Effects of calcitriol on peripheral endothelial progenitor cells and renal renovation in rats with chronic renal failure

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\textbf{ABSTRACT}

\textbf{Background:} The role of calcitriol (1,25-dihydroxyvitamin D\textsubscript{3} or 1,25-(OH)\textsubscript{2}D\textsubscript{3}) in physiological processes, such as anti-fibrosis, anti-inflammation, and immunoregulation is known; however, its role in the remodeling of the glomerular capillary endothelium in rats with chronic renal failure (CRF) remains unclear.

\textbf{Methods:} Here, we analyzed the role/number of endothelial progenitor cells (EPCs), renal function, and pathological alterations in rats with CRF, and compared the results before and after supplementation with calcitriol in vivo.

\textbf{Results:} Amongst the three experimental groups (sham group, CRF group, and calcitriol-treated group (0.03 µg/kg/d), we observed substantially elevated cell adhesion and vasculogenesis in vivo in the calcitriol-treated group. Additionally, lower levels of serum creatinine (Scr) and blood urea nitrogen (BUN) was recorded in the calcitriol-treated group than the CRF group (p > 0.05). Calcitriol treatment also resulted in an improvement in renal pathological injury.

\textbf{Conclusions:} Thus, calcitriol could ameliorate the damage of glomerular arterial structural and renal tubules vascular network integrity, maybe through regulating the number and function of EPCs in the peripheral blood of CRF rats. Treatment with it may improve outcomes in patients with renal insufficiency or combined cardiac insufficiency.

Calcitriol could ameliorate CRF-induced renal pathological injury and renal dysfunction by remodeling of the glomerular capillary endothelium, thus, improving the function of glomerular endothelial cells.

1. Background

Chronic kidney disease (CKD) is a worldwide public health problem, with a prevalence rate of about 5–15 % [1,2], and the rate is higher in economically backward areas than developed areas. In 2016, CKD was the ninth-leading cause of death in high-income countries [3], but years of life lost (YLLs) due to CKD are forecasted to more than double globally by 2040 [4]. In the developing world, however, the situation is more worrying. Therefore, the research on CKD has been getting more and more attention in the world. CKD is commonly characterized by peritubular capillaries, the progressive loss of kidney function, and ischemia-induced tubulointerstitial fibrosis [5-8]. The integrity of glomerular capillary clusters and peritubular capillaries formed by endothelial cells, having attracted more attention in the field of renal disease in recent years, is essential to protect renal function.

CD34\textsuperscript{+} stem cells differentiate into endothelial progenitor cells (EPCs), which form endothelial cells and is a pivotal role in endothelial regeneration and repair. EPCs are known to promote vascular healing,
and EPC-induced enhanced neovascularization has been shown to improve cardiac function after experimentally induced myocardial ischemia [9]. EPCs have become the focus of research and are considered as a potential therapeutic tool because of their favorable properties [10,11]. Eizawa et al. in 2003 were first to report a substantial decrease (41%) in the EPCs count in hemodialysis (HD) patients than control subjects, as determined by the culture assay [12]. Choi et al. reported that compared with healthy subjects, patients with chronic renal failure (CRF) exhibited a substantial decrease in the EPCs count (44.6%) along with the impaired function of colony, migratory, and angiogenesis [13]. Thus, a novel therapeutic approach might entail stimulation of the count and/or role of EPCs to treat patients with CKD undergoing hemodialysis [14,15].

Vitamin D₃, a steroid hormone that is converted to calcitriol in the kidney, is vital for bones, intestines, kidney, and parathyroid [16,17]. As glomerular filtration rate declines, a reduction in 1,25-(OH)₂D₃ concentrations is accompanied by hyperphosphatemia, secondary hyperparathyroidism, and microinflammatory state. Vitamin D is critical for endothelial cells and may have direct biological effects apart from anti-fibrosis, anti-inflammation, and immunomodulation [15,16]. Recent in vitro studies demonstrated that vitamin D is a trigger for endothelial colony-forming cells (ECFCs, a proliferative subpopulation of EPCs) mobilization and migration [20], and vitamin D treatment improves the angiogenic properties of EPCs [21] and EPCs barrier integrity through promoting the formation of VE-cadherin adhesion junctions [20]. It is reported that EPCs promotes the capillary-like tubule formation and migration of EPCs in culture, minimizing the negative effects of exposure to preeclampsia-related factors [22]. Thus, vitamin D could be a potential and promising tool for the field of human vascular health because of the neovasculogenic capacity of ECFCs and ability to repair damaged or diseased tissue.

