive urine

culture. In addition, each urine sample was analyzed for microalbuminuria using a commercially available species specific urine albumin ELISA by following instructions included in the package insert.[c] Finally, a bacterial urine culture was performed on urine collected via cystocentesis at initial visit, every 6 months thereafter, and at the clinician's discretion. All urine analysis and plating for urine culture was performed on the same day as the visit.

Blood pressure measurement

Systolic blood pressure measurements were obtained by use of an ultrasonic Doppler[d] monitor after the cat was acclimated to the hospital environment. The cat was placed in lateral recumbency. The up leg was used for pressure measurement with a neonatal #2 or #3 cuff (width approximately 35-40% the circumference of the leg) placed directly below the elbow. After shaving directly over the common digital branch of the radial artery, 3-4 readings were obtained and the mean of those readings was recorded.

Imaging

Thoracic radiographs, abdominal radiographs, and abdominal ultrasound were interpreted by a board certified veterinary radiologist or a veterinary radiology resident under the direct supervision of a board certified radiologist. When required to achieve diagnostic quality images, ketamine (5 mg/kg, 2.2 mg/lb, IV) and diazepam (0.5 mg/kg, 0.23 mg/lb, IV) was administered for sedation.

Diet

All cats were exclusively fed a commercially available renal specific diet. All cats were slowly transitioned to a renal specific diet[b] if not already consuming this diet. For cats that did not accept the first commercially available diet, one of two alternative commercially available diets[h,i] was offered. To facilitate compliance all diets were provided to the owner free of charge.

Treatment

Clinical management was at the discretion of the attending clinician, but certain guidelines for treatment were established and are described here. Although hypertension requiring treatment was an exclusion criterion, if a cat developed hypertension after inclusion into the study, amlodipine[j] was started at 0.625 mg, total dose, PO q 24 h. Dose was increased as

needed to maintain a systolic blood pressure below 160 mmHg. Severe hypokalemia was treated with intravenous potassium supplementation in the form of potassium chloride. Mild or moderate hypokalemia was treated on an outpatient basis when the potassium fell below the reference interval. Potassium gluconate in powder, tablet, or gel form was prescribed at 2 mEq, total dose, PO q 12h. This dose was increased as needed to maintain serum potassium within the normal range. Enteric phosphate binders were prescribed when serum phosphorous was found to be above the reference interval and the cat had been fed exclusively a renal specific diet for at least 1 month. Enteric phosphate binders prescribed included aluminum hydroxide[k] or Epakitin[l]. Enteric phosphate binders were given with food twice daily and dose was increased as needed to maintain a serum phosphorous within the reference range. If a cat was previously receiving sub-cutaneous fluids at inclusion in the study this treatment was continued. Cats that were found to be chronically clinically dehydrated, and deemed good candidates by the clinician and owner, were administered sub-cutaneous fluids with the dose determined on a case by case basis. Bacterial urinary tract infections were treated with an appropriate antibiotic based on culture and sensitivity testing. Treatment outcome was based on clinical signs and a repeat bacterial culture within 1 week of discontinuing antibiotic treatment. Uremic crisis and other illness not necessarily related to CKD were treated on a case by case basis at the discretion of the owner and attending clinician. To facilitate compliance all treatment(s) including uremic crisis and illness unrelated to CKD was provided free of charge to the owner.

Cause of death

Renal-related death (RD) was defined as death or euthanasia due to the progressive decline of renal function or the consequences thereof. This classification required documentation of an increased serum creatinine and clinical signs associated with azotemia including but not limited to anorexia, weight loss, gastrointestinal upset, and lethargy. Non-renal-related death (NRD) was defined as a documented cause of death or euthanasia, in which there was not a substantial increase in serum creatinine and/or an unrelated disease process was determined to play a substantial role in the patient's demise. All cause death (ACD) includes both RD and NRD.

Statistical analysis

Statistical analyses were performed on commercial software.[m,n] Descriptive statistics are presented at mean ± SD. Body weight, hematocrit, blood pressure, serum creatinine, and UPC were compared between groups at enrollment with ANOVA. In addition mean time of study enrollment was compared between groups with ANOVA. When appropriate a Sidak *post hoc* correction for multiplicity was used. Change in body weight, hematocrit, blood pressure, serum creatinine, and UPC were compared within groups and between groups over time with repeated measures ANOVA. Cats with less than four hospital visits as participants in the study, only due to recent inclusion into the study, were not included in the statistical analysis. Otherwise all participants were included in the statistical analysis. Significance was set at $p \leq 0.05$.

Target study numbers were based on a pre-study power estimate. It was estimated (based on expected population variance, mean, significant difference, and a within patient correlation of 0.75) that 6 cats in each group would detect a difference in serum creatinine of 0.4 mg/dL, UPC of 0.14, and systolic blood pressure of 10 mmHg at a power of 0.80. In other words there would be a 20% chance of a type II error or failing to detect a significant difference between or within treatment groups when in fact one exists.

