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Original Article

Therapeutic effects of CTLA-4-Ig on diabetic nephropathy in type 2 diabetes mellitus rats ascribed to protection of CTLA-4-Ig on podocytes

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Abstract: *Aim:* The aim of this study was to investigate the therapeutic effects of CTLA-4-Ig on diabetic nephropathy (DN) and potential mechanism in rats with type 2 diabetes mellitus (T2DM). *Methods:* Rats were randomly divided into the control group, the T2DM group and the CTLA-4-Ig group. In the CTLA-4-Ig group, rats were treated with CTLA-4-Ig for 8 weeks. *Results:* Fasting blood glucose, endogenous creatinine clearance, 24-h urinary albumin excretion rate, and glomerular hypertrophy index (GHI) in the T2DM and CTLA-4-Ig groups were significantly higher than in the control group ($P<0.05$). Endogenous creatinine clearance, 24-h urinary albumin excretion rate, and GHI in the CTLA-4-Ig group were significantly lower than in the T2DM group ($P<0.05$). Renal arterial peak blood flow velocities in the systolic and end-diastolic phases, and mean blood flow velocity were the lowest in T2DM group and highest in the control group, and significant differences were observed among three groups ($P<0.05$). The renal arterial accelerated velocity, pulsatility index, and resistance index in the systolic phase were highest in the T2DM group and lowest in the control group ($P<0.05$). Expression of podocin and nephrin was highest in the control group and lowest in the T2DM group, and significant difference was observed among three groups ($P<0.05$). Renal parenchymal structure and podocyte ultrastructure were significantly altered in the DN group and the CTLA-4-Ig group, and the pathological changes in DN group were more severe than in the CTLA-4-Ig group. *Conclusion:* Therapeutic effects of CTLA-4-Ig on DN are ascribed to the protection of CTLA-4-Ig on podocytes, but not related to the glomerular endothelial cells.

Keywords: Cytotoxicity T lymphocyte associated antigen 4, diabetic nephropathy, immunoglobulin, podocyte, type 2 diabetes mellitus

Introduction

The incidence of type 2 diabetes mellitus (T2DM) is increasing worldwide with the increase in the incidence of obesity [1]. In developed countries, about 25% of diabetes mellitus (DM) patients will develop end stage kidney disease [2]. In addition, diabetic nephropathy (DN) patients have a high risk for cardiovascular events [3, 4]. Angiotensin-converting enzyme inhibitors and angiotensin receptor antagonists may delay progression of kidney disease and reduce the morbidity of cardiovascular diseases in DM patients [5-12], and glucose lowering treatment is also helpful to prevent against DN and delay its progression [13-16]. However, drugs and therapeutic strategies targeting DN are still limited currently.

Cytotoxicity T lymphocyte associated antigen 4 immunoglobulin (CTLA4-Ig) is a clinically available fuse protein. It may bind B7-1 to block the transmission of its signals. CTLA4-Ig has been used in the treatment of autoimmune diseases. There is evidence showing that CTLA4-Ig is able to repair the physiological structure and activity of podocytes and combat with high glucose environment to attenuate proteinuria in rats with DN [17]. These therapeutic effects are different from those of CTLA4-Ig observed in the treatment of immune diseases. In addition, B7-1 expression in podocytes and the capability

Table 1. General conditions and biochemical parameters (x±S)

Parameters	Control group	CTLA-4-Ig group	DN group
FBG (mmol/L)	5.78±0.76	19.21±3.86[a]	20.13±3.78[a]
Ccr (ml/min)	5.04±0.69	21.09±3.69[a]	26.33±4.56[a,b]
UAER	0.62±0.18	5.86±0.66[a]	7.17±0.82[a,b]
KW/BW (mg/g)	3.31±0.41	5.68±0.58[a]	7.45±0.75[a,b]
ALT (U/L)	47.65±6.68	50.03±5.88	51.00±5.98
AST (U/L)	60.08±7.56	63.00±7.55	66.02±7.67

Note: [a]*P*<0.05 vs control group; [b]*P*<0.05 vs CTLA-4-Ig group. FBG: fasting blood glucose; Ccr: creatinine excretion rate; UAER: 24-h urine protein excretion rate; KW/BW: glomerular hypertrophy index (kidney weight/body weight); ALT: alanine aminotransferase; AST: aspartate transaminase.

of binding between CTLA4-Ig and B7-1 to attenuate proteinuria are still being explored, and therapeutic application of CTLA4-Ig in DN patients is needed to be confirmed in more preclinical studies and clinical trials.