Since patients with CRF, are significantly deficient in both 1,25-VitD₃ and EPCs, we hypothesized that 1,25(OH)₂D₃ might positively impact EPCs in CRF. Recently, almost all of the experimental data about vitamin D and EPCs are limited to the in vitro situation and need to be confirmed in a vascular in vivo system. Thus, post-supplementation with calcitriol in vivo, we analyzed the number and function of EPCs, renal function, and pathological changes in rats with CRF.

2. Methods

2.1. Study design

We procured 21 pathogen-free male SD rats (aged 6–8 weeks; mean weight: 200–250 g) from the Animal Experiment Center of Guizhou Medical University, Guiyang, China). The rats were housed at 21 ± 2 °C, with a relative humidity of 55 ± 2%, and were exposed to a 12 h light/dark cycle. Next, we randomly assigned these rats into three groups (n = 7 each); sham operation group, CRF group, calcitriol-treated group (0.03 μg/kg/d) by oral administration after 5/6 nephrectomy one week post-surgery, and were maintained for 6 weeks. The rats in the CRF group underwent right nephrectomy along with a surgical infarction of 2/3 of the left kidney [14]. The animals were anesthetized by administering intramuscular injections of xylazine (20 mg/kg, Virbaxil® 2% Virbac Laboratories) and ketamine (40 mg/kg), followed by decapsulation of the left kidney by making a left flank incision for resecting the lower and upper poles. After a week, the right kidney was excised via a right flank incision. This is a classic model of CKD, defined as stage IV of the CKD [23]. An identical procedure was performed on sham-operated rats (Sham group) except for kidney removal. All study procedures were conducted following the recommendations of the NIH Guide for the Care and Use of Laboratory Animals and were sanctioned by the Ethics Committee of Guizhou Provincial People’s Hospital and.

2.2. Sample collection

Seven weeks later, the bodyweight of these rats was recorded, followed by the collection of urine and blood samples as well as kidney specimens. Scr and BUN levels were assessed from the rat tail vein blood samples. Metabolic cages were used to collect urine samples, where rats had access to water ad libitum, 24 h before sampling. The 24-h urine albumin, 24-h total urinary volume, and 24-h Cr concentration were determined. After the animals were euthanized, parts of left renal tissues were obtained, fixed in 4 % paraformaldehyde, dehydrated, and embedded with paraffin for Periodic Acid Schiff (PAS) staining and Immunohistochemistry.

2.3. Histological analysis

Paraffin-embedded sections (2 μm) of kidney tissue specimens were treated with the PAS stain and analyzed by an independent pathologist, who was blinded to the experimental procedure, after counterstaining with H&E. For glomerulosclerosis, the first 20 randomly selected glomeruli at the kidney cortex were examined and graded as previously described [24,25]. The sections were scored as 0 = normal, 1 ≤ 10 %, 2 = 10–25 %, 3 = 26–75 % and 4 ≥ 75 % sclerosis and then the average of 20 individual scores was calculated to generate the glomerulosclerosis score.

2.4. Renal immunohistochemistry

Paraffin-embedded kidney tissues were used to detect the expression of CD31 antigens in the renal tissue specimens. Paraaffin sections (2 μm) were routinely dewaxed using xylene and hydrated with gradient ethanol and incubated with 3% H₂O₂ deionized water for 10 min. Endogenous peroxidase was blocked, followed by washing with PBS. The tissue specimen was incubated with the primary antibody (50 μL) at 37 °C for 2 h, and then with the secondary antibody at 37 °C for 30 min. After washing with PBS, the specimens were developed using DAB. PBS was used as the negative control instead of the primary antibody. The positive reactants were brown, and the nuclei were pale blue. At 400× magnification, five glomeruluses with no repeated field were randomly selected from each section at the cutaneous and medullary junction, and the pathological images were captured by the camera for data. The optical density (OD) value was calculated using the image analysis system Motic Images Advanced, and the average OD value was used for statistical analysis.

