

Effect of repeated administration of a parenteral feline herpesvirus-1, calicivirus, and panleukopenia virus vaccine on select clinicopathologic, immunological, renal histologic, and immunohistochemical parameters in healthy adult cats

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OBJECTIVE

To assess whether hyperinoculation of cats with a feline herpesvirus-1, calicivirus, and panleukopenia virus (FVRCP) vaccine could be used as a model to study interstitial nephritis and to assess humoral and cell-mediated immune responses toward vaccinal α -enolase.

ANIMALS

6 healthy young adult purpose-bred research cats.

PROCEDURES

Baseline renal cortical biopsies, whole blood, serum, and urine were collected prior to administration of a commercial FVRCP parenteral vaccine. Vaccine hyperinoculation was defined as a total of 8 vaccinations given at 2-week intervals over a 14-week period. Blood samples were collected immediately prior to each vaccination, and a second renal biopsy was performed 2 weeks after hyperinoculation (week 16). Renal histopathology, renal α -enolase immunohistochemistry, and assays to detect humoral and cell-mediated immune reactions against Crandell-Rees feline kidney (CRFK) cell lysates and α -enolase were performed. An α -enolase immunoreactivity score for renal tubules and glomeruli based on signal intensity was determined by a blinded pathologist.

RESULTS

Hyperinoculation with the vaccine was not associated with clinicopathologic evidence of renal dysfunction, and interstitial nephritis was not recognized by light microscopy in the time studied. The mean serum absorbance values for antibodies against CRFK antigen and α -enolase were significantly ($P < 0.001$) higher at weeks 4, 8, and 16 versus week 0. Renal tubular and glomerular α -enolase immunoreactivity scores were higher at week 16 compared to baseline.

CLINICAL RELEVANCE

Findings suggested that systemic immunological reactions occurred and renal tissues were affected by vaccine hyperinoculation; however, short-term FVRCP vaccine hyperinoculation cannot be used to study interstitial nephritis in cats.

Chronic kidney disease (CKD) is an irreversible and progressive disease with prevalence up to 80.9% in cats greater than 10 years of age.¹ On renal histology, chronic lymphoplasmacytic tubulointerstitial nephritis and fibrosis are the most common findings.² In many cases, an underlying etiology is not identified, and the disease is deemed idiopathic.³ In addition to age and severe periodontal disease, annual or frequent vaccination was found to be a risk factor for the development of azotemic CKD in cats.^{4,5}

Many organizations, like the American Association of Feline Practitioners and the World Small

Animal Veterinary Association, recommend administration of feline herpesvirus-1 (FHV-1)-, feline calicivirus (FCV)-, and feline panleukopenia virus (FPV)-containing core vaccines (FVRCP) to all cats.⁶ The goal of vaccination is to provide adequate immunization to protect against important pathogens, but not lead to inadvertent significant adverse effects.⁶ As an epidemiological association between frequent vaccination of cats and CKD exists, further research is needed to determine the level of concern. In addition, a model to induce renal inflammation, like that found in most cats with CKD, is greatly needed to

study ways to more accurately diagnose, treat, and prevent the disease.

All FVRCP vaccine (both parenteral and intranasal) viruses are grown in mammalian cell lines and the Crandell-Rees feline kidney (CRFK) cell line is frequently used.⁷ During virus purification for vaccine production, it is impossible to remove all the cell line proteins and thus low concentrations are likely contained in the vaccines. Therefore, immunized cats are exposed to cell line antigens and mount an immune response.^{8,9} It is possible that these proteins are associated with the development of CKD in cats. The CRFK cell line has been evaluated in studies^{8,10} attempting to determine why frequent vaccination could potentially be linked to CKD in cats. In short, hyperinoculation of healthy purpose-bred cats with a CRFK cell lysate (13 times over 2 year period) resulted in lymphoplasmacytic interstitial nephritis in 3 of 6 cats, and cats vaccinated 5 times over a 56-week period with an intranasal FVRCP vaccine had no such effects.¹⁰ Researchers⁸ also showed that hyperinoculated cats with a CRFK cell lysate or parenteral vaccine developed antibodies against the CRFK cell lysates as well as against a lysate of feline renal tissues.

