Role of Vitamin D3 in Regulation of T Helper Cell 17 and Regulatory T-Cell Balance in Rats With Immunoglobulin A Nephropathy

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Introduction. Dysregulation of CD4+ T cell subsets participates in the pathogenesis of immunoglobulin A nephropathy (IgAN). Vitamin D has immunomodulatory functions. This study aims to investigate the regulatory effect of vitamin D3 on T helper-regulatory T (Th17-Treg) cells balance in rats with IgAN.

Materials and Methods. Sprague-Dawley rats were randomly assigned to a normal group (n = 6), an IgAN model group (n = 5), a prednisone treatment IgAN group (n = 6), a 1,25-dihydroxyvitamin D3 IgAN group (n = 6), and prednisone plus 1,25-dihydroxyvitamin D3 treatment group (n = 6). At week 12, the 24-hour urine protein excretion and erythrocyte count and renal pathological changes were determined, and serum interleukin-17 and Treg cell levels were measured in blood.

Results. The urine protein content and the number of erythrocytes were lower in the vitamin D group than in the model group ($P < .01$), but higher than in the prednisone groups ($P < 0.01$). The pathological impairments in the glomerular mesangium, renal tubule, and renal interstitium decreased in response to treatment with prednisone with and without 1,25-dihydroxyvitamin D3. Serum interleukin-17 level in the vitamin D and prednisone plus vitamin D groups was lower than in the prednisone group ($P < .05$). The Treg cells in the vitamin D and prednisone plus vitamin D groups showed higher levels than in the prednisone group ($P < .01$).

Conclusions. Vitamin D3 can regulate the Th17/Treg balance and reduce the level of protein and blood in the urine of rats with IgAN.

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INTRODUCTION

Immunoglobulin A (IgA) nephropathy (IgAN) is one of the most common primary glomerular diseases. Its clinical manifestations are complex, being primarily recurrent hematuria with varying degrees of proteinuria. The renal pathology of IgAN includes characteristics of IgA granular- or bolus-like deposition in the glomerular mesangium, as well as various degrees of IgG, IgM, and complement C3 deposition, and mesangial cell and matrix hyperplasia, which are the primary causes of the end-stage renal disease. The mechanism underlying IgAN pathogenesis is not completely clear; however, some scholars have speculated that it is associated with immune disorders of T cells and abnormal reaction of B cells following abnormal signal transduction of T cells.[1,2]

The T helper cell 17 (Th17 cell) is a type of T

helper cell which primarily functions to secrete interleukin-17 and plays an important role in many immune diseases.[3] In contrast, the regulatory T cell (Treg cell) is a type of suppressor T cell, with fork-head box protein P3 as the main surface molecule. The regulatory T cell is involved in the inhibition of T cell activation, mediation of immune tolerance, and occurrence and development of autoimmune diseases, tumor, and allograft disease. The T helper 17 and Treg cells are restricted by each other in vivo and contribute to the maintenance of immune homeostasis. A previous study has shown that IgAN patients have reduced ability to inhibit their immune response because of reduced levels of Treg cells, and they consequently exhibit an excessive immune response against pathogens. This includes increased production of cytokines, and promotion of the mutual conversion between certain molecules of the immune system, which occur during the development of IgAN.[4] It is currently reported that IgAN patients present with a Treg-Th17 imbalance, which is possibly the leading cause of IgAN.[1]

Vitamin D is a fat-soluble vitamin essential for the human body. Jeffery and coworkers[5] showed that the bioactive form of vitamin D, 1,25-dihydroxyvitamin D3, inhibited the production of pro-inflammatory cytokines (such as interleukin-17, immunoreactive fibronectin-γ, and interleukin-21), stimulated the overexpression of cytotoxic T lymphocyte antigen-4 and fork-head box protein P3, promoted immune balance, and played a crucial role in resistance to infection and autoimmune disease. However, there is little evidence reporting the effects of vitamin D3 on IgAN and on the related mechanisms. In the current study, levels of Th17 and Treg cells were measured in a rat model of IgAN after treatment with 1,25-dihydroxyvitamin D3, in order to explore the regulatory effect of vitamin D3 on Th17 and Treg cells in IgAN rats, thus providing experimental evidence for the potential of vitamin D as a treatment for IgAN.

