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Original Article

Cytotoxic T lymphocyte associated antigen 4 immunoglobulin prevents the alteration of hemodynamics and the stiffness of kidneys in rats with type 2 diabetes

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Abstract: This study aimed to investigate the influence of Cytotoxic T Lymphocyte Associated Antigen 4 Immunoglobulin (CTLA-4-Ig) on the hemodynamics and stiffness of the kidneys in rats with type 2 diabetes mellitus (T2DM). Rats with T2DM were randomly divided into non-intervention group and CTLA-4-Ig group. There was also a normal control (NC) group. Rats in CTLA-4-Ig group were treated with 0.5 $mg{\cdot}kg^{-1}{\cdot}w^{-1}$ CTLA-4-Ig for 8 weeks. The hemodynamics, such as peak systolic velocity (PSV), end diastolic velocity (EDV), mean velocity (MV), systolic acceleration (SAC), pulsatility index (PI) and resistance index (RI) of renal arteries were measured. The stiffness of the kidneys was measured. Results showed that there were significant differences in PSV, EDV and MV among three groups, the lowest in non-intervention group and the highest in NC group ($P<0.05$); there were significant differences in SAC, PI and RI among three groups, highest in non-intervention group and the lowest in normal control group ($P<0.05$). There was significant difference in the elasticity scores of renal parenchyma among three groups, highest in non-intervention group, followed by CTLA-4-Ig group and normal control group ($P<0.05$). The fasting blood glucose (FBG), creatinine clearance rate (Ccr), urine albumin excretion rate (UAER) and KW/BW in non-intervention and CTLA-4-Ig groups were significantly higher than in NC group ($P<0.05$); Ccr, UAER and KW/BW in the CTLA-4-Ig group were significantly lower than in non-intervention group ($P<0.05$). In conclusions, CTLA-4-Ig can prevent the alteration of hemodynamics and the stiffness of kidneys in rats with T2DM to a certain extent.

Keywords: Cytotoxic T lymphocyte associated antigen 4 immunoglobulin, type 2 diabetes, color Doppler ultrasound, ultrasonic elastography, kidney, podocyte

Introduction

It was reported that prevalence of diabetes mellitus (DM) was 9.7% among adults over 20 years old in China, and 30%~70% of DM patients may suffer from diabetic kidney disease (DKD) [1]. DKD is one of the serious complications of DM and characterized by glomerular sclerosis and proteinuria [2]. The kidney injury secondary to DM is progressive and complicated during the period from the onset of DM to the occurrence of DKD [3]. A large number of clinical and basic studies have been carried out to investigate the mechanism underlying the occurrence and development of DKD. Recent studies have shown that podocyte injury and apoptosis are directly associated with proteinuria in DKD [4]. Cytotoxic T Lymphocyte Associated Antigen 4 Immunoglobulin (CTLA-4-Ig) has been extensively studied in immune system diseases [5]. Recent studies indicate that CTLA-4-Ig can effectively protect the podocytes