In a subsequent study,⁹ researchers identified by protein sequencing an immunodominant antigen α -enolase that induced antibodies against the CRFK lysates in cats, and found that cats administered parenteral FVRCP vaccines had rapidly increasing absorbance values to α -enolase. This glycolytic enzyme is widely distributed in the body and found in greatest concentration in the kidney and thymus.⁹ Because α -enolase is present in all mammalian cells, all FVRCP vaccines for cats are likely to have some level of α -enolase contamination. Based on these findings, α -enolase was chosen for continued study, including in the study described here.

In healthy humans, renal α -enolase is found primarily in the epithelial cells of the tubules and nearly undetectable in the glomeruli.¹¹ Within kidney cells, α -enolase is found in the cytoplasm and cell membrane.¹² Recently, the distribution of α -enolase in cats with and without kidney disease has been described using a α -enolase immunohistochemical stain on necropsy tissue specimens.¹³ In young cats (< 2 years), α -enolase immunoreactivity was found in the tubules and absent in the glomeruli. In senior cats (>10 years), α -enolase immunoreactivity was found in both the tubules and glomeruli. In cats with CKD, α -enolase immunoreactivity was decreased in atrophic tubules, similar to healthy cats in normal tubules, and increased in the glomeruli. The data suggested that α -enolase changes distribution in the kidney prior to development of CKD in cats.¹³

Since interstitial nephritis was noted in the previous CRFK lysate hyperinoculation study,¹⁰ we believed it reasonable to explore derivations of this model further, but within a more compressed time schedule. The primary aim of the study reported here was to determine if hyperinoculation with a market-leading FVRCP vaccine over a 16-week period would induce renal histological changes consistent with

feline interstitial nephritis and induce changes to the distribution of α -enolase as a pilot study to assess the protocol as a model to study the disease. The secondary aim was to determine whether the hyperinoculated cats would develop humoral and cell-mediated immune reactions against CRFK lysates and α -enolase.

Materials and Methods

This was a prospective study. A schematic of the study design and vaccine hyperinoculation schedule over the 16-week study period is shown (**Figure 1**). A control, unvaccinated group of cats was not used.

Selection of animals

Young (3-month-old) cats (3 males and 3 females) were purchased from an FHV-1-, FCV-, and FPV-free barrier facility. Prior to entering the study at 1 year of age, the cats were group housed in a barrier facility and the males were castrated. The cats were previously used as unvaccinated controls in an FHV-1 inoculation study at 5 months of age. In that study,¹⁴ the cats developed mild signs of FHV-1 infection but were normal with no clinical signs of upper respiratory infection at enrollment in the current study.

The cats were housed and cared for in accordance with a protocol that was approved by the Institutional Animal Care and Use Committee at the contract research facility that was used for the study (protocol No. 170.016). The approval included a rescue clause that allowed for vaccinations to be discontinued in any cat that developed a creatinine concentration of greater than 2.0 mg/dL, inappetence, vomiting, or signs of systemic hypersensitivity reactions. All cats were sedated with ketamine (10 mg, IV, once) for blood draws and cystocentesis procedures using the facility protocol.

