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Vitamin D Ameliorates Kidney Ischemia Reperfusion Injury via Reduction of Inflammation and Myofibroblast Expansion

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The incidence rate of Acute Kidney Injury (AKI) gets escalated each year. Kidney ischemia/reperfusion injury (IR injury) is the main cause of AKI after major cardiovascular surgery, trauma, or kidney transplantation. Reperfusion is considered essential for ischemic tissue. However, the evidence revealed that reperfusion itself has impact in cellular destruction. Vitamin D is not only known as calcium regulating hormone, but also as renoprotective agent. This study aimed to investigate the effect of vitamin D treatment on kidney IR injury in mice.

Kidney IR injury was performed using 30 minutes of bilateral clamping of renal pedicles, then released in male Swiss Webster mice (3 months, 30-40 grams, n=20), which were divided into three groups: sham operation (SO) group, IR injury (IRI) group, and IR injury with 0.25 µg/ kg body weight of vitamin D treatment (IR7+VD). Mice were terminated at day 7 post operation, kidneys were harvested and used for paraffin making, immunostaining and RNA extraction. Tubular injury was quantified based on Periodic Acid-Schiff's (PAS) staining. Immunostaining was done for quantification of macrophage (CD68) and myofibroblast (α-SMA). Reverse Transcriptase PCR (RT-PCR) was done to examine Monocyte Chemoattractant Protein-1 (MCP-1) and Toll-like Receptor 4 (TLR4) mRNA expression.

Kidney IR injury induced significant increase of tubular injury, which was associated with higher myofibroblast and macrophage number. Meanwhile, Vitamin D treatment significantly reduced tubular, myofibroblast and macrophage number. RTPCR revealed reduction of TLR4 and MCP-1 mRNA expressions after Vitamin D treatment ($p<0.05$ vs IR group).

Vitamin D ameliorates kidney IR injury through reducing inflammation and myofibroblast formation.

INTRODUCTION

The incidence rate of Acute Kidney Injury (AKI) gets escalated each year (1). Various strategies have been done, yet the morbidity and mortality rates are still inevitably high (2). In these days, the prognoses of patients with AKI hasn't showed any significant improvement (3,4). Ischemia/reperfusion injury (IR injury) is the main cause of AKI after major cardiovascular surgery, trauma, or kidney transplantation. Reperfusion is considered essential for ischemic tissue. However, the evidence revealed that reperfusion itself has impact in cellular destruction (2). The alteration of blood vessel endothelial cells, tubular epithelial cells, and leucocytes contribute to IR injury resulting in deterioration of kidney immune system homeostasis (5). Blood flow restoration and re-oxygenation could enhance the severity of tissue damage caused by ischemic injury and trigger inflammatory response; which furthermore called as reperfusion injury (6).

Kidney ischemic/reperfusion (I/R) injury commonly causes AKI and 70 % of the cases lead to chronic kidney diseases (3). Chronic Kidney Disease (CKD) becomes global health problem due to its bad prognosis. This condition leads to kidney fibrosis which is characterized by progressive injury of renal interstitial, extracellular matrix accumulation and myofibroblast formation (7). Deterioration of kidney functions in CKD causes Vitamin D deficiency (8). CKD also produces disruption of c*holecalciferol* in the skin, then reduces calcidiol amounts that enters the kidney (9). Reduction of 1-α-hydroxylase enzyme also induces limitation of the calcitriol production (10). Vitamin D is known not only in role of bones mineral metabolism, but also in its renoprotective effect (11). Vitamin D suppresses renal fibrosis by inhibiting TGF-β-Smad signal transduction through direct interaction with Smad3 (12). Vitamin D is also anticipated to prevent kidney myofibroblast formation by inducing the formation of Hepatocyte Growth Factors (HGF) by liver(13).

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Vitamin D has also been reported has beneficial effect in reducing kidney fibrosis, inflammation and epithelial cell apoptosis in Unilateral Ureteral Obstruction (UUO) model in mice (14). Some mechanisms of vitamin D action which targeting fibrosis prevention have been investigated yet still requiring further research to clarify the precise action mechanism (13). In this study we elucidated effect of vitamin D effect in chronic episode of kidney IR injury in mice focusing on inflammation, tubular injury and myofibroblast expansion.