MATERIALS AND METHODS

Establishment of Animal Models and Grouping

Thirty healthy male Sprague-Dawley rats, weighing 180 g to 220 g (mean, 200 ± 15 g), were provided by the Experimental Animal Center of the Second Xiangya Hospital of Central South University, China. The rats were randomly assigned to a model group (n = 24) and a normal (control) group (n = 6). The IgAN model was established according to previously described methods of Tang and colleagues,[6] with some modifications. Briefly, animals in the model group received a combined administration of lipopolysaccharide and bovine serum albumin and carbon tetrachloride as follows: intragastric administration of bovine serum albumin, 400 mg/kg, every second day for a total 12 weeks; tail vein injections of lipopolysaccharide, 0.05 mg, at the end of 6th and 8th weeks; and subcutaneous injections of carbon tetrachloride, 0.1 mL, plus castor oil, 0.5 mL, once a week for a total of 9 weeks. Rats in the control group were treated with an equal volume of distilled water via intragastric administration and equal amounts of physiological saline via tail vein injection (to match the lipopolysaccharide) and subcutaneous injection (carbon tetrachloride).

During the experiment, all rats were allowed free access to normal diet and distilled water. At week 9, 1 rat randomly selected from the model group was killed, and the kidney tissue was harvested for immunofluorescence and light microscopy, to confirm the successful establishment of the model. At week 10, the remaining 23 rats continued to receive intragastric administration of bovine serum albumin every 2nd day, and the rats from the model group were randomly assigned to either remain within the model group (n = 5) or receive prednisone (n = 6), 1,25-dihydroxyvitamin D3 (n = 6), or a combined treatment of prednisone plus 1,25-dihydroxyvitamin D3 (n = 6). The prednisone, vitamin D, and combined treatment groups were respectively fed with prednisone, 10 mg/kg body weight, and 1,25-dihydroxyvitamin D3, 200 ng/kg body weight, and prednisone, 10 mg/kg, plus 1,25-dihydroxyvitamin D3, 200ng/kg, while the model and control groups received an equal volume of distilled water daily, for a total of 2 weeks. At the end of the experiment, animals were anesthetized with 10% chloral hydrate, 3.5 mL/kg body weight, prior to collection of samples.

Main Reagents and Drugs

Reagents and drugs were obtained from the following suppliers: bovine serum albumin (Amresco LLC, Solon, OH, USA); Rocaltrol (1,25-dihydroxyvitamin D3, Shanghai Roche

Pharmaceutical Ltd, Shanghai, China); fluorescein isothiocyanate-labeled mouse anti-rat CD25 antibody (BioLegend Inc, San Diego, CA, USA); PE-labeled mouse anti-rat CD4 antibody (BioLegend Inc, San Diego, CA, USA); mouse anti-rat interleukin-17 monoclonal antibody (R&D Systems Inc, Minneapolis, MN, USA); interleukin-17 enzyme-linked immunosorbent assay kit (Changsha Jiahe Biological Technology Co Ltd, Changsha, Hunan Province, China); goat anti-rat IgA monoclonal antibody (Bethyl Laboratories Inc, Montgomery, TX, USA); and fluorescein isothiocyanate-labeled rabbit anti-goat IgG antibody (Proteintech Group Inc, Chicago, IL, USA).

Clinical Observation Index

The general condition of rats was observed, including diet, behavior, activity, and coat condition. The 24-hour urine sample was collected using a metabolic cage for detection of 24-hour urinary protein, and the number of urinary red blood cells was counted using a blood cell counting plate.

Pathologic Examination

Immunofluorescence and light microscopic examinations of the kidney specimens were performed. A sample of kidney tissue was fixed in 10% formaldehyde solution, dehydrated, embedded in paraffin, and used for periodic acid-Schiff staining under light microscopy. The other part of each frozen section was used for immunofluorescence IgA detection. The IgA immunofluorescence adopted the commonly used 5-level classification: nonvisible under low-power lens and visible under high-power lens, scored as "-"; slightly visible under low-power lens and visible under high-power lens, scored as "+"; visible under low-power lens and clearly visible under-higher power lens, scored as "++"; clearly visible under low-power lens and bright under high-power lens, scored as "+++"; and extremely bright under high-power lens, scored as "++++."

Determination of Interleukin-17 Cytokine

Three milliliters of cardiac blood was sampled from anesthetized rats and placed in a normal tube, then allowed to coagulate at room temperature for 10 to 20 minutes and centrifuged at 700 × g for 20 minutes. The supernatant was carefully collected and stored at -20°C. An enzyme-linked immunosorbent assay method was used to detect the serum level of interleukin-17.