Peripheral blood mononuclear cells (PBMCs) processed from whole blood collected from two 3-year-old, sexually intact female, specific-pathogen-free, unvaccinated control cats from a purpose-bred research colony were used as control samples in a lymphocyte proliferation assay. In addition, kidney

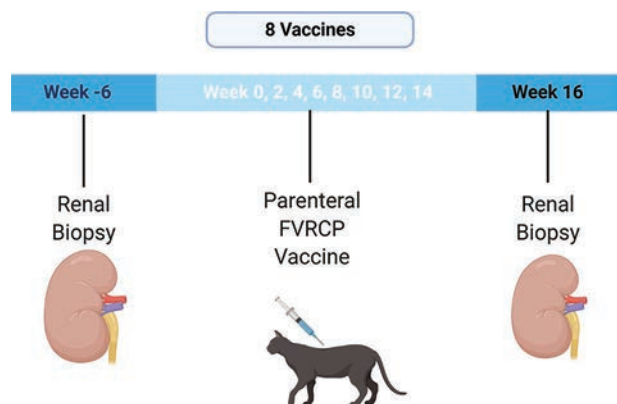


Figure 1—Schematic overview of the study timeline.

and spleen samples were collected during necropsy from a healthy young adult (approx 3 years of age) castrated male client-owned cat at the time of necropsy with owner consent; antigen collected from the tissue samples was used in a lymphocyte proliferation assay.

Vaccine

Measurement of total protein concentration after reconstitution was performed using a commercially available BCA protein assay (Pierce BCA Protein Assay Kit, Thermo Fisher Scientific Inc) on 5 market-leading FVRCP parenteral vaccines (Novibac Feline 3-HCP, Merck Animal Health Inc; Felocell 3, Zoetis Inc; Fel-O-Vax PCT + Calicivax, Boehringer-Ingelheim Vetmedica Inc; Ultra Fel-O-Vax FVRCP, Boehringer-Ingelheim Vetmedica Inc; and Purevax Feline 3, Merial Inc). A single aliquot from a single vaccine of each type was used for determination. The vaccine with the highest total protein concentration was used in the study (Felocell 3, Zoetis Inc).

Sample collection

Six weeks prior to vaccine hyperinoculation, the cats were anesthetized, and the 3 females were ovariectomized (male cats were castrated prior to enrollment in the study). A wedge of renal cortical tissue (5 mm wide X 3 mm deep X 3 mm long) from the left kidney was surgically obtained from each cat either during the ventral celiotomy (in females) or by flank approach (in males). Six weeks after surgery (week 0), the cats were administered the parenteral vaccine subcutaneously and then boosted on weeks 2, 4, 6, 8, 10, 12, and 14. On week 16, renal biopsies were performed on the right kidney as described via flank approach in all study cats. All cats were administered oral buprenorphine for 3 days after each surgery for postoperative pain control. Renal tissues were fixed in neutral buffered 10% formalin solution, paraffin embedded, and sectioned at 5 μ m.

Blood was collected via jugular venipuncture prior to vaccine administration on weeks 0, 2, 4, 6, 8, 10, 12, and 14 and on week 16. Blood was immediately placed into a sterile tube without anticoagulant. All samples were stored on ice after collection until processing within 3 hours of collection. Non-heparinized blood was centrifuged at 2,500 rpm for 10 minutes, and the serum was removed. The serum was aliquoted and stored at -80°C . At the time of blood collection, urine was collected by cystocentesis on weeks 0, 8, and 16. A serum aliquot and urine samples were submitted for serum biochemical panel, urinalysis, and urine protein-to-creatinine ratio at weeks 0, 8, and 16.

Histologic evaluation and enolase immunohistochemistry

The renal biopsy paraffin sections were stained with H&E stain, and α -enolase immunohistochemistry was performed as previously described.¹³ Each tissue section was immunohistochemically stained with H&E and α -enolase (1 from each cat 6 weeks

prior to vaccination and on week 16) and evaluated for histopathological evidence of interstitial mononuclear cell inflammation and for α -enolase immunoreactivity by a board-certified pathologist blinded to the timing of the biopsies. For α -enolase immunoreactivity, each tissue section was assigned a score based on signal intensity. Scoring was defined as 0 = no staining, 1 = light brown/tan, 2 = moderate brown staining, and 3 = dark brown, which may obscure the nucleus. Ten high-power fields (40X) were selected from a grid for each section of kidney. Twenty renal cortical tubules and twenty glomeruli from each section were scored. Tubules in the cortical labyrinth region that had morphologic features consistent with proximal tubules were scored.

