

## Calcitriol Ameliorates Kidney Injury Through Reducing Podocytopathy, Tubular Injury, Inflammation and Fibrosis in 5/6 Subtotal Nephrectomy Model in Rats

DWI CAHYANI RATNA SARI<sup>1</sup>, MAULIDA WIJAYA PUTRI<sup>1,2</sup>,  
TIARA PUTRI LEKSONO<sup>3</sup>, NOGATI CHAIRUNNISA<sup>3</sup>, GERRY NATHAN REYNALDI<sup>3</sup>,  
BENHARD CHRISTOPHER SIMANJUNTAK<sup>3</sup>, JOSEPHINE DEBORA<sup>1</sup>,  
JUNAEDY YUNUS<sup>1</sup>, and NUR ARFIAN<sup>1\*</sup>

<sup>1</sup> Department of Anatomy, Faculty of Medicine, Public Health and Nursing, Universitas Gadjah Mada;

<sup>2</sup> Postgraduate Student of Master Program in Biomedical Sciences, Faculty of Medicine, Public Health and Nursing, Universitas Gadjah Mada

<sup>3</sup> Undergraduate Student Faculty of Medicine, Public Health and Nursing, Universitas Gadjah Mada

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Chronic kidney diseases (CKDs) lead to end-stage renal diseases (ESRD) which are characterized by glomerulosclerosis, tubular injury, anemia, inflammation, and interstitial fibrosis. Vitamin D is known to have renal protective effects. However, its effects relate to low and high doses of Vitamin D in CKD model is still unknown. CKD was performed using 5/6 subtotal nephrectomy procedure in male Sprague Dawley rats (3 months old, 200-300 grams, SN group; n=6), then rats were sacrificed on day 14 after operation. Sham operation was used for control (SO group; n=6). Calcitriol was administered in two doses : 0.01 µg/mL/100 gramsBW/day (SND1 group; n=6) and 0.05 µg/mL/100 gramsBW/day (SND2 group; n=6) intraperitoneally for 14 days. Glomerulosclerosis and tubular injury score were examined using PAS staining, meanwhile, interstitial fibrosis area fraction was assessed with Sirius Red staining. RT-PCR was performed for assessing nephrin, podocin, IL-6, CD68, Collagen-1, and TGF-β1 mRNA expressions. Immunostaining (IHC) was carried out to observe macrophage (CD68) and myofibroblast (α-SMA). SN demonstrated CKD condition with higher tubular injury, glomerulosclerosis, interstitial fibrosis, and inflammation compared to SO. Calcitriol-treated group (especially SND2) demonstrated significant lower tubular injury, glomerulosclerosis, and interstitial fibrosis compared to SN. SND2 group showed not only significantly lower CD68, IL-6, Collagen-1, and TGF-β1 mRNA expressions, but also higher mRNA expressions of nephrin and podocin. SND2 group also demonstrated reduction of macrophages infiltration and myofibroblasts expansion based on its histopathological appearance. Vitamin D may have a renoprotective effect on 5/6 subtotal nephrectomy model by attenuating podocytopathy, tubular injury, inflammation and interstitial fibrosis.

### INTRODUCTION

Chronic kidney diseases (CKDs) have become one of the main non-communicable diseases around the world and lead to higher socio-economic burden to population. <sup>(1, 2)</sup> Prevalence of CKD is estimated to reach 8-16% worldwide thus made CKD the main cause of death in the last two decades. <sup>(3, 4)</sup> CKDs can be caused by progressive injuries in glomeruli, tubulointerstitial and vessels. <sup>(5)</sup> Exogenic factors, such as drugs and endogenous substances like glucose may also lead to CKD. <sup>(6)</sup> CKD is characterized by proteinuria, anemia, inflammation, glomerulosclerosis and tubulointerstitial fibrosis. <sup>(7)</sup> Other causes, such as inflammation and persistent hypoxia result in myofibroblast expansion and fibrosis, which are the main characteristic of CKD. <sup>(8)</sup> Nuclear factor kappa B or NF-κB regulates inflammatory cytokines and chemokines that contribute to CKD development. NF-κB activates Monocyte Chemoattractant Protein-1 (MCP-1) and induces macrophage infiltration. This sterile inflammation cascade may prolong tissue destruction and progressive injury. <sup>(7)</sup> Inflammation induces progressive renal injury and causes end-stage renal diseases (ESRD). ESRD lead to tubulointerstitial injury. <sup>(9)</sup> More than 90% of renal cortex mass are tubules, therefore tubulointerstitial requires lots of energy and prone to injury. This condition leads to tubulointerstitial damage as one of the main characteristics of CKD. <sup>(10)</sup>

Renal parenchymal damage and interstitial fibrosis are the main characteristics of ESRD. <sup>(11)</sup> Interstitial fibrosis decreases renal function, with reducing glomerular filtration rate (GFR) level. <sup>(12)</sup> The decrease in GFR will also affect the excretion of metabolism waste that later will have negative effects on the body. Interstitial fibrosis is related to the increase of TGF-β1, a strong pro-fibrotic cytokine. Pro-fibrotic effects of TGF-β1 include increase

the synthesis of extracellular matrix/ECM, decrease degradation of ECM, activate myofibroblast in the renal parenchyme, change tubules' epithelial cells into myofibroblast (epithelial to mesenchymal transition/EMT) and activate inflammation cascade.<sup>(13)</sup>