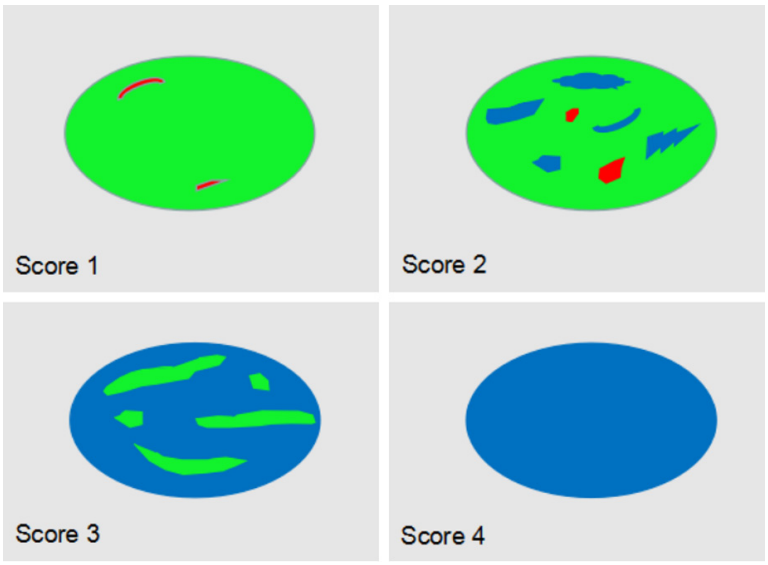

Figure 1. Classification of elasticity scores. A score of 1: shaded in green with some little red areas; a score of 2: a mosaic pattern of green, red and blue, most of area is green; a score of 3: a mosaic pattern of green and blue, most of area is blue; a score of 4: the entire lesion was blue.

in DKD. Thus, CTLA-4-Ig may be a new drug used for the treatment of DKD via preventing podocyte injury and apoptosis [6]. However, CTLA-4-Ig has never been used during the period from onset of DM to kidney injury. This study was to investigate whether the early intervention with CTLA-4-Ig can effectively prevent or alleviate the kidney injury in DM rats. The kidney injury was evaluated through blood and urine biochemical indexes, renal hemodynamics and elasticity of the renal parenchyma.

Materials and methods

Drugs and reagents

Recombinant CTLA-4-Ig (Abcam, United Kingdom); streptozocin (STZ, Sigma, USA); Urine protein detection kit (CBB method, Nanjing Jiancheng Biological Engineering Research Institute, China); Creatinine detection kit (Jaffé method, Nanjing Jiancheng Biological Engineering Research Institute, China); Cereal third transaminase detection kit (Colorimetric method, Shanghai Jining Industrial Co. Ltd., China); Glutamic oxaloacetic transaminase detection kit (Colorimetric method, Shanghai Jianglai Biological Company, China).

Induction of T2DM and grouping

45 specific pathogen free healthy male Sprague Dawley (SD) rats weighing 200±20 g and aged 6 weeks were purchased from Beijing Weitong Lihua Experimental Animal Technology Co. Ltd. and housed for one week before experiment. These 45 rats were divided into 3 groups: control group (rats were fed with normal food), non-intervention group and CTLA-4-Ig group. T2DM was induced in the rats of non-intervention group and CTLA-4-Ig group according to previously reported [7]. Then, rats in the CTLA-4-Ig group received intravenous injection of CTLA-4-Ig at 0.5 mg/kg/w for 8 weeks, and rats in non-intervention group and CTLA-4-Ig group received high-sugar and high-fat diets. Meanwhile, rats in the control group received normal diet. During the whole experiment, rats were given ad *libitum* access to water and were not treated with insulin or glucose lowering drugs. All animal care and experimental procedures were approved by the Animal Policy and Welfare Committee in Wenzhou Medical University (No. wydw2014-0117).

Detection of hemodynamics of renal artery and elasticity of renal parenchyma

A Biosound Esaote-MyLab 60 Xvision ultrasound instrument equipped with a 4-13 MHz transducer (LA523) was used for ultrasonography which was performed by the same physician one day before sacrifice. Room temperature was maintained at 25°C. The peak systolic velocity (PSV), end diastolic velocity (EDV), mean velocity (MV), systolic acceleration (SAC), pulsatility index (PI) and resistance index (RI) of the right main renal artery were measured. Measurements were repeated 3 times, and the averages were calculated. Sonographic elastography was then performed, and the scores were recorded. The elastography grade was classified from 1 to 4 according to the elasticity scoring system reported by Itoh et al [8]. A score of 1 indicated there was an even strain for the entire observed object (i.e., the observed object was shaded in green with little red areas); a score of 2 indicated there was strain in most of the observed object, with some areas that had no strain (i.e., the observed object had a mosaic pattern of green, red and

Table 1. Hemodynamics of the main renal artery in three groups ($\bar{x}$ ±s)