Results

A total of 46 cats were evaluated as possible participants in the study. Seventeen of these were excluded because of failure to fulfill inclusion criteria, specifically due to an inappropriate degree of azotemia (n=6 cats), systemic hypertension that required specific medical treatment (n=5), reno- or ureterolithiasis (n=3), illness from disease deemed not to be related to CKD (n=2), and fractious nature (n=1). The remaining 29 cats were enrolled in the study. Three cats that were enrolled in the study were not included in statistical analysis because of having less than four hospital visits as a participant in the study, due to recent inclusion, at the time of writing. Of the remaining cats, 8 cats were allocated to the rhubarb only group (Group 1), 9 cats to the benazepril only group (Group 2), and 9 cats to the rhubarb and benazepril group (Group 3). One cat originally allocated to Group 2 had a presumed severe cutaneous drug reaction to benazepril soon after inclusion into the study. This cat was placed in Group 1 after a 30 day "washout" period.

There were no significant differences between treatment groups at entry into the study for blood pressure, hematocrit, serum creatinine, or UPC (Table 1). There was a significant difference in body weights between group 1 and group 3 (5.65 ± 1.18 kg and 3.49 ± 1.13 kg, respectively; $P = 0.023$). The mean ± SD duration of time that cats were enrolled in the study, at time of statistical analysis for all cats was 17.0 ± 8.3 months (Table 3). The mean ± SD duration of enrollment for Group I, II, and III are 16.8 ± 9.78, 18.9 ± 8.52, and 15.1 ± 6.40 months, respectively (Table 3). There were no significant differences within groups over time for blood pressure, hematocrit, serum creatinine, change in body weight, or UPC (Figures 1-5). There were no significant differences between groups over time for blood pressure, hematocrit, serum creatinine, change in body weight, or UPC (Figures 1-5).

While enrolled in the study five cats died or were euthanized due to renal-related reasons. The number of cats from Group I, II, and III were one, 2, and 2, respectively. An additional 4 cats died or were euthanized unrelated to renal disease. The number of cats from Group I, II, and III were 2, none, and 3, respectively. Cause of death in this group of cats included 2 cats euthanized due to persistent inappropriate elimination, one cat with renal transitional cell carcinoma, and one cat with pancreatic carcinoma. One cat died of unknown causes. Based on contemporaneous blood work and clinical signs this death was determined to be non-renal-related. The number of AD cats in Groups I, II, and III were 3, 2, and 5, respectively. The mean duration between entry into the study and RD, NRD, and AD was 19.0 ± 4.56, 11.6 ± 4.03, and 15.3 ± 5.68 months, respectively (Table 2). Due to the low rate of mortality, survival analysis was not performed.

Discussion

This study is the first to investigate the effects of a commercially available rhubarb extract[a] on the progression of naturally occurring feline CKD. Feline CKD is the result of a predictable renal response to a heterogeneous group of renal insults. Likewise any given group of cats affected with CKD will be widely variable with regards to many parameters including: signalment, comorbidity, degree of renal dysfunction, and the consequences of said renal dysfunction.

The diagnosis of feline CKD is essentially one of exclusion. Azotemia due to ureteral obstruction, renal neoplasia, pyelonephritis, pre-renal or other post-renal causes must be ruled-out. The diagnosis usually includes small irregular kidneys on abdominal palpation and small, irregular kidneys with a loss of corticomedullary definition on abdominal ultrasound. It should be noted that this diagnosis usually does not include renal biopsy. As such it is possible for a specific underlying etiology to go undetected. One cat in this study was found to have renal transitional cell carcinoma on post-mortem examination. Although it is possible that this neoplasm was the cause of renal dysfunction in this patient from the beginning, it is also possible that this cat developed renal transitional cell carcinoma subsequent or in addition to feline CKD. The fact that this cat lived for almost 11 months with essentially stable renal function, that renal imaging was consistent with CKD at inclusion, and post-mortem examination showed both glomerulosclerosis and interstitial nephritis in addition to transitional cell carcinoma make the latter scenario more likely. For these reasons this cat was included in the final statistical analysis.

The results of this study failed to find any significant difference between treatment with rhubarb extract, benazepril, or both in regards to the progression of CKD as quantified by serum creatinine, hematocrit, change in body weight, systemic blood pressure, and UPC. One limitation to this study is that GFR, being a direct measure of renal excretory function was not measured. Instead serum creatinine was used. Serum creatinine indirectly correlates with GFR when certain factors such as body weight are held stable.[179] As a whole group the cats in this study, were expected to lose body weight over time. Although Group 1 cats had a significantly greater body weight than Group 3 cats at inclusion, there was no significant difference in the change in body weight over time between groups, indicating that across groups the effect of the change in body weight as it pertains to serum creatinine concentrations would be similar.

In this study with the use of client owned cats, and with client compliance in mind, repeated measurement of GFR was considered infeasible and unjustified. Other variables such as serum creatinine, change in body weight, hematocrit, systemic blood pressure, and UPC were chosen as these represent variables commonly affected by the loss of renal function.. Body weight is an important clinical variable as a combined indication of appetite and the catabolic

state of chronic uremia. This variable is likewise important to owners as a loss of lean body mass is easily observed and perceived negatively with regards to the patient's quality of life. There were no attempts in this study to further characterize loss of body weight in relation to caloric intake or lean body mass.

