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Original Article

Aldosterone blockade ameliorates vascular calcification in rats with chronic kidney disease

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Abstract: Vascular calcification (VC) is a common and serious cardiovascular complication in patients with chronic kidney disease (CKD). Aldosterone (Ald) plays a vital role in regulating cardiovascular function. It is known that patients with CKD suffer from hyperaldosteronism, suggesting Ald might regulate the development of VC in CKD. However, the role of endogenous Ald in the pathogenesis of VC in CKD is still unclear. In this study, we employed a model of adenine (ADN)-induced CKD in rats. The results showed that ADN-fed rats exhibited CKD, arterial media calcification, increased plasma levels of Ald and elevated serum biochemical parameters, including creatinine (Cr), blood urea nitrogen (BUN), phosphate and potassium. Serum calcium levels were not altered in rats with CKD. Compared with control aortas, alkaline phosphatase (ALP) activity and calcium content in calcified aortas were increased. The expression of osteogenic marker osteopontin (OPN) was up-regulated, and the expression of smooth muscle lineage marker alpha-smooth muscle actin (α-SMA) was down-regulated in aortas of rats with CKD. Furthermore, the Wnt/β-catenin pathway was activated in calcified aortas and its activation led to the phosphorylation of glycogen synthase kinase 3β (GSK3β) and the increase of β-catenin. Aldosterone antagonism with spironolactone (SPL) therapy mitigated aortic calcification, reduced serum Cr and BUN levels, and inhibited the activation of Wnt/β-catenin pathway as well. These results indicate that hyperaldosteronism could promote the progression of VC via Wnt/β-catenin pathway in rats with CKD. Ald blockade therapy may be effective in preventing the development of VC in patients with CKD.

Keywords: Aldosterone, vascular calcification, chronic kidney disease, GSK3β, β-catenin

Introduction

Chronic kidney disease (CKD) is a common and complex disease affecting millions of people [1]. Progression of CKD is accompanied by serious cardiovascular complications, which are the leading cause of death in patients with CKD [2]. Clinical studies have shown that vascular calcification (VC) is a strong predictor of high cardiovascular risk [3, 4]. It has been reported that chronic renal failure commonly accompanies with varying degrees of VC, and the extent of VC is associated with cardiovascular mortality in patients with CKD [5]. VC is a progressive disorder, which was once thought to be a passive process. But recent studies have demonstrated that VC is an active and highly regulated process similar to bone mineralization [6, 7]. VC in patients with CKD mainly affects the elastic lamellae in the artery wall to cause increased arterial stiffness. The pathogenesis of VC is complex and affected by various factors including increased calcium-phosphate products [8, 9], uremic toxins [10, 11], oxidative stress [12, 13], inflammation [14], etc. Currently, the available therapies for VC in patients with CKD are unsatisfactory. Therefore, further exploring the mechanisms of the VC is very important.

Aldosterone (Ald) is a steroid hormone regulating the body's salt and water balance, thereby helping to control blood volume and blood pressure. Ald plays an important regulatory role in cardiovascular events and renal damage. Excessive Ald levels are associated with hypertension, heart failure, atherosclerosis, and kidney disease [15-17]. Jaffe et al. [18] found that Ald promoted osteogenic transformation of human vascular smooth muscle cells (VSMCs) in vitro. Endogenous Ald is involved in the VC induced by

vitamin D_3 plus nicotine in rats [19]. These results indicate the potential role of Ald in VC. It has been known that patients with CKD suffer from hyperaldosteronism [20], suggesting Ald might regulate the development of VC in CKD. However, the role of Ald in the pathogenesis of VC in CKD is still unclear.

Adenine (ADN)-induced CKD rats with arterial calcification were used in experimental studies. The ADN is metabolized to 2,8-dihydroxyadenine, which forms crystals to obstruct the renal tubules, potentially leading to CKD [21]. The ADN-fed rats develop hyperparathyroidism, hyperphosphatemia and extensive arterial medial calcification in 6-8 weeks of feeding [22-24].

In this study, we employed an ADN-induced CKD model in rats to investigate the effect of Ald on VC in rats with CKD and explore the underlying mechanisms.