Chronic kidney disease causes many complications, one of which is anemia. Anemia was defined as serum hemoglobin levels  $\leq 12$  g/dL in women and  $\leq 13$  g/dL in men. Prevalence of anemia in people with CKD (15,4%) was twice higher compared to the general population (7,6%), and increased with stage of CKD.<sup>(14)</sup> Relative erythropoietin (EPO) deficiency is considered as the main cause of anemia in patients with CKD, other than disruption of iron (Fe) homeostasis and decrease in erythrocyte lifespan.<sup>(15)</sup> Normal or slightly higher erythropoietin level is usually found in anemia patients with CKD but is 10-100 times lower than EPO level measured in patients with anemia without CKD also known as relative erythropoietin deficiency.<sup>(16)</sup> Almost 90% of erythropoietin in the human body is produced by interstitial fibroblast in the renal cortex and outer part of the renal medulla.<sup>(17)</sup> Inflammation process induced by TGF- $\beta$  transforms fibroblast into myofibroblast, therefore, decreases erythropoietin that secreted by interstitial fibroblast. This results in a lower level of EPO produced by fibrotic kidney and contributes to the occurrence of anemia in CKD.<sup>(18)</sup>

Vitamin D has been known to have renoprotective effects. Vitamin D suppresses activation of NF- $\kappa$ B thus reducing inflammation and macrophage infiltration.<sup>(7)</sup> Calcitriol treatment also reduces interstitial fibrosis, epithelial cell apoptosis, and inflammation in Unilateral ureteral obstruction (UUO) or kidney fibrosis model.<sup>(8)</sup> In this study, we want to elucidate the role of Calcitriol in CKD model with 5/6 Subtotal Nephrectomy, which is one of the experimental model to obtain a widely used CKD model.<sup>(19)</sup> 5/6 subtotal nephrectomy procedure leads to a reduction in renal mass of  $> 85\%$  resulting in a decrease in the number of functional nephrons, increased intraglomerular pressure, glomerular distension and glomerular hypertrophy which ultimately leads to sclerosis.<sup>(11, 20)</sup> CKD patients generally have vitamin D deficiency.<sup>(21)</sup> Various populations in clinical studies have implicated vitamin D deficiency as potential risk factors for some other chronic diseases. Therefore, adequate vitamin D levels are a therapeutic goal for CKD patients.<sup>(22)</sup> We treated the CKD mice with two doses of Calcitriol, which represented a low and high dose of Calcitriol.

## MATERIALS AND METHODS

This was a quasi-experimental research with post-test only controlled group design. This study obtained permission from the Ethics Committee of Integrated Research and Testing Laboratory (LPPT) of Universitas Gadjah Mada, Yogyakarta based on the Certificate of Ethical Eligibility number: 00002/04/LPPT/II/2018.

### Animal Model of 5/6 Subtotal Nephrectomy

A total of 24 male Sprague Dawley rats age 3 months old were used in this study and placed in cages with a light-dark cycle of 12 hours. They were randomly divided into 4 groups: SO group (sham operation group), SN group (5/6 subtotal nephrectomy group), SND1 group (5/6 subtotal nephrectomy group with intraperitoneal injection of Calcitriol (Cayman) 10ng/100 grams BW/day), and SND2 group (5/6 subtotal nephrectomy group with intraperitoneal injection of Calcitriol (Cayman) 50ng/100 grams BW/day) for 14 days. During surgical procedure, rats were anesthetized using ketamine at a dose of 100 mg/kg BW intramuscularly. Unilateral nephrectomy was performed on the right kidney, followed by removal of superior and inferior poles of the left kidney in the following two days. Sham operation (SO) group were performed laparotomy in Flank's regions without removal of renal masses. Rats were terminated at day 14 after operation.

### Vitamin D Administration

We used the active form of vitamin D, calcitriol in powder form (Cayman®) dissolved in NaCl 0.9%, then divided into 2 doses: 10ng/100 grams BW (SND1 group) and 50ng/100 grams BW (SND2 group). Vitamin D was administered intraperitoneally for 14 days.

### Proteinuria score, creatinine level, hemoglobin measurement, and termination

Proteinuria was measured with a dipstick (YD Diagnostics, Seoul, Korea) just before sacrifice. Proteinuria score was assessed based on the value in the dipstick. The score was divided into 5 grades, from 0 (negative), and 1 to 4 based on color changes in the dipstick. Creatinine and hemoglobin were assessed from blood of the Retroorbital vein. For termination, mice were anesthetized using ketamine (Kepro Holland; K1356) at a dose of 100 mg/kg BW intramuscularly. In deep anaesthetization condition, abdomen and thorax were opened, perfusion was done from cardiac apex using NaCl 0.9 % solution, then kidneys were harvested. The kidney was divided into two halves, a half was kept in RNA Preservation solution (Favorgen; FARSS001) for RNA extraction and a half was kept in Normal Buffer Formalin (NBF) solution for paraffin making.