2.5. Isolation and cultivation of EPCs

After cervical dislocation, 5 mL of blood was collected from the heart of the SD rats and diluted with PBS. Ficol density gradient centrifugation was done to isolate the mononuclear layer, which was then suspended at a density of 1–3 × 10⁸ in 5 mL medium-199 (M199, Gibco, USA) supplemented with 20 % FBS (Gibco), vascular endothelial growth factor (VEGF, 10 μg/L, Peprotech, USA), penicillin (10⁵ U/L), and streptomycin (100 mg/L). Next, the cells were seeded on a human fibronectin-coated 12-well plate (hFN, Chemicon, USA). After 2 days, the nonadherent cells were washed away with M199 medium, and the remaining cells were cultured further. The medium was refreshed every 3–4 days.

2.6. Characterization of EPCs

2.6.1. Immuno-double fluorescence staining

The cells cultured on the 7th day were digested using trypsin (1 mL, 0.25 %) and EDTA (0.02 %) for 5 min, followed by suspension in M199 medium (2 mL). After seeding in a 24-well plate, the cells were incubated at 37 °C. Next, acetylated low-density lipoprotein (10 μg/mL; acLDL-DiI; 1:100 dilution) (Molecular Probe, Leiden, The
Netherlands) was added, mixed, and incubated at 37 °C for 4 h. Then, FITC-labeled Ulex europaeus agglutinin-1 (10 μg/mL; FITC-UEA-1,1:100 dilution) (Sigma, Munich, Germany) was added and kept at room temperature for 2 h. Finally, double-stained cells were visualized through laser confocal microscopy and regarded as EPCs.

2.6.2. Flow cytometry

We performed flow cytometry to evaluate surface markers of adherent cells (%). EPCs were cultured for 9 days, followed by lysis with 0.25 % EDTA for 2 min. Next, cells (1 × 10^5) were treated with phycoerythrin (PE)-conjugated anti-CD34, anti-CD31, and anti-VEGFR2 (Miltenyi Biotec) at 4 °C, followed by washing with 0.1 % BSA-containing PBS. The control used was the isotype-matched mouse IgG. FACS SCAN was used to assess binding to UEA-I (green, A) and acLDL (red, B) in MNCs. Differentiating EPCs were classified as yellow cells (C).

2.7. Adhesion of EPCs

EPCs were incubated with EDTA (0.5 mmol) to detach from the plates. Next, cells were centrifuged, and the pellet was resuspended in M199 medium and counted. Subsequently, EPCs (1 × 10^6) were seeded in 6-well plates coated with fibronectin, followed by incubation for 48 h. After washing with PBS, adherent cells were labeled for counting cells in three random fields.

2.8. EPCs in vitro angiogenesis test

24-well plate was coated with gelatin for 6 h, washed twice with PBS, then 0.25 % trypsin digested and adhered to EPC. Cells were beaten evenly, then suspended in M199 medium after cell count, and the number of cells was adjusted to 2 × 10^5/mL. Cells were incubated in 24-well plates and incubated in 37cc incubator for 12 h. The microscope was inverted. The formation of vascular lumen like structures was observed.

2.9. Statistical analysis

We used SPSS19.0 software for statistical analysis. Statistical analysis was performed after testing for normality distribution by Shapiro-Wilk normality test. One-way ANOVA was used to compare multiple groups. Data were represented as mean ± SD. Tukeys HSD test was used to compare differences between groups. p < 0.05 was considered as statistically significant.

3. Results

3.1. EPC characterization

EPCs are derived from CD34+ hematopoietic stem cells, which further differentiate into endothelial cells. Apart from morphologic features, these MNCs can be analyzed in the peripheral blood of the rat by flow cytometry using various stem cell surface marker proteins, such as CD34, CD133, and VEGFR-2 [17]. EPCs can be classified as adherent cells if they are double-positive for Dil-acLDL uptake and FITC-UEA-I binding. We observed a spindle-shaped morphology of the isolated mononuclear cells (MNCs). EPCs that were double-positive for FITC-UEA-I binding and Dil-acLDL uptake were characterized as adherent cells (Fig. 1). EPCs were further characterized based on the expression of CD31, CD34, and VEGFR-2. On day 9, the percentage of these surface markers was 4.14 %, 79.42 %, and 55.7 %, respectively (Fig. 2).