Materials and methods

Reagents

The ALP assay kit and the calcium assay kit were from Nanjing Jiancheng Bioengineering Company (Jiangsu, China). BCA protein assay kit was purchased from Beyotime Institute of Biotechnology (Jiangsu, China). Anti-osteopontin (OPN) and anti-alpha-smooth muscle actin (α-SMA) antibodies were purchased from Wuhan Boster Biological Technology, Ltd. (Wuhan, China). Anti-phosphorylation of glycogen synthase kinase 3β (p-GSK3β) and anti-glycogen synthase kinase 3β (GSK3β) antibodies were from cell signaling technology (Danvers, MA, USA), anti-β-catenin and anti-GAPDH antibodies were obtained from Santa Cruz Biotechnology Inc. (CA, USA). Spironolactone (SPL) was from Sigma Chemical Co. (St. Louis, MO, USA). All other chemicals were from Sigma or Santa Cruz.

Animals

Male Wistar rats (8 weeks old) were obtained from Changsheng Biotechnology Co., Ltd. (Liaoning, China). Commercial rat chow was provided by Beijing Keaoxieli Feed Co., Ltd, (Beijing, China). All animal experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals published by the China National Institutes of Health and approved by the Animal Care and Use Committees of Harbin Medical University.

Animal model and experimental groups

The rat model of CKD was performed as described previously [21] with minor modification. In brief, all rats were housed in a temperature-controlled room with a 12-hour light/dark cycle and were allowed free access to water and chow throughout the study. After one-week adaptation, Wistar rats (n=30) were randomly divided into three groups: (1) rats fed a standard diet for 8 weeks were used as control group (control); (2) rats fed a adenine-rich chow (0.75% adenine) for 8 weeks were used as adenine group (ADN); (3) rats fed the adenine-rich chow and administered spironolactone (40 mg/kg/day) by gavage for 8 weeks were used as adenine plus spironolactone treatment group (ADN+SPL). At the end of the experiment, the animals were sacrificed and the samples were collected.

Biochemical parameters and aldosterone assay

Blood was allowed to clot and serum was separated by centrifugation at 3000 rpm for 15 min at 4°C. Blood urea nitrogen (BUN), creatinine (Cr), calcium, phosphate and potassium levels were determined using a biochemical analyzer (Senlo, Zhuhai, Guangdong). Plasma was separated by centrifugation, and plasma aldosterone concentrations were determined by radioimmunoassay.

Histomorphology

The aortas were removed and fixed with 4% paraformaldehyde. Tissue blocks were embedded in paraffin, and cut into 5 μm-thick sections. Tissue sections were stained with H&E. Von Kossa staining were performed as described previously [25]. Briefly, Sections were deparaffinized, dehydrated, and then placed in 1% silver nitrate solution for 1 h under the sunlight. They were then put into 5% sodium thiosulfate for 5 min and counterstained.

ALP activity assay and determination of calcium content in aortas

Aortas were homogenized in ice-cold buffer (0.86% NaCl) to make a 10% homogenate.