Detection of Regulatory T Cells

Two milliliters of cardiac blood was sampled from anesthetized rats, placed in an anticoagulation tube and fully mixed. Fresh whole blood (100 µL) was collected to count the white blood cells using the erythrocyte counting plate, and 10^6 cells were incubated with the fluorescein isothiocyanate-labeled mouse anti-rat CD25 antibody (1 µg) and the phycoerythrin-labeled mouse anti-rat CD4 antibody (0.25 µg) in the dark for 20 to 30 minutes. Three volumes of erythrocyte lysis liquid was then added, and the sample was incubated at room temperature for a further 5 minutes, fully mixed, and then centrifuged at 850 × g for 5 minutes. After the supernatant was discarded, cells were washed with 1 mL of phosphate-buffered saline and centrifuged at 550 × g rpm for 5 minutes, and the before-mentioned procedure was repeated 3 times. Cells were finally washed with 300 µL of phosphate-buffered saline and immediately used for flow cytometry detection.

Statistical Analysis

All data were processed using the SPSS software (Statistical Package for the Social Sciences, version 17.0, SPSS Inc, Chicago, Ill, USA), and data are expressed as mean ± standard deviation. The homogeneity of variance was tested according to the data type; if P value was .05 and greater, a 1-way analysis of variance was used; if P value was less than .05, which indicates heterogeneity of variance, the Kruskal-Wallis test was used to compare the mean difference and the Nemenyi test was used to compare the two mean values. A P value less than .05 was regarded significant.

RESULTS

24-hour Urine Protein and Urine Erythrocytes

The protein content of 24-hour urine samples was lower in the vitamin D treatment group than the model group ($P < .01$), but higher than the prednisone and combined treatment groups ($P < .01$). The number of erythrocytes in urine of the rats was lower in the vitamin D treatment group than the model group ($P < .01$), but higher than the prednisone and combined treatment groups ($P < .01$; Table 1).

Table 1. Mean Changes in 24-hour Urine Protein and Erythrocyte Count of Rats

24-hour Urine	Rat Group					*F*	*P*
	Normal Control (n = 6)	Model (n = 5)	Prednisone (n = 6)	Vitamin D (n = 6)	Combined Treatment (n = 6)		
Protein, mg/d	8.90 ± 1.48*	47.34 ± 5.11†	11.75 ± 2.11*†	22.03 ± 3.09*	11.19 ± 2.35*†	151.203	< .001
Erythrocyte, × 10^{12}/L	8.46 ± 3.45*	492.31 ± 21.21†	12.49 ± 2.36*†	269.18 ± 49.25*†	10.77 ± 2.77*†	435.637	< .001

*P < .01 compared with the model group
†P < .01 compared with vitamin D group

Pathological Changes of Kidney Tissue

Pathological changes of kidney tissues of the rats are shown in Figure 1. Briefly, the vitamin D group showed no obvious changes compared with the model group (Figure 1D), the prednisone treatment group showed a reduction of impairment (Figure 1C), while the combined treatment had the greatest effect in reducing glomerular impairment (Figure 1E).

Immunoglobulin A Immunofluorescence Intensity in Kidney Tissue

The IgA immunofluorescence intensity in kidney tissue of rats is shown in Figure 2 and Table 2. The fluorescence intensity in rats from the control group showed significant differences compared with other groups (P < .01); after treatment with prednisone with and without 1,25-dihydroxyvitamin D3, IgA fluorescence intensity decreased; however, no significant difference was found between the model group and the vitamin D group, although the difference was significant when compared with the prednisone and combined treatment groups (P < .01). Fluorescence intensity showed a significant difference in the vitamin D group compared with the prednisone and combined treatment groups (P < .05 and P < .01, respectively), and the difference between the prednisone and combined treatment groups was also significant (P < .05).