Hematocrit was chosen as an indication of renal endocrine function. Although renal excretory function is commonly considered when dealing with chronic kidney disease, endocrine dysfunction in the case of a relative lack of erythropoietin can provide important therapeutic and prognostic information.[15,31] The failure to detect and appropriately treat anemia of CKD leads to lethargy and other signs of systemic illness readily recognized by the owner. It is accepted that anemia associated with CKD is multifactorial. In this study early signs of anemia especially when accompanied by gastric upset were treated with gastric acid suppression usually in the form of famotidine. While this treatment was directed towards gastric erosion or ulceration that may develop secondary to hypergastrinemia no further investigation such as gastric endoscopy was used to corroborate or refute this assumption. In addition bone marrow sampling in order to further investigate non-regenerative anemia was not performed in any cat. For this reason, although assumed to be unlikely, an undiagnosed comorbidity may have affected the hematocrit. An alternative to measuring hematocrit would have been quantifying erythropoietin directly, which would have been interpreted in light of the patient's hematocrit. At the time of this study a commercial erythropoietin assay was not available.

Systemic hypertension is a clinically important sequela- to feline CKD. As such, systemic blood pressure was monitored throughout the study. There is no evidence to support the fact that systemic hypertension is more prevalent as CKD progresses. In addition, there is no evidence that systemic hypertension negatively affects the prognosis associated with feline CKD. In fact there is evidence that any benefit to survival with regards to the control of systemic hypertension is actually related to the subsequent decrease in proteinuria.[30] With that being said the consequences of systemic hypertension including cardiomyopathy and acute blindness may indirectly affect survival in an individual patient. Certainly acute blindness is easily noticed by the owner and may lead to euthanasia. For this reason systemic hypertension that required specific medical management, as defined above, was an exclusion criterion. Making systemic

hypertension an exclusion criterion likely selected for a population of cats that were much less likely to develop systemic hypertension thereafter which may have contributed to finding insignificant changes in mean systemic blood pressure between and within treatment groups over time.

Quantification of urine protein concentration or the UPC provides prognostic information in feline CKD.[15,30,31] As such it would have been reasonable to either stratify or include a specific UPC as an exclusion criterion. The inclusion of proteinuric cats was deemed important as studies concerning rodent models of kidney disease have shown a decrease in urine protein content with the administration of rhubarb extract.[170,177] Cats seem less likely to have overt proteinuria associated with kidney disease when compared to dogs and humans, although this has not been directly investigated in any one study. In the 29 cats included in this study only 8 cats had a UPC > 0.2 and only 1 cat had a UPC > 1.0 at inclusion. It could be postulated that the infrequent severe proteinuria is in part responsible for the slow or clinically silent decline in renal function over long periods of time in this species. In attempts to ensure proteinuria was of renal origin, urine samples with pyuria, bacteriuria, gross hematuria, positive bacterial urine culture, or semen were excluded from statistical analysis.

Another limitation to this study is the length of time cats were enrolled in the study. The mean duration of enrollment for all cats in this study at the time of writing was approximately 17 months. As stated earlier 5 cats died of renal causes and 5 cats died of non-renal causes. At the time of writing, 9 additional cats had withdrawn from the study (not due to death/euthanasia). The most common reason for withdraw was owner relocation, which like most other reasons for study withdraw are inherent to long clinical studies. One cat was removed from the study due to acute ureteral obstruction and acute exacerbation of azotemia. This cat underwent successful treatment with ureterotomy and urolithectomy. At the time of writing, 9 cats were still active in the study. For case recruitment, referring veterinarians received a monetary incentive as did owners. The fact that a portion of the monetary incentive was given after 1 year of enrollment in the study likely improved compliance and participation in the study. Due to the monetary incentives provided, compliance as it relates to the individual treatments could not be

investigated. No attempts were made to assess owner compliance beyond questioning during obtaining historical information at each visit.

As expected, cats with IRIS Stage II or III CKD live a long time. It was for this reason that alternative dependent variables such as serum creatinine and hematocrit were used to assess response to treatment. In many countries, death associated with feline CKD is commonly due to euthanasia, usually due to a perceived poor quality of life.[10,15,16,44] This was shown in this study as all deaths were due to euthanasia. Out of the 5 cats that died due to non-renal-related causes, 2 were euthanized due to persistent inappropriate urination or periuria. It could be argued that this was related to CKD as these cats are polyuric. Other comorbidity, whether related to CKD or not, that might predispose a cat to periuria includes urinary tract infection, osteoarthritis, or cognitive dysfunction. Both cats had multiple negative bacterial urine cultures making bacterial cystitis an unlikely cause. Osteoarthritis was believed to be present in one case based on crepitus during joint manipulation. This cat failed to improve upon provision of an additional litter box that did not require a step-up and medical treatment for osteoarthritis. It was possible that osteoarthritis or the reluctance to step-up into the litter box initiated the periuria which then became behavioral, in which case treatment of the osteoarthritis may not lead to improvement. Cognitive dysfunction was believed to play a role in the second cat's periuria although this was at best speculative. Because other disease likely played a substantial role in the periuria and subsequent euthanasia in both cats they were classified as non-renal-related deaths. To investigate the effect of treatment as it relates to renal-related death in a study of this nature would require a substantially longer study duration, larger study population, or inclusion of cats with more advanced kidney disease.