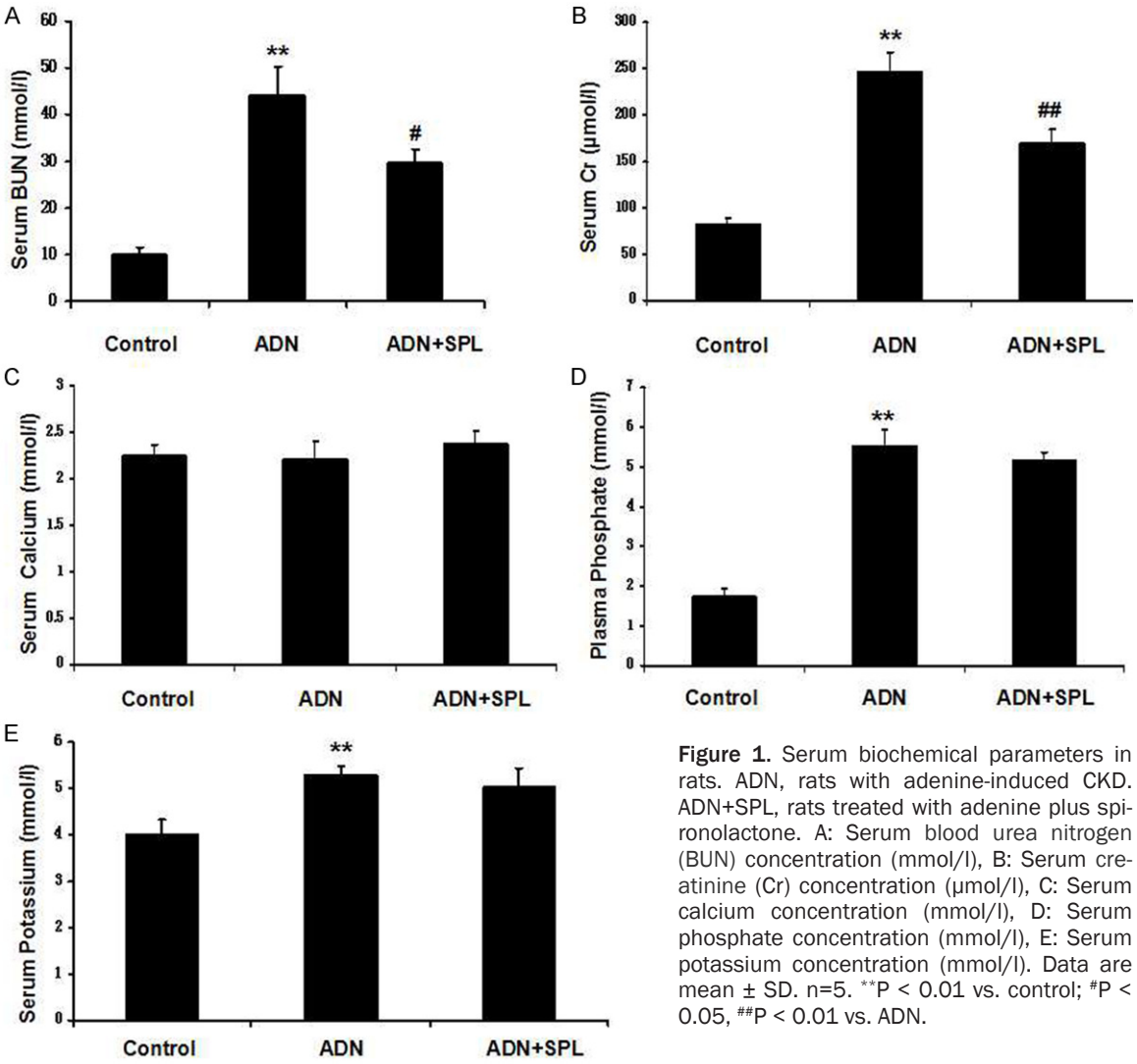

Figure 1. Serum biochemical parameters in rats. ADN, rats with adenine-induced CKD. ADN+SPL, rats treated with adenine plus spironolactone. A: Serum blood urea nitrogen (BUN) concentration (mmol/l), B: Serum creatinine (Cr) concentration (μmol/l), C: Serum calcium concentration (mmol/l), D: Serum phosphate concentration (mmol/l), E: Serum potassium concentration (mmol/l). Data are mean ± SD. n=5. $^{**}P < 0.01$ vs. control; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs. ADN.

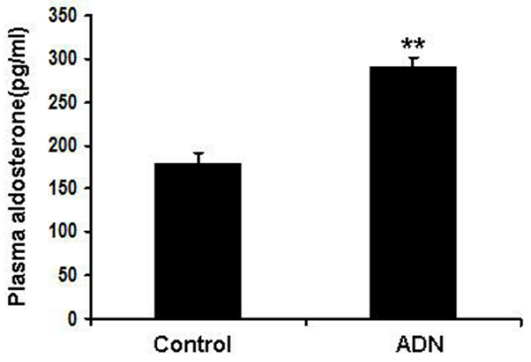

Figure 2. Levels of plasma aldosterone in rats. ADN, rats with adenine-induced CKD. Data are mean ± SD. n=5. $^{**}P < 0.01$ vs. control.

After centrifugation at 3000 rpm for 10 min, the supernatant was assayed. The ALP activity was measured by using the ALP assay kit according to the manufacturer's instructions. ALP activity was normalized to total protein determined with BCA method. Similarly, calcium content was measured by using the calcium assay kit.

