Resveratrol Ameliorates Diabetes-induced Renal Damage through Regulating the Expression of TGF-β1, Collagen IV and Th17/Treg-related Cytokines in Rats

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ABSTRACT

Objectives: *This study aimed at detecting the protective effects of resveratrol on diabetes-induced renal damage and on the expression of transforming growth factor-beta 1 (TGF-β1), collagen IV and Th17/Treg-related cytokines in streptozotocin-induced diabetic rats.*

Methods: *Twenty diabetic rats were further randomly divided into diabetic model group (DM group) and resveratrol group with 10 animals in each group. Another 10 non-diabetic rats served as control. The dia-betic rats in the resveratrol group were administered resveratrol for eight consecutive weeks (via gavage, 50 mg/kg daily, dissolved in saline). Rats in the control group and DM group received the same volume of saline only (via gavage). Renal function was measured. Histopathology changes of the kidney tissue were observed using haematoxylin and eosin staining. Levels of TGF-β1 and collagen IV in kidney homogenate were measured with enzyme-linked immunosorbent assay (ELISA). The level of Th17-related cytokines (IL-17A, IL-25) and Treg-related cytokines (IL-35, IL-10) in serum and in the supernatant of the kidney homogenate were determined using ELISA.*

Results: *Diabetic rats had damaged renal function, higher levels of TGF-β1, collagen IV, IL-17A and IL-25, as well as lower levels of IL-35 and IL-10, when compared to the control rats. Compared to the dia-betic rats without resveratrol treatment, application of resveratrol to the diabetic rats ameliorated the renal function, inhibited the expression of TGF-β1, collagen IV, IL-17A and IL-25, and increased the ex-pression IL-35 and IL-10.*

Conclusion: *Resveratrol might ameliorate diabetes-induced renal damage through mediating the balance of Th17/Treg-related cytokines and inhibiting the expression of TGF-β1 and collagen IV.*

Keywords: Diabetic nephropathy, renal damage, resveratrol, Th17/Treg-related cytokines

El Resveratrol Mejora el Daño Renal Inducido por la Diabetes a Través de la Regulación de la Expresión de TGF-β1, Colágeno IV y las Citocinas Relacionados con Th17/Treg en Ratas

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RESUMEN

Objetivos: *Este estudio estuvo encaminado a detectar los efectos protectores del resveratrol en el daño renal inducido por diabetes y en la expresión del factor de crecimiento transformante beta-1 (TGF-β1), el colágeno IV, y las citocinas relacionados con Th17/Treg en ratas con diabetes inducida por estrepto-zotocina.*

Métodos: *Veinte ratas diabéticas fueron divididas aleatoriamente en un grupo modelo diabético (Grupo MD) y un grupo de resveratrol, con 10 animales en cada grupo. A las ratas diabéticas en el grupo de resveratrol se les administró resveratrol durante ocho semanas consecutivas (mediante sonda nasogás-trica, 50 mg/kg diarios, disuelto en suero salino). Las ratas en el grupo control y el grupo MD recibie-ron el mismo volumen de solución salina solamente (vía sonda nasogástrica). Se midió la función renal. Se observaron cambios en la histopatología del tejido del riñón usando tinción con hematoxilina y eo-*

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sina. Se midieron los niveles de TGF-β1 y colágeno IV en un homogeneizado de riñón con ensayo por inmunoabsorción ligado a enzimas (ELISA). El nivel de las citocinas de Th17 (IL-17A, IL-25) y las citocinas de Treg (IL-35, IL-10) en suero y en el sobrenadante del homogeneizado de riñón, se determinaron mediante ELISA.

Resultados: *Las ratas diabéticas tuvieron daño de la función renal, niveles más altos de TGF-β1, colágeno IV, IL-17A y IL-25, así como niveles más bajos de IL-35 e IL-10, en comparación con las ratas control. En comparación con las ratas diabéticas sin tratamiento con resveratrol, la aplicación de resveratrol en las ratas diabéticas mejoró la función renal, inhibió la expresión de TGF-β1, colágeno IV, IL-17A y IL-25 y aumentó la expresión de IL-35 y IL-10.*

Conclusión: *El resveratrol podría mitigar el daño renal inducido por la diabetes mediante la mediación con el equilibrio de las citocinas relacionados con Th17/Treg, e inhibiendo la expresión de TGF-β1 y colágeno IV.*

Palabras claves: Nefropatía diabética, daño renal, resveratrol, citocinas de Th17/Treg

INTRODUCTION

Diabetes, a serious disease characterized by a group of metabolic disorders, affects a great fraction of people worldwide (1, 2). Diabetic nephropathy, a major complication of diabetes as the consequence of the metabolic disorders, is a leading cause of renal disease (3−5). Approximately 30% of diabetic patients in developed countries have diabetic nephropathy.