Anti-CRFK and anti-enolase ELISA

Two indirect ELISAs were used to measure the absorbance values of anti- α -enolase and anti-CRFK antibodies as previously described.^{8,9} Sera from the 6 cats prior to vaccination on week 0 and during the 16-week study period (weeks 4, 8, 12, and 16) were assessed in the assays.

PBMC isolation and lymphocyte proliferation assay

To evaluate the cell-mediated immune response toward α -enolase and CRFK antigen, a lymphocyte proliferation assay was performed using PBMCs. Eight weeks after vaccine hyperinoculation, whole blood was collected from 4 of 6 experimental cats. Whole blood collected from 2 unvaccinated purpose-bred cats was used as a control in the assay. The PBMCs were separated from EDTA-treated whole blood via Ficoll centrifugation (LSM lymphocyte separation medium, MP Biomedicals Corp) and washed with PBS solution. Cells were pelleted by centrifugation at 1,200 rpm for 5 min at 8°C . The medium was removed, and the cells were plated in a 96-well plate at a concentration of 5×10^5 cells/well. Then, 5-ethynyl-2'-deoxyuridine was added to each well at a final concentration of 1 μM . Fresh kidney and spleen samples were collected during necropsy from a young, healthy cat. The following experimental groups were used (in duplicate): PBMC control; PBMC and concanavalin A (ConA; Sigma-Aldrich); PBMC and α -enolase antigen; PBMC and CRFK antigen; PBMC and splenic cells; and PBMC and kidney cells. Cells were incubated for 72 h at 37°C , after which time cells were harvested, washed, and then resuspended in FACS buffer (2% fetal bovine serum, 0.05% sodium azide, 1X PBS solution). The amount of lymphocyte proliferation was analyzed via a Cyan ADP flow cytometer (Beckman Coulter Inc). For comparison, lymphocytes stimulated with ConA were set to 100% proliferation.

Statistical analysis

Statistical analysis was performed using statistical software (RStudio version 8.3.0). Normality of data were assessed by the Shapiro-Wilk normality test. Serum kidney values (creatinine and BUN) and anti-enolase and anti-CRFK serum absorbance levels

were compared between week 0 and week 16 using the paired Student *t* test for parametric data and Wilcoxon matched-pairs signed rank test for non-parametric data. Mean absorbance values for serum anti-CRFK and anti- α -enolase antibodies at weeks 4, 8, and 12 were compared to week 0 using a repeated measures 1-way ANOVA with Holm-Sidak multiple comparisons test. The mean tubular and glomerular immunohistochemical stain scores were compared between week 0 and week 16 using the Wilcoxon matched-pairs signed rank test (1-tailed). Statistical significance for all analyses was set at $P < 0.05$.

Results

Kidney clinicopathologic values

No cats developed clinicopathologic evidence of kidney disease during the study period based on urinalyses and biochemical profiles. Serum creatinine for each cat remained within the reference range (0.6 to 1.6 mg/dL) and did not significantly ($P = 0.13$) differ after hyperinoculation at week 16 (median, 1.2; range, 0.9 to 1.4 mg/dL) when compared to week 0 (median, 1.4; range, 1.0 to 1.4). The group serum BUN concentration was significantly ($P = 0.03$) lower at week 16 (median, 26; range, 23 to 29 mg/dL) compared to week 0 (median, 32; range, 25 to 34 mg/dL). For all samples collected during the study period,

the urine specific gravity remained above 1.045, urine protein-to-creatinine ratios were within reference range (< 0.15), and no urine casts suggestive of tubular injury were found on urine microscopy. Other biochemical parameters, including serum phosphorus, sodium, potassium, and globulin concentrations, remained within the reference range throughout the study period for all 6 cats.

