Vitamin D and hydroxychloroquine reduce renal injury and Ki67 expression in a rat model of IgA nephropathy via TLR4

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To the Editor: IgA nephropathy (IgAN) is of a great concern in China, where apprehensions regarding this condition are related to the fact that approximately 30% of IgAN patients experience a poor outcome and the tendency for IgAN to develop within younger patients. Moreover, 40% of IgAN patients developed end-stage renal disease (ESRD) within 20 years after diagnosis of this condition.[1] The pathogenesis associated with IgAN has not been clearly characterized, nor is there an effective treatment for this condition. Now, clinical treatment typically involves use of glucocorticoids and immune inhibitors, such as cyclophosphamide with mycophenolate mofetil. Therefore, a better understanding of the pathogenesis of IgAN along with more effective treatments represents critical issues requiring immediate attention.

Two agents having the potential to exert beneficial effects upon the kidney are Vitamin D (VitD) and hydroxychloroquine (HCQ). VitD is an essential fat-soluble vitamin that maintains blood calcium and phosphorus levels and exerts protective effects within the cardiovascular system and kidneys. It has been reported that VitD and tacrolimus treatment have excellent efficacy in reducing mesangial cell proliferation, glomerular basement membrane thickening, and glomerular inflammatory cell infiltration.[2]

As a common antimalarial drug, HCQ regulates immune responses by inhibiting inflammatory signaling pathways, which has been used in the clinical treatment of autoimmune diseases, such as systemic lupus erythematosus and rheumatoid arthritis. Therefore, in this report, we investigated the immunotherapeutic effects of VitD and HCQ alone and in combination upon renal histology in a rat model of IgAN. Such information may help to identify and improve protocols for the treatment of IgAN.

A total of 40 specific pathogen-free male Wistar rats (body weight [mean ± standard deviation] = 150 ± 10 g) were purchased from the China Medical University and were randomly divided into five groups (N = 8 rats/group): (1) Control, (2) IgAN, (3) IgAN + HCQ, (4) IgAN + VitD, and (5) IgAN + HCQ + VitD. The rats were maintained at the animal center of the China Medical University under controlled lighting (12 h light/dark cycle) and temperature (22–26°C) conditions and were permitted free access to food and water. All experiments complied with the ethical requirements of laboratory animal use and were approved by the Animal Care Committee (Approval No. 2018046).

To establish an IgAN model, rats in the four IgAN experimental groups received 400 $mg \cdot kg^{-1} \cdot d^{-1}$ bovine serum albumin (BSA; 1:10, Sigma-Aldrich Company, USA) for 8 weeks with a 1-day interval between two gavages. In addition, they received a weekly subcutaneous injection of castor oil (0.3 mL, Dalian Meilun Biological Technology Co., Ltd., China) and CCl4 (0.1 mL, Liaoning Xinxing Reagent Co., Ltd., China) for 9 weeks. Lipopoiysaccharides (LPS, 0.05 mg at 1:4000, Sigma-Aldrich Company) was injected in the 6th and 9th weeks via the caudal vein. The control group received 4 mL/kg distilled water every other day for 8 weeks, along with a subcutaneous injection of 0.4 mL normal saline that was administered weekly for 9 weeks, while 0.2 mL of normal saline was administered in the 6th and 9th weeks via the caudal vein.[3] At the end of the 9th week, one rat from each of the four experimental groups and one from the control group were randomly selected for assay of the kidney. These rats were injected with 10% chloral hydrate (0.35 mL/100 g); the kidney was removed and divided into two halves via a coronal section. One portion of the peripheral renal cortex was dissected and frozen at −80 °C, while the remaining portion was placed in a standard stationary liquid and embedded in paraffin for conventional sectioning. Immunofluorescence staining showed significant green fluorescence deposition in IgAN group, but not in Control group. And haematoxylin-eosin (HE) staining resulted in the outcome that the degree of glomerular atrophy in the IgAN group was significantly

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higher than that in the control group. The model of IgAN rats was successfully established.

After generation of this IgAN/control model as described above, the specific treatments for the five groups, Control, IgAN, IgAN + HCQ (SPH Zhongxi Pharmaceutical Co., Ltd., Shanghai, China), IgAN + VitD (Shanghai Roche Pharmaceuticals Co., Ltd., China), and IgAN + HCQ + VitD, were started at the 10th week. Control and IgAN rats received a gavage of distilled water at 4 $mL \cdot kg^{-1} \cdot d^{-1}$. Rats in the IgAN + HCQ group were treated with HCQ at 54 $mg \cdot kg^{-1} \cdot d^{-1}$ while those in the IgAN + VitD group received VitD at 0.2 $\mu g \cdot kg^{-1} \cdot d^{-1}$. These same doses of HCQ and VitD were used in the IgAN + HCQ + VitD group. Treatments were administered daily for the 8-week duration of the experiment. At the end of 17th week, we used the previous method to preserve the renal cortex of rats and treated them separately.