Western blot analysis

Total proteins from aortas were extracted. Western blot analysis was performed as described [26]. Equal amounts of the proteins from different experimental groups were boiled and separated on SDS-PAGE gel and transferred to a nitrocellulose membrane, The membranes were blocked in 5% skim milk for 1 h at room temperature and then were incubated overnight at 4°C with antibodies (anti-OPN 1:200, anti-α-SMA 1:400, anti-GSK3β 1:1000,

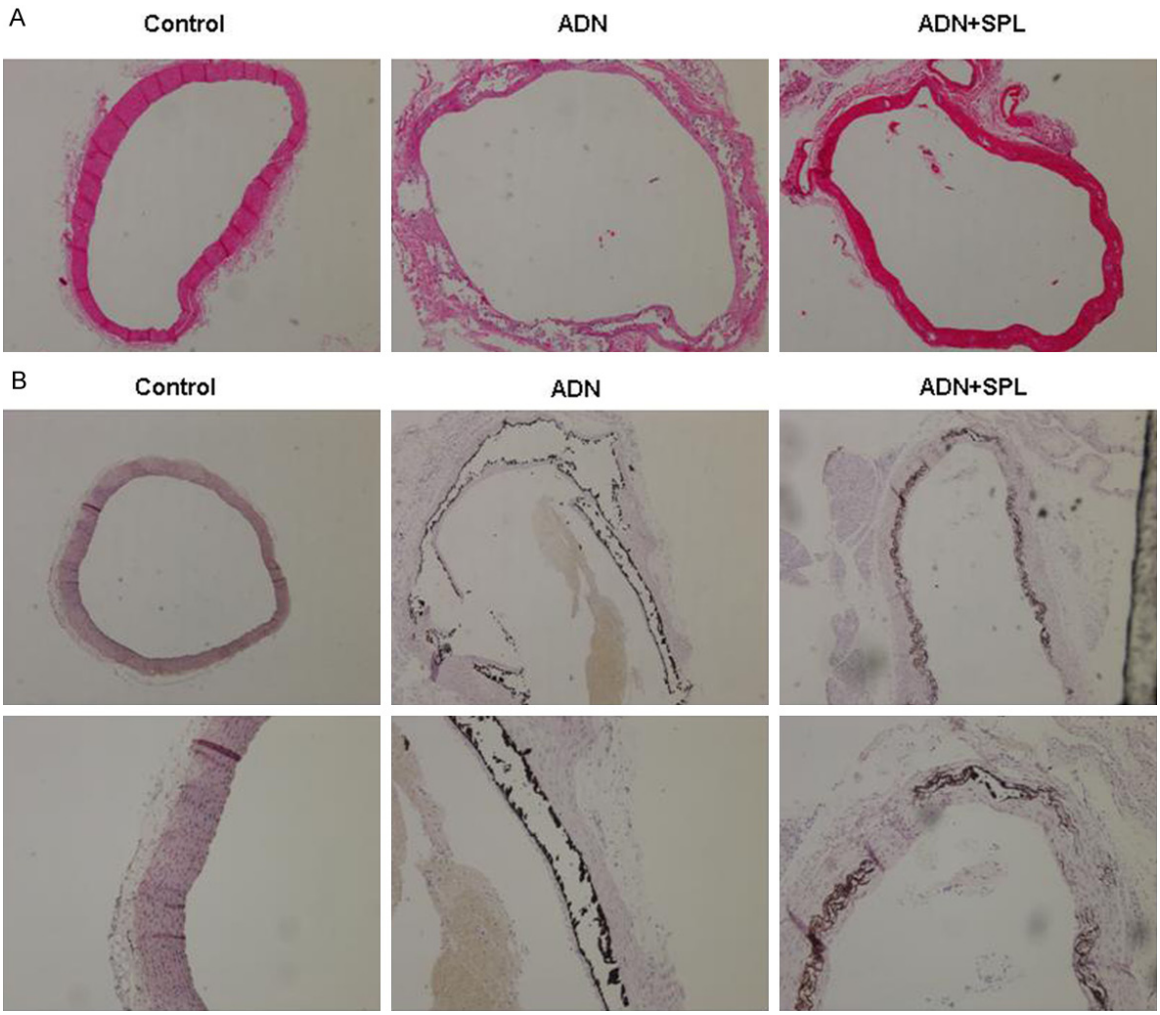

Figure 3. Histological changes in rat aortas. A: H&E staining. B: Von Kossa staining (top panels, original magnification, ×100) (bottom panels, original magnification, ×400). ADN, rats with adenine-induced CKD. ADN+SPL, rats treated with adenine plus spironolactone.

anti-p-GSK3β 1:1000, anti-β-catenin 1:500, and anti-GAPDH 1:500). The next day, the membranes were incubated with secondary AP-conjugated antibodies (1:500) for 1 h at room temperature. The intensity of the protein bands was quantified using the Scion densitometry program, GAPDH or total GSK3β was used as a control.

