Background. Current research on the progression of diabetic nephropathy (DN) suggests many important factors; metabolic disturbance, haemodynamic abnormality, chronic inflammation, oxidative stress, innate immune system activation and podocyte lesion. Triptolide, which is active diterpene purified from the traditional Chinese medicine *Tripterygium wilfordii* Hook F (*TwHF*), has anti-inflammatory, anti-oxidative, immunosuppressive and podocyte-protective effects. Herein, we investigated the therapeutic effects of triptolide on DN in *db/db* diabetic mice and studied the potential mechanisms.

Methods. *db/db* mice with DN were administrated with triptolide or valsartan. After 4, 8 and 12 weeks of treatment, 24-h urine albumin level, blood biochemical parameters and body weight were measured. Glomerulus area, glomerulus volume to Bowman’s capsule volume ratio, podocyte changes and inflammatory and oxidative stress markers were quantitatively determined to evaluate renal lesions.

Results. The albuminuria in *db/db* diabetic mice was markedly attenuated after triptolide treatment, accompanied with alleviated glomerular hypertrophy and podocyte injury. In addition, the inflammation and oxidative stress in the kidneys were also attenuated, accompanied with improved hyperlipidaemia and obesity. The efficacy increased with the prolonging of triptolide treatment, and the efficacy in high-dose triptolide group was superior to that in the low-dose group. The effect of triptolide on glomerular hypertrophy was similar to valsartan, but the effects of triptolide on renal inflammation and oxidative stress were more profound than those of valsartan.

Conclusions. Triptolide can dramatically attenuate albuminuria and renal lesion accompanied with dyslipidaemia.
and obesity in db/db diabetic mice. It is a new drug that exerts comprehensive protective effects on preventing DN progression.

Keywords: albuminuria; db/db mice; DN; triptolide; valsartan

Introduction

Diabetic nephropathy (DN) is one of the common complications in diabetics and major problems facing human health. Increased urine albumin excretion is not only an indication of diabetic renal injury but an important factor in the progression of DN [1]. Multiple factors are involved in the occurrence of albuminuria during DN. Not only do metabolic disturbance and abnormal renal haemodynamics play critical roles in the occurrence of albuminuria, but also chronic inflammation and the oxidative stress are crucial contributors in the continuous progression of DN [2]. These factors can cause extensive renal injury including glomerular, tubular and interstitial vascular lesions [3]. Rectification of metabolic disturbance is a basic strategy in the treatment of DN [4]. Angiotension converting enzyme inhibitors (ACEI) and angiotensin II receptor blockers (ARB) are internationally accepted drugs in the treatment of DN and can improve renal haemodynamics, resulting in attenuated albuminuria. However, in some patients with heavy albuminuria, satisfactory efficacy of ACEI/ARB cannot be observed. In recent years, more attention has been paid to the anti-inflammatory and anti-oxidative treatments as well as immunotherapy, which have been found to delay the progression of obesity-related glomerulonephropathy (ORG) and DN [2,5,6]. All these suggested above, rectification of metabolic disturbance and renal haemodynamics combined with anti-inflammatory and anti-oxidative treatments as well as immunotherapy may provide new strategies for comprehensive treatment to improve the therapeutic efficacy in patients with DN.

Triptolide is an active diterpene purified from the traditional Chinese medicine Tripterygium wilfordii Hook F (TwHF) which has been used in rheumatoid arthritis treatment for several centuries and nephritis treatment for two decades [7,8]. It has anti-inflammatory, anti-proliferative and immunosuppressive effects and can relieve albuminuria in several renal diseases. In addition, our previous in vivo and in vitro studies have indicated that triptolide could stabilize the cytoskeleton of podocytes [9,10], exerting protective effects on podocyte diseases including minimal change disease, focal segmental glomerular sclerosis and membranous nephropathy [9–11]. Evidence has confirmed that triptolide could inhibit inflammatory reaction to reduce the level of urinary monocyte chemoattractant protein-1 (MCP-1) and thus improve renal function [12]. To further investigate the anti-inflammatory, anti-oxidative, immunosuppressive and podocyte-protective effects of triptolide, which may delay the progression of DN, we explored the efficacy of triptolide in a DN mouse model using valsartan, a commonly used ARB drug, as a control.