Materials and methods

Animals and grouping

Specific pathogen free male SD rats (n=45) aged 6-8 weeks and weighing 200±20 g were randomly assigned into 3 groups (n=15 per group): 1) control group: animals were given ad libitum access to water and food, and no treatment was administered; 2) DN group: The DN animal model was established according to previously reported [18]. After establishment of the animal model, animals were fed a high sugar and high fat diet for 8 weeks and no other treatment was administered; 3) CTLA-4-Ig group: The DN rat model was established according to that previously reported [18], and then the rats were fed with high sugar and high fat diet and simultaneously received injection of CTLA-4-Ig at 0.5 mg/kg/w via the tail vein for weeks. In the study, animals were given *ad libitum* access to water and food. No insulin or other glucose lowering drugs were used. This study has been approved by the Ethics Committee of The Second Affiliated Hospital and Yuying Children's Hospital of Wenzhou Medical University. All institutional and national guidelines for the care and use of laboratory animals were followed.

Instrument and reagent

Color Doppler Ultrasound detector (Esaote, Italy), light microscope (Nikon, Japan) and transmission electron microscope (H-600, Hitachi, Japan) were used in the present study. Following reagents were used in this study: CTLA-4-Ig (Abcam, UK), Streptozotocin (Sigma, USA), urine protein quantitative detection kit (CBB method), creatinine detection kit (picric acid method), alanine aminotransferase detection kit (colorimetric method) (Shanghai Jining Industrial Co., Ltd), aspartate transaminase detection kit (colorimetric method) (Shanghai Jianglai Biotech Co., Ltd), rabbit anti-rat CD31 polyclonal antibody, rabbit anti-rat CD34 polyclonal antibody, neiphrin antibody, podocin antibody and B7-1 antibody (Shanghai Boyun Biotech Co., Ltd).

Detection of renal arterial blood flow parameters and renal parenchymal elasticity

Ultrasonography was performed one day before the end of CTLA-4-Ig treatment. The peak systolic velocity, end diastolic velocity, mean velocity, systolic acceleration, pulsatility index, and resistance index of the main renal artery were measured in 3 consecutive cardiac cycles, and means were calculated. With the elastic imaging mode, the parenchymal elasticity score of right kidney was determined according to the elasticity scoring system provided by Itoh et al. [19].

Blood and urine collection and biochemical detection

Urine was collected after 24-h one day before the end of CTLA-4-Ig treatment, and 24-h urine albumin (UAL) was measured. Before euthanasia, rats were weighed, and body weight (BW) was recorded. Blood was harvested for the biochemical detections (fasting blood glucose [FBG], alanine aminotransferase, aspartate transaminase, serum creatinine [Scr], urine creatinine (Ucr). The endogenous creatinine clearance rate (Ccr) was calculated on the basis of SCr and UCr as follow: Ccr=UCr/SCr × 1 min urinary volume. The urinary albumin excretion rate (UAER) was calculated on the basis of UAL and UCr as follow: UAER=UAL/UCr.

Renal histology and podocyte structure examination

After blood collection, kidneys were harvested and the weighed. A part of the right kidney was