Histological evaluation

Histological evidence of interstitial nephritis was not detected by light microscopy in any of the tissue biopsies collected 6 weeks prior to vaccine hyperinoculation or 2 weeks after vaccine hyperinoculation on week 16. Renal tubular and glomerular α -enolase immunoreactivity scores were significantly ($P = 0.02$) higher at week 16 (tubules: median, 1; range, 1 to 2; glomeruli: median, 2; range, 1 to 3) when compared to baseline (tubules: median, 0; range, 0 to 1; glomeruli: median, 1; range, 0 to 2; **Figure 2**).

Anti-CRFK and anti-enolase ELISA

Prior to vaccination on week 0, the mean serum absorbance values for anti-CRFK and anti- α -enolase antibodies were low and like the negative control. The mean serum absorbance values for antibodies against CRFK antigen and α -enolase were significantly ($P < 0.001$) higher at weeks 4, 8, and 16 versus week 0 (**Table 1**).

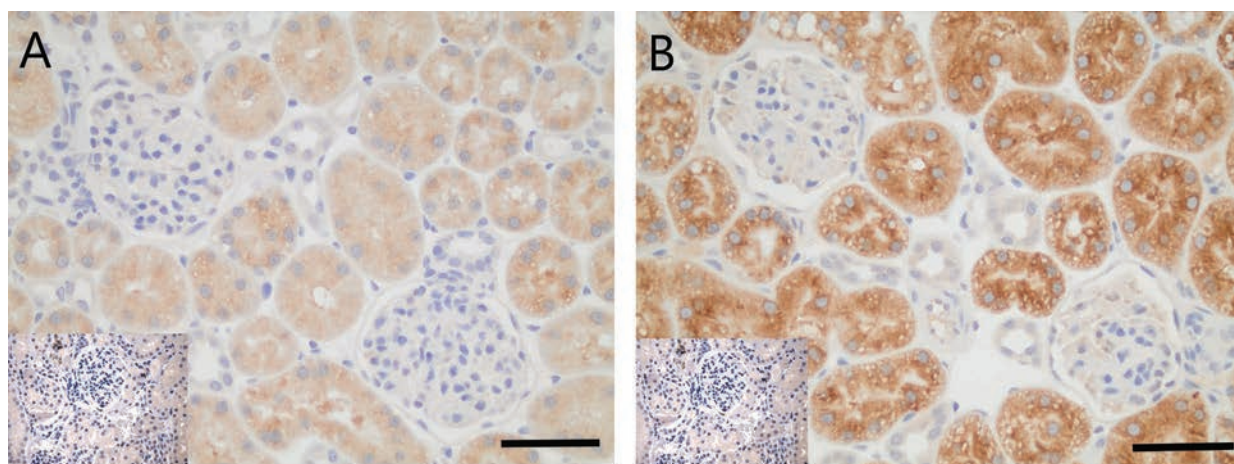


Figure 2—Representative photomicrographs showing α -enolase immunohistochemistry in a kidney section from a healthy research cat prior (A) and on week 16 after hyperinoculation (B) with a feline herpesvirus-1, calicivirus, and panleukopenia virus (FVRCP) vaccine. A—There is minimal staining in glomeruli and mild, monochromatic staining of tubules. B—There is minimal to mild staining in glomeruli with moderate, monochromatic staining of tubules. Bar = 50 μ m. Inset—negative control specimen.

Table 1—Mean \pm SD absorbance values for serum antibodies against α -enolase and Crandell-Rees feline kidney (CRFK) cell antigen prior to vaccination (week 0) and after vaccination at weeks 4, 8, 12, and 16 with a parenteral feline herpesvirus-1, calicivirus, and panleukopenia virus vaccine in 6 cats.

Antibody	Week 0	Week 4	Week 8	Week 12	Week 16
Anti- α -enolase	0.57 \pm 0.18	2.00 \pm 0.81	3.48 \pm 0.79	3.82 \pm 0.52	3.88 \pm 0.47
Anti-CRFK	0.28 \pm 0.04	0.66 \pm 0.25	1.38 \pm 0.43	1.63 \pm 0.41	1.81 \pm 0.44

Absorbance values for anti- α -enolase and anti-CRFK antibodies were significantly ($P < 0.001$) higher at weeks 4, 8, 12, and 16 versus week 0.

