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ORIGINAL ARTICLE

PTH suppression by calcitriol does not predict off-target actions in experimental CKD

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Abstract

Vitamin D receptor agonist (VDRA) therapy for PTH suppression is a mainstay for patients with severe CKD. Calcitriol (1,25-$(OH)_2D_3$) is a former first-line VDRA in CKD treatment. However, a consequence of its use in CKD is accelerated vascular calcification (VC). An experimental CKD model was used to determine whether altering the calcitriol delivery profile to obtain different PTH suppression levels could improve vascular health outcomes. High adenine diet (0.25%) was used to generate experimental CKD in rats. CKD rats were treated using different calcitriol dosing strategies: (a) 20 ng/kg SD (n = 8), (b) 80 ng/kg SD (n = 8), (c) 5 ng/kg QID (n = 9), or (d) 20 ng/kg QID (n = 9). Multiple targets of calcitriol were assessed which include arterial calcium and phosphate as well as circulating calcium, phosphate, PTH, FGF-23, VWF, and vitamin D metabolome. PTH suppression occurred dose-dependently after 1-week calcitriol treatment ($P < .01$), but the suppressive effect was lost over time. Both VC and circulating FGF-23 increased > 10× in all calcitriol-treated rats ($P < .05$ and $P < .001$, respectively); similarly, circulating VWF increased at all time points ($P < .05$). Ad-hoc analysis of CKD morbidities in treated rats indicated no differences in negative outcomes based on PTH suppression level (minimal-, target-, and over-). Comparing different calcitriol dosing strategies revealed the following: (a) despite initial calcitriol-influenced PTH suppression across all treatments, the ability to continually suppress PTH was markedly reduced by study conclusion and (b) PTH suppression level is not an adequate proxy for improvements in overall CKD morbidity. These findings show (a) a more holistic approach to evaluate CKD treatment efficacy aside from PTH suppression is needed and (b) that other VDRA therapies should be examined in CKD treatment.

KEYWORDS
Calcitriol, CKD, PTH suppression, vascular pathology

1 | INTRODUCTION

Vitamin D insufficiency, as measured by 25-OH-D_3 (calcifediol) levels below 30 ng/mL, is a hallmark of chronic kidney disease (CKD).[1] CKD also results in reduced conversion of calcifediol to its active form 1,25-$(OH)_2D_3$ (calcitriol), due to loss of expression and/or function of renal CYP27B1. This vitamin D deficiency commonly results in hypocalcaemia as calcitriol is the primary mediator of calcium absorption from the gastrointestinal tract. Together lower calcitriol levels accompanied by hypocalcaemia stimulate parathyroid hormone (PTH) release to restore calcium levels by stimulating resorption of bone calcium and phosphate stores.[1,2] Vitamin D levels further decrease as CKD progresses, leading to a worsening cycle of increasing secondary hyperphosphatemia and osteodystrophy.

The current Kidney Disease Improving Global Outcomes (KDIGO) recommendations for rectifying abnormalities in the vitamin D metabolome and mineral-bone axis focus on the supplementation of calcitriol and active vitamin D analogs to target severe and progressive hyperparathyroidism in patients with CKD G3a-G5, not on dialysis.[3] Depending on the jurisdiction, treatment options include calcitriol, paricalcitol, or precursors such as calcifediol or cholecalciferol.[3–5] The rationale behind using these precursors or analogues lies in directly rectifying the deficiency in circulating vitamin D as well as, ideally, acting on specific tissues to rectify abnormal decreases (eg, reduced circulating calcium due to lower gut absorption) or increases (eg, PTH in response to low calcitriol) to circulating factors. However, these guidelines do not provide advice on how to normalize vitamin D insufficiency in patients who have yet to progress to stage G3 or more severe.

Observational studies suggest that VDR agonist use associates with a reduction in the occurrence of cardiovascular events and left ventricular hypertrophy (LVH), and increases survival in ESRD patients with SHPT.[6–8] However, there are no randomized controlled trials evaluating these observations with patient-level outcomes such as cardiovascular events, hospitalization, and mortality. Although observational data suggest that VDR agonist use may be linked to survival, a number of clinical and animal studies suggest that using VDR agonists may promote cardiovascular disease (CVD) via off-target impact on mineral regulation. Furthermore, calcitriol acts to upregulate fibroblast growth factor-23 (FGF-23), a phosphaturic hormone that can also non-selectively stimulate left ventricular growth via FGF receptors (FGFR) in cardiac myocytes.[9,10] Taken together, these sequelae of calcitriol suggest further evaluation of its use is important.

The abnormalities in bone and mineral homeostasis that are a direct consequence of PTH overproduction strongly associate with frailty and relative risk of death in patients with CKD. These abnormalities are known as CKD mineral-bone disorders (CKD-MBD). Block et al[11] identified that even early PTH elevations prior to overt secondary hyperparathyroidism (SHPT) are associated with increased mortality. PTH reduction has long been a therapeutic target although no randomized controlled trials exist to define an optimal PTH level for patients with end-stage renal disease (ESRD). Present guidelines suggest that patients with levels of intact PTH (iPTH) that are progressively rising should be evaluated for modifiable factors including hyperphosphatemia, hypocalcaemia, and vitamin D insufficiency.[3]

An additional risk in using calcitriol for treatment of CKD is that it may promote vascular calcification (VC), a process where calcium-phosphate crystals form within the medial layer of arteries.[12,13] This process causes vascular stiffening, and associates with LVH and the development of CVD in CKD. By increasing gut absorption of calcium and phosphate to a level that potentially causes hyperphosphatemia and hypercalcemia, calcitriol can produce a pro-mineralization environment. Furthermore, calcitriol upregulates several pro-calcification genes in vasculature and can cause iatrogenic PTH over-suppression leading to adynamic bone disease. Together, these observations provide some indirect evidence for the link between calcitriol treatments and the development of calcification that has been reported in experimental models.[12,14–16] It is noteworthy that pre-clinical studies report discordant effects of calcitriol on VC that appear to be linked to dose. That is, low doses of calcitriol (eg, 20 ng/d) have been shown to inhibit VC progression in murine models while higher doses promote VC in rats with CKD.[6,8–10,12,15–20]