3.2. Effect of calcitriol on EPC count

The number of EPCs in CRF group, cultured 2 days after seeding, was much less than Control group (p < 0.001). However, the amount of EPCs extracted from peripheral blood of CRF rats treated by calcitriol was significantly higher than that in the CRF group (p =0.036, Fig. 3A and B). Post-phenotypic characterization of the EPC cultures, EPCs count obtained from the 7-day cultures derived from the peripheral blood MNC fraction of SD rats was evaluated. The number of EPCs in the CRF group decreased by approximately 50 % compared with the control subject. EPCs count was substantially elevated in cells isolated from CRF rats treated in vivo with calcitriol (p =0.042, Fig. 3C and D).

3.3. The effect of calcitriol on EPCs adhesion

We estimated the adhesion ability of EPCs cultured from peripheral blood. On the 14th day or so, the adherent cells showed the morphological characteristics of mature endothelial cells (ECs) like "paving stone", and the surrounding cells were fusiform and grew outward radially. That means, about 14 days of culture, all the EPCs converted to ECs.

We counted mature ECs derived from EPC adhesion. Fig. 4 shows that treatment with calcitriol promoted the adhesion of CRF rats’ EPCs, based on the substantial increase in the number of adherent cells in the calcitriol group than the CRF group (p =0.003, Fig. 4).

3.4. The effect of calcitriol on vasculogenesis of EPCs

The ability of vasculogenesis of EPCs, EPCs integration into the peripheral blood MNC-formed tubular networks, was used to assess the differentiation of EPCs. In the CRF group, considerably fewer EPCs were integrated into tubules than the EPCs from the control group (p < 0.001, Fig. 5). Tube-like structure formation was significantly stimulated by supplementation with calcitriol (p =0.002 vs CRF group Fig. 5). EPCs contributed to the development of the cellular network post-treatment with calcitriol.

3.5. Effect of calcitriol on Scr, BUN, and UPRO of EPCs

We observed elevated levels of Scr, BUN, and urine protein (UPRO) in SD rats than in the sham group (p < 0.05). Treatment with calcitriol for 6 weeks significantly lowered these levels than the CRF rats (p < 0.05, Table 1).

3.6. Renal morphology

Fig. 6 presents photomicrographs of the histopathology. The CRF group showed substantial vascular and glomerular damage. Large renal vessels exhibited onion-like vascular wall proliferation, consistent with human malignant nephrosclerosis. The surrounding afferent arterioles exhibited partial/complete glomerulosclerosis as well as hyalinosis and fibronoid necrosis. Additionally, we observed the focal rarefaction of the peritubular capillaries. Tubulointerstitial inflammation and fibrosis were observed along with the vascular changes. Treatment with calcitriol successfully attenuated these CRF-related changes. Calcitriol administration resulted in a reduced kidney tissue injury score than the sham group.
3.7. Renal immunohistology

Fig. 7 shows renal tissue staining for the anti-CD31 antibody. We found CD31 was more pronounced in calcitriol-treated animals. In the CRF group, glomerular capillaries had lower expression of CD31 in glomerular endothelial cells compared with calcitriol-treated animals 6 weeks after nephrectomy.

4. Discussions

Here, we treated CRF rats with calcitriol, a kind of active vitamin D3 intervention, which was found to be able to reduce 24-h urinary protein, improve renal function and reduce renal tissue damage, as well as regulate the number and function of circulating EPCs. EPCs are the precursor cells of mature endodermal cells, and belong to a stem cell population. EPCs originates from bone marrow primitive cells, similar to embryonic angioblast cells (angioblast). After hyperplasia, EPCs in bone marrow enters the blood circulation. Under the influence of certain factors, it adheres to and proliferates at the lesion site, and participates in endothelial regeneration and angiogenesis [26–28]. CRF is known to result in endothelial dysfunction and impaired angiogenesis, and fibrosis is a common consequence of inflammation- and oxidative stress-related endothelial dysfunction.