Statistical analysis

The data were presented as mean ± SD. SPSS 13.0 was used for data analysis. The difference between two groups was analyzed using the Student's t test. Comparisons among three groups were performed using one-way analysis of variance (ANOVA). *P* value < 0.05 was considered statistically significant.

Results

Biochemical parameters

Compared with control group, serum Cr and BUN concentrations were significantly increased in ADN group, a difference markedly modified by life-long treatment with the Ald receptor antagonist spironolactone (SPL) (**Figure 1A** and **1B**). The serum levels of calcium did not significantly differ among the three groups (**Figure 1C**). Serum phosphate concentrations were significantly higher in ADN group than in control group. Despite a tendency toward decreased phosphate levels following SPL treatment, no significant difference was observed between ADN group and ADN+SPL group (**Figure 1D**). Similarly, the serum potassium levels were sig-

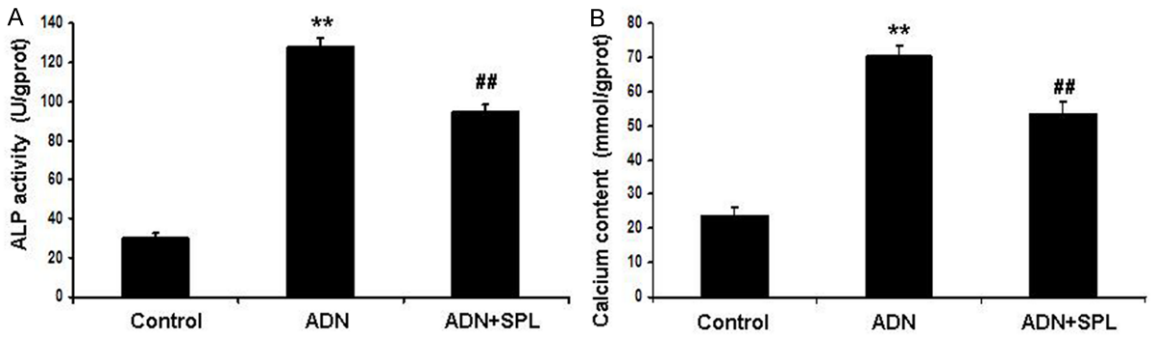

Figure 4. Alkaline phosphatase (ALP) activity and calcium content in rat aortas. $^{**}P < 0.01$ vs. control; $^{\#\#}P < 0.01$ vs. ADN. ADN, rats with adenine-induced CKD. ADN+SPL, rats treated with adenine plus spironolactone.

