Title**: Effects of calcitriol and paricalcitol on renal fibrosis in CKD.**

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Running title: calcitriol, paricalcitol and renal fibrosis.

Abstract:

BACKGROUND: In chronic kidney Disease (CKD) the activation of the renin-angiotensin-aldosterone system and renal inflammation stimulate renal fibrosis and the progression to end-stage renal disease. The low levels of vitamin D receptor (VDR) and its activators (VDRAs) contribute to worsen secondary hyperparathyroidism and renal fibrosis.

METHODS: The 7/8 nephrectomy model of experimental chronic renal failure (CRF) was used to examine the antifibrotic effects of treatment with two VDRAs (paricalcitol and calcitriol at equivalent doses (3/1 dose ratio) during 4 weeks.

RESULTS: CRF increased the activation of the renin-angiotensin-aldosterone system, renal inflammation and interstitial fibrosis. Paricalcitol treatment reduced renal collagen I and renal interstitial fibrosis by decreasing the activation of the renin-angiotensin-aldosterone system through renal changes in renin, ATR1 and ATR2 mRNAs levels and renal inflammation by decreasing renal inflammatory leukocytes (CD45), ADAM17 mRNA, TGF-β mRNA and protein and maintaining E-cadherin mRNA levels. Calcitriol showed similar trends without significant changes in most of these biomarkers.

CONCLUSIONS: Paricalcitol effectively attenuated the renal interstitial fibrosis induced by CRF through a combination of inhibitory actions on the renin-angiotensin-aldosterone system, inflammation and epithelial/mesenchymal transition.

Keywords: CKD, renal fibrosis, inflammatory infiltration, epithelial/mesenchymal-transition, VDRAs.

Bullet points:

What is already known about this subject:

- *In CKD patients the reductions of calcitriol and VDR aggravates the renal and cardiovascular damage.*

What this study adds:

- *Paricalcitol reduces renal fibrosis at least partly through the reduction of the activation of the renin-angiotensin-aldosterone system*
- *Paricalcitol reduces renal inflammation and leukocyte infiltration*
- *Paricalcitol impedes renal epithelial/mesenchymal transition decreasing TGF-β and increasing E-cadherin*

What impact this may have on practice or policy:

- *Paricalcitol has beneficial effects preventing renal fibrosis.*

ABBREVIATIONS

$1,25(OH)_2D_3$ Calcitriol

ADAM17   A desintegrin and metaloproteinase

ATR1   Angiotensin receptor 1

ATR2   Angiotensin receptor 2

CKD   Chronic Kidney Disease

CRF   Chronic Renal failure

EMT   Epithelial mesenchyme transition

GAPDH   Glyceraldehyde-3-phosphate dehydrogenase

LV   Left Ventricular

RAAS   Renin-angiotensin-aldosterone system

TGF-β   Transforming growth factor beta

TNF-α   Tumor necrosis factor alpha

Treg   T Regulatory Lymphocytes

VDR   Vitamin D Receptor

VDRAs   Vitamin D Receptor Activators

INTRODUCTION

In chronic kidney disease (CKD), hypertension and systemic inflammation are considered major stimuli for the progression of renal fibrosis to end stage kidney disease. In CKD patients the reduction of the $1,25(OH)_2D_3$ (calcitriol) and its receptor (VDR) contribute to trigger and worsen secondary hyperparathyroidism, but also impairs the hypertension and systemic inflammation aggravating renal and cardiovascular damage. Calcitriol directly reduces the activation of the renin-angiotensin system through the suppression of renin gene [1, 2] and influences the degree of systemic inflammation by attenuating the antigenicity of antigen presenting cells and inflammatory cytokines synthesis and by inducing protective T regulatory lymphocytes (Treg) [3].

All these protective actions of VDR activators (VDRAs) conceptually support their utility to prevent cardiac hypertrophy and fibrosis as shown in previous studies [4]. Recently, a higher cardioprotective efficacy of paricalcitol compared to calcitriol has been reported [5, 6], which has been also confirmed in the kidney [7]

In addition, some studies in renal fibrosis suggest that around a third of all disease-related fibroblasts have a tubular epithelial origin, thus, the epithelial/mesenchymal transition is considered an important contributor of renal fibrosis[8]. Treatment with active vitamin D attenuates TGF-β-driven reductions in E-cadherin [7], which is the main component of adherence junctions, essential for the maintenance of the epithelial tissue. Its down-regulation is considered a hallmark of epithelial/mesenchymal-transition (EMT) [9]. Despite these multiple beneficial effects of paricalcitol in experimental studies, there are some discrepancies with clinical trials. In fact, the PRIMO and OPERA studies showed no reductions in left ventricular (LV) mass in patient with CKD [10, 11].