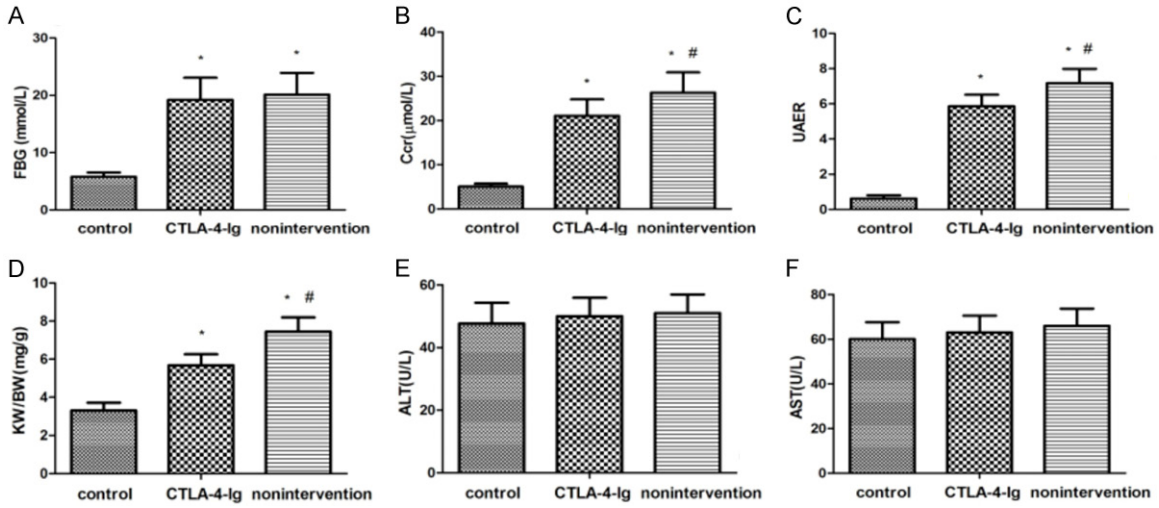

Figure 1. General conditions and biochemical parameters in three groups. Note: A. FBG in three groups; B. Ccr in three groups; C. UAER in three groups; D. KW/BW in three groups; E. ALT in three groups; F. AST in three groups. Non-intervention: DN group. Data are expressed as mean ± standard deviation. FBG: fasting blood glucose; Ccr: creatinine clearance rate; UAER: 24-h urine protein excretion rate; KW/BW: glomerular hypertrophy index (kidney weight/body weight); ALT: alanine aminotransferase; AST: aspartate transaminase. **P*<0.05 *vs* control group; #*P*<0.05 *vs* CTLA-4-Ig group.

Table 2. Main renal arterial blood flow parameters (x±s)

Blood flow parameters	Control group	CTLA-4-Ig group	DN group
PSV (cm/s)	55.42±10.21	33.85±7.63[a]	20.05±5.15[a,b]
EDV (cm/s)	18.65±3.16	9.05±2.84[a]	5.23±1.98[a,b]
MV (cm/s)	31.37±5.56	15.22±4.98[a]	10.14±3.67[a,b]
SAC (cm/s^2)	6.15±1.86	10.19±3.01[a]	13.32±3.11[a,b]
PI	1.08±0.21	1.56±0.25[a]	1.75±0.30[a,b]
RI	0.63±0.11	0.80±0.13[a]	0.90±0.10[a,b]

Note: [a]*P*<0.05 vs control group; [b]*P*<0.05 vs CTLA-4-Ig group. PSV: Peak systolic velocity; EDV: end diastolic velocity; MV: mean velocity; SAC: systolic acceleration; PI: pulsation index; RI: resistance index.

fixed in 4% paraformaldehyde for 48 h, followed by H&E staining, and the glomerular structure was observed under light microscope. In addition, remaining right kidney tissues were fixed in 4% glutaraldehyde for transmission electron microscopy, and the podocyte structure was observed.

Immunohistochemistry

Kidney sections were subjected to immunohistochemistry for CD31 and CD34, and the observed under light microscope. The CD31 and CD34 expression was quantitatively evaluated with Image-Pro plus.

Western blotting

Western blotting was employed to detect the protein expression of podocin, nephrin and B7-1 in the kidney, and quantification of protein expression was done with Image Lab 3.0 (Beta3).

Statistical analysis

Statistical analysis was performed with SPSS version 22.0. Quantitative data with normal distribution are expressed as mean ± standard deviation and analyzed with one way analysis of variance followed by post hoc LSD test. Qualitative data were compared with rank sum test. A value of *P*<0.05 was considered statistically significant.

Results

General condition and biochemical parameters

In the DN and CTLA-4-Ig groups, the FBG, Ccr, UAER, and glomerular hypertrophy index (kidney weight/body weight, KW/BW) were significantly higher than in control group (*P*<0.05). In the CTLA-4-Ig group, Ccr, UAER and KW/BW were significantly lower than in the DN group (*P*<0.05). There was no significant difference in FBG between the DN group and the CTLA-4-Ig group (*P*>0.05). In addition, no significant differences were observed in alanine aminotransferase and aspartate transamina-