MATERIALS AND METHODS

Materials

Experiments were done after approved by Ethical Committee of Faculty of Medicine, Public health and Nursing, Universitas Gadjah Mada, Yogyakarta, Indonesia with number KE/FK/259/EC/2016. Male Swiss Webster mice (n = 20) age 12-16 weeks old with 30-50 gr body weights were obtained from Experimental Animal Care Unit (UPHP) LPPT Universitas Gadjah Mada, Yogyakarta, Indonesia. Mice were housing in cages owned by Department of Anatomy, Faculty of Medicine, Universitas Gadjah Mada, Yogyakarta, Indonesia with the light-dark cycle of 12:12 hours.

Methods

Bilateral renal pedicles clamping was performed for 30 minutes to induce kidney ischemic/reperfusion (IR) injury. Briefly, mice were anesthetized with intraperitoneal injection of Sodium Pentobarbital (Somnopenthyl, 0.1mg/kg body weight). Anterior abdominal wall was opened; bilateral renal pedicles were visualized then clamped using non-traumatic vascular clamps. Sham Operation (SO, n=6) procedure was used for control group with the same procedure except for renal pedicles clamping. Subjects were divided into 3 groups i.e. SO (sham-IRI + ethanol 0,2%), IR7 (IRI + ethanol 0,2%, n=7), and IR7+VD (IRI + vitamin D 0,25 µg/ kg, n=7). Vitamin D treatment was done with intraperitoneal injection of Cholecalciferol diluted with Ethanol 0.2%. Mice got standard chow and access for free water *ad libitum*. Mice were terminated at day 7 post operation.

For sacrifice, mice were anesthetized with Sodium Pentobarbital (0.1mg/kg body weight, intraperitoneal), then the abdomen and thorax were opened. Organs were perfused with 0.9% NaCl from the left ventricle. Right kidney tissues were harvested; half side was kept in RNA Later for RNA extraction. Another half was fixated in 4% PFA in PBS for 24 hours, and used for paraffin embedded tissue process.

Paraffin sections with 4 mm thickness were used for following analyses. Paraffin sections were deparaffinized, and stained with Periodic Acid-Schiff's (PAS) to evaluate tubular injury. Immunohistochemistry (IHC) staining was done for these following antibodies: CD68 for macrophage (abcam, ab955, 1:200 dilution), and α-SMA for myofibroblast (Sigma, A2547, 1:500 dilution). Immunostaining was performed using Starr Trek Universal HRP Detection Kit (STUHRP700Hkit, Biocare Medical). Briefly, after deparaffinizing, paraffin sections were heated in citrate buffer, then incubated in 3% H_2O_2 in PBS. Nonspecific signal blocking was done using blocking sniper in the kit, then first antibodies were incubated overnight in 4^0. Secondary antibodies were incubated for 1.5 hours using Trekkie universal antibodies (Starr Trek Universal HRP Detection Kit (STUHRP700Hkit, Biocare Medical)). DAB and its buffer was used for examining the signal with hematoxyllin counterstaining.

Tubular injury was assessed using PAS staining based on previously described method (8). Scoring was done by grading tubular dilatation, epithelial simplification and brush border loss in 15 randomly chosen, non-overlapping fields (400x magnification). The lesions were graded on a scale from 0 to 4: 0 _ normal; 1 _ mild, involving less than 25% of the field; 2 _ moderate, involving 25 to 50% of the field; 3 _ severe, involving 50 to 75% of the field; and 4 _ extensive damage, involving more than 75% of the field. Myofibroblast (α-SMA positive cell) and macrophage (CD68 positive cell) number quantification were done using quantification of number in 400x magnification in 15 different fields. ImageJ software was used for quantification.