Parameters	NC group	CTLA-4-Ig group	Non-intervention group	*P*
PSV (cm/s)	42.05±8.18	28.62±6.33[a]	21.45±5.24[a,b]	0.000
EDV (cm/s)	16.75±3.26	8.15±2.92[a]	5.08±2.03[a,b]	0.000
MV (cm/s)	27.37±5.59	15.26±5.03[a]	10.44±3.75[a,b]	0.000
SAC (cm/s^2)	6.15±1.86	9.19±2.98[a]	11.83±3.09[a,b]	0.000
PI	1.01±0.21	1.33±0.26[a]	1.58±0.32[a,b]	0.000
RI	0.61±0.14	0.72±0.12[a]	0.84±0.11[a,b]	0.001

Note: [a]*P*<0.05 NC 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.

blue, but most of the area was green); a score of 3 indicated there was strain in a small part of the observed object, but a majority had no strain (i.e., the observed object had a mosaic pattern of green and blue, but most of area was blue); a score of 4 indicated there was no strain on the entire observed object (i.e., the entire lesion was blue) (**Figure 1**).

HE staining of kidney tissues

After blood collection, the kidneys were harvested and then weighed. Part of the right kidney was fixed in 4% paraformaldehyde for 48 h, followed by HE staining, and the glomerular structure was observed under light microscope.

Detection of blood and urine samples

All the rats were sacrificed after CTLA-4-Ig intervention for 8 weeks. Metabolic cages were used to collect urine, and blood was collected from the tail vein. The fasting blood glucose (FBG), alanine aminotransferase (ALT) and aspartate aminotransferase (AST), serum creatinine (Scr), urine creatinine (Ucr), 24-h urinary albumin (UAL), and the glomerular hypertrophy index (kidney weight/body weight, KW/BW) were determined. 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.

Statistical analysis

Data are expressed as mean ± SD. Statistical analyses were performed using the statistical package for the social software version 22 (SPSS Inc., Chicago, IL, USA). Comparisons of quantitative data were done using one-way analysis of variance (ANOVA) followed by post hoc LSD test Comparisons of qualitative data were done using the Nemenyi rank sum test. A value of *P*<0.05 was considered statistically significant.

Results

Comparison of hemodynamics of right main renal artery

Among three groups, the PSV, EDV and MV of the main renal artery were significantly different, and they were the lowest in non-intervention group and the highest in control group (*P*<0.01). The SAC, PI, RI of the main renal artery were significantly different among three groups, and they were as the highest in non-intervention group and the lowest in control group (*P*<0.01) (**Table 1**; **Figure 2**).

Comparison of elasticity of right renal parenchyma

There were significant differences in the elasticity scores of the renal parenchyma among three groups, and they were as the highest in non-intervention group and the lowest in control group (*P*<0.01) (**Tables 2** and **3**; **Figure 3**).

Comparison of HE staining of right kidney tissues

The structure of glomerulus was normal in control group, while there were mild changes in the structure of glomerulus in CTLA-4-Ig group. However, significant changes in the structure of glomerulus were found in non-intervention group (**Figure 4**).

Comparison of general parameters and biochemical parameters

The FBG, Ccr, UAER and KW/BW in non-intervention group and CTLA-4-Ig group were significantly higher than in control group (*P*<0.01). The Ccr, UAER and KW/BW in CTLA-4-Ig group were markedly lower than in non-intervention group (*P*<0.01). The FBG was comparable between CTLA-4-Ig group and non-intervention group (*P*>0.01). The ALT and AST were also similar among three groups (*P*>0.01) (**Table 4**).