Therefore, the aim of this study was to investigate the contribution of VDRA attenuation of the activation of the renin-angiotensin-aldosterone system and its downstream pro-inflammatory, pro-fibrotic signaling and/or of EMT to the renal antifibrotic effects of calcitriol and paricalcitol in an experimental chronic renal failure (CRF) model following the study design shown in Figure 1.

MATERIALS AND METHODS

Experimental studies

All rat protocols were approved by the Research Ethics Committee of the Oviedo University.

CRF was induced in male Wistar rats (350 to 400 g) by 7/8 nephrectomy as previously detailed [12]. With the use of this model it has been shown that is possible to achieve a more severe degree of renal failure than with 5/6 nephrectomy obtaining a more pronounced secondary hyperparathyroidism and bone and vascular lesions that better reproduce the findings observe in the severe CKD in humans [13].

The animals were fed a standard rodent chow containing 0.6% calcium, 0.6% phosphorus, 1000 IU vitamin D3 / Kg (Panlab, Barcelona, Spain), and were housed in wire cages, in a controlled environment with a 12-h light/dark cycle, with water and food ad libitum. One week after surgery, animals were divided into three groups. Group 1 (CRF + vehicle n=7) received vehicle (corn oil 0.8 ml/kg body weight/day), Group 2 (CRF+ calcitriol, n=7) received calcitriol (10 ng/kg body weight/day) and Group 3 (CRF + paricalcitriol, n=7) received paricalcitol (30 ng/kg body weight/day). The paricalcitol/calcitriol ratio (3/1) was used according previous studies [5, 14] in which it has been shown to achieve an equivalent PTH suppression without increasing vascular calcification. Compounds were dissolved in ethanol and diluted in corn oil to a final volume of 0.8 ml corn oil/kg body weight/injection and administered intraperitoneally 5 days/week over a period of 4 weeks. An additional group (SHAM + vehicle, n=7) served as reference group. (Figure 1)

After 4 weeks of treatment the rats were introduced in metabolic cages and 24-hour urine was collected. Rats were then sacrificed using CO_2 anesthesia. At sacrifice, blood was drawn and serum samples were frozen until analyses. The remnant kidney was removed, divided in 3 longitudinal sections: two sections were frozen in liquid nitrogen and stored at -80ºC for RNA analysis. The other section was fixed and embedded in paraffin for histological studies.

Analytical and technical procedures

Biochemical markers

Serum creatinine, calcium, phosphorus and creatinine were measured using a multichannel auto analyzer (Hitachi 717; Boehringer Mannheim, Berlin, Germany). Serum iPTH was measured by IRMA (Rat PTH kit Immutopics, San Juan Capistrano, CA), serum intact FGF23 and soluble Klotho were assessed with a Sandwich ELISA kit (Kainos Laboratories, Tokyo, Japan and USCN Life Science, China; respectively), ELISA kit were used to measure blood and urinary rat TNFα (Abcam plc, Cambridge, UK), chemiluminescent immunoassay was used to measure serum aldosterone and serum calcitriol (DiaSorin, Saluggia, Italy), following manufacturer's protocols.

Blood pressure measurement

During the 4th week, systolic and diastolic blood pressure were recorded using an automated, non-invasive tail-cuff method (LSI Letica, Barcelona, Spain). To minimize the procedure-induced stress, the animals were accustomed to the instrument for 4 consecutive days prior to the definitive measurements, which consisted in a set of a minimum of 10 repetitive measurements per rat.

Morphological and histological changes

Interstitial fibrosis was measured using an optical microscope (model DMRXA2, Leica Microsystems, Wetzlar, Germany) coupled to a digital video camera (model Dc-100, Leica Microsystems) in deparaffined 5 μm sections of the remained kidney stained with Masson's trichrome. Samples were blinded to the reader. Captured images were analyzed using an image analysis system and specific software (ImageJ). Results were expressed as percent of total kidney area measured.

Western blot analysis

Total protein extracts from kidney tissue were collected, quantified by the DC Protein Assay (Bio-Rad) and equal amounts of protein were separated by sodium dodecyl sulphate–polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride (PVDF) membranes (Amersham Hybond). Western blot analyses were performed following manufacturer's protocol with specific antibodies: fibronectin (1:500; AB 2033), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH)

(dilution 1:3000; Santa Cruz Biotechnology). Anti-fibronectin antibody (dilution 1:5000; Santa Cruz Biotechnology) and anti-GAPDH (dilution 1:5000 Santa Cruz biotechnologies), secondary antibody binding was detected Secondary antibody binding was detected with the Western Blotting Detection Kit Clarity ™ Western ECL substrate (BIO-RAD) and the ChemiDoc Gel Imaging System Model XRS (Bio-Rad) and quantified using Quantity One 1-D Analysis Software v4 (Bio-Rad).