**RNA extraction, cDNA making and Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)**

The RNA from kidney tissues were extracted using Genezol solution (GENEzol™, Cat No. GZR100) based on the manufacturer's protocol. RNA concentrations were quantified using a nanodrop. The synthesis of RNA to cDNA was done using ReverTra Ace® (Toyobo, Cat. No. TRT-101), deoxyribonucleotide triphosphate (dNTP) (Takara, Cat. No. 4030), and primary random (TAKARA, Cat No. 3801). Reverse Transcriptase-PCR (RT-PCR) was performed for these following gene with specific primers: nephrine (forward: 5' – ACTCAGGCTGACATCTGGGAT – 3'; reverse: 5' – AGAGCTGGAATGACAGTGATGG – 3'), podocin (forward: 5' – ATTCCGACTGGGACATCTGC – 3'; reverse: 5' – GGTTACCACCTCATGGAAAGGT – 3'), CD68 (forward: 5' – TGTGTCCTTCCCACAAGCAG – 3'; reverse: 5' – AAGAGAAGCATGGCCCGAAG – 3'), Interleukin-6 (IL6) (forward: 5' – TCCTACCCCAACTTCCAATGCTC – 3'; reverse: 5' – TTGGATGGTCTTGGTCCTTAGCC – 3'), Collagen-1 (COL1) (forward: 5' – CAACCTCAAGAAGTCCCTGC – 3'; reverse: 5' – AGGTGAATCGACTGTTGCCT – 3'), TGF-β1 (forward: 5' – CGAGGTGACCTGGGCACCATCC – 3'; reverse: 5' – GCTCCACCTTGGGCTTGCACC – 3'), and a house keeping gene, GAPDH (forward: 5' – GGCACAGTCAAGGCTGAGAAT – 3'; reverse: 5' – TCTCGTCCTGGAAGATGGTGA – 3'). For RT-PCR, we used Taq Master Mix (GoTaq®Green Master Mix, Cat No. M7122). PCR products were analyzed on 2% agarose gel with DNA ladder (Bioron, Germany, Cat No. 306009). Gene expression was quantified with densitometric analysis using ImageJ software and GAPDH used to normalize expression.

**Glomerulosclerosis score (GSC) and Tubular injury score measurement**

The paraffin was cut with a thickness of 4 mm and then deparaffinized and stained with Periodic Acid-Schiff (PAS) to examine the glomerulosclerosis and tubular injury score. Glomerulosclerosis scoring was classified from 0 to 4, (0= normal glomeruli, 1= sclerosis area <25%, 2= sclerosis area 25-50%, 3= sclerosis area 50-75%, 4= sclerosis area >75%), then calculated average. Tubular injury score was measured based on the histopathological appearance of tubules, that is tubular dilatation, tubular epithelial attenuation, intra-luminal cast, and loss of brush border. Tubular injury score was classified from 0 to 4, (0= normal tubules, 1= tubular injury <25%, 2= tubular injury between 25 – 50%, 3= tubular injury between 50 – 75%, 4= tubular injury >75%), then calculated average.

**Immunohistochemical (IHC) Staining of CD68 and αSMA**

The tissue slide that had been deparaffinized was then heated in 1x citrate buffer, incubated with 3% H<sub>2</sub>O<sub>2</sub> in PBS for endogenous peroxidase inhibition and incubated with blocking solution. Furthermore, the slides were incubated with anti-CD68 antibody (1/400 dilution; Abcam, ab955) and αSMA antibody (1/400 dilution; Sigma A2547) in humidity chamber at 4°C overnight. After slides were incubated with species-specific secondary antibodies (Biocare Medical;STU700H) for 1 hour at room temperature, slides were incubated complete antigen detection was used avidin-biotinylated complex-horseradish per oxidase (Biocare Medical;STHRP700L10) before DAB staining. Slides were counterstained with haematoxylin.