RNA was extracted from kidney tissue using Trizol (Invitrogen, 1559-018, Paisley, UK). cDNA was synthesized using ReverTra-Ace (TOYOBO Co., Ltd, TRT-101x10). Reverse Transcriptase PCR (RTPCR) was done for examining the expression of following genes: Monocyte Chemoattractant Protein-1(MCP-1) (forward, 5'- GGCATCACAGTCCGAGTCACA-3'; reverse,3'- CTACAGACAACCACCTCAAGCACTTC-5'), Toll-like Receptor 4 (TLR4) (forward,5'- GGGCCTAAACCCAGTCTGTTTG-3'; reverse, 3'-GCCCGGTAAGGTCCATGCTA-5'), and GAPDH (forward, 5'-TTGCTGTTGAAGTCGCAGGAG-3'; reverse, 5'-TGTGTCCGTCGTGGATCTGA-3') for housekeeping gene. The RT-PCR was done in the following temperature condition: 94°C for initial denaturation for 2 minutes, the followed by 35 cycles of 94°C denaturation phase for 10 seconds, 94°C annealing phase for 20 seconds, and 72°C extension phase for 1 minute. Then, ended with 72°C last extension phase for 10 minutes. Finally, densitometry analysis was performed based on electrophoresis of the genes using ImageJ software.

Data were presented in Mean ± SD and statistical analysis was done using SPSS software. $p<0.05$ was used for measuring significance among the groups.

RESULTS

Vitamin D treatment ameliorated tubular injury and reduced myofibroblast number

Microscopic examination showed tubular injury occurred after kidney IR injury which was characterized by tubular dilatation, intraluminal cash formation, and brush border loss. Tubular injury score was used to observe the degree of tubular injury in SO, IR7, and IR7+VD groups. As shown in Figure 1A, tubular injury was presented extensively in IR7 group but not in SO group. However, vitamin D treatment noticeably improved condition in IR7+VD group as shown by significant reduction of tubular injury score ($p<0.05$ vs IR7 group). With α-SMA positive staining, myofibroblast was a hallmark of interstitial fibrosis. We revealed that kidney IR injury group induced the increase of myofibroblast number compared to SO ($p<0.001$). Vitamin D treatment reduced myofibroblast cell number as shown by reduction of myofibroblast number in IR+VD group compared to IR7 group ($p<0.001$). However, IR+VD group still had significant higher myofibroblast number compared to SO group (Fig. 1B&D).