It should be noted that not finding a significant difference between treatment groups is only notable with an appropriately powered study. Pre-study power estimates showed that including 6 cats in each group would provide a power of 0.80 for the detection of a 0.4 mg/dL difference in serum creatinine, a 0.14 difference in UPC, or a 10 mmHg difference in systemic blood pressure. Differences in these variables to this degree were considered clinically relevant. This was assuming an intra-cat correlation over time of 0.75. This study consisted of at least 8 cats per treatment group. As such there is expected to be at least an 80% chance that if a

significant difference existed it would have been detected. With a β of anything but 1.0 there will always be a chance of not detecting a significant difference when one truly exists. When considering the comparison between two or more treatments, the only way of increasing β is to increase study number or decrease population variance (considering a certain dependent variable). To allow this population of cats to represent the domestic cat population as a whole, a certain degree of variability was necessary. There was no attempt to make post-study power estimates.

It has been suggested that an ACEi may slow the progression of feline CKD, although the evidence is minimal.[20,21] As previously discussed there is certainly evidence in other species that this is the case.[106,156,180-182] Not finding a significant difference between treatment groups would indicate that in this population of cats both benazepril and rhubarb extract affected the progression of CKD to the same degree (or possibly not at all). Rhubarb extract has been shown to decrease proteinuria in experimentally induced kidney disease in various rodents.[170,177] Alhough proteinuria was ucommon, the authors felt it was unethical to withhold appropriate treatment of proteinuria, acknowledging that evidence supporting the use of an ACEi in feline CKD remains minimal. In addition there is evidence that the therapeutic effects of an ACEi and rhubarb as they affect proteinuria and renal fibrosis act synergisticly.[170,177] For these reasons benazepril was chosen as the positive control and a third group of cats administered both drugs was included in the study. Without a negative control it is impossible to make any statement with regards to how either treatment compared to no treatment at all. Because neither owners nor investigators were blinded to the treatment groups some treatment bias is expected. The objective measures of kidney function or consequences thereof, such as serum creatinine, hematocrit, change in body weight, systemic blood pressure or UPC likely were not affected by this bias. Alternatively, it is safe to assume that the owner's perceived quality of life and the knowledge of lab values may have influenced the decision to euthanize for renal or non-renal related reasons.

Possible side effects of the rhubarb extract and benazepril were noted at each hospital visit as determined by historical findings provided by the owner and physical exam and laboratory findings. Two cats in Group 1 had reported possible side effects that included

transient lethargy and gastrointestinal upset. One cat in Group 2 had what appeared to be a severe cutaneous drug reaction which manifested as widespread erythema, pruritis, and alopecia most severe on the ventrum. Skin scrape and cytology were unremarkable. No further diagnostic investigation such as skin biopsy was performed. The apparent cutaneous reaction mostly resolved over the next 2 weeks after discontinuation of benazepril administration. Because this cat fit all inclusion criteria and had begun the study, at the owners request the cat was switched to Group 1. The fact that this group change contradicted the goals of random allocation was recognized, although the effect to the overall randomness of the study was considered minor. A total of 17 cats in this study received rhubarb extract for a mean duration of 15.9 ± 7.7 months. The fact that only 2 cats showed mild findings that were possibly associated with rhubarb administration would indicate that Rubenal® is safe to administer over a long period of time to cats with stage II or III CKD. It should also be noted that only one owner reported difficulty in administering the Rubenal® tablet.

Conclusion

The commercially available Chinese rhubarb extract (Rubenal®) is safe when administered to cats with Stage II or III CKD. In the studied population of cats, based on the analysis of easily measured clinical indicators of renal exocrine and endocrine function, this study failed to find a significant difference in the progression of feline CKD between cats administered oral benazepril, Rubenal®, or both at recommended doses. Further investigation into the effects of rhubarb extract as it relates to specific subsets of the cat population affected with CKD and as compared to no treatment may be indicated.