Immunohistochemistry

Five μm paraffin sections were deparaffinized and rehydrated. For CD45 immunohistochemistry (to determine leukocyte infiltration) samples were microwaved in 10 mM citric acid buffer pH 6,0 (Dako, REAL EnVision, Denmark) for 20 min. For the immunohistochemistry for E-cadherin the sample was incubated at 98ºC with buffer Tris EDTA PH=9 (Dako) for 20 min. For TGF-β immunohistochemistry five μm paraffin sections were placed in a stove at 58ºC for 1 hour. Samples were then incubated with 3% hydrogen peroxidase for 10min, washed with PBS and blocked with 3% of BSA in PBS for 1 hour. After an overnight incubation with primary antibody CD45 (1:50, Ab10558, Abcam), E-cadherin (1:10, anti-human E-cadherin DAKO) or TGF-β (1:100 AB92486) in 3% BSA in PBS at 4ºC, slices were washed and incubated with biotinylated secondary antibody following manufacturer's instructions (Dako). A negative control without primary antibody was used to set the level of the lowest detectable staining intensity.

Immune complexes with CD-45, E-cadherin and TGF-β were visualized with diaminobenzidine substrate (DAB) and samples were dehydrated and mounted with Entellan Merck. Semiautomatic image analysis software (ImageJ) evaluated protein expression Results were expressed as percent of total kidney area measured.

Total RNA isolation, cDNA synthesis, and quantitative RT-PCR

A kidney fragment was homogenized in an Ultraturrax (OmniHT) Total RNA was extracted by the TRIzol method (Sigma, Saint Louis, MO). Total RNA concentration and purity were quantified by UV-Vis spectrophotometry (NanoDrop Technologies, Wilmington, DE) measuring its absorbance at 260 and 280 nm. Reverse transcription was performed with a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) following the manufacturer's instructions.

Gene expression was measured in kidney by qRT PCR using an ABI Prism 7000 Sequence Detection System (Applied

Biosystems,). TaqMan Real-time PCR amplification was performed with gene-specific primer (Gene Expression Assays from Applied Biosystems) for renin, angiotensin receptor 1 (ATR1), angiotensin receptor 2 (ATR2), mineralocorticoid receptor (Nr3c2), collagen I, transforming growth factor beta (TGF-β), tumor necrosis factor alpha (TNF-α), Snail (a profibrotic transcription factor that inhibits E-cadherin), E-cadherin and ADAM17 (a desintegrin and metaloproteinase also called TACE for Tumor Necrosis factor α Converting Enzyme). Rat glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) was used as housekeeping gene. The relative quantitative evaluation of target gene was performed by comparing threshold cycles using the ΔΔCt method [15].

Statistical Analysis

Results were expressed as median and interquartile range. For all variables, the nonparametric Kruskal-Wallis test with Bonferroni correction was used [16]. Significant differences were considered when $p < 0.05$. The statistical program used was R 3.5.0

RESULTS

Biochemical markers and blood pressure

Table 1 shows that nephrectomy resulted in a similar decrease in creatinine clearance of more than 60% in all uremic groups compared with SHAM (p<0.05). Neither calcitriol nor paricalcitol treatment influenced significantly the reductions in the CRF induced by nephrectomy.

The CRF + vehicle group (group 1) did not show a significant increase of serum calcium compared with the SHAM, but calcitriol and paricalcitol groups did it compared with all groups. A marked suppression of PTH was also observed in the calcitriol and paricalcitol groups compared with the CRF + vehicle group. No significant differences were found in serum phosphorus levels and proteinuria among the different groups.

Increases in FGF23 were observed in all CRF groups (groups 1-3) compared to the SHAM group (p<0.05). The highest levels were observed in the calcitriol and paricalcitol groups (2-fold higher than Group 1).

Compared to the CRF + vehicle group, serum levels of calcitriol significantly increased in both VDRAs groups, particularly in the calcitriol group and with modest elevations in the paricalcitol group (Table 1).