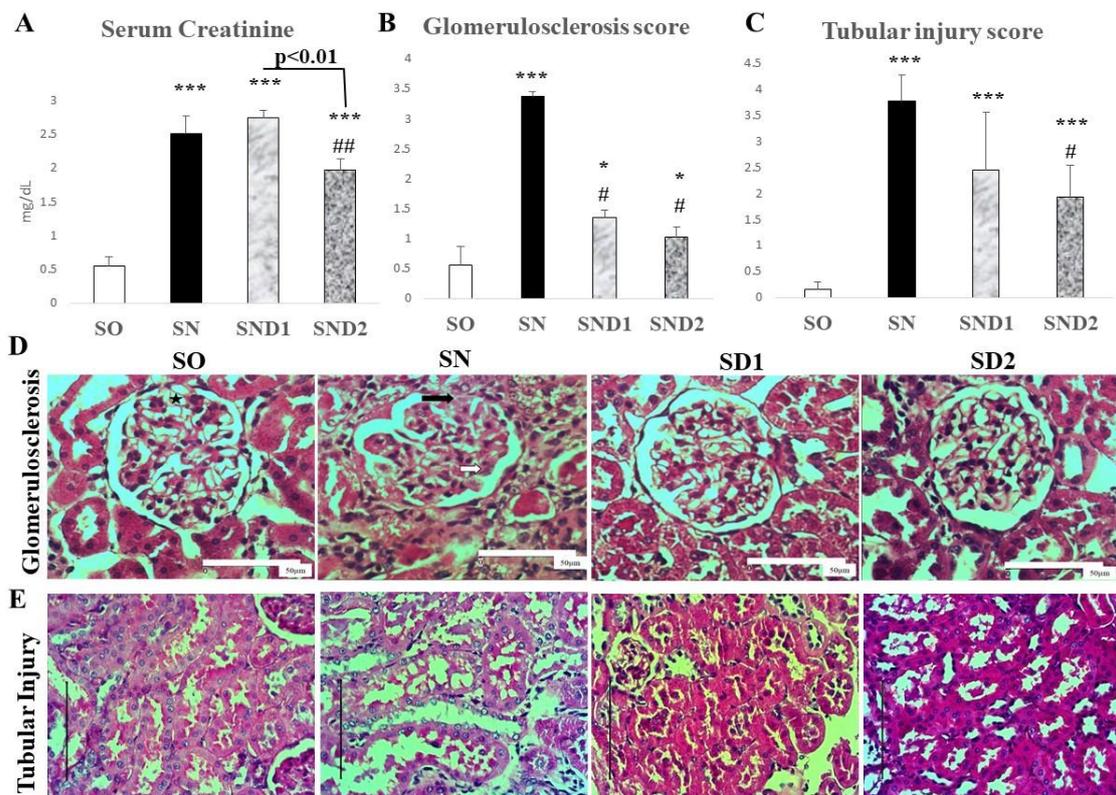
**Statistical Analysis**

Data were analyzed using SPSS 23 for Windows software. Test data normality using the Shapiro-Wilk test. If the data is normally distributed then the next analysis uses One-Way ANOVA test, whereas if the data is not normally distributed using Kruskal-Wallis test. A p-value <0.05 is used to determine the level of significance.

**RESULTS****Calcitriol treatment ameliorated glomerulosclerosis and tubular injury**

Subtotal Nephrectomy represented CKDs with reduction of renal function, as shown by significantly higher serum creatinine level in the SN group compared to SO group (Figure 1A). Calcitriol treatment in SND2 group demonstrated significantly lower serum creatinine level compared to SN and SND1 groups. However, it still had significantly higher serum creatinine level than SO group (Fig. 1A). Histopathological analysis revealed SN group demonstrated glomerulosclerosis as shown by the accumulation of matrix in the glomerulus, capillary loops closing and synechia in PAS staining (Fig. 1D). Glomerulosclerosis score (GSS) measurement revealed significant higher GSS in the SN group compared to SO. We did not find a significant difference between SND2 and SND1 in GSS, but both groups demonstrated significantly lower GSS compared to SN group (Fig.1B). PAS staining also demonstrated tubular injury after SN procedure. SN group revealed renal architecture damage based on histopathological appearance in PAS staining with brush border loss, epithelial cells effacement, tubular dilatation and intraluminal cast formation (Fig 1E). SN group demonstrated a significantly higher tubular injury score compared to SO group (Fig. 1C&E). Calcitriol-treated groups represented amelioration of tubular injury which was shown by brush border formation and lower tubular injury score, however, only SND2 group demonstrated

significant lower tubular injury score compared to SN group. We did not find any significant difference between SND1 and SND2 groups in tubular injury score.



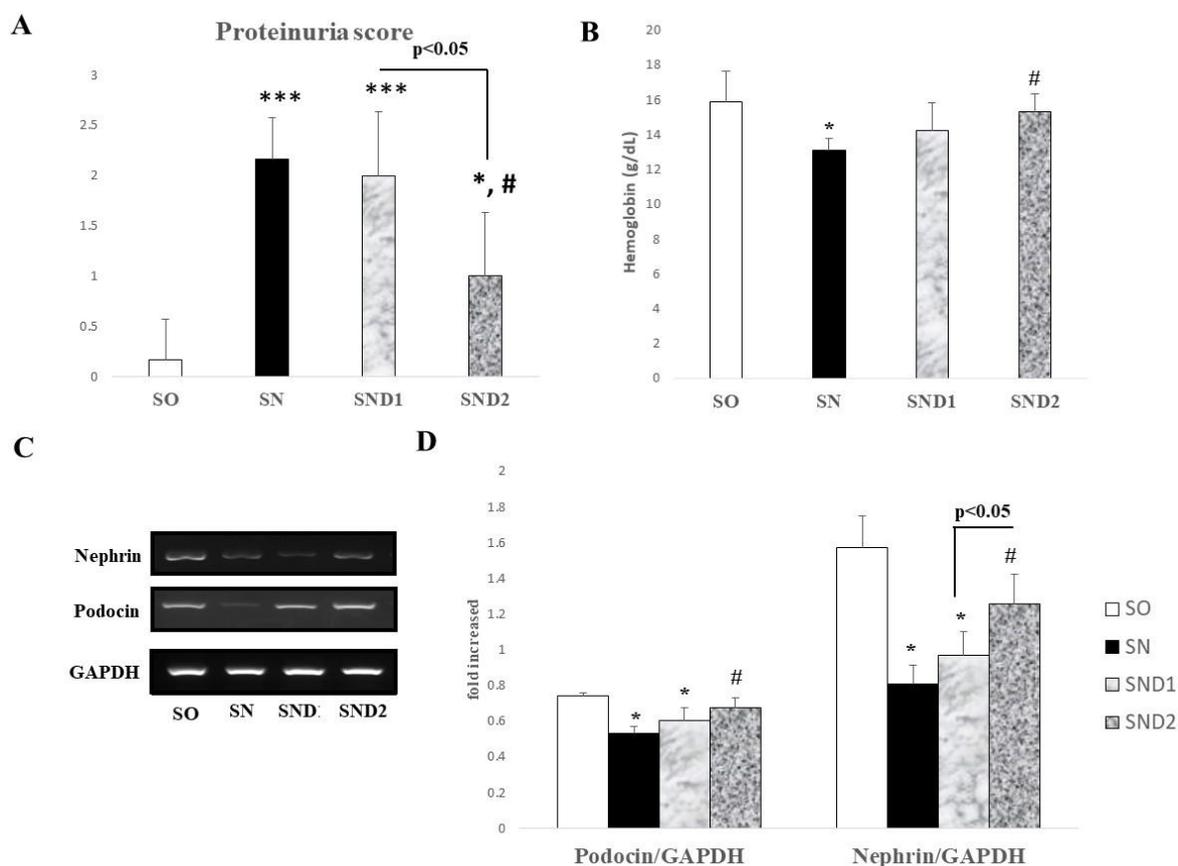
**Figure 1.** Calcitriol treatment ameliorated glomerulosclerosis and tubular injury. A. Bar charts showing serum creatinine level. B. Bar charts showing glomerulosclerosis score. C. Bar charts showing tubular injury score. D. Histopathological appearance of glomerulosclerosis shown in PAS staining. The star print showed normal capillary lumen of the glomerulus, the white arrow showed obliterated capillary, and the black arrow showed accumulation of matrix material E. Histopathological appearance of tubular injury shown in PAS staining. \* $p < 0.05$  vs SO; \*\*\* $p < 0.001$  vs SO, # $p < 0.05$  vs SN; ##  $p < 0.01$  vs SN.