Hypertrophy and fibrosis are characteristic in diabetic nephropathy. Among the factors that are relevant to the pathogenesis of diabetic nephropathy, multifunctional cytokines of the transforming growth factor-beta (TGF-β) family, especially TGF-β1 (6, 7), can regulate cell growth, differentiation, type IV collagen production, extracellular matrix production and apoptosis, which may lead to hypertrophy and fibrosis of the kidney.

There is also evidence that inflammation plays a role in the activation of TGF-β family and collagen family which deteriorates the pathogenesis of diabetic nephropathy in diabetic subjects. It is generally accepted that some cytokines, such as Th1/Th2-related cytokines interleukin (IL)-1, IL-6, tumour necrosis factor-alpha (TNF-α), are involved in the pathogenesis of diabetes and diabetic nephropathy (8−11). Recently, studies showed that some other kinds of immune cells such as Th17 cell and Treg cell also have a role in diabetes and diabetic nephropathy (12−16). However, to our knowledge, the changes and the role of Th17/Treg-related cytokines in subjects with diabetic nephropathy are not very well understood.

The treatments for diabetic nephropathy include blood pressure control, angiotensin-converting enzyme (ACE) inhibitors, angiotensin II type 1 receptor antagonists and so on, and are partially effective. Recently, resveratrol, a natural polyphenolic compound found in many plants, was introduced into the management of diabetes and diabetic nephropathy (17−19). Previous studies mainly focussed on its antioxidant effects and modulating angiogenesis effects by which it exerted its renal protective effects. A study conducted by Chang *et al* also showed resveratrol could retard the progression of diabetic nephropathy through modulating levels of inflammatory cytokines IL-1β, TNF-α and IL-6 (20). However, the effects of resveratrol on levels of Th17/Treg-related cytokines still remain unclear.

Thus, the present study mainly aimed to detect the protective effects of resveratrol on diabetes-induced renal damage and on the expression of Th17/Treg-related cytokines in streptozotocin-induced diabetic rats.

SUBJECTS AND METHODS
Animals

Experiments were performed on male Sprague-Dawley rats (180−220 g – initial body weight) purchased from Shandong University. The rats were housed in temperature-controlled 20 °C cages (five rats per cage) with a 12-hour dark-light cycle. All experimental procedures used in the project were conducted according to the National Institutes of Health's guidelines for the use and care of animals. The study was approved by the Animal Research Ethics Committee of Weifang Medical University.

Diabetes mellitus induction

Sprague-Dawley rats were intraperitoneally injected with a single dose of streptozotocin (Sigma Chemical Company, St Louis, MO, USA) [55 mg/kg body weight] which was dissolved in freshly prepared 0.01M citrate buffer (pH 4.5). The control animals received the same volume of fresh citrate buffer only. Three days after streptozotocin (STZ) injection, blood glucose level was measured using a Freestyle glucometer and the rats with marked hyperglycaemia (more than 300 mg/dL) were considered as the diabetic rats. Twenty diabetic rats were selected and further randomly divided into a diabetic model group (DM group) and a resveratrol group with 10 animals in each group. Another 10 Sprague-Dawley rats were taken as the control group.

Treatment

After the induction of diabetes mellitus, rats in the resveratrol group were treated daily with resveratrol (Zhuangtai Company,

Nanjing, China; dissolved in saline, *via* gavage] for eight consecutive weeks at a dose of 50 mg/kg body weight. Rats in the control group and DM groups daily received the same volume of saline only (*via* gavage).

Blood collection and preparation
The animals were deeply anaesthetized by intraperitoneal injection of ketamine and sacrificed after eight consecutive weeks of treatment. In order to perform the investigations, we collected the serum of the animals, obtained from the centrifugation (at 3000 rpm for 15 minutes) of the blood freshly collected from the rats, and immediately stored the serum at -80 °C until performing the investigations.

Kidney homogenate collection and preparation
Immediately after sacrifice of the animal, the right kidney was isolated and small parts of the kidney were collected, rinsed in ice-cold phosphate buffered saline (PBS) buffer and weighed. The tissue was cut into small pieces, immediately homogenized in PBS buffer (0.1 M, pH 7.4) using a homogenizer and centrifuged (3000 rpm for 15 minutes). The supernatant of the homogenate was aliquoted and stored at -80 °C.