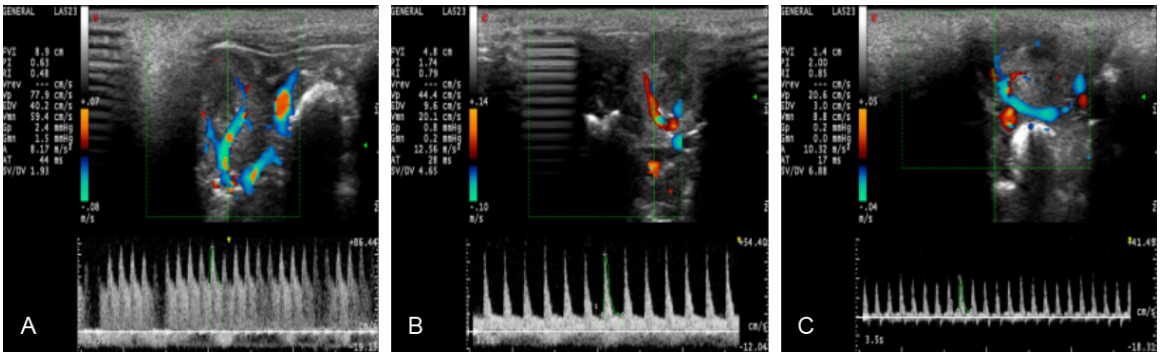

Figure 2. A. Pulse-wave Doppler of the right main renal artery of a rat from NC group. PSV: 77.9 cm/s; EDV: 40.2 cm/s; MV: 59.4 cm/s; SAC: 8.17 cm/s^2; PI: 0.63; RI: 0.48; B. Pulse-wave Doppler of the right main renal artery of a rat from CTLA-4-Ig group. PSV: 44.4 cm/s; EDV: 9.6 cm/s; MV: 20.1 cm/s; SAC: 12.56 cm/s^2; PI: 1.74; RI: 0.79; C. Pulse-wave Doppler of the right main renal artery of a rat from non-intervention group. PSV: 20.6 cm/s; EDV: 3.0 cm/s; MV: 8.8 cm/s; SAC: 10.32 cm/s^2; PI: 2.00; RI: 0.85.

Table 2. Elasticity scores of the renal parenchyma among three groups

Elasticity scores	NC group	CTLA-4-Ig group	Non-intervention group
1	14	6	-
2	1	9	11
3	-	-	4

Table 3. Elasticity scores of the renal parenchyma among three groups

Statistical parameters	NC group vs. CTLA-4-Ig group	CTLA-4-Ig group vs. Non-intervention group	Non-intervention group vs. NC group
χ^2	6.41	7.14	27.08
P	0.04	0.03	0.00

Discussion

DKD is a major complication of DM and characterized by the kidney damage [9, 10]. Currently, the current treatment for DKD has limited efficacy [11, 12], and thus early diagnosis and early treatment are important for controlling the development of DKD [13, 14]. Studies have shown that the pathological changes, such as proliferation of mesangial cells, thickening of the basement membrane, reduction of podocytes and damage to the renal tubules, may occur in the early stages of DKD, which ultimately result in glomerular sclerosis and renal interstitial fibrosis [15, 16]. Furthermore, protein filtration will increase once the structure of the slit diaphragm among the podocytes is impaired, which is one of the main factors affecting the prognosis of DKD patients [4, 17]. Some investigators have found that CTLA-4-Ig can effectively inhibit or reduce the damage to podocytes, suggesting that it may be used for the treatment of DKD [18]. Based on the available findings, this study was undertaken to investigate whether CTLA-4-Ig plays an important role in preventing or delaying renal injury if it is applied at the onset of DM.

With the progression of DM, glomerular sclerosis and renal interstitial fibrosis may occur in the kidneys [19], which leads to the alterations of renal hemodynamics and elasticity of the renal parenchyma [20, 21]. The time interval between the onset of DM and the occurrence of DKD is relatively long. In this study, rats in CTLA-4-Ig intervention group were treated with CTLA-4-Ig after the diagnosis of DM. Meanwhile, rats in non-intervention group had no CTLA-4-Ig treatment after the diagnosis of DM. Results showed that PSV, EDV and MV of the main renal artery in rats of CTLA-4-Ig intervention group were significantly higher than in non-intervention group, but they were lower than in control group (P<0.01). The SAC, PI and RI of the main renal artery of rats in CTLA-4-Ig intervention group were significantly lower than in non-intervention group, but they were higher than in control group (P<0.01). The decreases in PSV, EDV and MV imply a decrease in renal perfusion [22, 23]. The increases in SAC, PI and RI indicate an increase in the resistance of distal renal arter-