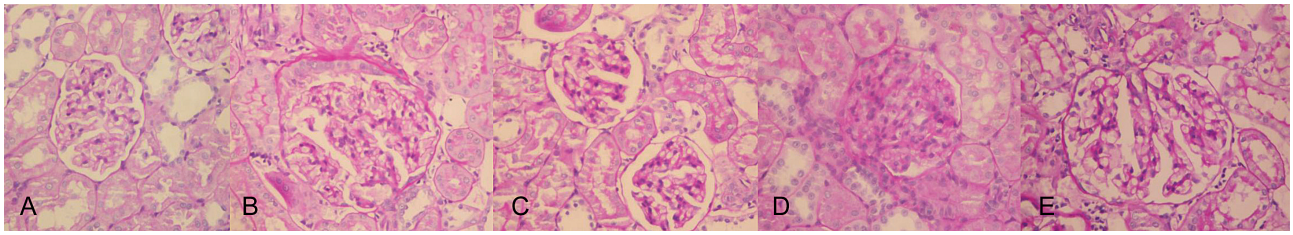

Figure 1. Kidney tissue in rats (periodic acid-Schiff, × 400). **A**, In the control group, glomerular morphology was normal, the mesangial cells and matrix were not significantly increased, and the renal tubule and interstitium showed no obvious abnormalities. **B**, In the model group, glomerular mesangial cells and matrix were significantly increased, some glomerular cells appeared in crescent form, and renal tubular epithelial cells showed vacuolar degeneration and brush border abscission. **C**, In the prednisone group, the number of glomerular mesangial cells and matrix was slightly increased, and there was some evidence of blood capillary lumen pressure. **D**, In the vitamin D group, the number of glomerular mesangial cells and matrix was significantly increased, and renal tubular epithelial cells showed degeneration with brush border abscission. **E**, In the combined treatment group, the number of glomerular mesangial cells and matrix was slightly increased, the capillary lumen opened well, and renal tubular epithelial cells showed vacuolar degeneration, but the renal interstitium showed no evidence of changes.

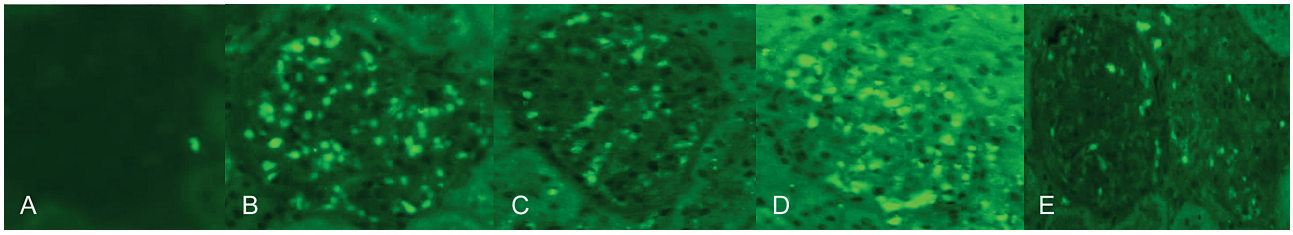

Figure 2. Immunofluorescence in kidney tissue of rats (× 400). **A**, In the control group, rat kidney tissue showed no IgA fluorescence. **B**, In the model group, kidney tissue showed strong IgA fluorescence as granular deposition, with intensity from ++ to +++. **C**, In the prednisone group, kidney tissue exhibited IgA fluorescence as granular deposition, with intensity from + to ++. **D**, In the vitamin D group, IgA fluorescence in kidney tissue showed both granular and linear deposition, with intensity from + to +++. **E**, In the combined treatment group, IgA fluorescence in kidney tissue showed granular deposition, with intensity was graded +; normal glomerulas were occasionally seen.

Table 2. Changes in Immunoglobulin A Fluorescence Intensity in Kidney Tissue of Rats

Fluorescence Intensity	Rat Group					*Hc*	*P*
	Normal Control* (n = 6)	Model (n = 5)	Prednisone*†‡ (n = 6)	Vitamin D§ (n = 6)	Combined Treatment*¶ (n = 6)		
+++	0	4	0	2	0		
++	0	1	3	3	0		
+	0	0	3	1	4		
-	6	0	0	0	2	23.002	< .01

*P < .01 compared with model group
†P < .05 compared with vitamin D group
‡P < .05 compared with combined treatment group
§P > .05 compared with model group
¶P < .01 compared with vitamin D group

Serum Interleukin-17 Levels

The serum level of interleukin-17 in the model group was significantly higher than the control, prednisone, vitamin D, and combined treatment groups (P < .01). The serum level of interleukin-17 in the prednisone group was significantly lower than those of rats in the model group (P < .01), but significantly higher than in the control group (P < .01). Compared with the prednisone group, the interleukin-17 levels were significantly lower in the vitamin D group (P < .05) and in the combined treatment group (P < .01; Table 3).