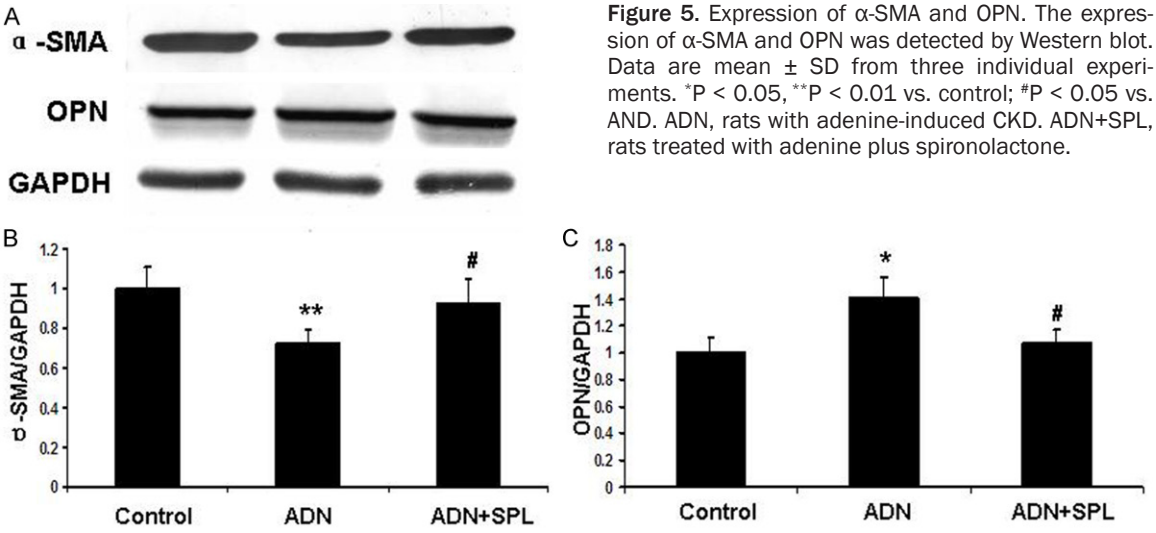

Figure 5. Expression of α-SMA and OPN. The expression of α-SMA and OPN was detected by Western blot. Data are mean ± SD from three individual experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. control; $^{\#}P < 0.05$ vs. AND. ADN, rats with adenine-induced CKD. ADN+SPL, rats treated with adenine plus spironolactone.

nificantly higher in ADN groups but not significantly affected by the SPL treatment (**Figure 1E**).

Levels of Ald in plasma

The plasma concentrations of Ald were determined by radioimmunoassay. As illustrated in **Figure 2**, the plasma Ald levels were markedly higher in ADN group than in control group.

Arterial histomorphology

H&E staining results revealed thickened vessel walls and disordered elastic fibers in ADN group in comparison with control group. SPL treatment improved the vascular structure (**Figure 3A**). As shown in **Figure 3B**, extensive medial calcification stained by Von Kossa was observed in aortas of ADN group but not of control group. The arterial calcification was significantly mitigated by SPL treatment.

Alkaline phosphatase (ALP) activity and calcium content in rat aortas

ALP activity in rat aortas was significantly higher in ADN group than in control group. The elevated ALP activity was reversed by SPL (**Figure 4A**). Similarly, the calcium content in rat aortas was significantly increased in ADN group and was also significantly affected by SPL treatment (**Figure 4B**).

Protein expression of α-SMA and OPN

In aortic tissue (**Figure 5A**), the protein expression of α-SMA was decreased in ADN group as compared with control group, a difference significantly blunted by SPL treatment. As illustrated in **Figure 5B**, the protein expression of OPN was increased in ADN group as compared with control group, a difference again blunted by SPL treatment.

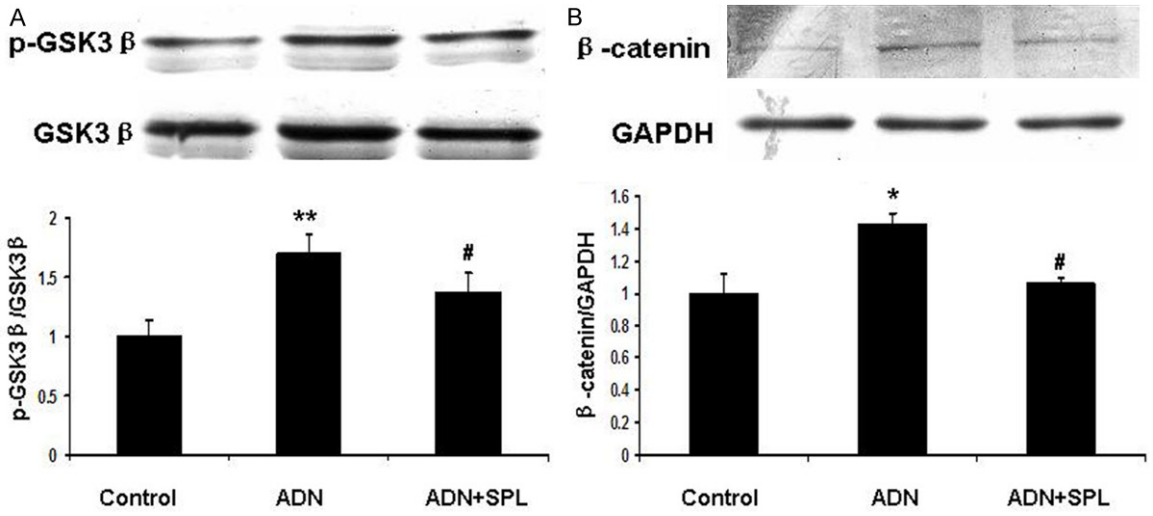

Figure 6. Change of Wnt/β-catenin signaling pathway. The expression of phospho-GSK-3β (A) and β-catenin (B) was analyzed by Western blot. Data are mean ± SD from three individual experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. control; $^{\#}P < 0.05$ vs. ADN. ADN, rats with adenine-induced CKD. ADN+SPL, rats treated with adenine plus spironolactone.