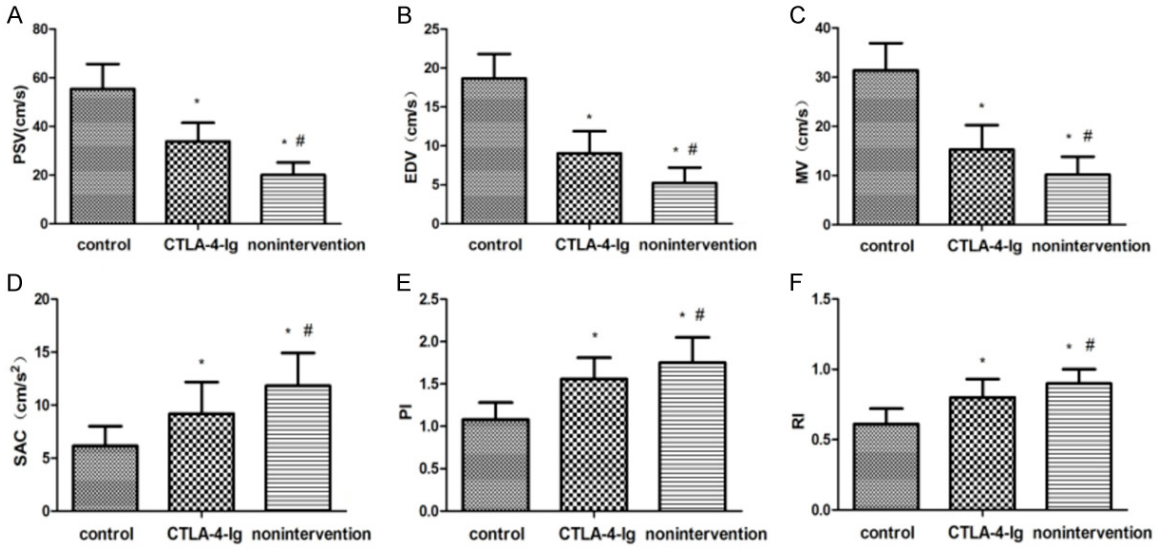

Figure 2. Main renal arterial blood flow parameters in three groups. Note: A. PSV in three groups; B. EDV in three groups; C. MV in three groups; D. SAC in three groups; E. PI in three groups; F. RI in three groups. Non-intervention: DN group. Data are expressed as mean ± standard deviation. PSV: Peak systolic velocity; EDV: end diastolic velocity; MV: mean velocity; SAC: systolic acceleration; PI: pulsation index; RI: resistance index. *P<0.05 *vs* control group; #P<0.05 *vs* CTLA-4-Ig group.

se among groups (P>0.05) (**Table 1** and **Figure 1**).

Main renal arterial blood flow parameters and kidney elasticity score

The peak systolic velocity, end diastolic velocity, and mean velocity of main renal artery were the lowest in DN group and the highest in the control group, showing significant differences among groups (P<0.05). Systolic acceleration, pulsatility index, and resistance index were the highest in the DN group and the lowest in the control group, showing significant differences among groups (P<0.05) (**Table 2**; **Figures 2**, **3A-C**).

The renal parenchymal elasticity score was the highest in the DN group and the lowest in the control group, showing significant difference among three groups (P<0.05) (**Tables 3**, **4** and **Figure 3D-F**).

Renal pathology

H&E staining showed the renal parenchymal structure was significantly altered in the DN group and the CTLA-4-Ig group, and the pathological change in the DN group was more severe than in the CTLA-4-Ig group (**Figure 4A-C**).

Podocyte ultrastructure

Podocyte ultrastructure showed significant change in the DN group and the CTLA-4-Ig group, and this change was more severe in the DN group than in the CTLA-4-Ig group (**Figure 4D-F**).

CD31 and CD34 protein expression in the kidney

The CD31 and CD34 protein expression in renal parenchyma was comparable between the CTLA-4-Ig group and the DN group (P> 0.05), but that in both group was significantly higher than in control group (P<0.05) (**Figure 5**).

Protein expression of podocin, nephrin, and B7-1 in the kidney

Protein expression of podocin and nephrin was significantly different among three groups (P<0.05): it was the highest in the control group and the lowest in the DN group. Significant difference was also observed in the B7-1 protein expression among three groups: it was the highest in the DN group and the lowest in the control group (P<0.05) (**Figure 6**).