In the present study, the accumulation of nitrogen metabolites, SCR, BUN and urinary protein were significantly increased in CRF rats. The main pathological changes were glomerulosclerosis, renal tubule atrophy and renal interstitial fibrosis. Meanwhile, we demonstrated that CRF was associated with a reduced number of circulating EPCs and reduced their abilities to adhere and form tube-like structures, indicating their impaired endothelial cells and angiogenic activity. Similarly, patients with advanced renal failure exhibit a reduced number of EPCs in peripheral blood than healthy subjects with comparable parameters [29]. The possible mechanisms are: ① In the case of chronic renal insufficiency, the mobilization of bone marrow EPCs is reduced, the consumption of EPCs in vitro is increased, the half-life of EPCs is shortened, and the oxidative low density lipoprotein accelerates the aging of EPCs [30], injuring its ability to repair endothelial cells and form tubular structures. ② In renal insufficiency, renal ischemia and hypoxia lead to oxidative stress and increased secretion of inflammatory cytokines, all of them could damage the EPCs [31]. The deterioration of renal function leads to the damage of EPCs, and impaired EPCs can not effectively repair the damage of glomerular capillaries and endothelial cells of the peritubule vascular network, which leads to increased capillary permeability, increased urinary protein, glomerular sclerosis and tubulointerstitial fibrosis. Thus, stimulating bone marrow mobilization of EPC and increasing the number of circulating EPC is an effective means to promote the repair after chronic kidney injury, which brings new hope for the treatment of patients with kidney disease.

We found that calcitriol significantly increased the number of EPCs in peripheral blood of rats with CRF, improved its function, and enhanced the ability of EPCs to form new blood vessels. It was demonstrated that Vitamin D3 is able to regulate EPCs in vivo. To our knowledge, at present, there are few studies on the effect of vitamin D3 on EPCs in vivo, especially in the field of kidney disease. The possible mechanisms are: ① Vitamin D-induced nitric oxide (NO) production maybe, in part, accountable for this physiological process. Because recent study demonstrated that the increase of plasma NO [32] levels could improve circulating/functional capacity of EPCs [33]. ② 1,25(OH)2D was reported to be able to increase endothelial progenitor adhesion by alleviating the inflammatory signals of TNF-α in vitro [34]. ③ Vitamin D upregulates VEGF expression [35], which in turn promotes EPC activity [36]. ④ Vitamin D has a protective effect on ECFCs in vitro by antagonizing inflammation [20], and it may have a direct effect on the mobilization of EPCs. It may be for these reasons that calcitriol promoted an increase in the number and function of circulating EPCs in CRF rats.

And we also observed that, at the same time, in the calcitriol treatment group, the urine protein decreased, renal function improved, the
glomerular sclerosis index decreased, and renal interstitial fibrosis changes were alleviated. The mechanism may be as follows: ① High perfusion, high filtration and high pressure of residual nephron in the glomerulus during CRF, which causes cumulative damage to glomerular microvascular endothelial tissue, leading to glomerular sclerosis accompanied by tubulointerstitial injury caused by ischemia. Studies have shown that transplanting EPCs may help improve vascularization and renal perfusion [37].

Calcitriol may effectively maintain the integrity of the peritubule vascular network by increasing the number and function of EPCs in CRF rats, as well as promoting the ability of EPC to form new vessels, thus effectively maintaining the renal interstitial structure. ② The increased ability of EPCs to angiogenesis increased the number of glomerular capillaries and the density of capillary endothelial cells; EPCs also maintains endothelial functional activity, resulting in improved glomerular filtration membrane permeability, decreased albumin filtration, and decreased urinary protein. The reduction of urinary protein also reduces further damage to renal function. ③ Additionally, we observed calcitriol-treated animals exhibited a higher blood capillary density as glomerular endothelial cells exhibited stronger CD31 staining than CRF rats. EPCs contributed considerably to the cellular network, and Vitamin D3 levels maybe an independent predictor of EPC levels in CKD. Thus, we think calcitriol could delay the progression of chronic kidney disease, possibly by promoting the increase of EPCs and the improvement of adhesion function and neovascularization function.