The exact mechanism by which VDR activation modulates VC in a dose-dependent manner has yet to be elucidated, but one hypothesis, being tested in the present study, is that the adverse effects of calcitriol (hypercalcemia, elevated FGF-23, and PTH over-suppression) are a consequence of the pharmacologic strategy of once-daily bolus dosing. Studies examining the use of a modified release calcifediol dosing regimen demonstrated marked reductions in PTH and higher levels of circulating calcitriol without causing hypercalcemia/hyperphosphatemia or significantly increasing FGF-23.[21–23] To test whether similar benefits could be derived with calcitriol dispersed over a broader time period, this study examined if altering the calcitriol dose or frequency of administration so as to moderate peak levels would improve outcome measures of PTH, FGF-23, and VC. Two different dosing levels were tested (20 and 80 ng/kg/day) using strategies of either once a day (SD) or divided into smaller doses four times a day (QID) in rats with an experimental form of CKD. The hypothesis was that treatment with lower dose of calcitriol, given in four divided doses (4 × 5 ng/kg/day), would be the most effective strategy for achieving target levels of circulating PTH while minimizing negative effects on vascular health and other mineral-hormonal factors. Given that VDR agonists are the mainstay of SHPT management in many countries, strategies that could enhance their safety profile are warranted.

2 | MATERIALS AND METHODS

2.1 | Animal model

All animal procedures were performed in accordance with the guiding principles of the Canadian Council on Animal Care and were approved by the Queen's University Animal Care Committee.

Adult male Sprague Dawley rats (n = 34, 14 weeks of age; Charles River®, Montreal, QC) were individually housed and maintained on a 12-hour light/dark cycle. Animals were acclimatized for 1 week prior to study commencement. From the beginning of the study (Week 0, Day 0) onwards, CKD was generated using a specially formulated diet (0.25% adenine, 1% phosphate, 1% calcium, and 6% protein),[12] Navid[24] that was provided throughout the entirety of the experiment. After 3 weeks of CKD induction, rats were stratified (Week 4, Day 22) into one of five calcitriol treatment groups: 0 ng/kg (CKD-Untreated, n = 8), 20 ng/kg SD (20D SD, n = 8), 5 ng/kg QID (5D QID, n = 9), 80 ng/kg SD (80D SD, n = 8), and 20 ng/kg QID (20D QID, n = 9) based on serum creatinine to ensure equivalent CKD status across groups. Rats were provided with calcitriol for three full weeks (Week 6, Day 41), then during the fourth week on treatment (Week 7) rats were anesthetized and sacrificed with blood and tissues collected for analysis. A control group (n = 6) was given standard rat chow (LabDiet 5001, Ren's Pets Depot, Oakville, ON, Canada) for the duration of the experiment and sacrificed with CKD animals. All rats received water ad libitum for the duration of the study.

2.2 | Calcitriol dose

Calcitriol dosage is approximately 15-50 ng/kg/day in the rat to mimic human clinical therapeutic levels. From our previous studies, the 80 ng/kg/day was selected to generate a phenotype consisting of vascular calcification, mild hypercalcemia, and over-suppression of PTH.[15,16] The 20 ng/kg/day dosage previously appeared to avoid these adverse outcomes. The dosing schedule of SD vs QID was to determine whether the magnitude of bolus or 24-hour exposure changed outcomes, with QID being chosen as to best approximate to a steady-state dosing based off of calcitriol's half-life of approximately 6 hours.[25]

2.3 | Serum biochemistries

Blood samples from saphenous vein were collected in capillary tubes for serum and heparin plasma at baseline, 3 and 5 weeks of treatment, and at sacrifice. Plasma PTH and C-terminal FGF-23 (cFGF-23) levels were measured using enzyme-linked immunosorbent assays (ELISA; 60-2500, 60-6300, Immutopics®). Circulating VWF levels were measured via ELISA per manufacturer's protocol (DAKO, Carpinteria). Creatinine levels were measured with QuantiChrom Creatinine Assay Kit (DICT-500; BioAssay Systems) while serum calcium and phosphate were determined colorimetrically using the o-cresolphthalein complexone assay (540 nm, Sigma-Aldrich Canada Co.) and malachite green methods (650 nm), respectively.[16] In brief, the O-cresolphthalein colour reagent forms a purple complex with the calcium in the samples and the malachite green reagent involves the formation of a green complex between malachite green, molybdate, and free phosphate.

2.4 | Vessel calcification

Aorta were demineralized in 50 µL/mg tissue 1.0 N hydrochloric acid at 4°C for 24 hours. Tissue was then removed, and homogenate analysed for calcium and phosphate content using the assays for serum calcium and phosphate.[15,16,26]

2.5 | Measurement of 1,25-(OH)2-D3 and metabolites

Serum 25-(OH)-D_3 and 24, 25-$(OH)_2$-D_3 were quantified by LC-MS/MS, using previously published methods[27,28] (except that the starting volume of serum was reduced to 25 µL and diluted with 275 µL of water after addition of internal standard (mixture of d_6-25-OH-D_3 and d_6-24-25-$(OH)_2$-D_3) where an equivalent of 19 µL of serum was analyzed per injection. Vitamin D metabolite levels were determined in individual animals. 1,25-$(OH)_2D_3$ levels were similarly quantified using LC-MS/MS as previously established.[29,30]

2.6 | Statistical analysis

Statistical analysis was performed using GraphPad Prism 7.0 (GraphPad Software). Two-way ANOVA was performed with ad-hoc Bonferroni correction to identify interactions while linear regressions were employed to identify associations. Significance was defined as $P < .05$.

3 | RESULTS

3.1 | Induction of chronic kidney disease

We used a modification of the standard adenine rat model of CKD for these studies.[31] Providing adenine in the diet over 7 weeks (0.25% adenine, 1% phosphate) increased circulating creatinine (Table 1), phosphate (Table 1), PTH (Figure 1A), and FGF-23 (Figure 1B) to levels indicative of moderate (<300 umol/L creatinine) to severe CKD (>300 umol/L creatinine). This model generates a stably progressing CKD phenotype through the accumulation of adenine metabolite crystals, 2-dihydroxyadenine, within the nephric tubular interstitium. These crystals progressively accumulate and damage nephrons, reducing kidney function. In these studies, there were no significant differences between treatment groups in terms of CKD generation, including the non-treated CKD (Figure 1D). As expected, this modified adenine-based dietary protocol caused a slight reduction in bodyweight during the induction of CKD (Table 1).[15,16,26,32] After 4 weeks on the adenine diet, CKD rats were stratified, based on circulating creatinine, into the five calcitriol treatment groups with similar overall severity of CKD: (a) No treatment (0 ng/kg), (b) 5 ng/kg 4 times/day (5D QID, n = 9), (c) 20 ng/kg/ once/day (20D SD, n = 8), (d) 20 ng/kg 4 times/day (20D QID, n = 9), and (d) 80 ng/kg once/day (80D SD, n = 8).

