Int J Clin Exp Med 2017;10(6):9117-9123
www.ijcem.com /ISSN:1940-5901/IJCEM0055230

Original Article

Protective effects and mechanisms of acteoside on renal injury in streptozotocin (STZ)-induced diabetic mice

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Received April 12, 2017; Accepted May 13, 2017; Epub June 15, 2017; Published June 30, 2017

Abstract: Objective: To investigate the protective effects and mechanisms of acteoside on renal injury in diabetic mice. Methods: Forty mice were divided into the control group, the diabetic mice group (DM), the acteoside-treated diabetic mice group (DM + A), and acteoside group (A), involving 10 mice in each group. Diabetic models were established by intraperitoneal injection with streptozotocin (50 mg/kg) for 5 days. Model establishment would be regarded as successful if the blood glucose concentration of mice were greater than 14 mmol/L at 72 hours after modeling. Eight weeks later, the fasting blood-glucose (FBG), 24 h urinary volume and urine protein quantitation (UAlb/24 h), serum creatinine (Scr), blood urea nitrogen (BUN), kidney weight/body weight (KW/BW) of mice in each group were measured. The expression level of malondialdehyde (MDA), γ-glutamylcysteine synthetase (Y-GCS) and superoxide dismutase (SOD) in serum and kidney homogenate were measured as well. And the fluorescent quantitation PCR was adopted to detect the expression level of renal inflammatory factors (IL-1, IL-6 and TNF-α). Results: Eight weeks later, the blood glucose, 24 h urine protein quantitation, Scr, BUN, KW/BW and MDA content in DM group all increased compared to the control group. The level of Y-GCS and SOD was significantly decreased while the inflammatory factors increased. Although the blood glucose in DM + A group didn't decrease markedly compared to the DM group, the 24 h urine protein quantitation, Scr, BUN, KW/BW and MDA content were decreased, the antioxidant molecules (Y-GCS and SOD) were increased significantly and the expressions of all renal inflammatory factors were obviously decreased. Conclusion: Oxidative stress plays a critical role in the occurrence and development of diabetes. Acteoside can reduce the oxidative stress level and relieve the renal inflammatory reactions of diabetic mice, meanwhile, it has protective effects on the renal injury caused by diabetes.

Keywords: Acteoside, diabetic renal injury, oxidative stress, inflammatory injury

Introduction

Diabetes mellitus is a very common metabolic disorder disease in clinic. It is caused by a variety of risk factors, such as genetic factor, mental factor, microbial infection, immune dysfunction and so on, all these factors can lead to pancreatic islet hypofunction and insulin resistance, and ultimately cause the metabolic disturbance of the glucose in body, fat, protein, water, electrolyte and so on [1]. The morbidity of diabetes has been increasing with the economic and social development [2]. The diabetic nephropathy (DN), as a severe complication in advanced diabetes [3], has the characteristics of renal injury like proteinuria, edema, hypertension etc. In recent years, the number of patients with DN is growing gradually, and DN has become one of the main risk factors of end-stage renal disease [4]. The pathogenesis of DN is very complex without distinct conclusion yet. At present, the dominant view is that the process of oxidative stress and inflammatory reaction may play a critical role in the occurrence and development of DN [5-8]. The life qualities of patients with DN have been seriously affected due to the lack of effective medication.

Acteoside, a kind of phenylpropanoid glycoside, exists extensively in various plants, and has many physiological activities such as anti-inflammatory, antiviral, adjusting proliferation and apoptosis of cells and lipase inhibition etc. [9-12]. Recent studies have shown that acteoside can provide neuroprotection when combined with caspase-3 [13] and inhibit the formation of liver cancer by suppressing oxidative stress and inhibiting apoptosis [14], and can also alleviate the inflammatory injury in acute lung injury [15].

Acteoside has a wide range of applications as well as important research value. However, relevant studies of its functions in diabetic renal injury are still limited. This study was designed to investigate the effects and possible mechanisms of acteoside on diabetic renal injury by exploring its influence on the oxidative stress and inflammatory reactions.

Materials and methods

Experimental animals and major reagents

The healthy male C57BL/6 mice under SPF conditions, 6-8 weeks old, weighing 18-22 g, were purchased from Shanghai SLAC Laboratory Animal Co., Ltd. STZ was purchased from Sigma Company in USA; acteoside, with purity greater than 98%, was purchased from Chengdu Ruifensi Biological Technology Co., Ltd. MDA, Y-GCS, SOD, Scr, BUN and albumin detection kit were all purchased from Nanjing Biological Engineering Institute. And Trizol reagent was bought from Ambion Life Technologies in USA, and reverse transcription kits (PrimeScript 1stStrand cDNA Synthesis Kit) were bought from TaKaRa in Japan.

Preparation and grouping of diabetic mice models

Ten mice were assigned to four groups and fasted for 12 hours before modeling. The modeling groups were induced by the intraperitoneal injection of STZ at a dose of 50 mg/kg for 5 days. The control group was injected with equal dose of normal saline. The DM + A group was treated with acteoside (30 mg/kg) every day after modeling. Besides, the constant gavage was performed for 6 weeks in DM + A group. The tail vein blood of mice in four groups was collected at 72 hours after the modeling, then the blood glucose was measured. Blood glucose concentration greater than 14 mmol/L indicated successful modeling.

Detection of fasting blood glucose

The mice in each group were fasted for 8 hours before the end of trial and the tail vein blood was collected to detect the fasting blood glucose with blood glucose analyzer.