Footnotes

a. Rubenal, Vetoquinol USA, Fort Worth, TX

b. k/d, Hill's Pet Nutrition, Topeka, KS

c. Benazepril, Teva Pharmaceuticals USA, North Wales, PA

d. E.R.D. Health Screen Urine Tests, Heska, Loveland, CO

e. Ultrasonic Doppler Flow Detector, Parks Medical Electronics Inc., Aloha, OR

f. Bili Labstix, Bayer Healthcare LLC., Morristown, NJ

g. Hitachi 911, Roche Diagnostics, Indianapolis, IN

h. NF Kidney Function, Purina Veterinary Nutrition, St. Louis, MO

i. Renal LP, Royal Canin USA, St. Charles, MO

j. Amlodipine, Mylan Pharmaceuticals Inc., Canonsburg, PA

k. Aluminum hydroxide, Rugby Laboratories Inc., Corona, CA

l. Epakitin, Vetoquinol USA, Fort Worth, TX

m. STATA 11, STATA Corp LP, College Station, TX

n. SAS on Demand, SAS, Cary, NC

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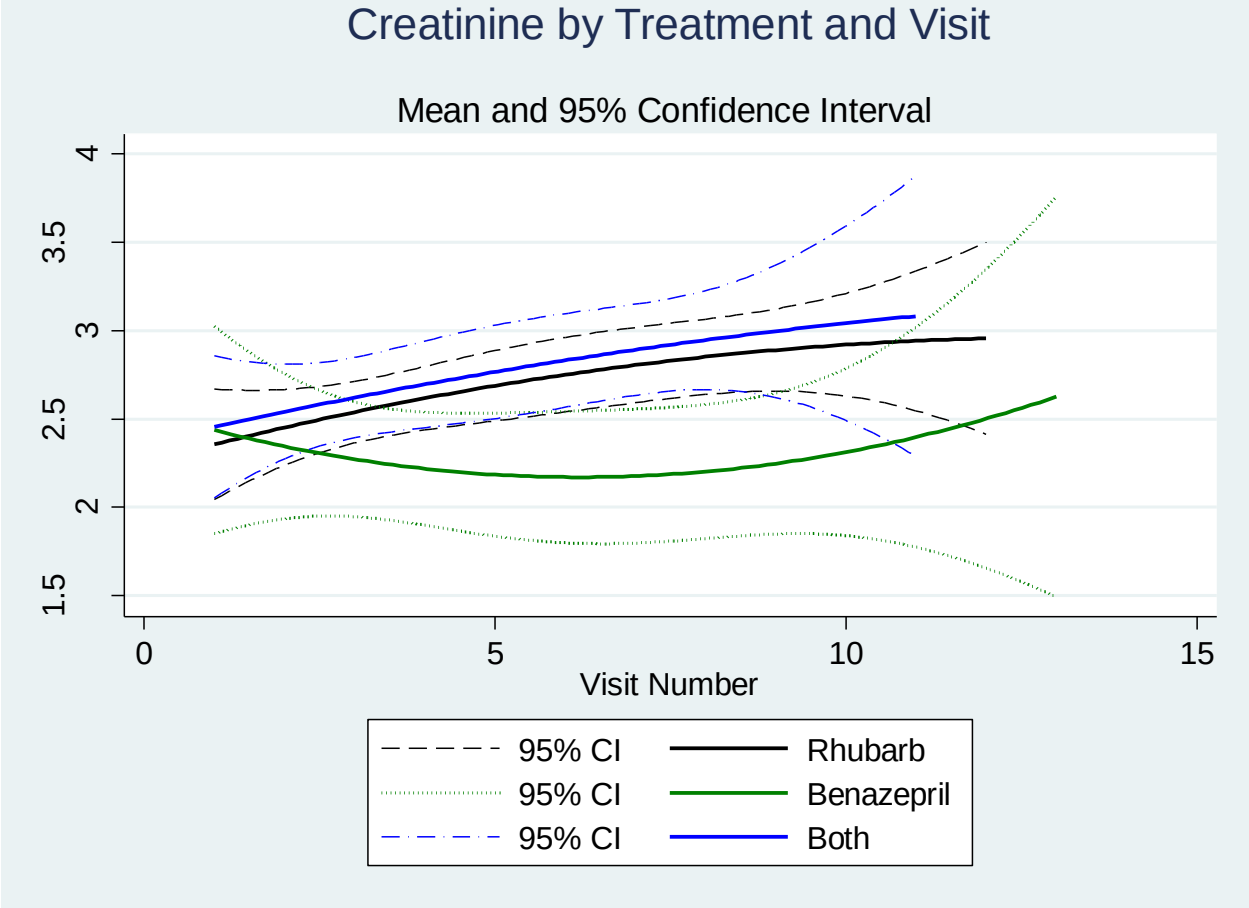
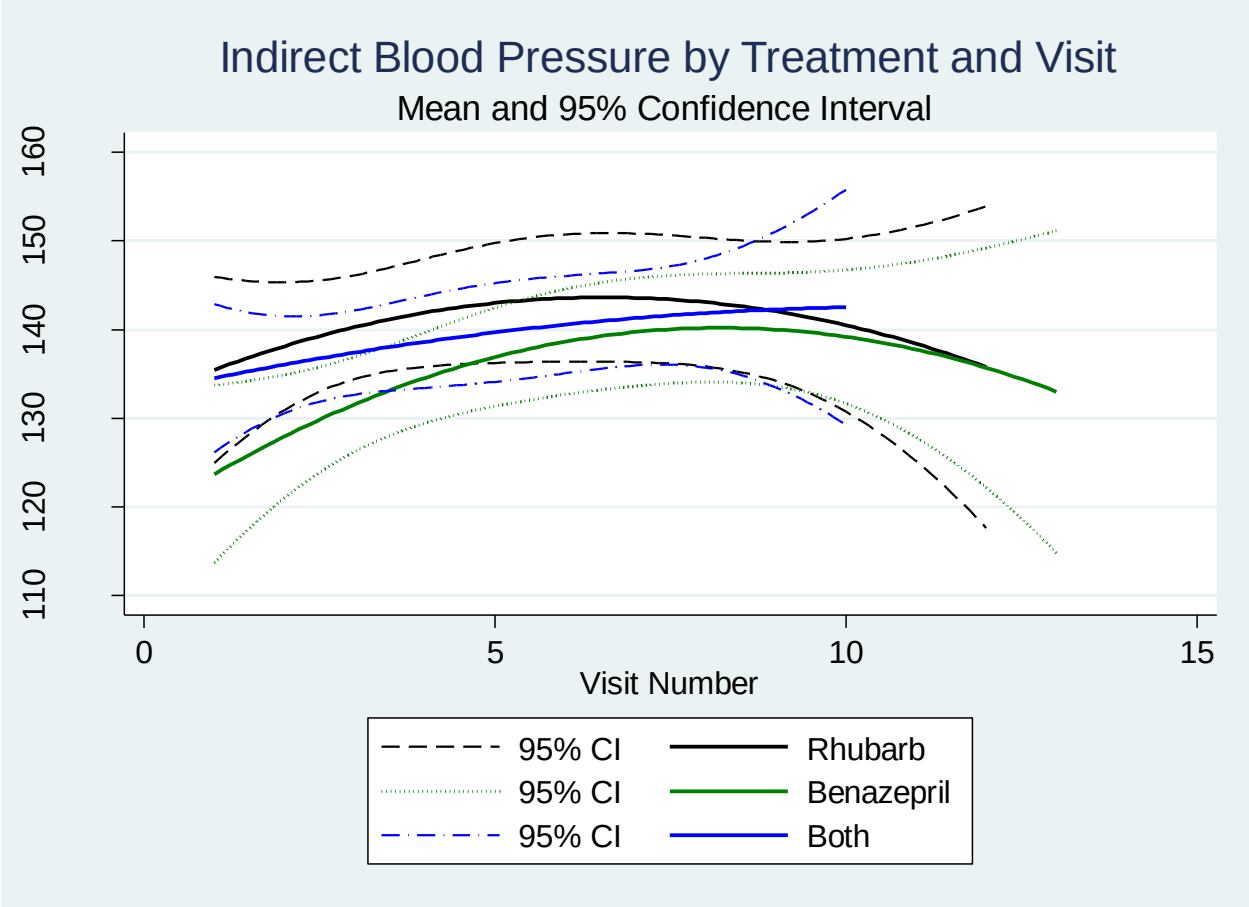
Figures