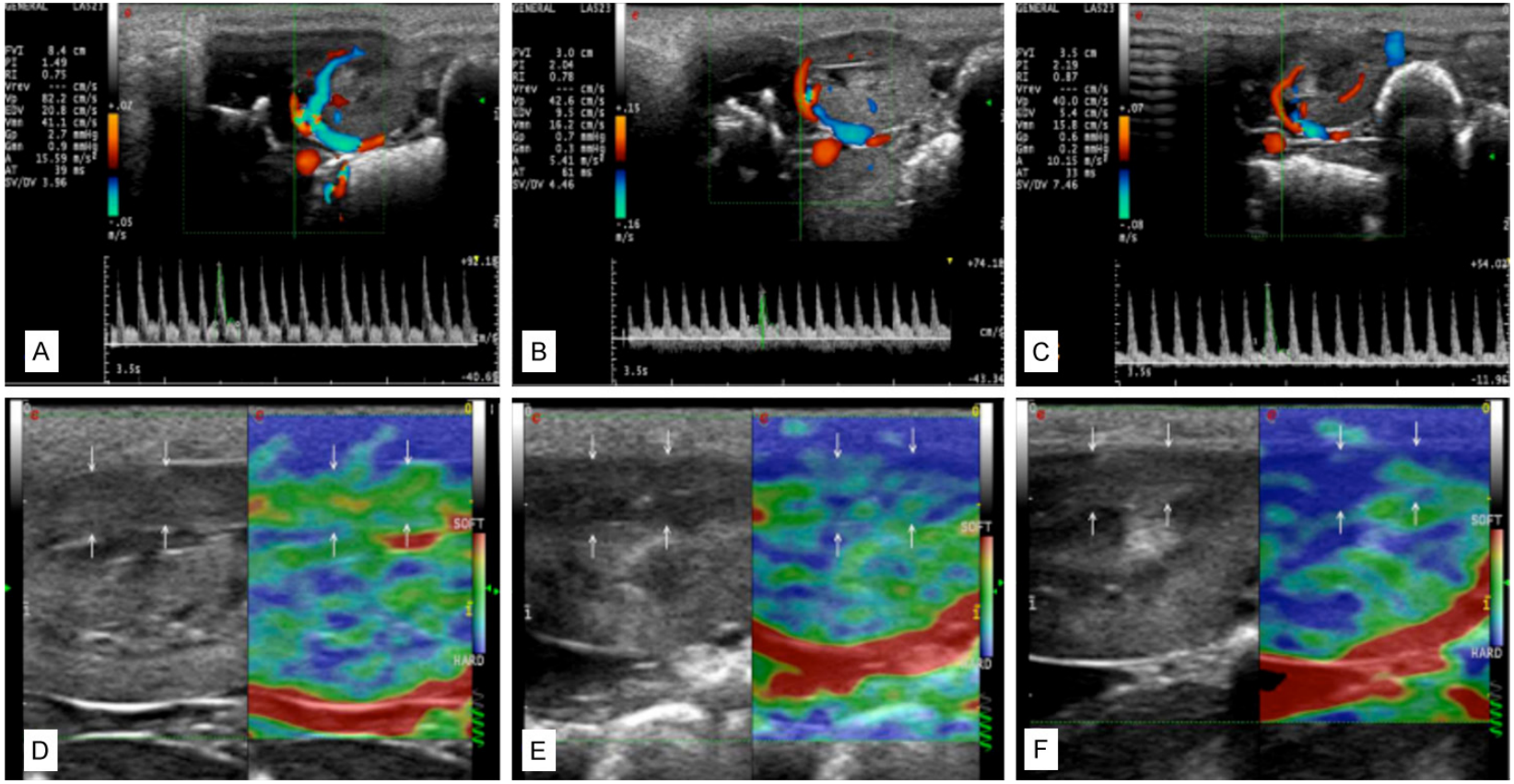

Figure 3. Pulse Doppler flow spectrum and renal parenchymal elasticity images in three groups. Notes: A. Control group: PSV=82.2 cm/s; EDV=20.8 cm/s; MV=41.1 cm/s; SAC=15.59 cm/s^2; PI=1.49; RI=0.75; B. CTLA-4-Ig group: PSV=42.6 cm/s; EDV=9.5 cm/s; MV=16.2 cm/s; SAC=5.41 cm/s^2; PI=2.04; RI=0.78; C. DN group: PSV=40.0 cm/s; EDV=5.4 cm/s; MV=15.8 cm/s; SAC=10.15 cm/s^2; PI=2.19; RI=0.87; D. CONTROL group: renal parenchymal elasticity image (score 1); E. CTLA-4-Ig group: renal parenchymal elasticity image (score 2); F. DN group: renal parenchymal elasticity image (score 3). White arrow: rat right renal parenchyma.

Table 3. Renal parenchymal elasticity score in three groups

Elasticity score	Control group	CTLA-4-Ig group	DN group
1	14	6	-
2	1	7	9
3	-	2	6

Table 4. Paired comparison of renal parenchymal elasticity score

Statistics	Control vs CTLA-4-Ig	CTLA-4-Ig vs DN	Control vs DN
Z	-3.057	-2.607	-4.847
P	0.010	0.016	<0.001

Discussion

Studies have shown that pathological changes (such as mesangial hyperplasia, basement membrane thickening, podocyte reduction and renal tubular injury) may occur at early stage of DN, finally causing glomerulosclerosis and renal interstitial fibrosis [20, 21]. The damage to the slit diaphragm between podocytes may cause the filtration of proteins, leading to proteinuria, which is one of factors affecting the prognosis of DN [22]. There is evidence showing that CTLA-4-Ig is effective to inhibit or attenuate podocyte injury, which is protective on DN. This study aimed to investigate the protective effects of CTLA-4-Ig on DN and the potential mechanism.

Our results show that CTLA-4-Ig treatment in DN rats can be effective to improve the kidney function (reduction in Ccr, increases in UAER and KW/BW), but has no significant influence on FBG, which may be ascribed to the action site of CTLA-4-Ig. In addition, CTLA-4-Ig was also effective to improve the main renal arterial blood flow parameters of DN rats, which was characterized by the increase in blood flow velocity and reduction in blood flow resistance in DN rats. Moreover, CTLA-4-Ig was able to reduce the renal parenchymal hardness in DN rats.