Circulating soluble Klotho levels were reduced by 50% in all uremic groups compared to Sham, without reaching statistical significance (Table 1). All uremic groups had significantly decreased renal Klotho mRNA levels compared to Sham group (Sham: 0.82 [0.51 – 1.37]; CRF + vehicle: 0.33 [0.17 – 0.38]; CRF + Calcitriol: 0.16 [0.07 – 0.25]; CRF + Paricalcitol: 0.07 [0.06 – 0.26]), without any differences among uremic groups.

Renin-angiotensin-aldosterone system

Table 1 shows that systolic blood pressure increased with uremia (group 1), meanwhile there were no differences in diastolic blood pressure. There were no significant differences in the blood pressure control between the CRF rats treated with VDRAs and group 1, although the paricalcitol group showed a homogeneous but not significant decrease of systolic blood pressure compared with the CRF + vehicle group.

The CRF + vehicle group showed the highest renin gene expression. Paricalcitol significantly prevented the median increases in renin gene expression induced by uremia (Group 1, Figure 2 A). In addition, both VDRAs attenuated the increase in ATR1 gene expression induced by uremia, calcitriol by 36% and paricalcitol by 40% (Figure 2 B). A positive and significant correlation between mRNA renin and FGF23 levels was found (R=0.550, p=0.041) in the rats without VDRAs treatment (Sham and CRF groups). A trend was observed between AT1R mRNA and FGF23 levels but differences were not significant (R=0.308, p=0.305).

A significant increase in the ATR2 gene expression in the paricalcitol group was observed: 2.98 [2.79, 3.68] not only compared with CRF + vehicle group: 0.99 [0.29, 1.74], p=0.0023 but also with the calcitriol group: 1.67 [0.83, 1.85], p= 0.038.

A significant increase in the serum levels of aldosterone was observed in the CRF + vehicle group compared to the Sham group. This increase was partially attenuated in the groups treated with either VDRAs (Figure 2 C).

By contrast, the mRNA expression of the mineralocorticoid receptor in the kidney did not show any significant differences amongst groups, although the paricalcitol group showed the lowest value. (paricalcitol group: 0.49 [0.43–0-64], calcitriol group: 0.95 [0.72–1.23], CRF + vehicle group: 1.24 [0.54-1.39]).

Renal inflammation, epithelial/mesenchymal transition and fibrosis

The CRF + vehicle group did not show increase in ADAM17 compared with the SHAM, but a statistically significant reduction in the ADAM17 gene expression was observed in the paricalcitol group (54% compared to the CRF + vehicle group; p=0.03) (Figure 3 A), this decrease was not significant in the calcitriol group. Neither calcitriol nor paricalcitol were effective to significantly decrease the TNF-α gene expression induced by uremia (47% reduction in paricalcitol, Figure 3 B). Unfortunately, the TNF-α protein expression in serum and urine using the most adequate ELISA kit showed undetectable levels in either of these two fluids (results not shown). The immunohistochemical analysis of renal leukocyte CD45 expression showed 7 times higher infiltration

in the CRF + vehicle group compared with the SHAM. Calcitriol was ineffective to reduce the CD45 increase, but paricalcitol significantly reduced it (Figure 3 C, D).

The CRF (group 1) increased more than 3 times the TGF-β gene and protein expression compared with the SHAM. Paricalcitol significantly reduced the TGF-β mRNA and protein levels induced by uremia, calcitriol did not (Figure 4 A, B, C). Changes in E-cadherin expression were opposed to those of TGF-β gene expression as shown in Figure 5 A, the uremia (group 1) significantly reduced E-cadherin by 67% compared with SHAM and paricalcitol effectively prevented the reduction of E-cadherin induced by CRF (p<0.001) (Figure 5 C). Snail mRNA significantly increased 3.5 times in the CRF + vehicle group, but neither calcitriol nor paricalcitol significantly affected Snail levels (Figure 5 B).

The CRF + vehicle group showed a 3 fold increase in collagen I and 8 fold increase in interstitial fibrosis compared with the SHAM group. Calcitriol did not significantly reduce the collagen I mRNA nor the fibrosis induced by the uremia, but paricalcitol did it (Figure 6 A, B, D), p<0.001 and p<0.05 respectively). The CRF + vehicle group showed an increase in fibronectin protein expression compared with SHAM group, calcitriol group did not reduce significantly the fibronectin protein expression but paricalcitol did it. (Figure 6 C)

DISCUSSION

The present study shows that paricalcitol was effective to attenuate the renal interstitial fibrosis induced by CRF through a combination of inhibitory actions on the renin-angiotensin-aldosterone system, inflammation and epithelial/mesenchymal transition. Calcitriol showed a similar trend but without significant changes in most of these positive effects. These beneficial effects of VDRAs on renal fibrosis, particularly with paricalcitol, could partly explain some of the positive actions of the VDRAs in the amelioration of the progression of CKD at cardiac and vascular level.[17].