TABLE 1 Characteristics of control, untreated CKD, and CKD rats treated with calcitriol at study endpoint. (7 wks CKD; 0.25% adenine diet; 5D QID: 5 ng/kg 4×/day; 20D SD: 20 ng/kg/d; 20D QID: 20 ng/kg 4×/day; 80D SD: 80 ng/kg/d)

	Control (n = 6)	CKD-Untreated (n = 8)	CKD 5D QID (n = 9)	CKD 20D SD (n = 8)	CKD 20D QID (n = 9)	CKD 80D SD (n = 8)
BWt	461.8 ± 20.8	394.6 ± 46.2	385.6 ± 22.4	398.3 ± 45.4	370.9 ± 31.5	400.3 ± 25.6
Cre	41.6 ± 4.3	472.3 ± 81.6	374.8 ± 117.3	372.9 ± 122.4	383.2 ± 156.0	365.7 ± 111.0
Ca	1.9 ± 0.3	1.5 ± 0.2	2.2 ± 0.1**	2.3 ± 0.3***	1.9 ± 0.4	2.1 ± 0.4**
PO_4	2.0 ± 0.3	5.0 ± 1.2	4.1 ± 0.6	4.6 ± 0.8	4.2 ± 1.0	4.2 ± 0.8

Data expressed as mean± SD.

Abbreviations: 20D QID, 20 ng/kg calcitriol 4×/day; 20D SD, 20 ng/kg calcitriol 1×/day; 5D QID, 5 ng/kg calcitriol 4×/day; 80D SD, 80 ng/kg calcitriol 1×/day; BWt, bodyweight (g); Ca, calcium (mmol/L); CKD, chronic kidney disease; Cre, creatinine (μmol/L); PO_4, phosphate (mmol/L).

**$P < .01$,

***$P < .001$ significantly different than CKD-Untreated.

Following the sorting of groups according to creatinine, there were no between-group differences regarding bodyweight, PTH, or FGF-23.

3.2 | Influence of calcitriol on the CKD phenotype

Compared to untreated CKD animals, calcitriol treatment produced significant elevations (27%-53%) in serum calcium (Table 1) without significantly changing serum phosphate (Table 1).

Within 1 week of calcitriol treatment, PTH was significantly suppressed in all calcitriol groups relative to untreated rats (Figure 1A). With 80 ng/kg/day treatments providing significantly greater suppression than the 20 ng/kg/day treated groups (Figure 1A). The effectiveness of calcitriol to suppress PTH in all treatment regimens was significantly reduced between 1 and 3 weeks.

In contrast, there was no marked attenuation of the effect of calcitriol on FGF-23, which significantly increased over time for both daily doses (Figure 1B). Notably, rats receiving 80 ng/kg/day had significantly higher overall FGF-23 levels compared to 20 ng/kg/day (Figure 1B).

3.3 | Effect of dividing calcitriol doses on circulating biomarkers of CKD

No significant differences in elevated circulating phosphate (Table 1), calcium (Table 1), PTH (Figure 1A), or FGF-23 (Figure 1B) were observed comparing single daily dose (20 or 80 ng/kg SD) vs subdivision into four smaller doses (5 or 20 ng/kg QID). All groups suppressed PTH to a greater extent at the earlier time point compared to the 3-week time point. That is, despite continuous dosing the capacity to suppress PTH for each dose similarly declined regardless of dosing regimen.

3.4 | Measures of vascular damage and endothelial dysfunction

Aortic tissue accrual of both calcium and phosphate was significantly increased in all calcitriol-treated groups compared to untreated CKD (Figure 2A,B). However, no differences were detected between the 20 and 80 ng/kg total dose groups or between the single vs four-time dosing regimens (SD vs QID). Furthermore, all calcitriol-treated rats had a significantly greater proportion of animals with von Kossa stainable VC (phosphate > 50 nmol/mg tissue, calcium > 80 nmol/mg tissue) compared to untreated CKD rats. The increase in vessel mineral levels corresponds to the development of medial layer vascular calcification as visualized via von Kossa stain (Figure 3).

Von Willebrand Factor (VWF), an endothelial factor released, in part, in response to shear force, was significantly elevated as CKD progressed (Figure 2C). Treatment with calcitriol led to significant elevations in VWF compared to non-treated CKD rats after only 1 week of treatment, with further near two-fold increases compared to untreated-CKD as treatment continued to study endpoint. Linear regression analysis revealed there was a significant association ($r^2 = .32$, $P < .001$, Figure 2D) between aortic phosphate content and the corresponding increased level of VWF at the end of the experiment.

3.5 | Alterations to CKD biomarkers and vascular health relative to PTH suppression status

The therapeutic target for PTH suppression is two to nine times above the upper limit of the reference range for the PTH assay being used.[3] As a proxy, we employed a therapeutic target that was two to nine times the circulating PTH average in healthy control rats (mean = 224.12 pg/mL; range: 448.24-2017.07 pg/mL), as to create an internal standardized reference range. Neither altering the magnitude of the daily dose (20 vs 80 ng/kg/day) nor changing the frequency of administration (SD vs QID) led to differences in attaining therapeutic PTH suppression, where 41.2% of animals on 20 ng/kg/day vs 46.7% on 80 ng/kg/day attained therapeutic PTH target.