Nephrin is a podocyte specific protein. The presence of nephrin in the urine is related to the podocyte injury secondary to DN and suggestive of DN progression [23]. In a study on T2DM, results showed nephrin was detectable in 54% of patients with proteinuria in normal range and it was negatively related to the proteinuria. These findings indicate that nephrin may serve as a marker of DN in early stage [24]. Podocin is a member of stomatin family and another important component of podocyte slit diaphragm in. For DN patients, the increase in urine podocin means the excretion of a large amount of podocin into the urine. It has been confirmed that nephrin and podocin are specific markers of glomerular podocytes in DN patients and their expression increases in the urine of DN patients [25]. On the other hand, there is evidence showing that B7-1 expression increases in podocytes of DN patients. Podocytes exposed to high glucose (30 mmol/L) for a specific duration show PI3K mediated increase in B7-1 expression, which is related to loss of synaptopodin, activation and expression of integrin α3β1, and subsequent changes in cytoskeletons and podocyte movement. Addition of CTLA4-Ig is able to prevent or reverse the B7-1 expression and then affect the above pathological changes [26]. High glucose and B7-1 expression may also induce the podocyte apoptosis and necrosis *in vitro*, but CTLA4-Ig significantly reduces or controls the death of podocytes. Up-regulated expression of B7-1 is related to kidney dysfunction and deterioration of proteinuria, which has been confirmed in type 1 DM and type 2 DM animal models. In addition, CTLA4-Ig is effective to prevent the deterioration of proteinuria and attenuate pathological changes of the kidney [26]. Of note, to reverse the proteinuria is realized in case of B7-1 expression in podocytes. For the B7-1 deficient animal model, CTLA4-Ig fails to attenuate the pathological change of DN. This implies that B7-1 expression is a marker of kidney dysfunction and also a premise for the therapeutic effects of CTLA4-Ig. B7-1 is mainly expressed in podocytes, and the up-regulated expression of B7-1 is accompanied by the deterioration of kidney function.

Our results show that protein expression of nephrin and podocin was the highest in control group and the lowest in DN group whereas B7-1 protein expression was the lowest in control group and the highest in DN group. These indicate that CTLA-4-Ig is able to significantly increase nephrin and podocin protein expression and reduce B7-1 expression to protect the podocytes.