Materials and methods

Drugs and reagents

Triptolide was purchased from the National Institute for Control of Pharmaceutical and Biological Products. The purity of triptolide was as high as 99% as demonstrated by HPLC. Triptolide was dissolved in DMSO before use, and designed concentrations were prepared with normal saline. Valsartan was purchased from Novartis. Albumin kit was purchased from Excocell Inc, USA. Anti-nephrin antibody was kindly provided by Prof. Shankland. Anti-desmin (Neomarkers, USA), MCP-1 (abcam, UK), 4-HNE (Biomarkers, USA) and CD68 (SeroTec, UK) antibody were used in the present study.

Animals and grouping

C57BL/KsJ db/m normal and db/db diabetic mice were purchased from Jackson Research Laboratory (USA) and housed in our animal center. Mice aged 9 weeks were randomly divided into five groups (male to female ratio of 1:1; n = 18 per group): (a) db/m mice with saline (normal control); (b) db/db mice with saline (diabetic control); (c) db/db mice with valsartan (20 mg/kg day); (d) db/db mice with low-dose triptolide (25 μg/kg day); (e) db/db mice with high-dose triptolide (50 μg/kg day). During the experiment, animals were given ab libitum access to food and housed in the laminar flow cabinet with a 12 h/12 h dark/light cycle. After 4, 8 and 12 weeks of treatment, six mice per group (male to female ratio: 1:1) were randomly taken out for body weight determination and urine sample collection. After that, mice were sacrificed, and blood samples and kidneys were collected.

Determination of urine albumin

Mice were housed in metabolic cages throughout 24 h. Urine specimens were obtained, and the volumes were determined. Urine albumin concentrations were measured by ELISA according to manufacturer’s instructions.

Determination of biochemical parameters

EDTA anti-coagulated orbital venous blood specimen was obtained. The levels of albumin, cholesterol (Chol), triglyceride (TG), high-density lipoprotein (HDL), low-density lipoprotein (LDL), alanine transaminase (ALT), aspartate transaminase (AST) and creatinine (Cr) in the plasma were determined using a automatic biochemical analyzer (Hitachi7080).

Pathological examination

Light microscopy. The kidneys were fixed in 10% formaldehyde, embedded in paraffin, cut into 2-μm sections and stained with periodic acid–Schiff (PAS). The pathological changes were observed under light microscope. Photographs were obtained and quantitatively analyzed with SPI analysis software for morphology. About 10 glomeruli from each slide were randomly selected, and the mean glomerular area as well as the glomerulus volume to Bowman’s capsule volume (G/B) ratio was determined.

Electron microscopy. The kidney was cut into tissues of 1 mm³ and fixed in 3.75% glutaraldehyde followed by 1% osmic acid. The foot processes were observed under electron microscope and detected by Gatan Digital Micrograph software. The average podocyte foot process width [FPW bpm] = (π/4) x (ΣGBM length/Σnumber of foot process) [13]. The area density of podocyte nuclei in each glomerulus was determined by the method of White [14].

Immunofluorescence. Cryosections of the kidney tissues were obtained by the method of White [14]. The kidney was cut into sections of 1 mm³ and fixed in 10% % H2O2 for 10 min to inactivate endogenous peroxidase. Microwave antigen re-
retrieval was performed with citric acid solution (pH = 6.0) for 10 min. The slides were incubated with primary antibodies (desmin, MCP-1 or 4-HNE) at room temperature for 1 h. Envision immunohistochemical staining was used, and sections were developed with diaminobenzidine (DAB) 30 min later followed by counterstaining with haematoxylin. The slides were observed under light microscope. The MCP-1 or 4-HNE positive area was quantitatively determined with Image Pro Plus 6.0 software.

Statistical analysis
Quantitative data were presented as means ± SD. ANOVA followed by Student–Newman Keul’s test was used to analyze the significance of differences between groups. P < 0.05 was considered statistically significant, and P < 0.01 was considered highly statistically significant for all comparisons.

Results
Abnormal status of db/db mice in every group is similar before drug intervention.