An ad-hoc sub-analysis of all calcitriol-treated CKD rats was performed to determine the effects of differing levels of PTH suppression. Treated rats were stratified based on circulating

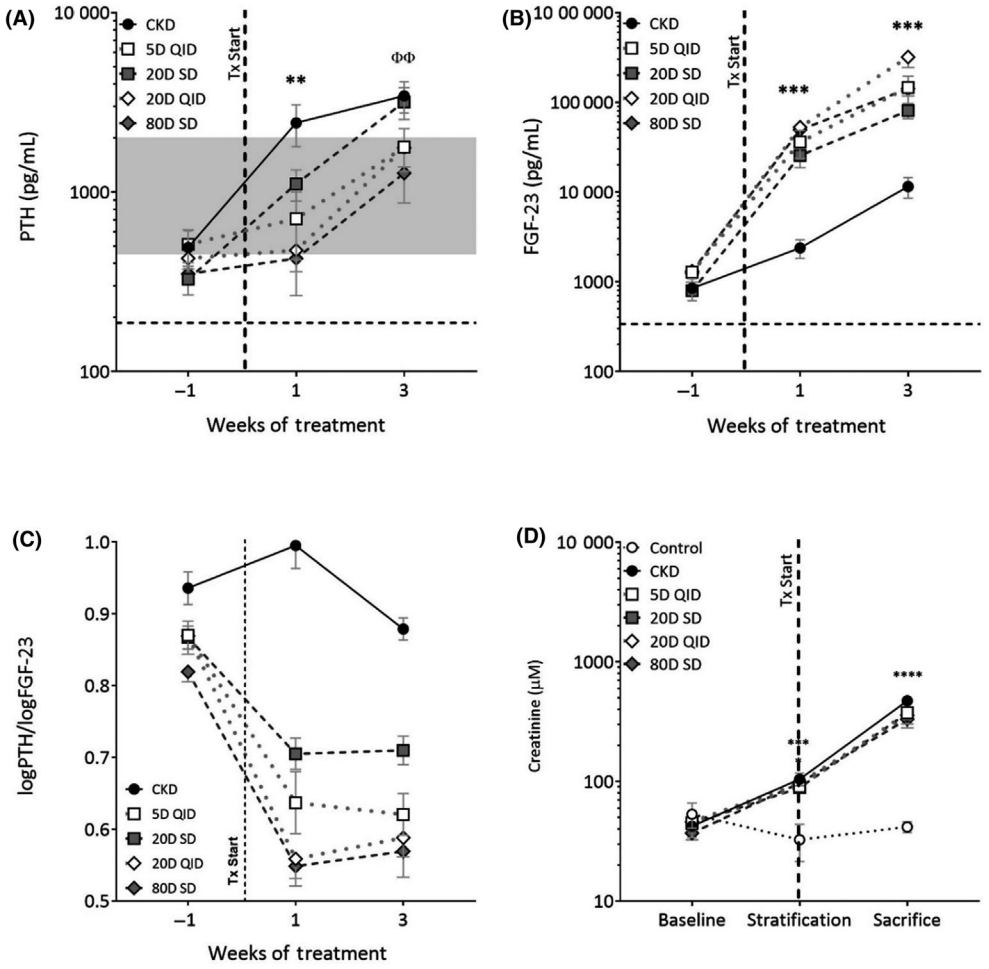

FIGURE 1 Changes to circulating PTH (A), FGF-23 (B), PTH to FGF-23 (C) and circulating creatinine (D) during CKD generation and calcitriol treatment. Calcitriol treatment after 1- and 3-week decreases circulating PTH (A) while increasing circulating FGF-23 (B). CKD-inducing diet (0.25% adenine) equivalently increased circulating creatinine in all CKD groups (D). Groups consisted of untreated CKD (black circle, n = 8), 5D QID (white square, n = 9), 20D SD (gray square, n = 8), 20D QID (white diamond, n = 9), 80D SD (gray diamond, n = 8). $^{**}P < .01$, $^{***}P < .001$ all treatment groups significantly different than untreated CKD at the same time period; $^{\Phi\Phi}P < .01$ 80D SD significantly different than untreated CKD at the same time period. Dashed horizontal line represents levels of circulating PTH and FGF-23 levels in healthy Controls (mean ± SD; 186.90 ± 52.67 and 338.93 ± 30.33, respectively). Shaded area represents therapeutic target PTH range (448-2017 pg/mL). Data represented as mean ± SD

PTH levels: Minimal Suppression (MS, PTH > 2017.17 pg/mL, n = 13), Target (448.24 < PTH <2017.07 pg/mL, n = 14), and Over-Suppression (OS, PTH < 448.24 pg/mL, n = 5). In comparing these groups, CKD rats attaining Target or OS levels were more likely to have significantly lowered serum creatinine than untreated CKD and MS groups (Table 2). Despite the differences in PTH suppression across the various calcitriol dose groups, the impact on FGF-23 was consistently and similarly elevated across all treatment groups (Figure 4B). The increased development of VC during calcitriol treatment was also not altered when assessed according to PTH suppression status (Figure 4C,D). Furthermore, there were no between-group differences to increases in circulating VWF based on suppression status (MS = 3.03 ± 0.25 U/mL, Target = 3.02 ± 0.19 U/mL, OS = 3.15 ± 0.15 U/mL).

3.6 | Calcitriol dosing effect on Vitamin D and Vitamin D-related metabolites

Compared to untreated CKD, calcitriol treatment resulted in marked decreases to both circulating 25-OH-D_3 and 24, 25-$(OH)_2D_3$ (Figure 5A,B). Furthermore, the ratio of 24, 25-$(OH)_2D_3$ to 25-OH-D_3 was mildly decreased for the two higher doses of calcitriol: with significant suppression in the 80 ng/kg per day vs 20 ng/kg per day treated rats (Figure 5C). Circulating 1,25-$(OH)_2D_3$ was significantly elevated in all calcitriol-treated rats compared to untreated CKD with significantly higher levels in rats given 80 vs 20 ng/kg/day (Figure 5D).