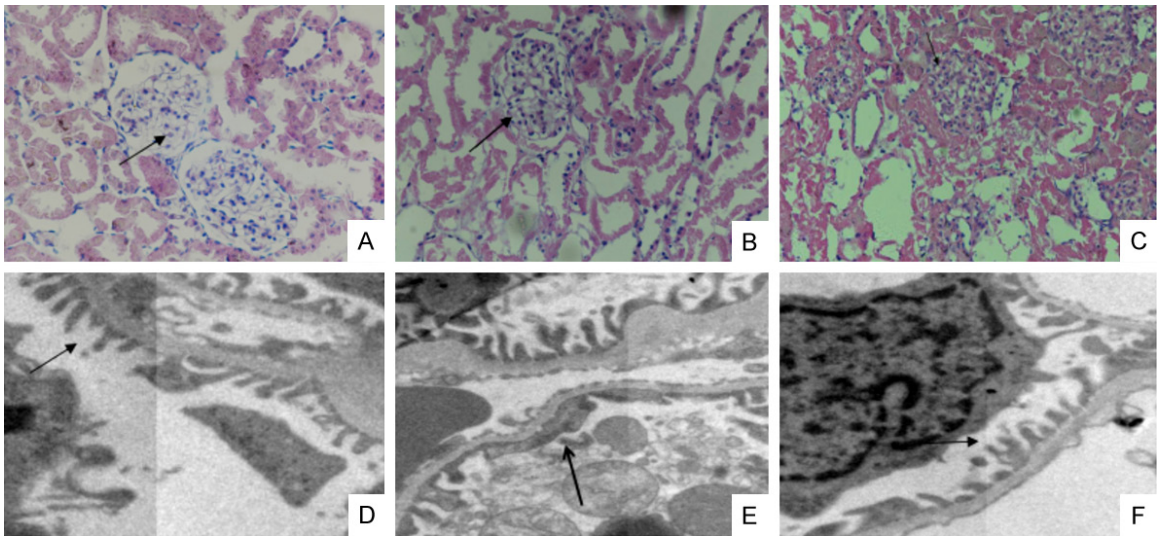

Figure 4. H&E staining of renal parenchyma and electron microscopy of podocytes in three groups. Note: A. H&E staining of renal parenchyma in control group (black arrow: no congestion in glomerular capillaries, no cell proliferation in vascular glands and no basement membrane thickening, clear glomerular balloon); B. HE staining of renal parenchyma in CTLA-4-Ig group (black arrow: narrowing or even complete occlusion of glomerular capillaries, enlargement of vascular glands, slight cell proliferation, and basement membrane thickening [coil like], focal occlusion of glomerular balloon); C. HE staining of renal parenchyma in DN group (black arrow: enlargement of vascular glands, evident cell proliferation, and basement membrane thickening, narrowing and focal occlusion of glomerular capillaries, occlusion of glomerular balloon); D. ETM of podocytes in control group (black arrow: normal podocytes with even distribution of non-fused processes); E. ETM of podocytes in CTLA-4-Ig group (black arrow: podocytes with different sizes, fused processes and irregular arrangement, and basement membrane thickening); F. ETM of podocytes in DN group (black arrow: podocytes with different sizes, massive fused processes, and some processes with incomplete structure or even absence of some processes).

CD31 is mainly expressed in endothelial cells. Newly generated blood vessels may be found in the glomeruli and interstitium in case of DN, which is related to the increase in CD31 expression [27, 28]. The change in CD31 expression may reflect the angiogenesis. CD34 is mainly distributed in the glomeruli and capillaries around the renal tubules. Studies have shown that the CD34 expression on the cell membrane of glomeruli increases significantly in aged animals and DM animals, which is consistent with the elevated expression of CD34 in case of injury or pathological condition [29]. In cases of glomerulus nephritis, endothelial CD34 expression increases, which implies the proliferation of endothelial cells. Increased CD34 expression in the glomeruli is related to the age and DM.

In summary, CD31 and CD34 expression in the renal parenchyma of the CTLA-4-Ig group and the DN group was significantly higher than in the control group ($P<0.05$), but it was comparable between the CTLA-4-Ig and DN groups ($P>0.05$). Therefore, CTLA-4-Ig has no influence on the expression of CD31 and CD34 in the renal parenchyma.

Conclusions

In this study, DN rats were treated with CTLA-4-Ig and the renal hemodynamics, renal parenchymal elasticity, and biochemical parameters were evaluated, as well as expression of CD31, CD34, podocin, nephrin, and B7-1 in the renal parenchyma. Our results indicate that CTLA-4-Ig is effectively to improve the kidney function, reduce main renal arterial resistance, increase the main renal arterial flow velocity, decrease renal parenchymal hardness, and improve the podocyte structure. In addition, CTLA-4-Ig had no influence on the CD31 and CD34 expression in the renal parenchyma, but was able to significantly increase podocin and nephrin expression, and reduce B7-1 expression in the kidney. Thus, we speculate that the therapeutic effects of CTLA-4-Ig on DN are ascribed to the protection of CTLA-4-Ig on podocytes and not related to the glomerular endothelial cells.