Regulatory T Cells

The levels of blood Treg cells in the model group were significantly lower compared with the control, prednisone, vitamin D, and combined treatment groups (P < .01). Levels in the vitamin D group were significantly higher compared with the prednisone group (P < .01), but showed no significant difference compared with the combined treatment group. There were significantly more Treg cells in the combined treatment group than in the prednisone group (P < .01), which in turn had significantly more than did rats in the model group (P < .01; Table 3 and Figure 3).

DISCUSSION

In recent years, Th17-Treg immune balance has received increasing attention in the field of autoimmune diseases and it has been reported that this balance is also important in many kidney diseases. Shao and coworkers[7] showed that the number of Th17 cells increased, while the number of Treg cells decreased, in children with nephrotic syndrome, and interleukin-17 expression in the kidney tissue was upregulated, which indicated the occurrence of Th17 and Treg disorders. Increasing evidence has shown immune disorders of T cells in IgAN patients.[8-10] Huang and colleagues[11] have hypothesized that IgAN patients exhibit attenuated immune inhibition due to a decrease in Treg cell number, thus triggering excessive immune response against pathogens, increasing the production of inflammatory cytokines, and promoting mutual conversion between immune molecules (for example, from IgM into IgA), leading to the development of IgAN. Subsequent research has verified this hypothesis.[4] Recent studies have reported the presence of Treg-Th17 immune imbalance in IgAN patients.[1] Our experimental findings have also shown Th17-Treg disorders in rat models of IgAN, through evidence of increased levels of serum interleukin-17 and reduced levels of blood Treg cells.

As a novel immunomodulator, vitamin D3 acts to regulate the immune response in a variety of diseases.[12] In the current study, after treatment

Table 3. Mean Serum Levels of Interleukin-17 and Regulatory T Cells

Parameter	Rat Group					*F*	*P*
	Normal Control (n = 6)	Model (n = 5)	Prednisone (n = 6)	Vitamin D (n = 6)	Combined Treatment (n = 6)		
Interleukin-17, pg/mL	35.13 ± 2.97*†	61.04 ± 3.96	41.43 ± 2.74*	37.27 ± 3.04*‡	34.49 ± 2.51*†	68.761	< .001
Regulatory T cell, %	5.53 ± 1.03*	1.20 ± 0.49†	3.28 ± 0.71*	4.71 ± 0.38*†	5.30 ± 0.68*†	32.444	< .001

*P < .01 compared with model group
†P < .01 compared with prednisone group
‡P < .05 compared with prednisone group