Assessment of renal function
In order to collect urine, animals were put in a metabolic cage for 24 hours. The levels of urinary albumin, serum and urinary creatinine (Cr), and serum blood urea nitrogen (BUN) were measured using an automatic biochemistry analyser.

  Urinary albumin = urinary albumin concentration × 24-hour urine volume

  Ccr = (urinary creatinine concentration × urine volume per minute)/n serum creatinine concentration

Histopathology analysis
The left kidney was removed and small samples of the kidney were fixed in 4% paraformaldehyde solution and embedded in paraffin. Embedded kidneys were sectioned at 5 μm, stained with haematoxylin and eosin and examined with a light microscope for morphological evaluation.

Assessment of TGF-β1 and collagen IV
Transforming growth factor-beta 1 (TGF-β1) and collagen IV play roles in the pathophysiology of renal fibrosis which may result in the damage of the renal function. Levels of TGF-β1 and collagen IV homogenate of the kidney tissue were deter-

mined using enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturer's guidelines (Boster Company, Wu Han, China).

Assessment of Th17/Treg-related cytokines
Levels of IL-17A, IL-25, IL-35 and IL-10 in serum and homogenate of the kidney tissue were determined using ELISA kits according to the manufacturer's guidelines (R&D Systems, USA) to detect the effects of resveratrol on Th17/Treg-related cytokines. The serum and the supernatant of the homogenate which were stored at -80 °C were centrifuged again before investigations at 3000 rpm for 15 minutes.

Statistical analysis
Results were presented as mean ± SD. Statistical analysis was performed to determine differences between groups using SPSS 11.0. The results were compared by one-way analysis of variance (ANOVA) and post-hoc comparisons. *P*-values < 0.05 were considered to be statistically significant.

RESULTS
Effects of resveratrol on renal function
We examined the renal function of the rats after the treatment. We found that there was a significant increase of urine volume in diabetic rats from the DM group when compared with the control animals. After eight weeks of treatment with resveratrol, the urine volume of diabetic rats from the resveratrol group significantly decreased compared to the DM group ($p <$ 0.05). The results also showed that urine protein was significantly more in the diabetic rats from the DM group than the control rats ($p < 0.05$), and Ccr level of the DM group was lower than the control group ($p < 0.05$). With resveratrol treatment, there was a marked reduction of urine protein and increase of Ccr level in diabetic rats from the resveratrol group compared to the DM group (both $p < 0.05$). The data suggested that streptozotocin injection which induced diabetes mellitus in rats caused damage to the renal function, and resveratrol management could ameliorate the diabetes-induced renal damage (Table 1).

Effects of resveratrol on histopathology changes
Histological examination revealed marked hypertrophy of the kidney with bigger glomerular diameter in the diabetic rats from the DM group than the animals in the control group ($p < 0.05$). Diabetic rats that received resveratrol had smaller

Table 1:   Evaluation of the renal function and histopathology changes of the rats

Group	Urine volume (ml/24 hour)	Ccr (ml/min)	Urinary albumin (mg/24 hour)	Glomerular diameter (μm)
Control	21.07 ± 3.09	1.89 ± 0.11	0.88 ± 0.12	82.09 ± 7.56
DM	67.35 ± 9.21$^\Delta$	0.78 ± 0.10$^\Delta$	4.37 ± 0.53$^\Delta$	115.73 ± 8.31$^\Delta$
Resveratrol	32.88 ± 5.90$^{\Delta\star}$	1.25 ± 0.13$^{\Delta\star}$	2.63 ± 0.46$^{\Delta\star}$	98.06 ± 7.18$^{\Delta\star}$

Data are expressed as mean ± SD
$^\Delta$Significantly different compared with control group at $p < 0.05$; *Significantly different compared with DM group at $p < 0.05$; DM = diabetes mellitus, Ccr = urinary creatinine

glomerular diameter than the diabetic rats without resveratrol management ($p < 0.05$). The data showed that resveratrol could inhibit the hypertrophy of the kidney of the diabetic rats (Table 1).

Effects of resveratrol on TGF-β1

Transforming growth factor-beta 1 is believed to play a key role in the pathophysiology of renal fibrosis which may result in the damage of the renal function. We determined its levels in kidney homogenate using ELISA. We found streptozotocin injection induced higher levels of TGF-β1 in the homogenate from diabetic rats of the DM group in comparison to the rats of the control group ($p < 0.05$). The resveratrol application significantly decreased the TGF-β1 levels compared to the diabetic rats without resveratrol treatment ($p < 0.05$) [Table 2].