Further analysis, based on PTH suppression status, showed significant decreases in circulating 25-$(OH)D_3$ in Target and OS groups, but not the MS group (Figure 6A). Although a similar pattern was

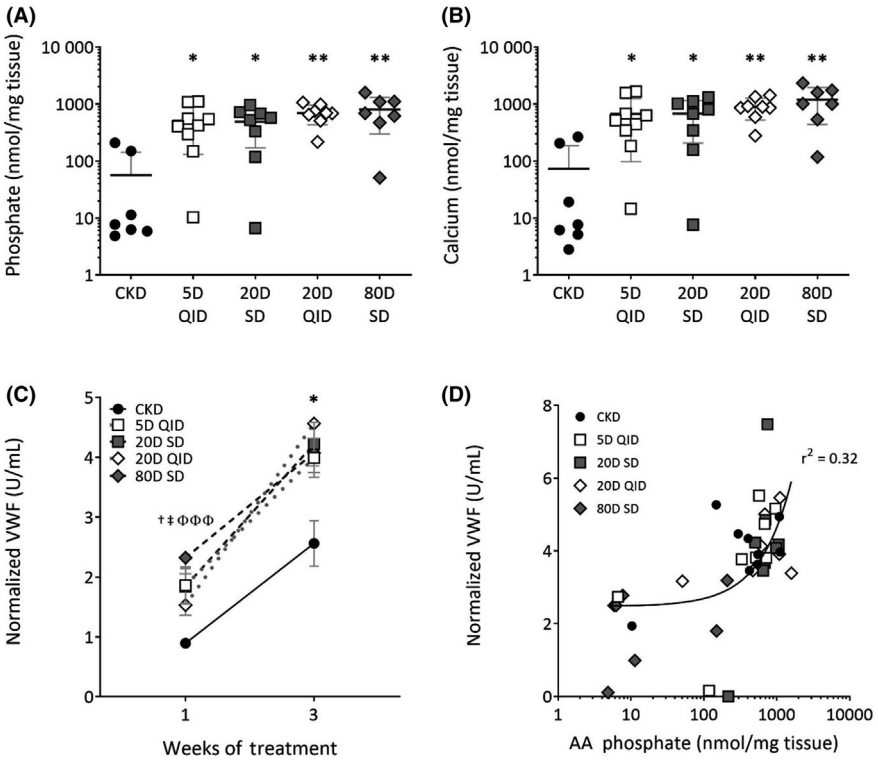

FIGURE 2 Impact of calcitriol on vascular calcification and health. Accrual of phosphate (A) and calcium (B) in abdominal aorta, changes to circulating VWF after 1 and 3 wks of treatment (C) and relationship between circulating VWF and abdominal aortic phosphate content in: untreated CKD (black circle, n = 8), 5D QID (white square, n = 9), 20D SD (gray square, n = 8), 20D QID (white diamond, n = 9), 80D SD (gray diamond, n = 8). Multiple comparisons test with Bonferroni correction to test within-group differences; $^{\dagger}P < .05$ 20D SD significantly different than untreated CKD, $^{\ddagger}P < .05$ 5D QID significantly different than untreated CKD, $^{\Phi}P < .05$ 80D SD significantly different than untreated CKD, $^{\theta}P < .05$ 20D QID significantly different than untreated CKD. Chi-squared test to compare proportion calcified (phosphate > 50 nmol/mg, calcium > 80 nmol/mg); $^{*}P < .05$, $^{**}P < .01$ significantly different than untreated CKD-control. Data represented as mean ± SD

noted for 24,25-$(OH)_2D_3$, only the decline in the OS group was significant compared to CKD (Figure 6B). The ratio of 25-OH-D_3 to 24,25-$(OH)_2D_3$ was not significantly different between the three groups divided by suppression status (Figure 6C). Interestingly, 1,25-$(OH)_2D_3$ levels were similarly elevated in all calcitriol treatment groups regardless of the impact on PTH suppression (Figure 6D).

4 | DISCUSSION

This study sought to determine whether implementing a divided-dose strategy for calcitriol, the direct-acting vitamin D receptor (VDR) agonist, in the management of SHPT in experimental CKD, would ameliorate the adverse changes of this treatment to the mineral-bone disease phenotype. The findings revealed that all calcitriol treatment protocols increased vascular calcification (VC), circulating fibroblast growth factor-23 (FGF-23) and von Willebrand Factor (VWF), as well as similarly modifying the vitamin D metabolome, but without providing proportional and sustained parathyroid hormone (PTH) suppression. Specifically, comparing two daily single dosing strategies (20 vs. 80 ng/kg/day) to a divided dosing strategy (QID) revealed that (a) despite all treatments producing significant short-term suppression of PTH, in a dose-dependent manner, there was marked attenuation of PTH suppression in all groups by week three of treatment and (b) stratification by level of PTH suppression (minimal-, target-, and over-) did not differentiate for the impact on VC, endothelial dysfunction, hypercalcemia or increased FGF-23, although there was a moderate differential impact on the vitamin D metabolome.

A critical finding was the significant loss of PTH responsiveness with calcitriol treatments between 1 and 3 weeks. Previous studies suggest that a potential cause of this progressive attenuation of response is a CKD-induced decline of VDR density in the parathyroid glands possibly mediated by hyperplasia. Specifically, in experimental models of CKD, pharmacological inhibition of parathyroid gland hyperplasia preserved both VDR levels and the associated calcitriol-based PTH suppression.[33,34] Experimentally, the stimulus for parathyroid gland hyperplasia has been reported to be a combination of uremia and hyperphosphatemia.[33,35–37] The current findings agree with this concept since both the severity of hyperphosphatemia and serum creatinine elevation were associated with a greater loss of calcitriol-mediated PTH suppression. The present findings differ from other studies in that calcitriol was still able to suppress PTH at 4 weeks of CKD.[33] The more prolonged responsiveness