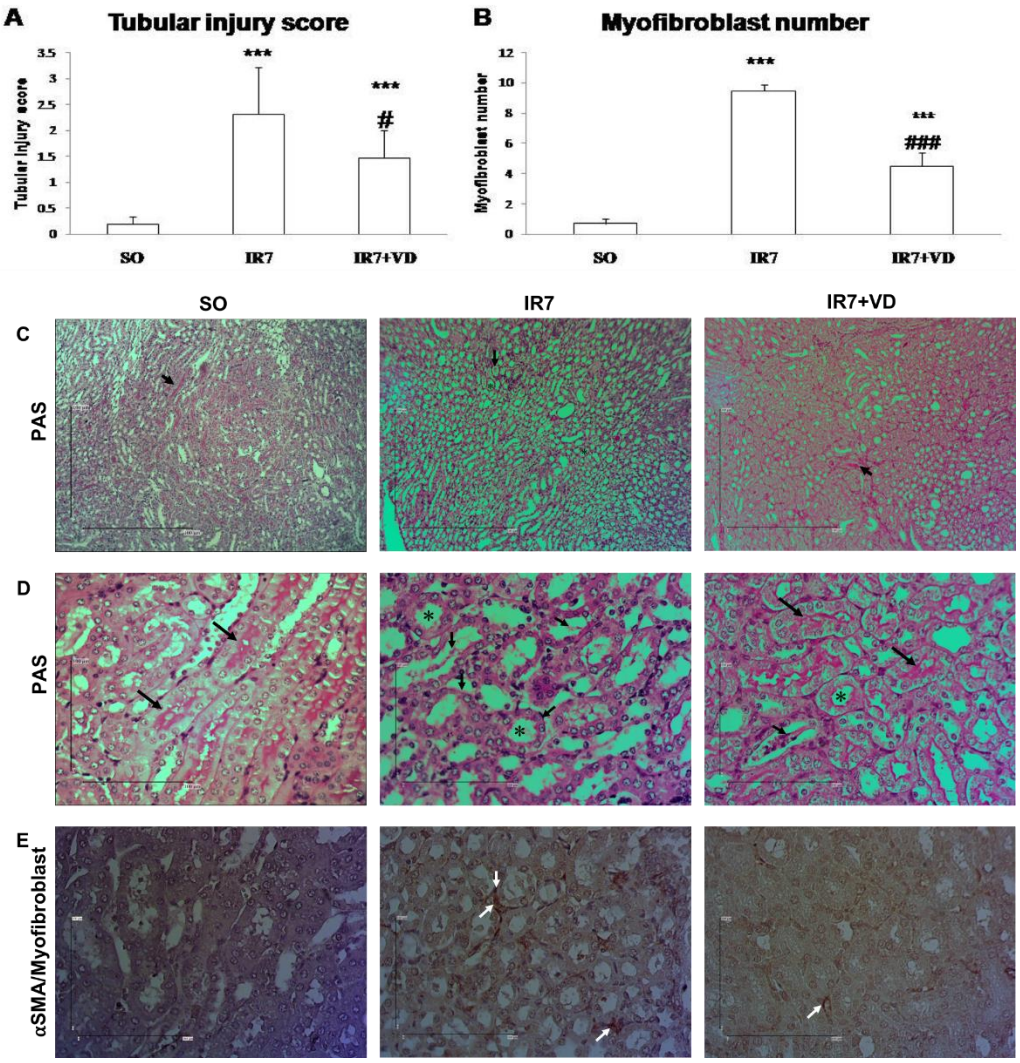

Figure 1.
(A) Tubular injury scores of the groups based on PAS staining. (B) Quantification of myofibroblast number based on αSMA immunostaining. (C) Representative picture of tubular injury based on PAS staining in 100x magnification. Black arrows were used to show tubular atrophy, cast formation and brush border loss. Asterisks showed tubular dilatation(D) Representative picture of αSMA to show myofibroblast (White arrows) in 400x magnification. *** P<0.001 VS SO group; # P<0.05 vs IR7 group.

Vitamin D lowered macrophage number associated with reduction of TLR4 and MCP-1 mRNA expressions

We observed inflammation through quantification of macrophage using CD68 immunostaining. CD68 positive staining was also positive in SO group which was positive for resident dendritic cells. Kidney IR injury induced positive staining of CD68 showing high macrophage number (Fig 2A). Macrophage number was significant lowered in vitamin D treated group compared to IR7 group (Fig. 2A&B). RTPCR result revealed high expression

of inflammatory mediators in IR7 group as shown by upregulation of TLR4 and MCP1 after kidney IR injury compared to SO (Fig 2C&D). Vitamin D treatment showed significant reduction of TLR4 and MCP1 expression compared to IR7 group.