### Calcitriol treatment attenuated filtration disruption and anemia

Subtotal Nephrectomy procedure induced filtration disruption as shown by significant higher proteinuria score ( $p < 0.001$ ) in the SN group compared to SO group. SND2 group demonstrated attenuation of filtration disruption with significant lower of proteinuria score in SND2 group compared to the SN group ( $p < 0.05$ ). There was no difference between the SND1 and SN group (Fig. 2A). SN group also demonstrated a reduction of hemoglobin level and had significantly lower hemoglobin level compared to SO group (Fig. 2B). SND2 represented amelioration of anemia with significantly higher hemoglobin level compared to SN, however, we did not find any significant difference between SND2 and SO groups.

Then, we examined the effects of vitamin D on renal podocyte's markers, nephrin and podocin mRNA expressions. The expression of nephrin mRNA in the SN group ( $0.80 \pm 0.10$ ) was lower than the SO group ( $1.57 \pm 0.17$ ) indicating that there was an injury at the podocyte. Meanwhile, the mRNA expression of nephrin in the SND1 group ( $0.96 \pm 0.13$ ) and SND2 ( $1.26 \pm 0.16$ ) was higher than the SN group. There was significant higher mRNA nephrin expression in SND2 group compared to SND1 group (Fig. 2C-D). The decrease in nephrin mRNA expression in the SN group associated with significantly lower podocin mRNA expression in the SN group ( $0.53 \pm 0.04$ ) compared to the SO group ( $0.74 \pm 0.01$ ). SND1 and SND2 groups demonstrated higher podocin mRNA expression compared to SN, on the other hand, only SND2 group had significantly higher podocin than the SN group. We did not find any significant difference between SND2 group and SO group in podocin mRNA expression (Fig. 2C-D).