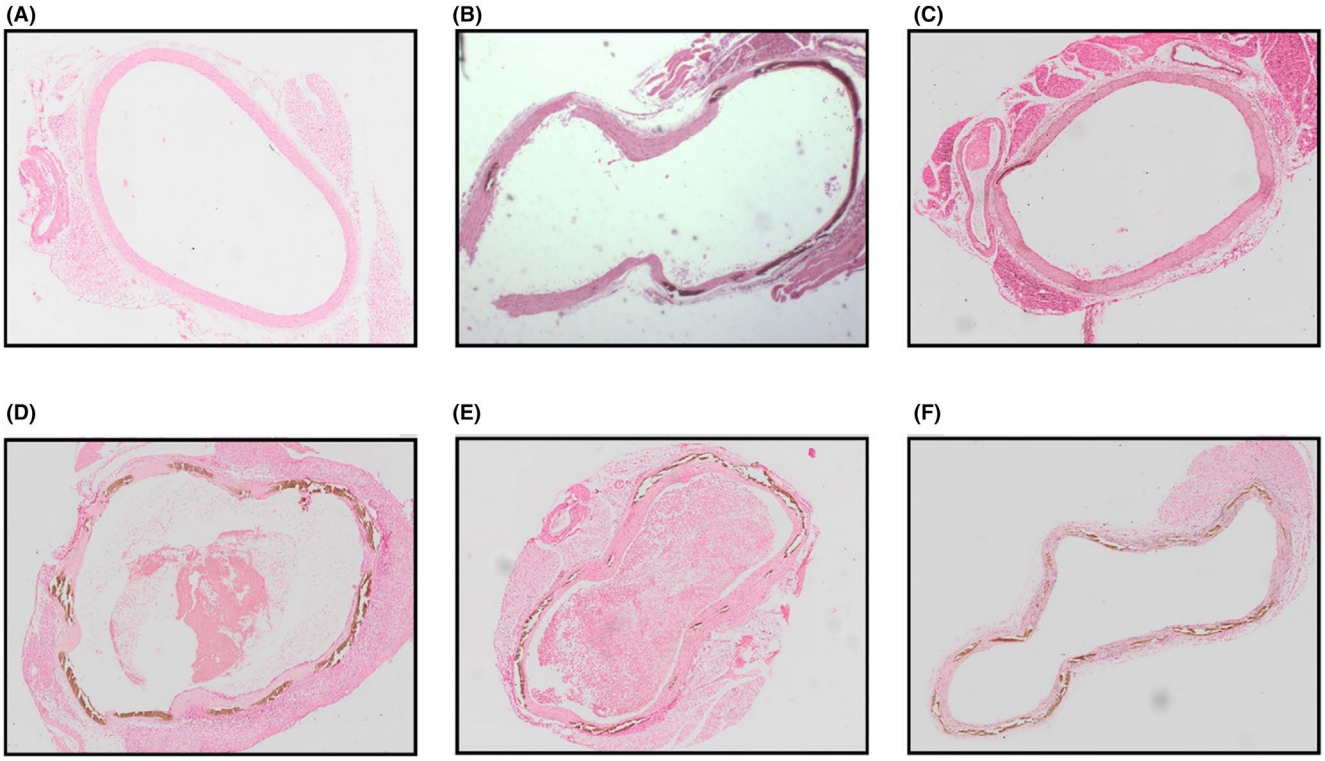

FIGURE 3 Von Kossa staining of medial layer calcification in harvested rat aorta. Visualized mineral accrual of calcium and phosphate in the aorta of Control (A), CKD-untreated (B), 20D SD (C), 5D QID (D), 80D SD (E), and 20D QID (F) rats. Mineral accrual visible as brown-stained areas in tissues

TABLE 2 Stratification based on different levels of PTH suppression after 3 wks of treatment

	CKD-untreated (n = 6)	Minimal suppression (PTH > 2017; n = 13)	Target (PTH = 448-2017; n = 14)	Over suppression (PTH < 448; n = 5)
BWt	394.6 ± 46.2	379.8 ± 42.8	391.6 ± 24.7	401.2 ± 21.1
Cre	472.3 ± 81.6	454.3 ± 120.7	339.2 ± 87.4*†	260.5 ± 66.5**††
Ca	1.5 ± 0.2	2.2 ± 0.3**	2.1 ± 0.4**	2.2 ± 0.3**
PO_4	5.0 ± 1.2	4.8 ± 0.6	4.1 ± 0.7	3.6 ± 0.5*

Note: Data expressed as mean ± SD.

Abbreviations: BWt, bodyweight (g); Ca, calcium (mM); Cre, creatinine (μM); PO_4, phosphate (mM); PTH, parathyroid hormone (pg/mL).

*$P < .05$,

**$P < .01$ significantly different than CKD-Untreated;

†$P < .05$,

††$P < .01$ significantly different than the Moderate Suppression group.

could be because the generation of CKD through adenine induction is achieved more gradually than that obtained using 5/6 nephrectomy.[38] In a previous study using this adenine model,[15] calcitriol treatment was given to animals with less severe disease (according to serum creatinine levels) and was found to have greater effectiveness. In the present study, the diminished suppression is likely multifactorial but could include changes within the parathyroid gland itself. For example, hyperplasia of the parathyroid gland can result in a decline in VDR density and be a primary cause of the attenuated PTH response.[39] Despite that the current treatments were initiated during a responsive phase, none of the four calcitriol protocols were able to prevent this decline in effectiveness. Further studies to characterize the changes in underlying mechanisms of this phenomenon in relation to CKD severity are needed.

All calcitriol treatments, regardless of the dosing profile, increased the VWF-associated endothelial dysfunction, enhanced FGF-23 levels, and exacerbated the severity of VC in all blood vessels. The significant increases in FGF-23 and VC were expected as similar findings were previously reported in this adenine model.[15,16] The differences in the ratio of PTH to FGF-23 provided evidence of a dose-response profile within the treatment groups. Specifically, the ratio declined progressively from once a day 20 ng/kg, to the divided