Changes in 24-h urine albumin level after treatment
After 4 weeks of treatment, triptolide and valsartan markedly decreased the urine albumin level (P < 0.05, vs diabetic control mice). The therapeutic effects were more evident with the prolonging of treatment (Figure 1).

Changes in blood lipid after treatment
The levels of Chol, TG and LDL were markedly reduced by triptolide and valsartan. The effect of triptolide on reducing plasma Chol level was superior to valsartan. Moreover, the level of HDL in diabetic mice was significantly higher than normal mice. After administration with triptolide or valsartan, the HDL level was reduced but still higher than in normal mice (Figure 2A–D).

Changes in blood glucose level after treatment
Low-dose triptolide had little effect on the blood glucose level. At the early stage of treatment, transiently increased blood glucose level was noted after administration with high-dose triptolide followed by a decrease.

Changes in body weight after treatment
The obesity of db/db mice was markedly improved after 12 weeks of triptolide treatment. During the experiment, no evident changes in food (Supplementary material 1) and water intake were observed, and no diarrhoea was noted (Figure 2E).

Pathological changes of kidneys
Light microscopy. The glomerulus volume was markedly increased accompanied by increased cell number and widened mesangial region. After administration with triptolide or valsartan, the number of cells in the glomeruli decreased, and the widened mesangial region was alleviated (Figure 3). Quantitative analysis showed glomerulus area was decreased significantly after 12 weeks treatment, and no significant difference was noted among treated groups (Figure 3B). Furthermore, the G/B ratio was found to be dramatically increased in diabetic mice, which could be sharply reduced to nearly normal level by triptolide or valsartan treatment (Figure 3C). In addition, Oil-O-red staining showed that the increased staining in db/db mice was markedly reduced by triptolide or valsartan treatment (Supplementary material 2).

Electron microscopy. Widened foot process was noted in diabetic mice accompanied by partial fusion of glomerular capillary loops and widened thickness of glomerular basement membrane (GBM) (Figure 4). Quantitative analyses indicated the following (10 loops from each mouse were determined):

(1) The density of podocytes in diabetic mice aged 13 weeks, which decreased with age, was lower than normal mice. The podocyte density was markedly increased after triptolide or valsartan treatment (Figure 4B).

(2) The podocyte foot process width in diabetic mice aged 13 weeks, which elevated with age, was wider than normal mice. The decreased foot process width was noted after triptolide or valsartan treatment, which was more evident after high-dose triptolide treatment (Figure 4C).

Immunofluorescence. The nephrin-positive staining is lined along the glomerular capillary loops with even distribution in normal mice. However, the expression of nephrin was markedly decreased, and the absence of nephrin expression was noted in partial region. The expression of nephrin was dramatically increased after 4 weeks of triptolide or valsartan treatment, which was near to normal level (Supplementary material 3).
Immunohistochemistry

1. Expression of desmin protein. Low expression of desmin was found in the mesangial region of normal mice, which, however, was markedly increased in diabetic mice. The desmin positive area was also found along the glomerular capillary loops in diabetic mice. After 4 weeks of administration with triptolide or valsartan, the desmin positive area along mesangial region and capillary loops was markedly decreased and would not profoundly elevate with the increase in age. The decreased expression of desmin in high-dose triptolide group was most significant (Supplementary material 4).

2. Expression of MCP-1 protein. Low expression of MCP-1 was noted at multiple sites including glomerular endothelial cells, mesangial cells, renal tubular epithelial cells and tubulointerstitium in normal mice. The MCP-1 positive staining was markedly increased at multiple sites in the kidney of diabetic mice. After administration of triptolide or valsartan, the expression of MCP-1 was significantly decreased. The decreased expression was most profound in high-dose triptolide group (Figure 5I-III).

3. Expression of CD68-positive macrophages. CD68-positive macrophages increased in interstitium of db/db diabetic mice kidney, which could be reduced by triptolide or valsartan. The effects of triptolide on reduc-
ing CD68-positive macrophages were more powerful than valsartan (Figure 5IV).

(4) Expression of 4-HNE protein. The expression of 4-HNE was rarely noted in normal mice, which, nevertheless, was markedly increased in glomerular mesangial region and tubules in diabetic mice. Triprolide or valsartan dramatically reduced the expression of 4-HNE in glomerular mesangial region and tubules, especially high-dose triprolide (Figure 6).