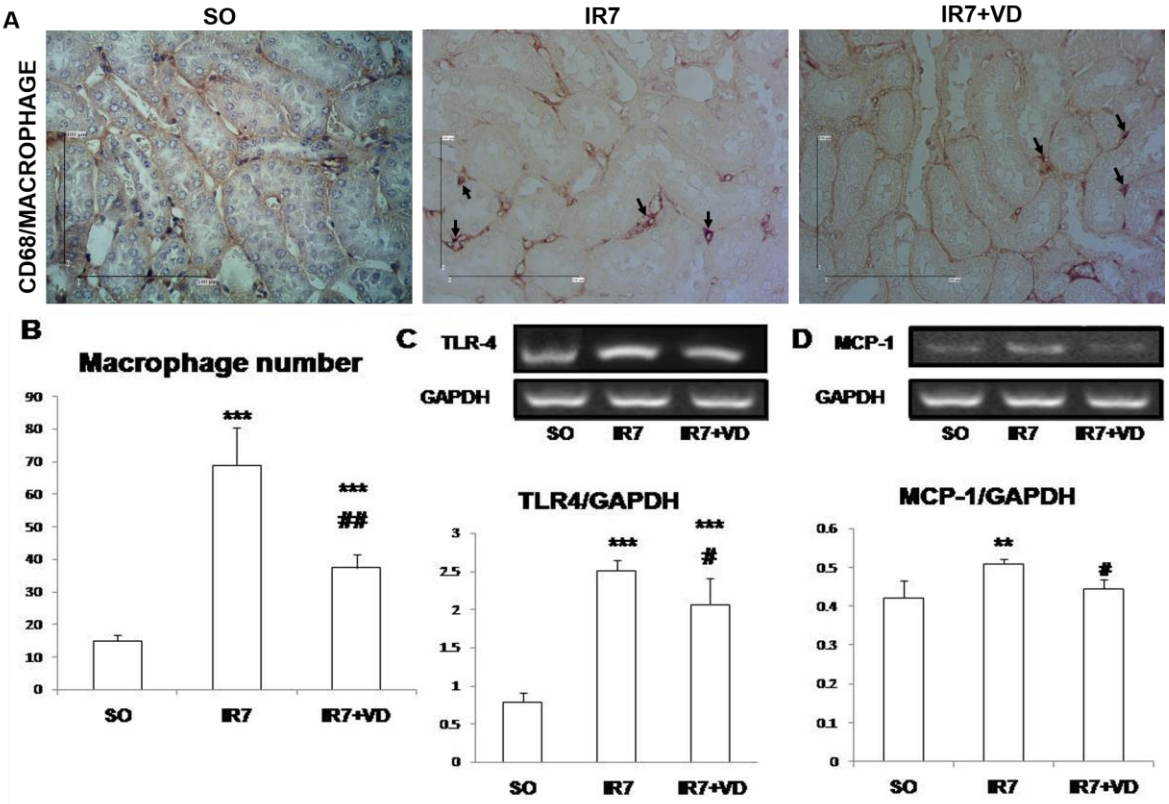

Figure 2.
(A) Representative picture in 400x magnification of CD68 immunostaining SO, IR7 and IR7+VD. Arrow showed positive staining for macrophage. (B) Quantification of macrophage number. (C-D) Reverse transcriptase PCR (RT-PCR) measurement of TLR4 and MCP1 expression, and densitometry analyzes using ImageJ software. **P<0.01 vs SO group; *** P<0.001 vs SO group; # P<0.05 vs IR7 group; ##P<0.01 vs IR7 group.

DISCUSSION

We revealed renoprotective effect of vitamin D in kidney IR injury through ameliorating tubular injury, reducing myofibroblast, and inflammation in this study. Termination experiment in day7 after kidney IR injury revealed chronic damage still occurred in Acute Kidney Injury model, such as kidney IR injury. Even though the total kidney blood flow returned to a former condition during 1-4 weeks of recovery period, it is assumed there is chronic damage of medullary vascular function after AKI indicating reduced vascular regenerative capacity of tubular epithelial cells where hypoxia condition is considered as the biggest contributor in interstitial fibrosis (15). This condition might be developed further, and advanced fibrosis will disrupt microvascular function and drop the tubular nutrition and oxygen supply. Therefore, these are causing tubular stress level elevation, epithelial cell damage, and disrupt the natural regeneration process leading to further fibrosis. Those deterioration was shown by higher tubular injury in day7 after kidney IR injury. Functional analysis should be quantified to examine the effect of the supplementation, especially serum creatinine level. Vitamin D treatment reduced those tubular injury as shown by reduction of tubular injury score in IR7+VD group (Fig 1A&C).

IR injury is one of the main cause of acute kidney injury (16). IR injury model in rat has been proven to cause fibrosis post ongoing kidney injury (17). Myofibroblast with αSMA positive staining is hallmark of kidney fibrosis. A conducted study on collagen-I,α1 showed a result which revealed the indication of pericyte as subendothelial cell that regulates microvascular integrity on peritubular capillary network and also perivascular fibroblast is the main resource of myofibroblast (18). As the response to kidney injury, pericyte showing detached appearance, increasing collagen expression, migrating way far from endothelial cell, and activating back the NG2 pericyte marker (19). We emphasized the renoprotective effect of vitamin D is through its inhibiting effect of myofibroblast formation.1,25(OH)2D3 has the effect of reducing in vitro matrix production and myofibroblast activation as the main effector to inhibit extracellular matrix pooling in kidney fibrosis through dependent Hepatic Growth Factor

mechanism with ischemic reperfusion method (13). The research finding is corresponded with former conducted research that there is reduction of kidney fibrosis effect after vitamin D administration in mice. Calcitriol administration reduces the expression of TGFβ1-induced α-SMA and increasing the type I collagen and thrombospondin-1(13).