The number of EPCs in peripheral blood in healthy adults is very small, with a percentage of 0.01 %, that is, only 70–210 EPCs in 1 mL of peripheral blood. Under pathological conditions, the number and

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**Fig. 3.** The effect of calcitriol on EPCs. (A) The effect of calcitriol on the number of EPCs (200×, 2d after seeding) (B) EPCs number per high-power field (200×, 2d) The number of EPCs in CRF group, cultured 2 days after seeding, was much less than Control group (p < 0.001). However, the amount of EPCs extracted from peripheral blood of CRF rats treated by calcitriol was significantly higher than that in the CRF group (p =0.036, Calcitriol group vs. CRF group). (C) The effect of calcitriol on the number of EPCs in fluorescent chemical assay is shown through phase contrast images (7d) (D) EPCs number per high-power field (75 μm), the number of EPCs cultured for 7 d, only which expressing both red and green fluorescence were included in the count, was decreased significantly in CRF group compared with control group (p < 0.001), but treatment with calcitriol resulted in rapid increasing than CRF group (p =0.042, Calcitriol group vs. CRF group).
activity of EPCs in patients with ischemic cardiovascular risk factors, including nephropathy, diabetes, hypertension, etc., will be significantly reduced, and the ability to form lumen-like structures in vitro culture will be significantly impaired. However, in vitro transplantation via EPC takes more time and money, as well as increased risks. Our results suggested that supplementation with calcitriol not only improves renal bone disease in CRF patients who lack vitamin D3, but also protects the kidney by promoting EPCs. Therefore, supplementation of active vitamin D3 is a good method for the treatment of CRF.

For patients with renal insufficiency, especially end-stage renal disease, cardiovascular events are a high risk factor and most deaths are due to complications of atherosclerosis, which have been associated with reduced EPC levels and function [38]. Patients with CRF are often complicated with chronic heart failure (CHF), present with cardiorenal syndrome, and have high cardiovascular morbidity and mortality [39]. In CHF, a negative correlation between cultured EPC and functional New York Heart Association (NYHA) class has been reported [40]. Therefore, we conclude that calcitriol treatment in CRF patients may protect the kidney and heart by increasing the number and activity of

| Group    | Scr (μmol/L) | BUN (mmol/L) | UPRO (mg/24 h) |
|----------|--------------|--------------|----------------|
| Sham     | 29.35 ± 7.48 | 5.9 ± 1.03   | 10.66 ± 3.02   |
| CRF      | 92.13 ± 12.45 | 17.67 ± 3.1  | 78.33 ± 14.86  |
| Calcitriol | 63.74 ± 13.49 | 12.31 ± 1.5  | 46.2 ± 11.32   |

* P<0.05 VS Control group.

* P<0.05 VS CRF group.
EPCs, thereby improving the quality of life of patients, reducing cardiovascular accidents and reducing mortality.

5. Conclusions

Thus, these results showed that calcitriol could regulate the number and function of EPCs in the peripheral blood of CRF rats. And maybe through this effect, calcitriol repaired the damaged glomerular capillary endothelial cells, effectively prevented the damage of glomerular arterial structural and renal tubules vascular network integrity, improving renal function. It may improve outcomes in patients with renal insufficiency or combined cardiac insufficiency.

Ethics approval and consent to participate

All animal experimental procedures were performed in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The current study was approved by the Animal Ethics Committee of Guizhou Provincial People’s Hospital.

Consent for publication

Not applicable.

Availability of data and materials

The datasets created during and/or analysed during the current study will be available from the corresponding author on reasonable request. There are no security, licensing, or ethical issues related to these data.
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Authors' contributions

XY and YZ designed, performed the experiment, analyzed the data, wrote the manuscript and final approved the manuscript. JY, JXW, RD, and ZLS performed the experiment and collected the data and read the manuscript and finally approved the manuscript. All authors have read and approved the manuscript.

Declaration of Competing Interest

The authors report no declarations of interest.

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