The main objective for the use of VDRAs in CRF has been the control of secondary hyperparathyroidism. In our study this important effect of VDRAs has been observed and a similar PTH suppression was achieved using equivalent doses of paricalcitol and calcitriol (ratio: paricalcitol 30 ng/day / calcitriol 10 ng/day). Like in other studies [18, 19], a minor and not significant decrease in creatinine clearance was observed with the use of the VDRAs and there were no differences between paricalcitol and calcitriol regarding their hypercalcemic effects [5, 20]. In our study the 3/1 paricalcitol/calcitriol (dose-ratio) was based in previous studies [14, 20, 21], but the serum calcium and PTH levels achieved indicates the use of 3/1 dose-ratio possibly exceeded the optimal dose, a finding also in keeping with the increments of FGF23 serum levels observed [22, 23]. Several studies have shown undetectable levels of serum calcitriol following paricalcitol treatment, an effect attributed to the stimulation of CYP24A1 activity to degrade excessive circulating calcitriol levels [24]. In our study, both VDRAs significantly increased serum levels of calcitriol compared to those in the uremic control group, an effect more remarkable in the calcitriol group supporting the adequacy of the doses used [25].

Apart from the PTH control, the use of VDRAs seems to produce other additional benefits, which at the end could contribute to reduce the fibrosis. It is well known that the activation of the renin-angiotensin system, currently present in CRF, together with the low levels of $25(OH)D_3$ and calcitriol contribute, among others, to the impairment of the renal damage [26] [27-30]. Calcitriol and paricalcitol directly inhibits the transcription of the renin gene [1, 31], in fact VDR knockout mice and 1-α-hydroxylase-deficient mice exhibit high blood pressure [1]. In addition, paricalcitol have shown to

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be able to inhibit the renin-angiotensin system, reducing glomerular and tubulointerstitial damage, blood pressure and proteinuria [31].

Our results confirm these findings. Paricalcitol treatment significantly reduced both the renal renin and AT1R gene expression induced by one month of CRF (group 1) by 80% and 40%, respectively. Calcitriol was similar to paricalcitol at reducing ATR1 but failed to suppress renal renin expression. In addition, a significant increase of ATR2 gene expression was observed in the paricalcitol group compared not only with the CRF + vehicle group, but also with the calcitriol group. Probably the differential effect of paricalcitol with calcitriol found in our study might be attributed to the high ATR2 gene expression in the paricalcitol group promoting vasodilation, and reducing renal fibrosis and inflammation [32], through a reduction in the TGF-β in renal proximal tubular cells [33].

Circulating aldosterone, a profibrotic factor [34], increased with nephrectomy. This increase was similarly attenuated by both VDRAs [35, 36]. However, VDRAs treatment did not alter the mRNA levels for the mineralocorticoid receptor. Despite the ability of VDRAs to mildly reduce aldosterone, which could contribute to renal klotho reductions, neither the protection against aldosterone elevations nor the prevention of klotho reductions reached statistical significance. Furthermore, in agreement with reports demonstrating that high FGF23 increases RAAS activation in the kidney [37], this study demonstrates a correlation between FGF23 and renin in untreated uremic rats. Both high FGF23 and high renin are recognized markers of a higher susceptibility to CKD progression. Interestingly, this correlation was lost upon VDRA treatment. Since both VDRA markedly increased FGF23 and effectively reduced renin mRNA, our results demonstrate that the efficacy of VDRA to reduce renin expression prevailed over their induction of FGF23 in the control of the RAAS.

VDRA could have an additional anti-fibrotic effect attenuating FGF23 deleterious effects by decreasing FGFR4 receptor at cardiac and vascular level [17]. Neither VDRAs was effective to maintain circulating levels of soluble klotho or prevent the decreases in renal klotho gene expression induced by the progressive deterioration of renal function in a month [38]. As in other studies, calcitriol and paricalcitol were not able to reduce blood pressure [39], a result which is not surprising as the kidney protective effects of VDRAs are independent of their effects on blood pressure control.