Lymphocyte proliferation assay

Lymphocytes from hyperinoculated cats proliferated significantly more in the presence of α -enolase compared to the unvaccinated control cats ($P = 0.03$). No significant difference in lymphocyte proliferation between hyperinoculated study cats and unvaccinated control cats was found for the feline spleen, kidney, and CRFK cell antigen.

Discussion

This study evaluated whether hyperinoculation of young adult cats with a commercial FVRCP vaccine over a 14-week period induces histological changes consistent with interstitial nephritis as a preliminary investigation. The purpose was to assess the protocol as a model to study the disease in cats as well as to continue to assess vaccine involvement with kidney disease in cats. Inflammatory cells on renal histology consistent with interstitial nephritis were not detected by light microscopy, and results of the serum biochemical panels and urinalyses remained within reference range during the period studied. However, the tubular and glomerular α -enolase immunoreactivity scores increased significantly in the renal biopsies obtained 2 weeks after hyperinoculation on week 16 compared to baseline renal biopsies in these cats. The results of the study described herein suggest that hyperinoculation was associated with changes in α -enolase immunoreactivity at the renal tissue level but did not cause clinicopathologic or histologic changes consistent with interstitial nephritis.

In addition, this study aimed to assess the immune response toward α -enolase contained in the FVRCP vaccine. Results of the indirect ELISA and lymphocyte proliferation assays confirmed that hyperinoculation with a parenteral FVRCP vaccine over a 14-week period leads to a humoral immune response against α -enolase and CRFK cell proteins and to a cell-mediated immune response against α -enolase. The serological results were similar to what has previously been reported⁸⁻¹⁰ after parenteral administration of FVRCP vaccines. However, to the authors' knowledge this is the first study to document that cell-mediated immune responses against α -enolase occurs after FVRCP vaccine hyperinoculation. In people, α -enolase autoantibodies are nephrogenic by inducing endothelial cell injury and cell death through an apoptotic process.¹⁵⁻¹⁷ Anti- α -enolase antibodies are detected in 67% to 80% of people with autoimmune nephritis associated with systemic lupus erythematosus (SLE) and approximately 70% of people with membranous nephropathy, and are rarely documented in healthy controls.¹⁸⁻²⁰ The significance of vaccines sensitizing lymphocytes to α -enolase antigen and inducing the formation of anti- α -enolase antibodies in cats is unknown, and a direct link between α -enolase autoantibodies and kidney injury in cats has not been documented.

We found that tubular and glomerular α -enolase immunoreactivity scores increased significantly in the week 16 renal biopsies obtained 2 weeks after

hyperinoculation compared to the baseline renal biopsies. Similar to findings^{11,13} in young adult cats and cats > 10 years of age without CKD, normal human kidneys have α -enolase in tubules and almost undetectable α -enolase in glomeruli based on immunohistochemistry. In humans, renal biopsies from SLE patients have increased α -enolase enzyme expression in tubules and have some expression in the glomeruli in variable sites.²¹ In addition, the data^{21,22} in SLE patients show that α -enolase enzyme expression is present in sites of active renal inflammation and that anti-enolase serum antibodies correlate with active renal disease. The mechanism behind changes in α -enolase staining patterns and mechanisms of renal injury induced by anti-enolase antibodies in people and cats is unclear but in people is hypothesized to be due to direct cell damage or interference with normal α -enolase function by autoantibodies.²¹ Additional work is needed to determine the significance of altering α -enolase immunoreactivity within renal tubules with vaccine hyperinoculation.