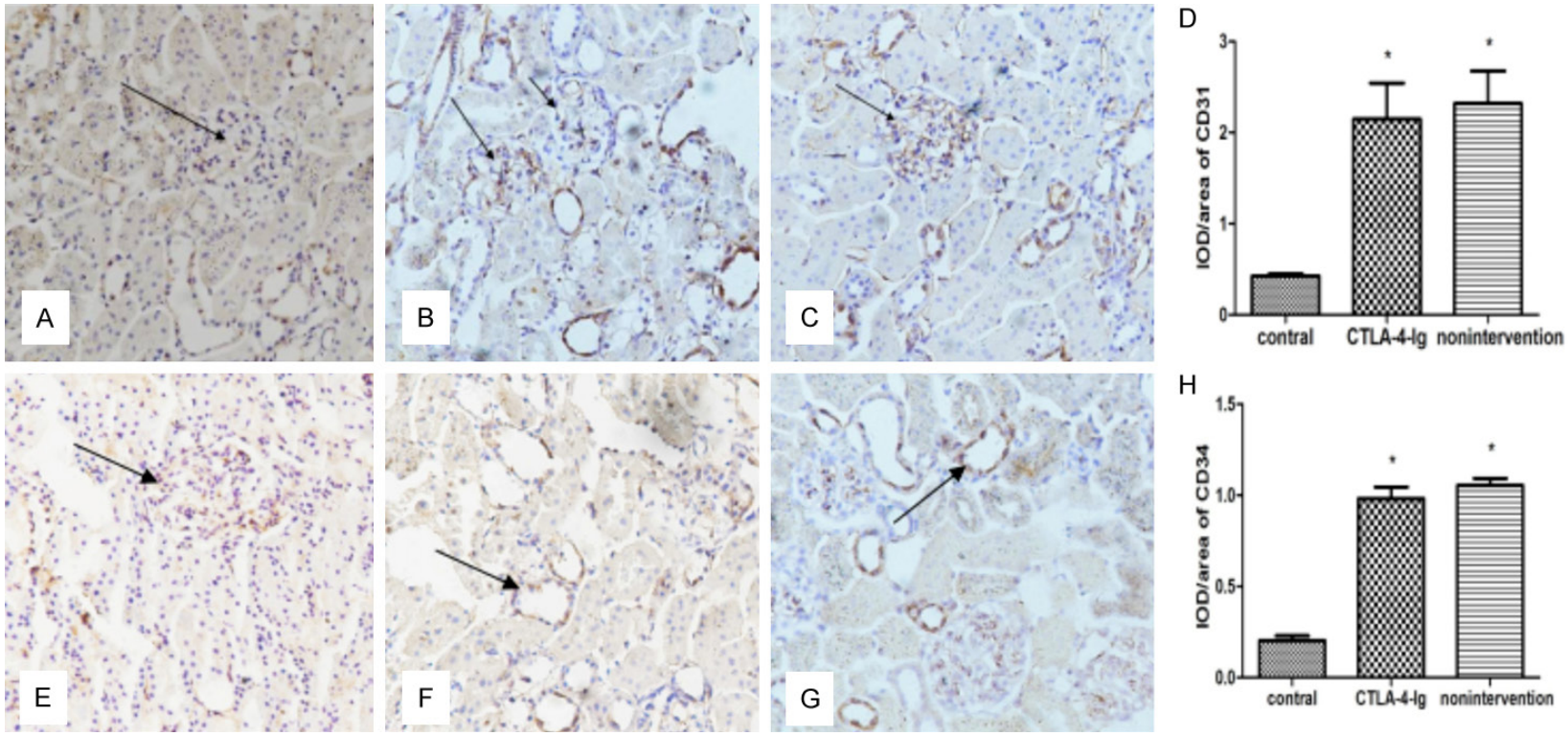

Figure 5. CD31 and CD34 protein expression in the renal parenchyma of three groups (immunohistochemistry). Note: A. CD31 expression in control group (black arrow: CD31 negative glomeruli; B. CD31 expression in CTLA-4-Ig group: (black arrow: mild angiogenesis in glomeruli and positive expression of CD31); C. CD31 expression in the DN group (black arrow: evident angiogenesis in glomeruli and positive expression of CD31); D. CD31 protein in the renal parenchyma of three groups. Nonintervention: DN group. Data are expressed as mean ± standard error (IOD/area of CD31: Integral optical density of CD31 positive area; *P<0.05 vs control); E. CD34 expression in the control group (black arrow: CD34 negative glomeruli); F. CD34 expression in CTLA-4-Ig group (black arrow: mild angiogenesis in glomeruli and positive expression of CD34); G. CD34 expression in the DN group (black arrow: evident angiogenesis in glomeruli and positive expression of CD34); H. CD34 protein in the renal parenchyma of three groups. Nonintervention: DN group. Data are expressed as mean ± standard error (IOD/area of CD34: Integral optical density of CD34 positive area; *P<0.05 vs control).

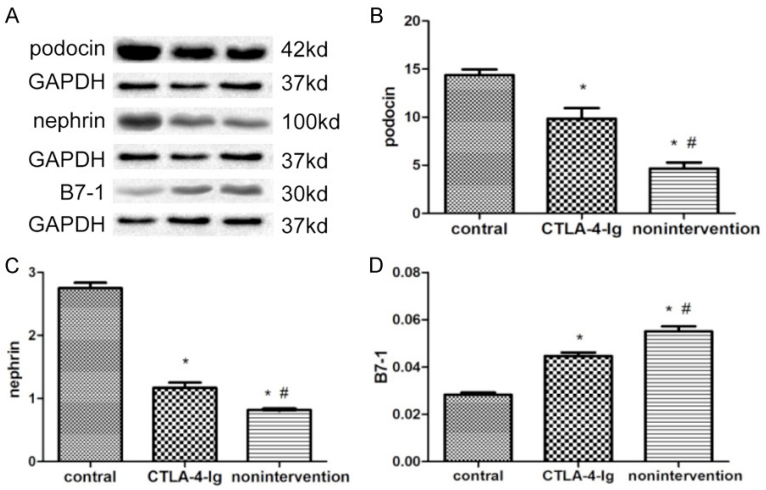

Figure 6. Protein expression of podocin, nephrin, and B7-1 in the kidney of three groups. Note: A. Protein expression of podocin, nephrin, and B7-1 in the kidney; B. Podocin protein expression; C. Nephrin protein expression; D. B7-1 protein expression. Nonintervention: DN group. Data are expressed as mean ± standard error. **P*<0.05 *vs* control group; #*P*<0.05 *vs* CTLA-4-Ig group.

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Disclosure of conflict of interest

None.

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