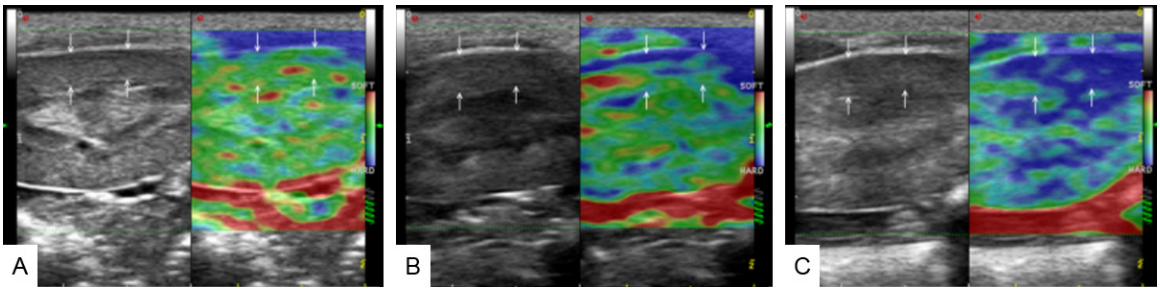

Figure 3. A. Gray scal and ultrasonic elastography of the right renal parenchyma (arrows) of a rat from NC group. Elastography score is 1; B. Gray scal and ultrasonic elastography of the right renal parenchyma (arrows) of a rat from CTLA-4-Ig group. Elastography score is 2; C. Gray scal and ultrasonic elastography of the right renal parenchyma (arrows) of a rat from non-intervention group. Elastography score is 3.

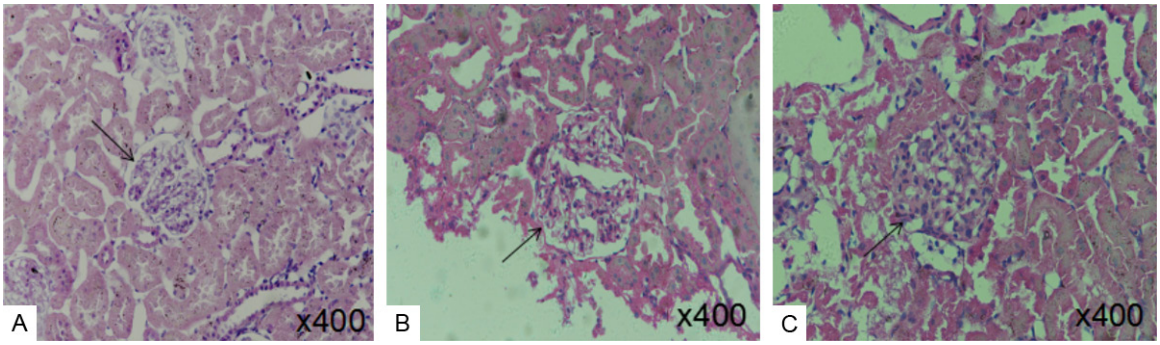

Figure 4. A. There was no congestion in capillary lumen of glomerulus, and no cell proliferation or basement membrane thickening inside glomus in rats of NC group. And the sacculus was clear (black arrow). B. There was no congestion in capillary lumen of glomerulus in rats of CTLA-4-Ig group. The glomus enlarged. There was mild cell proliferation and basement membrane thickening. But the sacculus was still clear (black arrow). C. In non-intervention group, the glomus enlarged. There was significant cell proliferation and basement membrane thickening. The capillary lumen was decreased or obstructed. And the sacculus was blocked (black arrow).