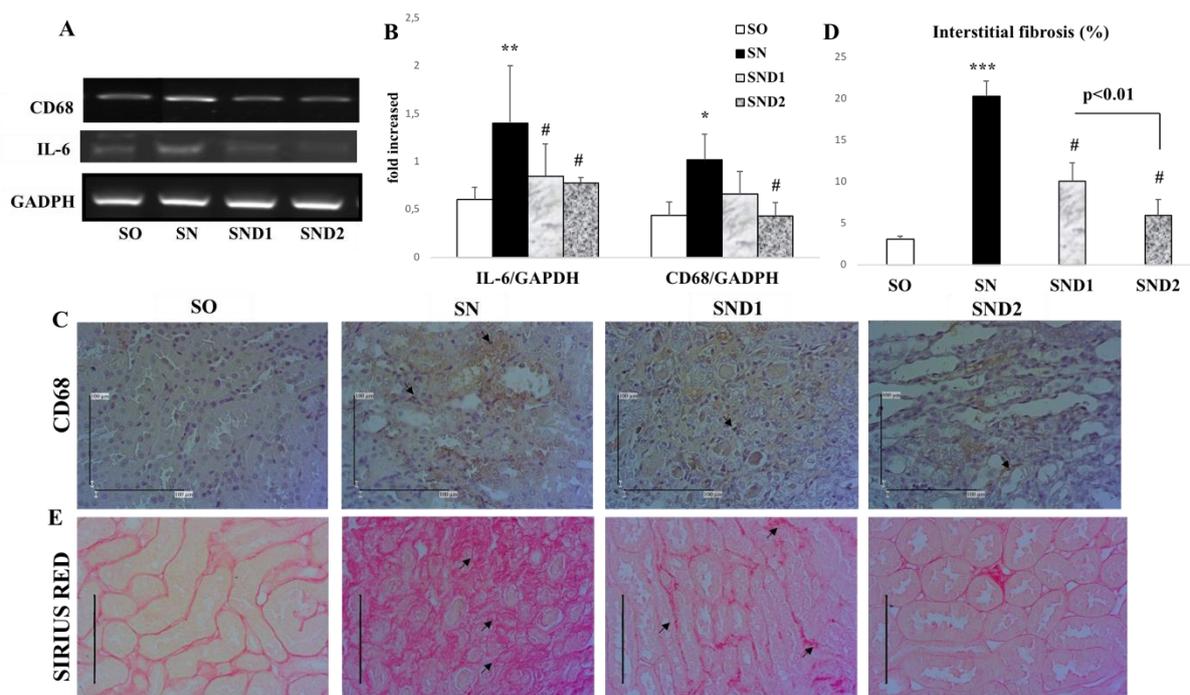
## CALCITRIOL AMELIORATES KIDNEY INJURY IN SN MODEL



**Figure 2.** Calcitriol treatment attenuated filtration disruption. A. Quantification of proteinuria score. B. Quantification of hemoglobin level showed a higher hemoglobin level in SND2 group. C-D. Representative picture of Nephrin and Podocin mRNA expression and densitometry analysis of Nephrin and Podocin RT-PCR. Densitometry analysis based on RT-PCR revealed amelioration of podocyte injury in SND2 group with higher Podocin and Nephrin mRNA expressions. \* $p < 0.05$  vs SO; \*\*\* $p < 0.001$  vs SO, # $p < 0.05$  vs SN.

### Calcitriol treatment attenuated inflammation and fibrosis

Further, we investigated inflammation and interstitial fibrosis. Subtotal nephrectomy induced significantly higher IL-6 and CD68 (macrophage marker) mRNA expressions in the SN group compared to SO (Fig. 3 A-B). RT-PCR analysis revealed that calcitriol-treated groups had significantly lower IL-6 mRNA expression compared to SN, but no significant difference was found between the SND1 and SND2 group. However, SND2 had significantly lower expression of CD68 compared to SN group. Immunohistological staining of CD68 (macrophage) demonstrated positive in the interstitial area of the SN group (Figure 3C). This positive staining represented macrophage infiltration that associated with higher IL-6 and CD68 mRNA expression in the SN group. SND2 group showed lower macrophage number based on the impression of its immunohistological features. Sirius red staining revealed positive staining for collagen accumulation in interstitial areas which demonstrated extracellular matrix expansion. Interstitial fibrosis area fraction assessment revealed significant higher area fraction in the SN group compared to SO group (Fig. 3D-E). Meanwhile, Calcitriol treated groups had significantly lower interstitial fibrosis area fraction compared to the SN group. On the other hand, SND2 represented significant lower interstitial fibrosis area fraction compared to SND1.

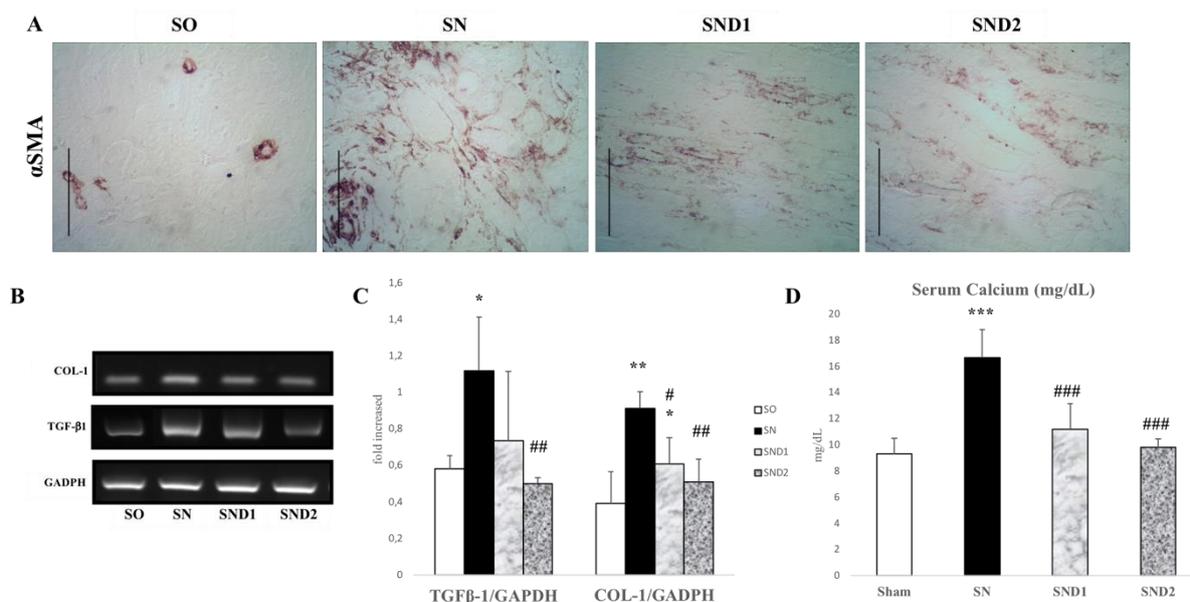


**Figure 3.** Calcitriol treatment attenuated inflammation and fibrosis. A. Gel electrophoresis results of RT-PCR analysis showing *CD68*, *IL-6*, and *GAPDH* mRNA expressions. B. Bar charts showing semiquantitative analysis of *IL-6* and *CD68* mRNA expression relative to *GAPDH*. C. Immunohistological features of renal glomeruli. Brown stained interstitial represented macrophage infiltration. The black arrows showed CD68-positive cells. D. Bar charts showing semiquantitative analysis of interstitial fibrosis. E. Histopathological appearance of each group shown in sirius red staining. The black arrows showed interstitial fibrosis. \* $p < 0.05$  vs SO; \*\* $p < 0.01$  vs SO; \*\*\* $p < 0.001$  vs SO, # $p < 0.05$  vs SN.