Effects of resveratrol on collagen IV

Over-expression of collagen IV can lead to fibrosis of the kidney tissue. Levels of collagen IV in homogenate of the kidney tissue were measured using ELISA. We found markedly elevated levels of collagen IV in the rats of the DM group when compared to the control animals ($p < 0.05$). Levels of collagen IV in the diabetic rats with eight weeks of resveratrol treatment markedly decreased compared to the diabetic subjects without resveratrol application ($p < 0.05$), which suggested that resveratrol attenuated the over-expression of collagen IV (Table 2).

Table 2: Levels of TGF-β1 and collagen IV in kidney of the rats

Group	TGF-β1 (µg/g)	Collagen IV (µg/g)
Control	0.15 ± 0.02	0.31 ± 0.02
DM	$0.48 \pm 0.06^{\Delta}$	$0.85 \pm 0.11^{\Delta}$
Resveratrol	$0.33 \pm 0.02^{\Delta\star}$	$0.53 \pm 0.08^{\Delta\star}$

Data are expressed as mean ± SD
$^{\Delta}$Significantly different compared with control group at $p < 0.05$;
*Significantly different compared with DM group at $p < 0.05$;
TGF-β1 = transforming growth factor-beta 1, DM = diabetes mellitus

Effects of resveratrol on levels of Th17/Treg-related cytokines

Th17 cell and Treg cell, which mainly exert their function *via* their related cytokines, are thought to be involved in the pathogenesis of diabetic nephropathy. Levels of Th17-related cytokines (IL-17A, IL-25) and Treg-related cytokines (IL-35, IL-10) in serum and homogenate of the kidney tissue were determined using ELISA. The results showed significantly higher levels of IL-17A and IL-25 and significantly lower levels of IL-35 and IL-10 in the diabetic rats of the DM group in comparison to the rats of the control group (all $p < 0.05$). Furthermore, application of resveratrol significantly decreased levels of IL-17A and IL-25 and significantly increased levels of IL-35 and IL-10 when compared to the DM group (all $p < 0.05$). The results suggested that resveratrol could regulate the balance of Th17/Treg-related cytokines both in serum and homogenate of the kidney tissue of the diabetic rats (Tables 3 and 4).

Table 3: Levels of Th17/Treg-related cytokines in serum

Group (ng/L)	IL-17A (ng/L)	IL-25 (ng/L)	IL-35 (ng/L)	IL-10 (ng/L)
Control	$19.7\,2\pm 2.63$	15.82 ± 2.60	28.41 ± 4.72	24.18 ± 2.64
DM	$51.69 \pm 6.20^{\Delta}$	$39.10 \pm 5.03^{\Delta}$	$13.37 \pm 2.75^{\Delta}$	$11.53 \pm 2.07^{\Delta}$
Resveratrol	$30.04 \pm 5.31^{\Delta\star}$	$22.28 \pm 4.62^{\Delta\star}$	$20.08 \pm 2.40^{\Delta\star}$	$18.53 \pm 2.10^{\Delta\star}$

Data are expressed as mean ± SD

$^{\Delta}$Significantly different compared with control group at $p < 0.05$;
*Significantly different compared with DM group at $p < 0.05$; IL = interleukin, DM = diabetes mellitus

Table 4: Levels of Th17/Treg-related cytokines in the homogenate of the kidney

Group (ng/L)	IL - 17A (ng/L)	IL - 25 (ng/L)	IL - 35 (ng/L)	IL - 10 (ng/L)
Control	31.58 ± 6.31	27.52 ± 3.61	46.94 ± 6.02	38.53 ± 5.37
DM	$89.13 \pm 10.96^{\Delta}$	$64.26 \pm 8.92^{\Delta}$	$21.30 \pm 3.16^{\Delta}$	$17.41 \pm 2.28^{\Delta}$
Resveratrol	$51.23 \pm 7.16^{\Delta\star}$	$43.52 \pm 6.76^{\Delta\star}$	$31.62 \pm 4.80^{\Delta\star}$	$26.67 \pm 2.35^{\Delta\star}$

Data are expressed as Mean ± SD
$^{\Delta}$Significantly different compared with control group at $p < 0.05$, *Significantly different compared with DM group at $p < 0.05$; IL = interleukin, DM = diabetes mellitus

DISCUSSION

The study provides evidence for changes of Th17/Treg-related cytokines in rats with diabetes-induced renal damage and the possible pathways by which resveratrol exerts its renal protective effects.

Diabetic nephropathy is one of the most serious complications of diabetes. The main current treatments of DN include glycaemic, lipid and blood pressure controls, renin-angiotensin-aldosterone system blockade [ACE inhibitors or angiotensin receptor blockers] (21). However, even with those managements regimes, many diabetic nephropathy patients still progress into end-stage renal disease. Thus, new therapeutic medication is urgent.