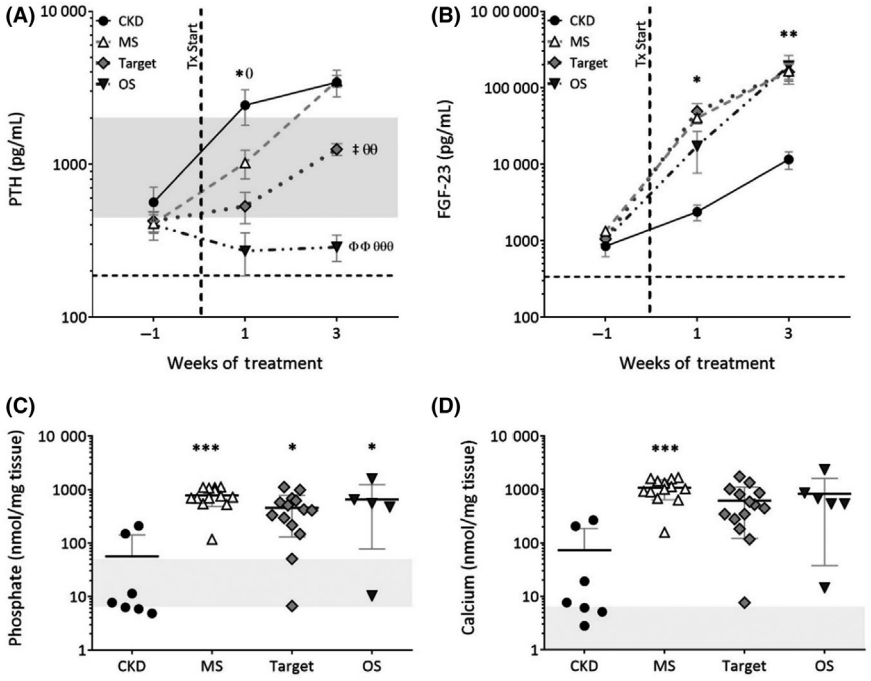

FIGURE 4 Sub-analysis of serum PTH (A), FGF-23 (B), aortic tissue phosphate (C), and calcium (D) after calcitriol treatment in CKD rats based on stratification of PTH response. Untreated CKD rats (black circles, n = 7), Minimal Suppression (MS, white upward triangle, n = 13), Target Suppression (Target, gray diamond, n = 14), Over Suppression (OS, black downward triangle, n = 6). Multiple comparisons test with Bonferroni correction to test within-group differences. $^{‡}P < .05$ Target significantly different than untreated CKD; $^{ΦΦ}P < .01$ and $^{ΦΦΦ}P < .001$ OS significantly different than untreated CKD; $^{θθ}P < .01$ and $^{θθθ}P < .001$ Target significantly different than MS; $^{*}P < .05$ and $^{***}P < .001$ all groups significantly different than untreated CKD. Dark shaded area represents therapeutic target PTH range (A, 2-9× normal control range), light shaded area represents control range (C, D). The horizontal line represents levels of PTH and FGF-23 in healthy Controls (mean ± SD; 186.90 ± 52.67 and 338.93 ± 30.33, respectively). Data represented as mean ± SD

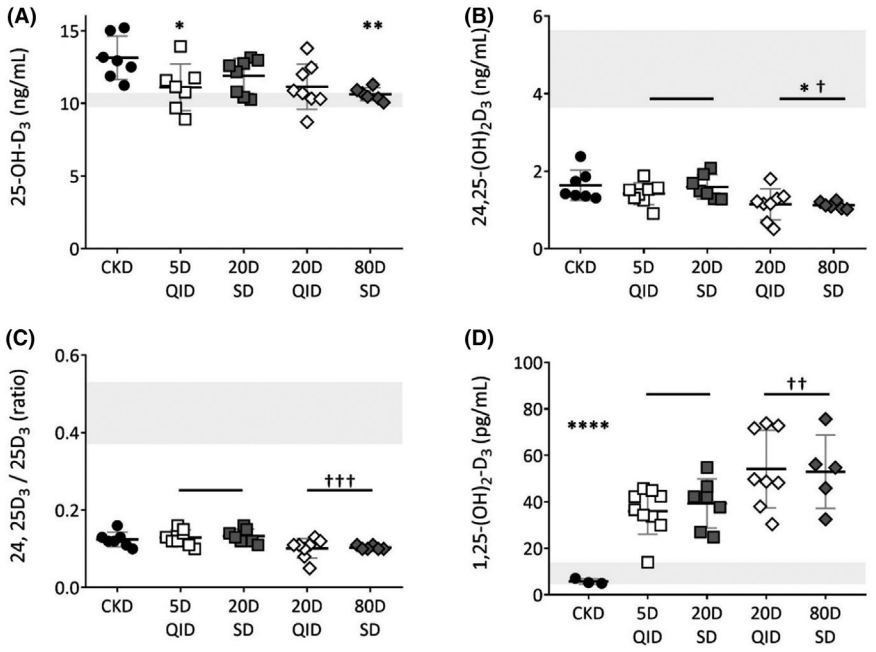

FIGURE 5 Circulating vitamin D metabolites at study endpoint. Total 25-OH-D3 levels (A), total 24,25-(OH)2D3 levels (B), ratio of 24,25-(OH)2-D3 to 25-(OH)2-D3 levels (C), 1,25-(OH)2D3 levels (D). Shaded area represents range of values from healthy animals. Multiple comparisons test with Bonferroni correction to test within-group differences. $^{*}P < .05$, $^{**}P < .01$ significantly different than untreated CKD. $^{†}P < .05$, $^{††}P < .01$, $^{†††}P < .001$ 20 ng/kg/day significantly different than 20 ng/kg/day. Data represented as mean ± SD. Shaded area represents range of values from healthy animals (Mean ± SD)

dose calcitriol to the two high-dose paradigms. What was also evident was that unlike the rising trajectory of the individual hormones the ratio did not significantly change at any dose between 1 and 3 weeks. This latter finding suggests that a pharmacological steady state had been reached but the disease progression continued to drive hormonal changes. An unexpected finding was the minimal difference found between the different dosing regimens. The half-life of calcitriol in rats is 5-8 hours[40,41] such that the lower dose (5 and 20 ng/kg, QID) given four times per day was expected to produce a different pattern compared to the single high doses (20 and 80 ng/