This study also highlighted beneficial effect of Vitamin D treatment in reducing inflammation as shown by decreasing macrophage and inflammatory mediators. Hypoxia condition which occurred during ischemic injury causing damage on barrier function of endothelial cell due to the decreased activation of intracellular adenylyl cyclase and cAMP which leads to increased vascular permeability and leakage (6). Vascular leakage provides possibility for macrophage to immigrate towards kidney interstitial cells so that the amount of CD68 positive cells is increased in kidney interstitial of IR7 group compared to SO. Vitamin D could reduce macrophage number in this study which was associated with downregulation Toll-like Receptor 4 (TLR4) mRNA expression. Wu et al., reported that normal kidney tissue expresses TLR-4 in basic level, and yet the mRNA TLR-4 level is elevating on first and third day, furthermore it gets significantly even higher even on fifth and ninth day after IR injury (20). Ligand bond with toll-like receptor activates downstream signal through NF-κB signal, mitogen-activated protein kinase (MAPK), and type I interferon that induced pro-inflammatory cytokines and chemokines (6). The attenuation of macrophage infiltration was not only associated with downregulation of TLR-4, but also Monocyte Chemoattractant Protein 1 (MCP1) mRNA expression (Fig 2). MCP-1 is one of the expressed chemokines and induces macrophage infiltration towards interstitial tissue (20) From this research also revealed the increase of MCP-1 expression in IR7 group compared to SO. This was associated with the elevated number of macrophage accumulation in interstitial tissue. This finding corresponds to a former research done by Stroo et al., (21), stated that the MCP-1 mRNA expression of MCP-1+/+mice is significantly increased on the first day, reaches its highest point on the day 7, and remain high until 14 days post IR injury. A research conducted by Braganca et al., also stated the significant number of ED1+ cells infiltration/field in IRI group compared to control group. Several studies show that the antigen recognized by rat ED1 is homologue with human CD68 (12).

The discovery of numerous tissues and cells in human body has vitamin D receptor (VDR) an enzyme whose ability to transform 25-hydroxyvitamin D as the circulating form of vitamin D into the active form, which is 1,25-dihydroxyvitamin D giving a new perspective about the function of this vitamin (22). It has been reported that paricalcitol as an analogue of synthetic vitamin D has renoprotective effect on AKI which induced by IR injury. The feasible involved mechanism involved is paricalcitol prevents the activation of inflammation through TLR-4-NF-κB pathway. NF-κB is a transcription factor and activated when TLR-4 bonded with its ligand. NF-κB will subsequently regulate several genetic expression including the one which regulate cytokine, adhesion molecule, and chemokine (23). Based on the previous results, we assumed that NF-κB signal which is TLR-4 downstream signaling will be reduced, thus downregulated MCP-1 and attenuated macrophage infiltration in our study.

Another factor that contributes to Vitamin D deficiency in CKD is the upregulation of *Fibroblast Growth Factor 23* (FGF-23), that leads to suppression of 1α-hydroxylase expression and activity. This may influence the increased of serum phosphate level (24). The phosphate retention occurs persistently, thus reduces 1α-hydroxylase enzyme's activity and reduces calcitriol concentration (8). This phenomenon reveals correlation between CKD and Vitamin D deficiency, furthermore many researches demonstrated renoprotective effects of Vitamin D in CKD. However, other side effects of Vitamin D treatment may occur such as, hypercalcemia or hyperphosphatemia which induced vascular calcification. Elucidating those side effects may provide beneficial understanding in Vitamin D metabolism. Vitamin D has been known to ameliorate proteinuria, glomerulosclerosis, inflammation and oxidative stress in kidney (9). This study also demonstrated renoprotective effects of Vitamin D in CKD after AKI condition. Many factors related to Vitamin D metabolism should be elucidated in the future study, especially role of 1α-hydroxylase enzyme in this study. This becomes a limitation of this study. Calcium and phosphate level also should be measured to evaluate the effects of Vitamin D treatment in this model.

CONCLUSION

To sum up, vitamin D treatment could ameliorate kidney ischemia reperfusion injury through reducing inflammation and myofibroblast formation.

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