### Calcitriol reduced profibrotic substance upregulation and fibroblast expansion

RT-PCR analysis also revealed significantly higher of COL-1 and TGF- $\beta$ 1 mRNA expression in the SN group compared to SO group (Fig. 4 A&B). On the other hand, calcitriol-treated groups had significantly lower COL-1 and TGF- $\beta$ 1 mRNA expression compared to the SN group. SND2 group had significantly lower TGF- $\beta$ 1 mRNA expression compared to the SN group, however, there was no significant difference between SND1 and SND2. Furthermore, we found significantly lower of COL-1 mRNA expression in SND1 and SND2 groups than the SN group. SND2 group represented significantly lower compared to SND1 group. Immunostaining using  $\alpha$ SMA as myofibroblast marker revealed positive stain in smooth muscle cells of the vessel in SO group. Meanwhile in SN group positive stained cells was found in interstitial areas which represented myofibroblast formation (Fig. 4C). SND1 and SND2 group showed lower myofibroblast expansion based on the impression of their immunohistological features. To examine effect of SN and calcitriol treatment, we measured serum calcium level. SN group had significant higher serum calcium level compared to SO group. Meanwhile, calcitriol treated group showed lower calcium level than SN group. There was no statistically different between SO and calcitriol treated groups (Fig. 4D).

## CALCITRIOL AMELIORATES KIDNEY INJURY IN SN MODEL



**Figure 4.** Calcitriol reduced profibrotic substance upregulation. A. Immunohistochemical staining of  $\alpha$ SMA demonstrated smooth muscles cells and myofibroblast positive staining. Myofibroblast formation occurred in the interstitial areas of SN groups. Brown stained interstitial represented myofibroblasts and blood vessel. B. Gel electrophoresis results of RT-PCR analysis showing *COL-1*, *TGF- $\beta$ 1* and *GAPDH* mRNA expressions. C. Bar charts showing semiquantitative analysis of *TGF- $\beta$ 1* and *COL-1* mRNA expression relative to *GAPDH*. D. Serum calcium level demonstrated higher of Calcium level in SN group, meanwhile lower in Calcitriol treated groups. \* $p < 0.05$  vs SO; \*\* $p < 0.01$  vs SO; # $p < 0.05$  vs SN; ###  $p < 0.01$  vs SN.

## DISCUSSION

This study revealed calcitriol treatment attenuated injury in CKD model with Subtotal Nephrectomy. It attenuated tubular injury, inflammation, and fibrosis, shown through reduced profibrotic substance upregulation and reduced interstitial fibrosis in SN model. SN shows similar findings to CKD characteristics, includes increased in serum creatinine. Increased in serum creatinine level is proportional with reduction of renal function found in chronic kidney disease.<sup>(23, 24)</sup> Low GFR decreases creatinine filtration thus increases serum creatinine level. Creatinine is chosen as a filtration marker because it is not protein bound, not metabolized by renal and is freely filtered.<sup>(25)</sup> High dose of calcitriol in this study demonstrated attenuation of renal functions with ameliorating glomerular filtration barrier and renal parenchymal damage. Calcitriol treatment in CKD model after Type-2 Diabetes reported its effects on reducing creatinine level, lowering tubulointerstitial fibrosis and improving renal architecture<sup>(26, 27)</sup> Lower tubular injury score possibly results of its antifibrotic, antiapoptotic, and anti-inflammatory effects.<sup>(27-29)</sup>

Attenuation of glomerular filtration disruption was showed with upregulation of podocyte's marker in SND2 group (Fig. 2). Vitamin D can directly protect podocyte function by increasing the expression of nephrin mRNA by binding to VDR in renal podocytes.<sup>(30)</sup> The study is also in line with other studies suggesting that analogous vitamin D may stimulates nephrin expression in podocytes by working on VDRE in proximal nephrin gene promoters.<sup>(31)</sup> Then, using a mouse model of hypertensive nephropathy it was found that vitamin D can decrease proteinuria as well as increase nephrin expression significantly.<sup>(32)</sup> Meanwhile, vitamin D is known to restore expression of WT-1 protein in animal model adriamycin nephropathy.<sup>(33)</sup> Vitamin D administration could improve hyperglycemia and nephrin signaling restorations through the PI3K / p-Akt signaling pathway in diabetic nephropathy model mice<sup>(34)</sup>, then through VDR expression upregulation in podocytes may upregulate nephrin expression.<sup>(35)</sup>

This study also demonstrated calcitriol treatment ameliorate anemia in SN model. Anemia is an early complication of CKD that also shown in SN model.<sup>(36)</sup> Calcitriol treatment was reported to contribute in increasing hemoglobin and hematocrit level in CKD patients, decreasing erythropoietin resistance, and lowering myofibroblast fraction area.<sup>(27, 42, 43)</sup> Vitamin D may attenuates anemia in patients with CKD due to its roles in erythropoietic process. Vitamin D also reduce hepcidin expression and increases ferroportin thus prevents iron deficiency anemia cases in CKD patients. Furthermore, Vitamin D downregulates proinflammatory cytokine expression and hepcidin that increases resistance to erythropoietin.<sup>(41)</sup> In addition to the effects on tubular injury and anemia, calcitriol possibly also has effects on inflammation and fibrosis.<sup>(7, 44-46)</sup> We showed reduction of