Figure 1. Serum creatinine by treatment and visit.



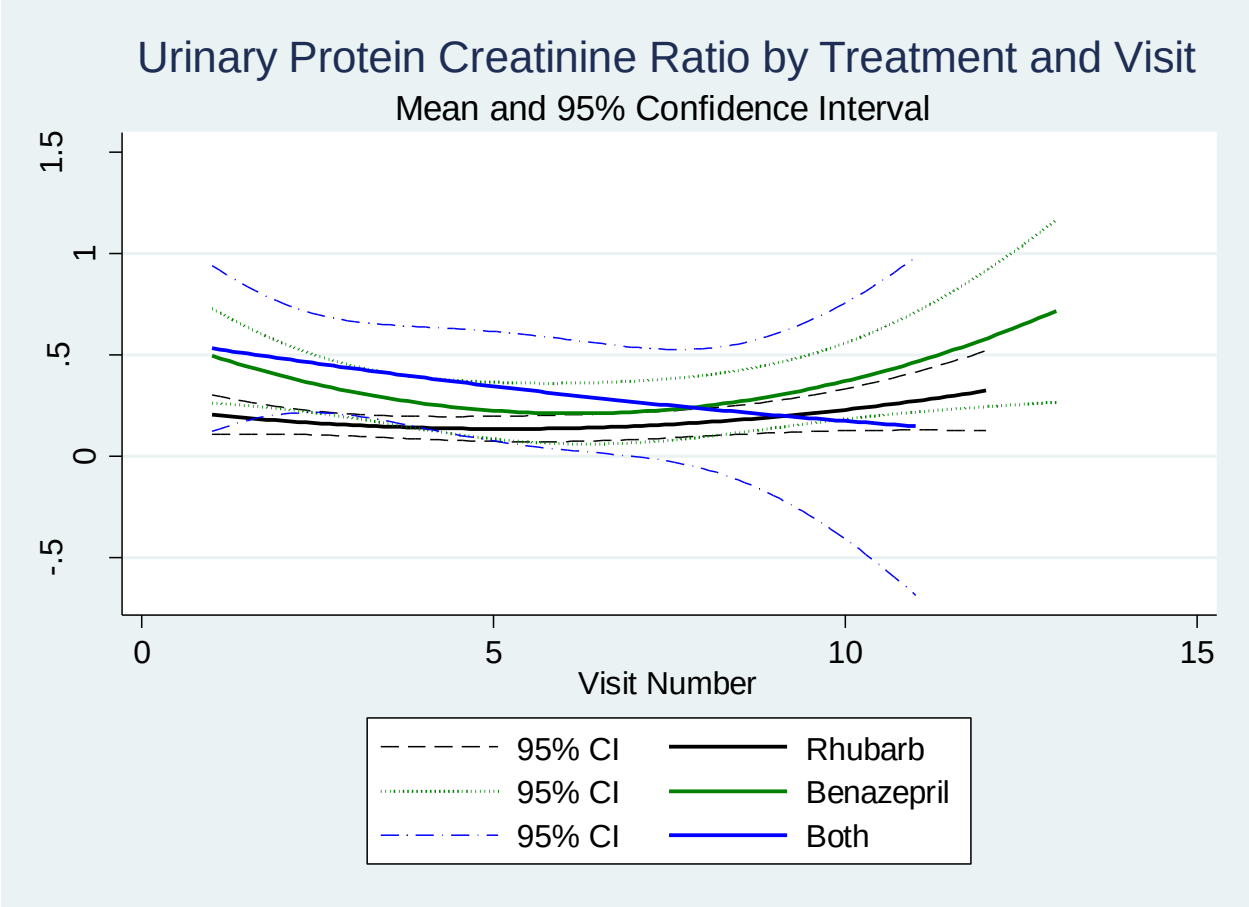
A chart of serum creatinine concentrations (mg/dL) over time. Solid lines are group means and dotted/dashed lines are 95% confidence intervals. There was not a significant difference over time between or within groups.