In CRF, other key aspect in the control of the fibrosis is the prevention of inflammation and epithelial/mesenchymal transition. Several inducers of inflammation are currently increased in CKD of all etiologies. Conversely, in our study, the uremia itself (group 1) did not show a significant increase in the renal ADAM17 expression, one of the core inflammation molecules. However, in spite of no increases in ADAM17 with the uremia, paricalcitol significantly reduced ADAM17. Different to what happened with ADAM17, the uremia did induce a significant increase in TNF-α (group 1, Figure 3 A), but this increase was not significantly reduced by none of the VDRAs used. Finally, the leukocyte infiltration, as expected, showed a significant increase in the non-treated uremic rats (Figure 3 C-D) which was significantly reduced by paricalcitol.

Taking all the previous results together, we may speculate that simultaneous suppression of renin, RAAS activation and ADAM17 gene expression by paricalcitol could have contributed to attenuate the induction of ADAM17 activity by angiotensin II at the renal cell surface which causes the activation of the epithelial growth factor receptor (EGFR) signaling including inflammatory cell infiltration as well as pro-fibrotic and pro-inflammatory signals [40]. Unfortunately, as in our study the uremia did not induce significantly increases in ADAM17 we cannot strongly support the influence of ADAM17 in the increase of fibrosis observed in group 1. Nevertheless, despite of that, paricalcitol induced significant decreases of ADAM17 in parallel of its effect on TGF-β, leukocyte infiltration and renal interstitial fibrosis. In fact, recently some authors have proposed the anti-inflammatory actions of paricalcitol preventing the activation of the noncanonical NF-kB2 pathway through the reversion of TNF receptor-associated factor 3 (TRAF3) down regulation, in a vitamin D receptor-independent manner [41].

To reduce the changes in epithelial/mesenchymal transition is other of the important targets to reduce fibrosis. Paricalcitol reduced TGF-β and consequently attenuated the 80% decreases in renal E-cadherin induced by uremia. These results are in agreement with previous studies [7], which found that paricalcitol largely preserved E-cadherin in vivo and abolished TGF-β–mediated E-cadherin suppression in tubular epithelial cells in human tumors and in vitro [7, 9, 42, 43]. All these beneficial

effects of the VDRAs, particularly those observed with paricalcitol, may help to maintain the E-cadherin levels reducing the possibilities of the development of renal fibrosis.

The combination of the down-regulation of the 3 factors; renin-angiotensin system activation, renal inflammation and epithelial/mesenchymal transition by paricalcitol helps to explain the remarkable reduction observed in collagen I gene expression, fibronectin protein expression and renal interstitial fibrosis. This protective effect has been also demonstrated in models of unilateral urethral obstruction by other authors [7, 44, 45].

In all experiments, calcitriol was either not effective or less effective than paricalcitol, this differential and weaker effect of calcitriol could have been partly due several factors such as the higher doses of calcitriol used and the different experimental model to induce renal damage as some authors have observed [18]. The choice of a 10 ng/kg body weight/day dose of calcitriol was based on its effective suppression of secondary hyperparathyroidism without increasing aortic calcium deposition, as reported by our group and others [4, 5, 46].

In agreement with the beneficial effect of paricalcitol observed in kidney fibrosis, similar results have been reported for the prevention of myocardial fibrosis using the same experimental model [5, 6] and also in a model of aortic constriction [4]. This effect of paricalcitol has been partly attributed to an increase of the collagen degradation promoted by increases in MMP1, an interstitial collagenase that degrades types I, II and III structural collagens.

 Other additional beneficial action of paricalcitol could be due to its capacity to down-regulate miR-29b and miR-30c, previously reported as possible responsible of the reductions of cardiac fibrosis [6]. In clinical studies like PRIMO [10] and OPERA [11] paricalcitol did not reduce the left ventricular mass, however part of the discrepancies between the results from clinical (PRIMO, OPERA) and experimental studies, can be due to the fact that the image technique used in PRIMO to measure the left ventricular mass (MRI), does not differentiate muscle from fibrotic tissue. In fact, in experimental studies, using two histological techniques to stain muscle and fibrotic tissue, the main differential advantage observed with the use of paricalcitol [5], was not the reduction of the ventricular mass but

its capacity to reduce the percentage of ventricular myocardial fibrosis, maintaining the normal muscle/fibrotic ratio of the left ventricle. These experimental morphological findings could explain the functional benefits observed in the PRIMO trial, where without reductions in the left ventricular mass the diastolic left ventricular function and BNP levels were preserved.

As strengths of this study we can highlight the comparison of the efficacy of two VDRAs at counteracting 3 main determinant of renal fibrosis: Reductions in inflammatory markers, the renin-angiotensin-aldosterone system and epithelial/mesenchymal transition, with identification of the earliest biomarkers of these changes. One likely limitation is the short 4 week period of treatment. A longer treatment could have increased differences between VDRA treatments with those in the CRF + vehicle group, particularly with those in the calcitriol group. As limitations, we cannot exclude the use of doses of VDRAs that increased serum calcium, even though there were insufficient to significantly increase aortic calcium deposition or the vascular phenotype, as demonstrated by no reductions in aortic alpha-actin content, at least after a 4 week treatment.