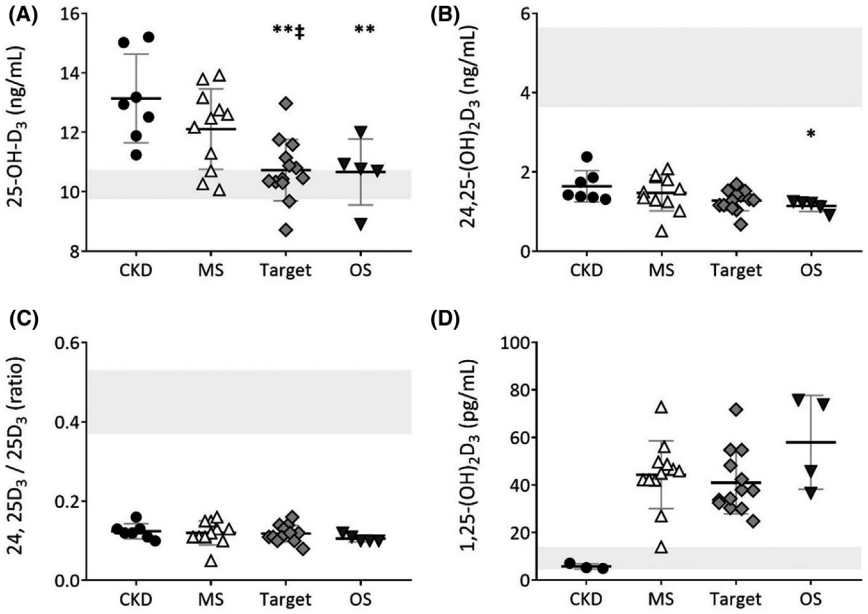

FIGURE 6 Sub-analysis of circulating vitamin D metabolites at study endpoint stratified based on PTH response to calcitriol treatment Total 25-OH-D3 levels (A), total 24,25-(OH)2D3 levels (B), ratio of 24,25-(OH)2-D3 to 25-(OH)2-D3 levels (C), 1,25-(OH)2D3 levels (D). Multiple comparisons test with Bonferroni correction to test within-group differences. $^{*}P < .05$, $^{**}P < .01$ significantly different than untreated CKD. $^{‡}P < .05$, significantly different than Minimal Suppression (MS). Data represented as mean ± SD. Shaded area represents range of values from healthy animals (Mean ± SD).

kg, SD) given once per day. There have been conflicting reports from studies using calcitriol in vivo, with respect to alterations in the prevalence of VC in experimental CKD. Some studies have found protective effects, whereas others have found accelerated pathogenesis using similar doses of calcitriol.[12,14–16,42]

VWF is released in response to shear force and damage to the vascular endothelial. Supporting the present findings, increased VWF with progressing VC has previously been shown in experimental CKD.[43] Although the present findings conflict with previous studies suggesting that VDR activation is associated with better endothelial function,[44] taken together these data suggest that the specifics of the condition need to be taken into account before assuming either a benefit or an adverse effect will occur. The present results provide strong evidence that the increased VWF levels are associated with the calcitriol-mediated progression of aortic mineralization and not from an off-target action of calcitriol. The similar impact on VWF across all the stratified suppression states demonstrates a lack of direct impact of calcitriol on endothelial release of VWF. The increases are more likely due to the magnitude of the VC acting to change the hemodynamic properties of the vessel. Although further studies will be required to confirm the basis of this change, the early approximately two-fold increase in VWF in all calcitriol groups after only 1 week suggests this endothelial effect may indicate a yet unknown insult to the endothelium brought on by calcitriol treatment or an earlier initiation of VC. As such, the present study does provide further indication that elevations in circulating VWF in CKD can be used to identify not only endothelial dysfunction but also the occurrence of medial layer VC and that calcitriol treatment potentiates these developments experimentally.

Although there was a similar pathogenic impact of the four calcitriol protocols on VC, VWF, and FGF-23, changes within the vitamin D metabolome appeared to be much more dose-dependent. Specifically, calcitriol treatment increases circulating levels of 1,25-$(OH)_2D_3$, as expected, and reduced that of the endogenous precursor, 25-OH-D_3, and associated metabolite 24,25-$(OH)_2D_3$ in a dose-dependent manner. The calcitriol-mediated reduction in the levels of 25-OH-D_3 likely results from a negative feedback response producing changes in the levels of enzymes involved in synthesis and/or degradation.[45] Given that the ratio of 25-OH-D_3 to 24,25-$(OH)_2D_3$ was not altered suggests that a full analysis of the pathways will be needed to determine which components of the vitamin D metabolome are affected by calcitriol treatments.

Using a proxy of the KDIGO paradigm,[3] stratification by minimal-, target-, and over-suppression revealed that focus on PTH alone does not predict changes in the development of VC or FGF-23 elevation. Specifically, despite achieving a wide range of suppression for PTH with the various calcitriol dosing strategies, there were no significant differences in the levels of FGF-23 induction or VC according to this stratification. These findings suggest that modifying calcitriol treatment strategies in this experimental CKD is insufficient to gain additional benefit beyond PTH suppression.

One conclusion of this study is that PTH suppression status alone does not sufficiently assess the impact of calcitriol treatment in CKD with mineral-bone disorder (CKD-MBD). Given the variety of changing factors that occur with CKD-MBD using multiple serum indicators (PTH and FGF-23 alone and/or ratio, calcium, and phosphate) as well as vascular health status (eg, CAC imaging, VWF levels, lumbar spine X-ray) be integrated to determine calcitriol treatment efficacy in CKD-MBD. Additionally, given the effects of PTH on bone resorption, examining changes to bone health and function may need to be incorporated; an area not examined within the present study due to experimental design limitations. VDR agonist use remains a prominent strategy for managing progressive and severe SHPT in CKD.[3] However, the potential for adverse effects of this therapy suggests that further refinement is clearly needed.[46,47] Although retrospective clinical studies report survival benefits and reduced CVD in patients given VDR agonists,[48,49] there are no prospective randomized clinical

trials, particularly in pre-dialysis patients,[47] to show a benefit of direct-acting VDR agonists. As with rodent studies, the balance of results does not indicate cardiovascular benefit.[12,42]

In conclusion, the present findings demonstrate that all calcitriol treatment strategies, regardless of changes to the treatment profile, lost the ability to sustain long-term PTH control as well as promoting adverse outcomes (increased VC, VWF and FGF-23). Thus, the concept that a benefit could be achieved by decreasing the peaks in circulating levels of calcitriol was not borne out. That is, the potential for a sustained release formulation of a direct-acting VDR agonist to provide greater benefit in terms of PTH control as well as decrease negative off-target outcomes was not supported. Specifically, the loss of efficacy at suppressing PTH in combination with the untoward effects on CKD-MBD suggests a very different approach may be required to manage SHPT such as providing a precursor to facilitate appropriate and feedback controlled local production of active vitamin D or treatments which modify the sensitivity of the parathyroid gland sensitivity to calcium.

DATA SHARING AND DATA ACCESSIBILITY

The results in this paper were attained via an in vivo study and did not employ the use of a public repository. Said results have not been uploaded or made accessible to a public repository.

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CONFLICTS OF INTEREST

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AUTHORS' CONTRIBUTIONS

Technical assistance was provided by Cynthia Pruss, Martin Kaufmann, and Glenville Jones pertaining to serum/plasma analysis. Jason Zelt helped with study design. Kimberly Laverty provided technical assistance with animal handling, daily checks, and tissue collection. Martin Petkovich assisted with manuscript editing. Rachel Holden and Michael Adams assisted with study design, analysis, and editing of the manuscript.

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