Change of Wnt/β-catenin signaling pathway

Our results showed that the levels of phosphorylation of GSK3β were increased in ADN group as compared with control group, a difference significantly affected by SPL (**Figure 6A**). The β-catenin protein expression was further observed. Compared with control group, the expression levels of β-catenin were increased in ADN group. The elevated β-catenin levels were also reduced by SPL treatment (**Figure 6B**).

Discussion

In this study, rats were fed an ADN-rich diet for 8 weeks to develop the CKD model. Similar to human CKD, serum Cr, BUN, phosphate and potassium concentrations were elevated in ADN-fed rats. The CKD rats showed elevated Ald levels and typical arterial medial calcification. According to the present observations, Ald promoted the activation of Wnt/β-catenin signaling pathway and the transforming VSMCs to osteoblast-like cells in calcified aortas. To better evaluate the role of Ald in VC in rats with CKD, the Ald receptor antagonist SPL was introduced to the study. SPL mitigates the effects of Ald and reduces the pro-calcification signaling.

In CKD patients, calcium and phosphate metabolism disorders facilitate the progression of VC [27]. In present study, we observed that serum phosphate levels were increased in CKD model, but serum calcium levels were no difference among three groups. Despite a tendency toward reduced phosphate levels following SPL treatment, no significant difference was observed between ADN group and ADN+SPL group. The data suggest that Ald blockade could not affect the metabolism of blood calcium and phosphate in CKD. It is known that Ald antagonism is related to the risk of hyperkalemia in patients with and without remaining renal function [28]. In present study, it was found that the serum potassium levels were elevated in ADN groups, but were not significantly affected by treatment with SPL (40 mg/kg/day), which is consistent with the results of a clinical study [29].

VC is related to reduced and fragmented elastic fibers in the vessel wall, leading to decreased arterial elasticity, arterial hypertension, and vascular stiffening [30]. In this study, H&E staining and Von Kossa staining were performed to show VC. SPL significantly reduced the arterial medial calcification in ADN group. In addition, ALP activity and calcium content in aortas were investigated to further confirm the calcification. Our results showed that ADN-increased ALP activity and calcium content were significantly suppressed by treatment with spironolactone. The results suggest that high-plasma Ald concentrations could promote the progression of VC in rats with CKD.

The phenotypic transformation of VSMCs to osteoblast-like cells is particularly striking during VC, whereby the VSMCs cease to express smooth muscle lineage markers (α-SMA and SM22α), and instead express osteogenic markers (OPN, OCN and RUNX2) [31, 32]. Therefore, we detected the expression of α-SMA and OPN in aortas by Western blot. As compared with control aortas, the expression of α-SMA was markedly decreased while the levels of OPN were significantly increased in ADN aortas. The changes were rescued by SPL treatment, indicating that Ald promotes the development of VC by stimulating the osteogenic transition of VSMCs.

The osteoinductive signalling triggered excessive VC [33, 34]. In recent years, the role of Wnt/β-catenin signaling in the induction of osteoblast differentiation and bone formation has attracted considerable interest [35, 36]. The central player in Wnt/β-catenin signaling cascade is β-catenin. The activation of the Wnt/β-catenin signaling pathway results in the phosphorylation of GSK3β and the increased expression of β-catenin. The β-catenin enters to the nucleus and regulates the expression of target genes [37]. Our results showed that the expression levels of p-GSK3β and β-catenin were increased in aortas of ADN group. The activation of Wnt/β-catenin signaling was inhibited by SPL treatment, indicating that Ald could act via Wnt/β-catenin signaling to promote VC.

Taken together, our findings suggest that hyperaldosteronism could promote the progression of VC via Wnt/β-catenin pathway in rats with CKD. The findings contribute to understanding the role of Ald in the pathogenesis of VC in CKD. Ald blockade therapy may be effective in preventing the development of VC in patients with CKD.

Acknowledgements

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Disclosure of conflict of interest

None.

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