While the systemic immunological responses and changes in renal α -enolase staining patterns suggested that renal inflammation may have been induced by the model of the present study, such inflammation was below the limit of detection by light microscopy, serum biochemical tests, and urinalyses. In contrast, a previous study¹⁰ showed that interstitial nephritis was induced in 50% of cats hypersensitized with purified CRFK cell lysate 13 times over a 2-year period. The reason why the current study did not lead to histologic evidence of interstitial nephritis is likely multifactorial. First, it is possible that the cats in our study received too little CRFK cell lysate or α -enolase over the 14-week period with vaccination compared to the previous study¹⁰ that hyperinoculated cats with CRFK cell lysates. Also, the amount of α -enolase contained within vaccines may vary between vaccine aliquots or lots. Second, formation of interstitial nephritis secondary to an immune-mediated reaction toward CRFK cell lysates may be idiosyncratic and a consequence of the strength of the immune response mounted by the individual cat. Third, 16 weeks may not be enough time to identify histopathologic evidence of interstitial nephritis. Lastly, renal lesions can be segmental, so it is possible we simply missed lesions in our tissue sections.

While frequent vaccination has been associated with the development of CKD in cats in an epidemiological study,⁵ to date, there has been no documented direct link between the parenteral administration of FVRCP vaccines and feline interstitial nephritis or CKD. While intranasal FVRCP vaccine administration did not induce antibodies against CRFK lysates and α -enolase in a previous study,⁸ it is unknown if this route of administration is safer. To highlight, the hyperinoculation protocol used in this study is drastically different than the parenteral FVRCP vaccine protocol recommended for client-owned cats to infer protection against viral infections.⁶ For this purpose, a parenteral FVRCP vaccine is recommended every 3 years starting 6 to 12 months after the initial booster series (1 or 2 vaccines 3 to 4 weeks apart). Therefore,

findings of this study cannot be applied to client-owned cats receiving FVRCP vaccines in accordance with the recommended vaccination protocol.⁶

The present study had limitations. As previously stated, this preliminary investigation lacked a control group of cats that were not vaccinated yet had a renal biopsy performed at the same time as the study cohort. It is unknown whether previously performing kidney biopsies is a source of increased α -enolase activity. Therefore, the increased α -enolase staining in the renal tubules and glomeruli cannot definitively be attributed to vaccine hyperinoculation. Second, we did not quantify the amount of CRFK cell components or α -enolase contained in the lot of vaccines that the cats were administered. This makes it difficult to make direct comparisons between our study and previous studies¹⁰ utilizing CRFK cell lysates, although the authors believe that the propensity to develop renal inflammation may be idiosyncratic. Although we have shown that cats vaccinated with parenteral FVRCP vaccines develop increasing anti-CRFK and anti- α -enolase antibody absorbance levels, we have not directly documented CRFK cell antigens, including α -enolase, in commercially available FVRCP vaccines, including the vaccine used in this study.^{8,9} Third, the study was designed to be intentionally shorter in duration than in previous studies in order to evaluate the protocol for development of a model to study interstitial nephritis in cats.^{8,10} The long-term effects of this short-term vaccine hyperinoculation protocol were not evaluated, and as a consequence, it is not possible to know whether the same alterations are still identifiable over time and/or any kind of evolution of the pathologic mechanisms occurs. Fourth, the 3 female cats were ovariectomized at the time of the first renal biopsy. It is unknown to what degree (if at all) changes in hormonal influences would have had on study results. Finally, the use of purpose-bred research cats makes it difficult to ascertain the clinical significance of the study findings in client-owned cats.

In conclusion, hyperinoculation with a commercial FVRCP parenteral vaccine did not induce histopathologic findings of interstitial nephritis in cats within the 16-week study period. Cats developed a humoral and cell-mediated immune response toward α -enolase contained in the vaccine, and renal α -enolase immunoreactivity scores increased in tubules and glomeruli. Findings suggested that systemic immunological reactions occurred and renal tissues were affected by vaccine hyperinoculation; however this short-term FVRCP vaccine hyperinoculation protocol cannot be used to study interstitial nephritis in cats.

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has current research or donation relationships with 16 companies. These relationships were unrelated to this research.

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