inflammatory mediator, Interleukin-6 (IL-6) in this study due to calcitriol treatment. Furthermore, expression of CD68 mRNA as macrophage's marker in SND1 and SND2 group were significantly lower compared with the SN group. Vitamin D also has an anti-inflammatory effect by downregulating pro-inflammatory mediators such as Toll-Like Receptor 4 (TLR4), Inter-Cellular Adhesion Molecule 1 (ICAM1), and Monocyte Chemoattractant Protein 1 (MCP1).<sup>(8, 27)</sup> TLR4 is expressed in many tissues, such as tubular epithelial cell and mesangial cell as a response to injury.<sup>(51)</sup> Improvement in renal architecture in the vitamin D group decreases the expression of TLR4 and other pro-inflammatory mediators. Vitamin D also inhibits activation of NF- $\kappa$ B by binding with IKK $\beta$  protein, which blocks the production of TNF $\beta$ -induced IKK complex.<sup>(52)</sup>

Reduction of inflammation and fibrosis due to calcitriol treatment in this study may associate with attenuation of anemia. Vitamin D suppresses activation of NF- $\kappa$ B, one of the transcription factors that regulate immune response and secretion of inflammatory cytokines and chemokines, one of which is MCP-1 that induces macrophage infiltration.<sup>(7)</sup> MCP-1 increases proinflammatory cytokine – IL-6, and adhesion molecule – ICAM-1. MCP-1 stimulates IL-6 expression through NF- $\kappa$ B by degrading I- $\kappa$ B $\alpha$  – an inhibitory protein of NF- $\kappa$ B and activator protein-1/AP-1. It stimulates ICAM-1 expression through activation of NF- $\kappa$ B.<sup>(62)</sup> IL-6 modulates monocytes differentiation into macrophages and dendritic cells in peripheral blood. Local macrophages become dendritic cells when exposed with granulocyte macrophage colony stimulation factor (GM-CSF) and IL-4. Later, IL-6 will transform dendritic cells into macrophages through enhancement of receptor regulator M-CSF IL-6 in monocytes.<sup>(63)</sup>

Amelioration of anemia in calcitriol treated groups might not only associate with reduction of inflammation, but also reduction of fibrosis in this study. Vitamin D works in non-classical pathway where vitamin D is able to suppress renal fibrosis through inhibition of transduction signal of TGF $\beta$ -SMAD, which is one of the fibrosis and apoptosis pathways, without activating vitamin D receptor (VDR)-mediated transcription.<sup>(50)</sup> Anemia in CKD is usually caused by erythropoietin deficiency. Almost 90% of erythropoietin in human is secreted by interstitial fibroblast in the renal cortex and outer part of the medulla.<sup>(17)</sup> Interstitial fibroblast that changes into myofibroblast decreases EPO secretion,<sup>(40)</sup> therefore lowers EPO level that produced by fibrotic renal and contributes into anemia events in CKD.<sup>(18)</sup> Myofibroblast is one of the main cells that produce ECM in fibrotic renal other than glomerular mesangial cell.<sup>(39)</sup> Reduction of fibrosis was showed by downregulation of TGF- $\beta$ 1 and Collagen-1 mRNA expression, and interstitial fibrosis area fraction reduction. Tubular cells also secrete profibrotic growth factors such as platelet-derived growth factor, connective tissue growth factor, and TGF- $\beta$ 1, that stimulate accumulation and activation of fibroblast, and further cause interstitial collagen deposition. TGF- $\beta$ 1 also secreted by macrophage as a feedback mechanism to facilitate the resolution of pro-inflammatory response. TGF- $\beta$ 1 triggers activation of fibroblasts and development of myofibroblasts that facilitate tissue repair and fibrosis<sup>(65, 72)</sup>, inhibit PAI expression, and increase TIMPS<sup>(57)</sup>. Myofibroblasts secrete extracellular matrix, includes collagen I, III, and fibronectin that cause interstitial fibrosis and further damage. Another profibrotic factor, Angiotensin II, stimulates TGF- $\beta$  production and stimulates renal cells to produce ECM.<sup>(12, 39, 72)</sup>

This study showed lower interstitial fibrosis fraction area and lower expression of mRNA TGF- $\beta$  was found in calcitriol-treated group compared with SN group. This may result from vitamin D effects in renal fibrosis which are: 1) Vitamin D prevents signal transduction of TGF- $\beta$ -Smad by inhibiting pSmad3<sup>(50)</sup>, 2) it induces hepatocyte growth factor (HGF) that inhibits myofibroblast activity and inhibits epithelial to mesenchymal transition.<sup>(73)</sup> This cascade will lower extracellular matrix production, such as collagen I, III, and fibronectin in renal interstitial space. Last, vitamin D also inhibits renin-angiotensin system through lowering renin gene transcription.<sup>(74)</sup> Angiotensin II also known to stimulate TGF- $\beta$  production and stimulate renal cells to produce ECM.<sup>(72)</sup>

Elucidating effects of Calcitriol treatment, we checked serum calcium level and it showed that SN induced significant higher serum calcium level compared to SO. CKD induced secondary hyperparathyroidism with renal osteodystrophy which is followed by elevation of serum calcium level<sup>(75)</sup>. However, calcitriol groups showed no significant difference of calcium level compared to SO group. Measurement of serum vitamin D level may be needed to examine the side effects of the treatment such as vascular calcification<sup>(75)</sup> although the dose which induces intoxication is more than  $\geq 100\text{ng}/100\text{grBB}/\text{day}$  in rat<sup>(76)</sup>.

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