**Effects of triprolide on liver and renal functions**

No significant difference in plasma ALT and AST levels after 12 weeks of treatment were observed between the high-dose triprolide group and untreated diabetes group [(100.5 ± 30.7 vs 125.8 ± 55.9) and (130.0 ± 22.6 vs 165.5 ± 74.0) U/L, respectively; P > 0.05]. The same trend was also noted in the plasma Cr levels between high-dose triprolide group and untreated diabetes group [(48.0 ± 3.6 vs 47.2 ± 4.2) μmol/l; P > 0.05].

**Discussion**

Metabolic disturbance, chronic inflammation, oxidative stress, innate immune system activation and podocyte injury play important roles in the progression of DN. Meanwhile, triprolide has been proved to have anti-inflammatory, antioxidative, immunosuppressive and podocyte-protective effects. Herein, we investigated the therapeutic effects of triprolide on DN. Our results indicated triprolide to
be a potential new drug that can alleviate albuminuria and protect DN from progression in a different fashion from valsartan.

The innate obese db/db diabetic mouse provides an ideal model for type 2 diabetes, and the disease course is extremely similar to that of humans [15]. The significant metabolic disturbance has been observed in db/db mice aged 8 weeks with increased albuminuria and impaired renal function [16]. Therefore, db/db diabetic mice aged 9 weeks were chosen for experiment. Triptolide could markedly decrease the urine albumin excretion in a time- and dose-dependent manner. The protective effect of low-dose triptolide on albuminuria was comparable to valsartan. But the protective effect of high-dose triptolide on albuminuria was superior to valsartan. The alleviated albuminuria reflected the protective effects of triptolide on renal lesion.

Besides the protective effects of triptolide on albuminuria, improved metabolic disturbance, alleviated glomerular hypertrophy and podocyte injury, suppressed inflammation and oxidative stress were also observed. The improved metabolic disturbance by triptolide was characterized by decreased levels of Chol, TG and LDL and attenuated obesity. Previous studies showed that high HDL level in db/db diabetic mice [17] was markedly decreased after administration of peroxidase proliferator-activated receptor (PPARα/γ) agonist, rhein, simvastatin or benazepril [16,18,19]. The decreased HDL level was also one of characteristics of improved metabolic disturbance. Triptolide improved dyslipidaemia and obesity without affecting food intake. The improved dyslipidaemia and obesity by triptolide may be related to its immuno-effects. CD4+ T cells were found to be implicated in the regulation of obesity-related metabolic disturbance and played crucial roles in the regulation of body weight and adipocyte hypertrophy. The predominant T-cell effect on glucose homeostasis, revealed by CD4+ T cell reconstitution studies in lymphocyte-free mice, was improvement of glucose toler-