In summary, in rats with CRF, the VDRAs, particularly paricalcitol, attenuated the uremia-induced renal fibrosis. Graphical abstract summarize the likely mechanisms involved in the amelioration of kidney fibrosis that can be attributed to the vitamin D hormonal system. The beneficial effects of paricalcitol seems to be multifactorial involving at least the control of renin-angiotensin-aldosterone system, renal inflammation and epithelial/mesenchymal transition. Several of these favorable actions of vitamin D observed in the kidney may also explain some of the benefits attributed to this hormone in the cardiovascular system.

DISCLOSURES

The authors declare no conflict of interest

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TABLES

Table 1. Biochemical parameters and Systolic Blood pressure (SBP) and Diastolic Blood pressure (DBP) in the different groups of rats. SHAM-operated (SHAM); CRF rats + vehicle (CRF+Vehicle (Group 1)), CRF rats+ calcitriol (CRF+1,25VD (Group 2), CRF rats + paricalcitol (CRF+PARI (Group 3). Data represent median and interquartile range. *p<0.05 vs. CRF + vehicle.

	SHAM (n=7)	CRF + Vehicle (Group 1) (n=7)	CRF + 1,25VD (Group 2) (n=7)	CRF + PARI (Group 3) (n=7)
Creatinine clearance (mL/min)	2.06 [1.85-2.23]*	0.81 [0.63-0.89]	0.57 [0.52-0.67]	0.67 [0.48-0.79]
Calcium (mg/dL)	10.8 [10.8-11.1]	11.9 [11.5-12.0]	14.5 [13.0-15.0]*	14.2 [13.7-14.6]*
Phosphorus (mg/dL)	6.70 [6.30-7.75]	5.40 [4.80-5.55]	7.00 [5.60-7.70]	7.40 [7.10-7.40]
PTH (pg/mL)	2.90 [1.55-5,75]	8.40 [4.45-15.00]	0.50 [0.5-1.40]*	0.50 [0.5-1.90]*
Proteinuria (mg/24 hours)	12.9 [7.24-14.18]	8.26 [7.26-9.56]	5.83 [2.95-9.01]	7.60 [5.10-9.93]
FGF23 (pg/mL)	355 [327-396]*	681 [671-820]	1257 [1252-1267]*	1186 [1179-1196]*
Calcitriol (pg/mL)	8.32 [7.93-9.73]	6.37 [5.23-7.42]	30.4 [18.3-43.1]*	13.8 [13.3-26.1]*
Soluble Klotho (ng/mL)	5.82 [2.47-10.2]	3.21 [1.30-4.07]	2.02 [1.25-3.06]	2.40 [2.21-2.56]
Aldosterone (pg/mL)	53.1 [48.1-60.1]*	298 [230-362]	69.8 [58.8-339]	144 [52.5-154]
SBP (mg Hg)	128 [124-137]*	147 [144-148]	147 [141-154]	135 [133-137]
DBP (mg Hg)	94 [87-97]	107 [105-121]	105 [93-108]	102 [97-109]

Figure 1. Experimental design of animal model of chronic renal failure. SHAM-operated (SHAM); CRF rats + vehicle (CRF + Vehicle (Group 1)), CRF rats+ calcitriol (CRF + 1,25VD (Group 2)), CRF rats + paricalcitol (CRF + PARI (Group 3)). Nx= 7/8 Nephrectomy.

Figure 2. Renin-angiotensin-aldosterone system expression evaluated by qPCR. SHAM-operated (SHAM); CRF rats + vehicle (CRF+Vehicle (Group 1)), CRF rats+ calcitriol (CRF+1,25VD (Group 2), CRF rats + paricalcitol (CRF+PARI (Group 3) (A) Renin mRNA (B) ATR1 mRNA (C) Serum levels of aldosterone. Data represent median and interquartile range. *$p<0.05$ vs. CRF + vehicle.

Figure 3. Renal inflammation markers expression evaluated by qPCR. SHAM-operated (SHAM); CRF rats + vehicle (CRF+Vehicle (Group 1)), CRF rats+ calcitriol (CRF+1,25VD (Group 2), CRF rats + paricalcitol (CRF+PARI (Group 3). (A) ADAM17 mRNA (B) TNF-α mRNA. (C) CD45 IHC quantification in all rats. (D) Representative photo of CD45 IHC 20x. Data represent median and interquartile range. *$p<0.05$ vs. CRF + vehicle (Figure en grayscale, see Supplementary Figure 1 for colour).