Atrophic glomeruli were observed in the IgAN groups. As compared with the IgAN group, pathological manifestations were significantly improved within the three drug treatment groups. The glomerulus of HCQ and HCQ + VitD groups showed mild atrophy, and the degree of atrophy was slightly less than that of VitD group. Maximal reductions in the degree of atrophy were observed in the HCQ + VitD group; however, when compared with that of the Control group, kidneys of rats within the HCQ + VitD group did not fully return to normal [Supplementary Digital Content, Figure 1, http://links.lww.com/CM9/A651].

IgA deposition intensity in the three drug treatment groups were significantly lower than that observed in the IgAN group (577.293 ± 168.973 ng/L, ++ to ++++; $P < 0.05$). When comparing IgA deposition intensity among the three drug treatment groups, we found that the HCQ + VitD combination therapy (27.337 ± 15.998 ng/L, − to +) resulted in significantly lower intensity of IgA deposition than that obtained with either the HCQ (136.750 ± 54.373 ng/L, + to ++) or VitD (71.338 ± 12.793 ng/L, +) alone treatment groups ($P < 0.05$). However, none of these treatments completely reduced the IgA deposition to that of the levels observed in the kidneys of Control rats (0, −)[Supplementary Digital Content, Figure 2, http://links.lww.com/CM9/A651].

As compared with that of Control ($0.624 \pm 0.173\%$), the ratio of fibrosis area in the IgAN ($2.468 \pm 0.312\%$), HCQ ($1.494 \pm 0.315\%$) and VitD ($2.311 \pm 0.400\%$) groups was significantly increased ($P < 0.05$). The fibrosis area ratio in the HCQ and HCQ + VitD ($0.888 \pm 0.208\%$) groups was significantly decreased as compared with that in the IgAN group ($P < 0.05$). The ratio of fibrosis area within the HCQ + VitD group was significantly decreased than that in the HCQ group ($P < 0.05$) [Supplementary Digital Content, Figure 3, http://links.lww.com/CM9/A651].

Compared with the Control group ($0.143 \pm 0.018\%$), Ki67 expression rate was significantly increased in other groups ($P < 0.05$). Ki67 expression rate within the kidneys of the three drug treatment groups was significantly reduced ($P < 0.05$) compared with that of the IgAN group ($0.603 \pm 0.134\%$). Expression rate of Ki67 within the HCQ + VitD group ($0.290 \pm 0.073\%$) was significantly lower than that of the HCQ group ($0.370 \pm 0.087\%$) and VitD group ($0.450 \pm 0.078\%$) ($P < 0.05$) [Supplementary Digital Content, Figure 4, http://links.lww.com/CM9/A651].

As compared with that of the IgAN group ($0.754 \pm 0.166\%$), expression rate of TLR4 within the kidneys of the three drug treatment groups was significantly reduced ($P < 0.05$). TLR4 expression rate of the HCQ + VitD group ($0.322 \pm 0.247\%$) was significantly lower than that of the HCQ ($0.299 \pm 0.073\%$) and VitD groups ($0.310 \pm 0.039\%$; $P < 0.05$). In spite of these reductions, all TLR4 expression rates obtained within these three treatment groups remained significantly higher than that obtained in the control group ($0.098 \pm 0.023\%$; $P < 0.05$; Figure 1).

In this study, IgAN group of rats showed glomerular atrophy with crescent formation, excessive proliferation of mesangial cells, and large amounts of IgA deposition in the mesangial region; all substantiated that these rats had IgAN. And significantly increased levels of Ki67 and TLR4 expression in this group were found. In particular, Ki67 represents a notable biomarker as it is a nuclear antigen expressed during cell proliferation but not expressed in resting cells, and is commonly used to detect the proliferation of normal cells and various malignant tumor cells.

A growing number of studies have shown that TLR4 plays an important role in IgAN. It regulates the expression of pro-inflammatory cytokines and chemokines through MyD88 dependent and independent pathways producing effective mediators to promote inflammation and fibrosis. Li *et al*[3] found that inhibiting the TLR4-mediated NF-κB/MAPK signaling pathway can reduce the occurrence of inflammatory cells and decrease the accumulation of extracellular matrix and the degree of renal fibrosis in mice with unilateral ureteral obstruction.