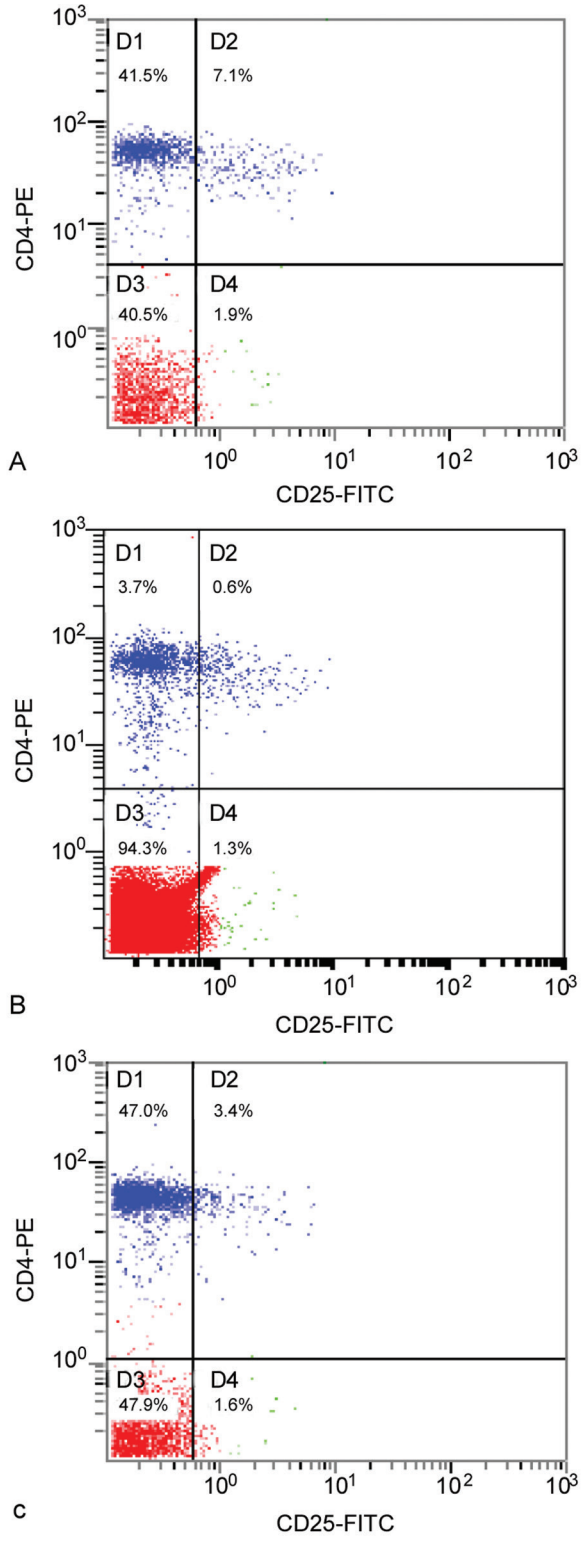

Figure 3. Treg cells level in rat blood (%). **A**, control group; **B**, model group; **C**, prednisone group; **D**, vitamin D group; and **E**, combined treatment group.

with prednisone, vitamin D3, or a combination of the two, the level of serum interleukin-17 in IgAN rats was reduced, with the lowest level seen in the combined treatment group. In addition, the interleukin-17 content of rats in the vitamin D treatment group was lower than that of rats in the prednisone treatment group. Blood Treg cell levels were elevated after treatment, with the highest levels seen in the combined treatment group, while levels of these cells in the vitamin D treatment group were higher than in the prednisone treatment group. These experimental findings indicate that prednisone and vitamin D_3 both have immunomodulatory effects on Th17-Treg in IgAN rats, with vitamin D_3 showing stronger regulatory effects than prednisone. However, the combined application of prednisone and vitamin D3 was more effective than vitamin D3 alone, suggesting

that the combined treatment has synergistic effects.

In addition to effects on the immune system, the increase in 24 hour urine protein and urine red blood cell count in the model rats was reduced in the combined treatment, prednisone, and vitamin D group. This indicates that vitamin D3 may reduce hematuria and proteinuria in IgAN patients, and combined use with prednisone may achieve better effects. A randomized controlled experiment of Agarwal and colleagues[13] showed that in chronic kidney disease patients, vitamin D replacement therapy significantly increased the probability of decreased proteinuria compared with the placebo group, which was not associated with sex, age, race, or use of angiotensin-converting enzyme inhibitors or angiotensin receptor blockers. This conclusion was confirmed by Fishbane and colleagues.[14] A recent clinical trial demonstrated that urinary protein was significantly reduced, and the ratio of urine protein and urine creatinine decreased, after twice-weekly vitamin D_3 treatment in ten IgAN patients who showed persistent proteinuria and poor therapeutic effects of angiotensin-converting enzyme inhibitors or angiotensin receptor blockers. In addition, serum levels of tumor growth factor-β were decreased, while renal function and blood pressure were maintained, over the course of the experiment.[15] An Open-label, small, non-placebo-controlled, randomized study demonstrated that the addition of calcitriol to a renin-angiotensin system inhibitor resulted in a safe decrease in proteinuria in patients with IgAN.[16] The above experiments show that vitamin D3 can reduce urinary protein levels, but its long-term effects require further exploration. Current evidence suggests that its mechanisms may be associated with inhibition of the renin-angiotensin-aldosterone system, regulation of immune cell functions, or protection of renal inherent cells such as potocyte.[17] Existing studies regarding the role of 1,25-dihydroxyvitamin D3 on IgAN are generally of small sample size, and there is little evidence on its role in hematuria. In the current experiment, we have observed renal pathology in experimental IgAN model rats. Following various treatments, renal pathological changes and IgA fluorescence intensity showed varying degrees of reduction, with the greatest reduction seen in the combined treatment group, followed by prednisone treatment, while pathological changes of kidney tissue in the vitamin D group showed no obvious change compared with the model group. The reason for this may be the relatively short medication time, different targets in kidney tissue or alleviating clinical symptoms through other mechanisms.

CONCLUSIONS

Our results suggest that vitamin D3 can regulate Th17-Treg immune imbalance and play a protective role in the kidney, thus reducing hematuria and proteinuria.

CONFLICT OF INTEREST

None declared.

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