Figure 4. TGF-β gene expression evaluated by qPCR and IHC. SHAM-operated (SHAM); CRF rats + vehicle (CRF+Vehicle (Group 1)), CRF rats+ calcitriol (CRF+1,25VD (Group 2), CRF rats + paricalcitol (CRF+PARI (Group 3). (A) TGF-β mRNA. (B) TGF-β IHC. (C) Representative photo of TGF-β IHC 10x. Data represent median and interquartile range. *$p<0.05$ vs. CRF + vehicle; #$p<0.001$ vs.CRF + 1,25VD (Figure en grayscale, see Supplementary Figure 2 for colour).

Figure 5. Inmunohistochemistry renal epithelial to mesenchymal transition: E-cadherin IHC and E-cadherin and Snail expression evaluated by qPCR. SHAM-operated (SHAM); CRF rats + vehicle (CRF+Vehicle (Group 1)), CRF rats+ calcitriol (CRF+1,25VD (Group 2), CRF rats + paricalcitol (CRF+PARI (Group 3). (A) E-cadherin mRNA (B) Snail mRNA. (C). Representative photo of E-cadherin IHC 10x. Data represent median and interquartile range. *$p<0.05$ vs. CRF + vehicle.

Figure 6. Collagen I gene expression evaluated by qPCR and renal interstitial fibrosis: Masson´s Thricromic staining. SHAM-operated (SHAM); CRF rats + vehicle (CRF+Vehicle (Group 1)), CRF rats+ calcitriol (CRF+1,25VD (Group 2), CRF rats + paricalcitol (CRF+PARI (Group 3) (A) Col I

mRNA. (B) representative Western blot of fibronectin and GAPDH and Fibronectin western blot quantification (C) Masson's trichrome staining interstitial fibrosis representative photo of Masson´s trichrome staining 10x and quantification in all rats. Data represent median and interquartile range. *p<0.05 vs. CRF + vehicle; #p<0.05 vs. CRF + 1,25VD (Figure in grayscale, see Supplementary Figure 3 for colour).

Graphical abstract. Interactions between Vitamin D and the kidney. Gray lines indicate direct interaction, and black lines indicate mediated interaction. RAAS = Renin-angiotensin-aldosterone system, EMT= epithelial mesenchyme transition.

SUPPLEMENTARY FIGURE LEGENDS

Supplementary Figure 1 (Figure 3 in colour). Renal inflammation markers expression evaluated by qPCR. SHAM-operated (SHAM); CRF rats + vehicle (CRF+Vehicle (Group 1)), CRF rats+ calcitriol (CRF+1,25VD (Group 2), CRF rats + paricalcitol (CRF+PARI (Group 3). (A) ADAM17 mRNA (B) TNF-α mRNA. (C) CD45 IHC quantification in all rats. (D) Representative photo of CD45 IHC 20x. Data represent median and interquartile range. *p<0.05 vs. CRF + vehicle.

Supplementary Figure 2 (Figure 4 in colour). TGF-β gene expression evaluated by qPCR and IHC. SHAM-operated (SHAM); CRF rats + vehicle (CRF+Vehicle (Group 1)), CRF rats+ calcitriol (CRF+1,25VD (Group 2), CRF rats + paricalcitol (CRF+PARI (Group 3). (A) TGF-β mRNA. (B) TGF-β IHC. (C) Representative photo of TGF-β IHC 10x. Data represent median and interquartile range. *p<0.05 vs. CRF + vehicle; #p<0.001 vs. CRF + 1,25VD.

Supplementary Figure 3 (Figure 6 in colour). Collagen I gene expression evaluated by qPCR and renal interstitial fibrosis: Masson´s Thricromic staining. SHAM-operated (SHAM); CRF rats + vehicle (CRF+Vehicle (Group 1)), CRF rats+ calcitriol (CRF+1,25VD (Group 2), CRF rats + paricalcitol (CRF+PARI (Group 3) (A) Col I mRNA. (B) representative Western blot of fibronectin and GAPDH and Fibronectin western blot quantification (C) Masson's trichrome staining interstitial fibrosis representative photo of Masson´s trichrome staining 10x and quantification in all rats. Data represent median and interquartile range. *p<0.05 vs. CRF + vehicle; #p<0.05 vs. CRF + 1,25VD.