Table 4. General parameters and biochemical indictors among three groups($\bar{x}\pm s$)

Parameters	NC group	CTLA-4-Ig group	Non-intervention group	*P*
FBG (mmol/L)	5.12±0.61	20.08±3.89[a]	19.49±3.27[a]	0.000
Ccr (ml/min)	5.14±0.64	18.89±2.01[a]	20.13±3.05[a,b]	0.000
UAER (mg/24 h)	0.58±0.16	4.78±0.53[a]	6.17±0.57[a,b]	0.000
KW/BW (mg/g)	3.12±0.38	5.08±0.47[a]	6.82±0.65[a,b]	0.000
ALT (U/L)	45.56±6.81	49.13±5.26	48.65±5.74	0.299
AST (U/L)	57.28±7.03	58.39±6.55	61.21±6.67	0.616

Note: [a]$P<0.05$ vs. control group; [b]$P<0.05$ vs. CTLA-4-Ig group. FBG: fasting blood glucose; Ccr: creatinine clearance rate; UAER: Urinary albumin excretion rate; KW/BW: kidney weight/body weight; ALT: alanine aminotransferase; AST: aspartate aminotransferase.

ies [24, 25]. The elasticity scores in CTLA-4-Ig intervention group were significantly lower than in non-intervention group, but the scores were higher than in control group ($P<0.01$). The increase in the elasticity score showed an increase in the stiffness of the renal parenchyma. All these results indicate that early CTLA-4-Ig intervention can effectively improve the hemodynamics and the elasticity of parenchyma via inhibiting the decreases in PSV, EDV and MV, the increases in SAC, PI and RI and the elevation in elasticity of the renal parenchyma, leading to the alleviation of kidney injury. However, the intervention with CTLA-4-Ig in DM rats failed to reverse the kidney function to the control group as shown in this study, and whether this is related to the dose of CTLA-4-Ig and the frequency of CTLA-4-Ig treatment is unclear. The conventional and biochemical indicators, such as FBG, Ccr, UAER, and KW/BW measured in rats of non-intervention group and CTLA-4-Ig intervention group

were significantly higher than in control group ($P<0.01$). The Ccr, UAER, and KW/BW in CTLA-4-Ig intervention group were markedly lower than in non-intervention group ($P<0.01$), but there was no significant difference in FBG between CTLA-4-Ig intervention group and non-intervention group ($P>0.01$). The ALT and AST were also comparable among three groups ($P>0.01$). These indicate that early CTLA-4-Ig intervention can effectively protect the kidney function in DM rats by inhibiting the increases in Ccr, UAER, and KW/BW, but it cannot inhibit the increase in FBG. This may be explained as than CTLA-4-Ig has no significant effect on the liver function. Growing attention has been paid to the protection of podocytes in the treatment of DKD. Recent studies have found that B7-1 can be expressed in podocytes under pathological conditions [6]. Yu et al found that podocytes with B7-1 expression displayed morphological and functional changes through combination with the integrin β1. Adhesion to the surrounding matrix also decreased, which led to the detaching of podocytes from the glomerular basement membrane. As a result, urinary protein content increased [18]. CTLA-4-Ig can be synthesized with recombinant deoxyribonucleic acid technology and includes a biological immune inhibitor because CTLA-4-Ig retains the extracellular domain that can integrate with B7-1. CTLA-4-Ig can bind to B7-1 on antigen-presenting cells, which inhibits the binding of B7-1 to integrin β1, which prevents the detachment of podocytes from the basement membrane. Therefore, CTLA-4-Ig treatment may be promising in the treatment of DKD by targeting podocytes with B7-1 expression [6, 18].

In this study, early CTLA-4-Ig intervention is administered in DM rats, and the renal hemodynamics, stiffness of renal parenchyma and biochemical indicators were determined to evaluate the therapeutic effects of CTLA-4-Ig on DKD in DM rats. Results indicate that early CTLA-4-Ig intervention may effectively attenuate kidney injury. However, the underlying mechanisms are not investigated in detail, and additional experiments are needed to characterize these mechanisms.

Conclusions

The renal hemodynamics, stiffness of the renal parenchyma and biochemical indicators are determined to evaluate the therapeutic effects of CTLA-4-Ig on DKD in DM rats, and results indicate early CTLA-4-Ig intervention is effective to alleviate kidney injury. CTLA-4-Ig may be promising in the prevention and treatment of DKD.

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Disclosure of conflict of interest

None.

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