Resveratrol is a natural polyphenolic compound rich in grapes and some other plants (22). Recently, resveratrol was used to manage diabetes and some other chronic diseases. A growing body of reports showed resveratrol had a hypoglycaemic effect on DM subjects and was beneficial for some complications of diabetes (17–19). It had also been shown to alleviate renal dysfunction in diabetic animals. In the present study, the results showed that the rats who received streptozotocin injection had higher levels of urine protein and lower Ccr levels than the control rats, which suggested that the diabetic rats had damaged renal function. With resveratrol treatment, urine protein levels decreased and Ccr levels increased. The data suggest that resveratrol attenuated the renal damage of the diabetic rats. The previous studies mainly focussed on its antioxidant effects and modulating angiogenesis effects by which resveratrol exerted its renal protective effects. However, further studies are still required to detect the precise mechanisms of resveratrol's action on diabetic nephropathy.

Hypertrophy and fibrosis are the main pathological changes of diabetic nephropathy. Accumulating evidence shows that TGF-β1 is a key factor in the pathogenesis of diabetic nephropathy. The TGF-β family, especially TGF-β1 (6, 7), can regulate cell growth, differentiation, type IV collagen production, extracellular matrix production and apoptosis, which will lead to hypertrophy and fibrosis of the kidney, resulting in diabetic nephropathy. In our study, the diabetic rats had higher levels of TGF-β1 and collagen IV in the kidney than the control rats. With eight weeks of resveratrol treatment, levels of TGF-β1 and collagen IV in diabetic rats decreased, suggesting that resveratrol inhibited the over-expression of TGF-β1 and collagen IV in kidney of the diabetic rats. Histological examination in this study revealed marked hypertrophy of the kidney with bigger glomerular diameter in the diabetic rats. Resveratrol management inhibited the hypertrophy of the kidney of diabetic rats.

Diabetic nephropathy is increasingly considered an inflammatory state characterized by leukocyte infiltration at every stage of renal involvement (23). Studies have also shown that inflammation plays a role in the activation of the TGF-β family and collagen family, resulting in hypertrophy and fibrosis. The roles of Th1/Th2-related cytokines have been well demonstrated in the pathogenesis of diabetes and diabetic nephropathy (8−11). Recently, some studies showed that some other kinds of immune cells, such as Th17 cell and Treg cell, were also involved in diabetes and diabetic nephropathy (12−16). Th17 is a new subset of T helper cells and has been shown to play important roles in the pathogenesis of some autoimmune diseases. Recent evidence demonstrated that Th17 cell-related cytokine IL-17 was involved in the development and progression of diabetes. However, little is known about the relationship between Th17 cell-related cytokines and diabetic nephropathy. There is evidence that diabetic patients with or without diabetic nephropathy might have elevated levels of IL-17. Treg cell is another subset of T helper cells and has also been shown to be involved in diabetes. Clinical and animal studies have demonstrated that the diabetic subjects usually had decreased Treg cell activity and low levels of Treg-related cytokines (24). Enhancement of Treg cell activity, either by adoptive transfer or supplementation of supporting cytokines, such as IL-2, is considered as one of the therapeutic methods to diabetes (25, 26). Yet, whether Treg cells play roles in diabetic nephropathy still requires further investigation. According to our results, the diabetic rats had higher levels of Th17 cell-related cytokines IL-17A and IL-25 and lower levels of Treg-related cytokines IL-35 and IL-10 in serum and homogenate of the kidney tissue, when compared to the control rats. Application of resveratrol for eight weeks significantly decreased levels of IL-17A and IL-25 and significantly increased levels of IL-35 and IL-10 when compared to the DM group. Taking the changes of renal function together, the results suggest that the inflammatory cytokines IL-17A, IL-25, IL-35 and IL-10 might take part in the renal damage in diabetic rats.

In conclusion, diabetic rats with renal dysfunction had elevated levels of Th17 cell-related cytokines IL-17A and IL-25 and decreased levels of Treg-related cytokines IL-35 and IL-10. Resveratrol could ameliorate diabetes-induced renal damage and the mechanism of the renal protective effects might be partially associated with the down-regulation of TGF-β1, collagen IV and Th17 cell-related cytokines IL-17A and IL-25, as well as up-regulation of Treg-related cytokines IL-35 and IL-10. However, the precise roles and mechanisms of Th17/Treg cells in diabetic nephropathy still require further investigation.

Authors' note

The authors report no conflicts of interest regarding the contents of this work.

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