Figure 2. Indirect blood pressure by treatment and visit.



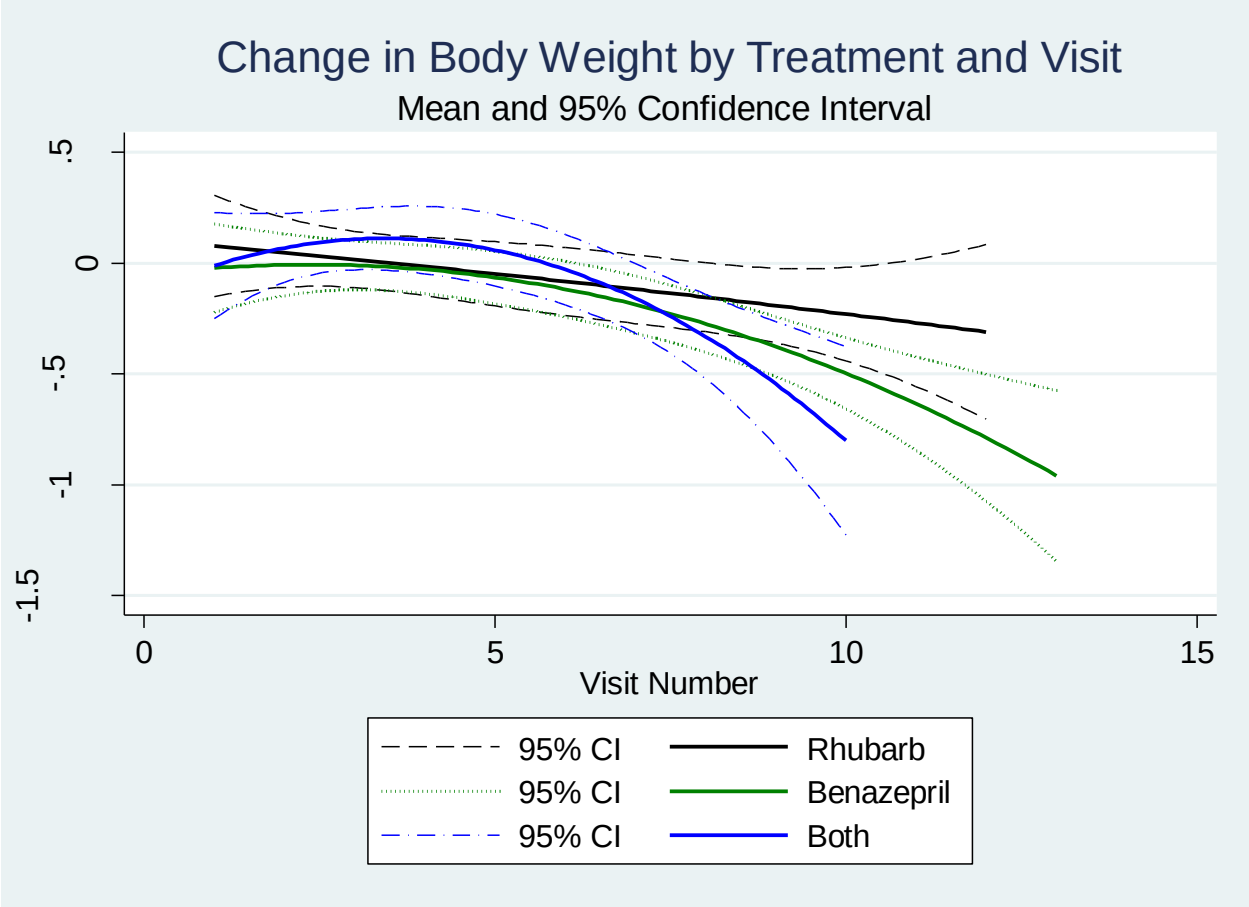
A chart of systolic blood pressure over time. Solid lines are group means and dotted/dashed lines are 95% confidence intervals. There was not a significant difference over time between or within groups.

Figure 3. Urine protein creatinine ratio by treatment and visit.