Fig. 4. Triptolide and valsartan lessen podocyte injury in db/db mice. (A) A1, A2, A3, db/m mice after 4, 8 and 12 weeks of saline treatment; B1, B2, B3, db/db diabetic mice after 4, 8 and 12 weeks of saline treatment; C1, C2, C3, db/db diabetic mice after 4, 8 and 12 weeks of valsartan treatment; D1, D2, D3, db/db diabetic mice after 4, 8 and 12 weeks of low-dose triptolide treatment; E1, E2, E3, db/db diabetic mice after 4, 8 and 12 weeks of high-dose triptolide treatment. (B) Changes in podocyte density after treatment (×10^6/μm^3). (C) Changes in foot process width after treatment (nm). §P < 0.01 vs db/m normal mice; *P < 0.05 vs db/db diabetic mice; #P < 0.05 vs valsartan treatment mice.
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Fig. 5. Triptolide and valsartan lessen the expression of inflammatory markers in db/db mice (IH, ×400). (I) MCP-1 expression after 12 weeks treatment (IH, ×400). A db/m mice with saline; B db/db diabetic mice with saline; C db/db diabetic mice with valsartan treatment; D db/db diabetic mice with low-dose triptolide treatment; E db/db diabetic mice with high-dose triptolide treatment. (II) The quantitative analysis results of MCP-1 positive area in glomerulus (%). (III) The quantitative analysis results of MCP-1 positive area in tubule and interstitium (%). (IV) The positive index of CD68-positive macrophages in interstitium (cells/mm²). §P < 0.01 vs db/m normal mice; *P < 0.05 vs db/db diabetic mice; #P < 0.05 vs valsartan treatment mice.
There is increasing evidence that chronic inflammation and oxidative stress are major factors in the progression of DN. Reactive oxygen species (ROS) acts as a signal amplifier in diabetes [32]. Triptolide not only decreases chronic inflammation but improves oxidative stress, which is characterized by decreased expression of MCP-1 and 4-HNE. These effects of triptolide were superior to valsartan. MCP-1 is a chemokine which stimulates migration of monocytes. It induces renal interstitial fibrosis, participates in macrophage infiltration and accelerates the progression of DN. MCP-1 has been a new diagnostic marker and therapeutic target for progressive renal injury in DN [33,34]. Triptolide reduced MCP-1 expression accompanied with CD68+ macrophages. 4-HNE is an α,β-unsaturated hydroxyalkenal which is produced by lipid peroxidation in cells. Triptolide reduced the staining of Oil-O-red in the kidney accompanied with 4-HNE. Our previous study also found that triptolide could suppress the production of ROS and the activation of p38-mitogen-activated protein kinase (MAPK) in PAN nephrosis rats [10]. Triptolide can directly inhibit the activation of nuclear factor-kappa B (NF-κB) and the production of superoxide anion and NO [35]. Decreased production of tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), IL-6 and interferon-γ (IFN-γ) as well as reduced mRNA expression of iNOS and other inflammation-related cytokines by triptolide are also noted in a previous study [36]. Chronic inflammation and oxidative stress played important roles in metabolic-syndrome-related nephropathy [23–25]. The anti-inflammatory and anti-oxidative effects of triptolide may take part in ameliorating albuminuria and renal injury.

Podocyte injury is a typical characteristic in DN. Drugs that have beneficial effects on podocytes can improve our ability to treat DN. In the present study, results showed that, after administration with triptolide, the density of podocytes was increased, foot process width decreased and the expression of nephrin increased, accompanied by decreased expression of desmin (a marker of podocyte lesion), suggesting the protective effects of triptolide against podocyte injury. Our previous studies indicated that triptolide treatment could stabilize the cytoskeleton of podocytes, exerting direct protective effects against podocyte injury [10]. Recently, we found that triptolide could stabilize the intercellular junction between podocytes and protect podocytes against AngII-induced injury, which was associated with the production of ROS and p-38 as well as ERK and MAPK signaling pathway (unpublished data). Furthermore, glomerular hypertrophy and hyperlipidaemia are also detrimental to podocytes [37]. Triptolide treatment can attenuate glomerular hypertrophy and improve hyperlipidaemia, resulting in protective effects against podocyte injury. Taken together, triptolide has direct protective effects against podocyte injury and improved dyslipidaemia and obesity, alleviated glomerular hypertrophy, suppressed chronic inflammation and oxidative stress; all take part in podocyte protection. Evidence indicated valsartan also exerted protective effects against podocyte injury through improving haemodynamics and suppressing glomerular hypertrophy [38]. In our study, no significant difference on decreased glomerular hypertrophy was observed between triptolide group and valsartan group. However, the improved podocyte density, foot process width and expression of nephrin were more profound with triptolide than in the valsartan group. Triptolide has multiple protective effects against podocyte injury.

During the experiment, no adverse effects of triptolide on AST, ALT and Cr levels were observed, which suggested that triptolide treatment in diabetic patients might be safe
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without liver or kidney toxicity. The albuminuria in db/db diabetic mice was markedly attenuated by triptolide, accompanied by alleviated renal injury. The protective effect of triptolide on glomerular hypertrophy was similar to valsartan, a widely used ARB drug. However, the curative effects of triptolide on chronic inflammation, oxidative stress and podocyte injury were superior to valsartan. Therefore, we postulated that triptolide exerts comprehensive protective effects on DN which is different with valsartan.

Supplementary data

Supplementary data mentioned in the text is available to subscribers in Nephrology Dialysis Transplantation online.

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