In addition to ensuring a normal state of the human skeleton, VitD serves as an effective anti-inflammatory agent which can protect the kidney. In IgAN patients, the addition of active VitD, as achieved with valsartan treatment, significantly reduced urinary protein excretion, especially in IgAN patients with moderate proteinuria who were intolerant to glucocorticoids or immunosuppressants.[4] Moreover, the addition of HCQ reduced proteinuria in IgAN patients receiving conventional immunosuppressive (IS) therapy whose proteinuria was maintained at >1 g/day.[5]

Here, we found that beneficial effects were significantly enhanced with the combined treatment of VitD and HCQ. Rats in the HCQ + VitD group showed no significant glomerular atrophy, the least amount of IgA deposition within the mesangial region, and significantly decreased expressions of Ki67 and TLR4 within the kidney as compared with that observed in either the VitD or HCQ groups ($P < 0.05$). This report shows that Ki67 is related to the proliferation of IgAN mesangial membrane. Accordingly, it appears that the beneficial effects resulting from VitD + HCQ treatment, in part, reside in its capacity to reduce inflammatory responses via TLR4 inhibition and thus delay

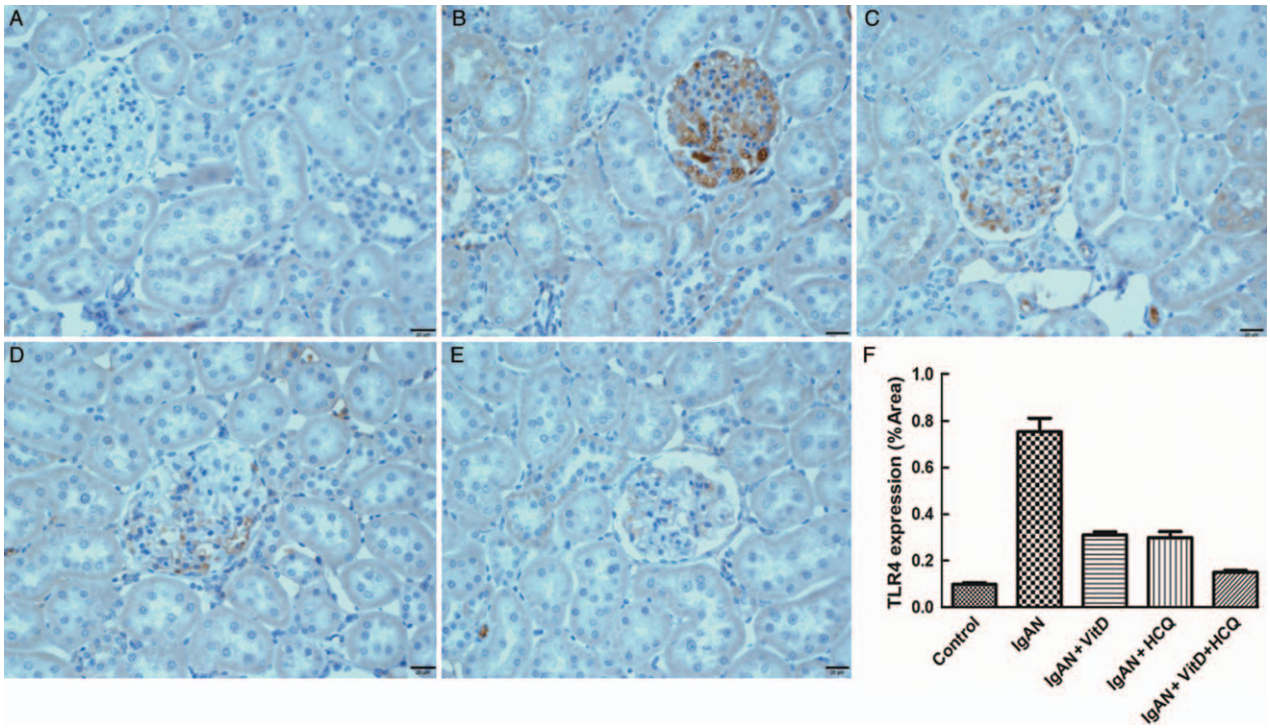

Figure 1: TLR4 expression of renal tissue in (A) Control, (B) IgAN, (C) IgAN + VitD, (D) IgAN + HCQ, (E) IgAN + HCQ + VitD groups. Immunohistochemistry staining (Original magnification×400). (F) Comparison of TLR4 expression rate in five groups. HCQ: Hydroxychloroquine; IgAN: IgA nephropathy; VitD: Vitamin D.

the progression of IgAN disease. But no specific literature has reported the correlation between Ki67 and TLR4 pathway, as well as the expression of Ki67 in glomeruli of IgAN.

In this report, we reveal that HCQ + VitD treatment results show protective effects within the kidney of IgAN rats through inhibiting TLR4 expression. Therefore, it will be necessary to evaluate more long-term, quantitative measures involving kidney glomerular injury in response to these treatments. These findings have potentially important clinical significance as they suggest that this combined HCQ + VitD treatment may be beneficial in patients with IgAN.

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Conflicts of interest

None.

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