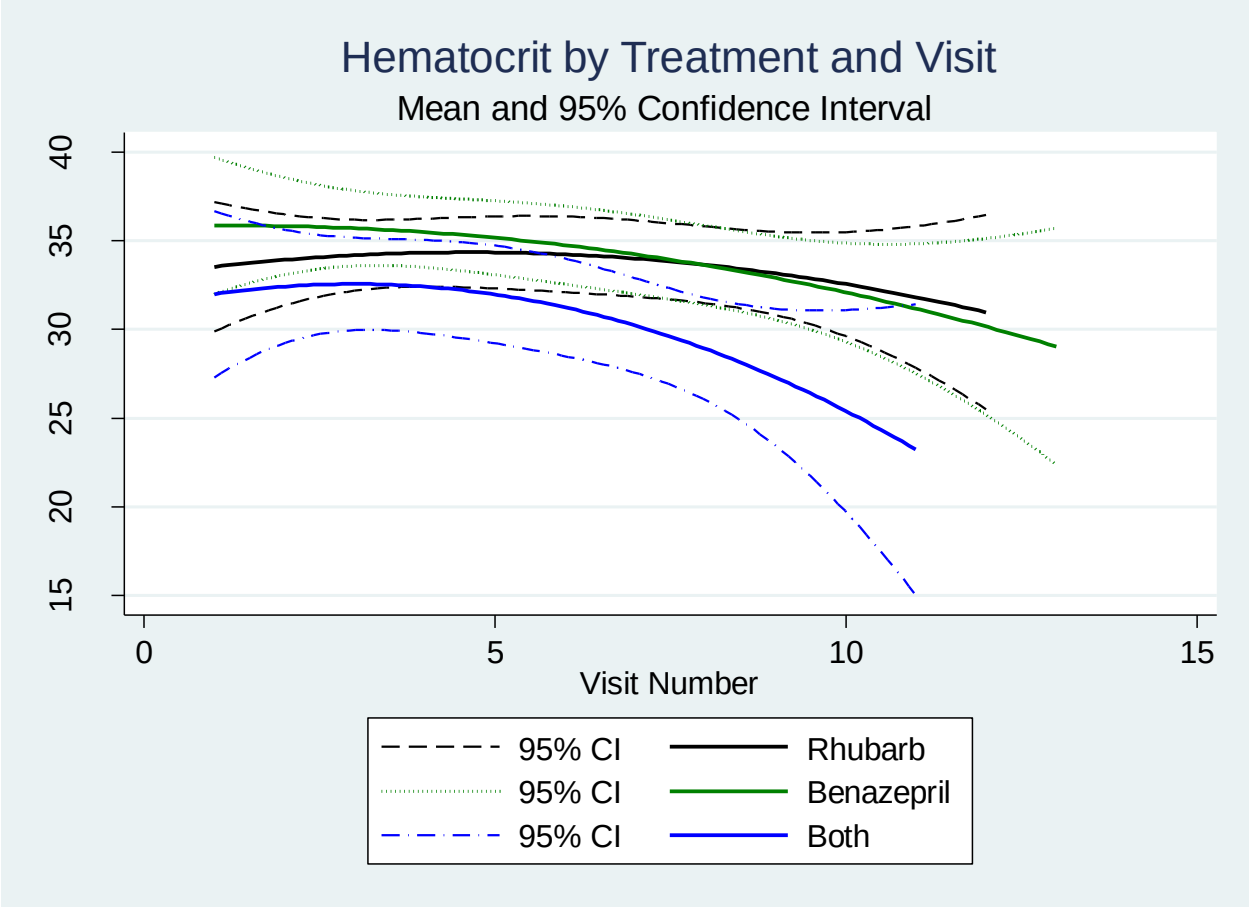
A chart of UPC over time. Solid lines are group means and dotted/dashed lines are 95% confidence intervals. There was not a significant difference over time between or within groups.

Figure 4. Change in body weight by treatment and visit.



A chart of change in body weight (kg) over time. Solid lines are group means and dotted/dashed lines are 95% confidence intervals. There was not a significant difference over time between or within groups.

Figure 5. Hematocrit by treatment and visit.



A chart of change in body weight over time. Solid lines are group means and dotted/dashed lines are 95% confidence intervals. There was not a significant difference over time between or within groups.

Tables

Table 1. Select parameters at study inclusion

Variable	Overall mean ± SD	*P*-value
Serum creatinine (mg/dL)	2.43 ± 0.66	0.887
Indirect blood pressure (mmHg)	129 ± 16.5	0.173
UPC	0.54 ± 1.11	0.656
Hematocrit (%)	34.5 ± 5.91	0.390
Body weight (kg)	4.67 ± 1.65	0.022
Group 1 vs. Group 3		0.021
Group 1 vs. Group 2		0.592
Group 2 vs. Group 3		0.125

There were no difference between groups at entry into the study for serum creatinine, indirect blood pressure, UPC, or hematocrit. Group 1 cats weighed significantly more than Group 3 cats.

Table 2. Duration time to death

Cause of death	Overall time from inclusion to death (months, mean ± SD)
Renal related death (RD)	19.0 ± 4.56
Non-renal related death (NRD)	11.6 ± 4.03
All cause death (AD)	15.3 ± 5.68

The overall time from inclusion into the study to death for all cats included in the study. Due to a relatively low mortality rate statistical analysis was not attempted.

Table 3. Duration of enrollment

Group	Duration (months, mean ± SD)	*P*-value
Group 1 (rhubarb only)	17.0 ± 9.78	0.631
Group 2 (benazepril only)	18.9 ± 8.52	
Group 3 (both)	15.11 ± 6.39	
All groups	17.0 ± 8.28	

There was no significant difference in enrollment time between groups.

Appendix A – IRIS Staging Schemes

a. IRIS staging scheme for feline CKD*[5]

IRIS Stage	Plasma Creatinine (mg/dL)	Comments
I	<1.6	No clinical signs
II	1.6-2.8	Usually no clinical signs
III	2.9-5.0	May have clinical signs
IV	>5.0	Clinical signs present

*Proteinuria and systemic hypertension or lack of is noted as sub-stages.

b. IRIS systemic blood pressure risk scheme[5]

IRIS Stage	Systolic BP (mmHg)	Diastolic BP (mmHg)
0 – minimal risk	<150	<95
1 – low risk	150-159	95-99
2 – moderate risk	160-179	100-119
3 – high risk	>180	>120

c. IRIS proteinuria staging scheme[5]

UPC Value	Sub-stage
<0.2	Non-proteinuric (NP)
0.2-0.4	Borderline proteinuric (BP)
>0.4	Proteinuric (P)