Detection of serum biochemical index

The experiment was ended after 8 weeks of modeling. The blood extracted from orbital venous of mice in each group was placed still for 1 hour until the serum separated. Then, the serum obtained was centrifuged at 3000 rpm for 10 minutes and the upper layer of the serum was collected. Samples and reagents were added successively in accordance with the kit instructions of MDA, Y-GCS, SOD, Scr, BUN. The optic density (OD) of each sample was detected by spectrophotometer and all indexes were quantitatively calculated according to the values of OD.

Detection of MDA, Y-GCS and SOD in renal tissues

Equal amounts of renal cortex tissue (50 mg) from each mouse of each group were collected and pre-cool normal saline was added to yield a 10% tissue homogenate. Then, the suspension was centrifuged at 1500 rpm for 15 minutes at low temperature. The supernatant was extracted for detection. The corresponding assay kit was chosen to detect the levels of MDA, Y-GCS and SOD in tissue homogenate. All indexes were quantitatively calculated according to the values of OD measured by spectrophotometer.

Twenty-four hour urinary volume and urine protein quantitation

Mice in all groups were placed in metabolism cages, the 24 h urine was collected weekly at the same time point and the urinary volume was measured. The protein concentration was determined by enzyme-linked immunosorbent assay, reagents were added in accordance with the instructions of assay kits. Standard curve was established and the protein concentration was worked out according to the OD values of samples, then multiplied by 24 h urinary volume to get the data of 24 h urine protein.

Table 1. Specific primers (5'->3')

	Forward primer	Reverse primer
IL-1β	GCAACTGTTCCTGAACTCAACT	ATCTTTTGGGGTCCGTCAACT
IL-6	CTCTGGGAAATCGTGGAAAT	CCAGTTTGGTAGCATCCATC
TNF-α	TCTCTTCAAGGGACAAGGCT	GGCAGAGAGGAGGTTGACTT
β-actin	AGTGTGACGTTGACATCCGT	GCAGCTCAGTAACAGTCCGC

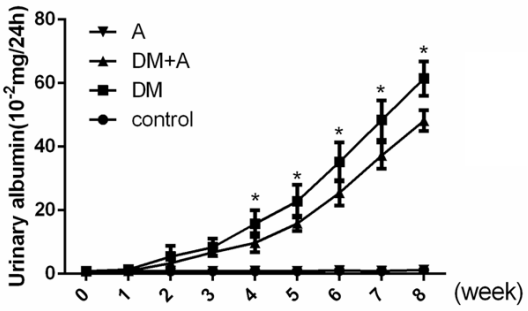

Figure 1. The effect of acteoside on 24 h urinary albumin in diabetic mice. *P<0.05 compared with DM group.

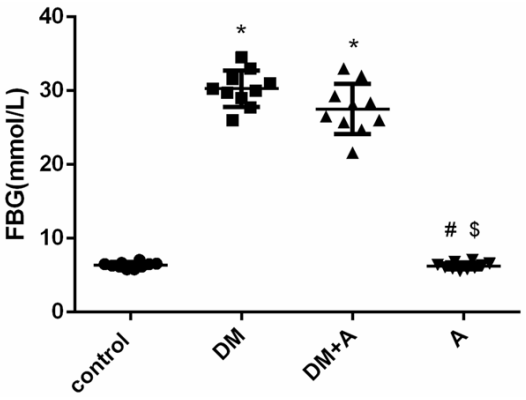

Figure 2. The effect of acteoside on FBG in diabetic mice. *P<0.05 compared with control group; #P<0.05 compared with DM group; $P<0.05 compared with DM + A group.

Measurement of KW/BW

The mice in each group were weighed up after cervical dislocation. Both kidneys were removed and weighed after getting rid of the capsular. The ratio of both kidneys weight to body weight was the specific value of KW/BW.

Fluorescent quantitative PCR assay

The total RNA was extracted from fresh renal tissues of mice by Trizol and was reverse-transcribed to cDNA using the Takara reverse transcription kit. Then, the cDNA was analyzed by ABI PRISM 7500 fluorescent quantitative PCR (brought from Applied Biosystems, USA) with β-actin as the internal reference. The conditions of amplification were as follows: pre-denaturation was performed at 95°C for 20 seconds, followed by 40 cycles of denaturation for 3 seconds at 95°C, and for 30 seconds at 60°C. The Ct values of each sample were calculated and compared with the Ct values of internal reference gene, and $2^{-\Delta\Delta CT}$ was used to work out the relative quantification of genes in samples. All primers were synthesized by Shanghai Shenggong Company and the sequences of specific primers were listed in **Table 1**.

Statistical methods

All the data were analyzed by the SPSS17.0 software and the measurement data were expressed as mean and standard deviation ($\overline{x}$ ± SD). The comparison of groups was performed by using one-way ANOVA. P<0.05 was considered statistically significant.

Results

Twenty-four hour urine protein quantitation

With the continuous proceeding of diabetes modeling, the content of 24 h urine albumin in DM group was increased gradually, which was significantly different from the control group (P<0.05). Compared with DM group, the content of 24 h urinary albumin in DM + A group was decreased significantly, while there was no significant difference between the A group and the control group in this aspect (P<0.05, **Figure 1**).

Fasting blood glucose

Compared to the control group, the application of acteoside alone had no obvious influence on blood glucose, but the blood glucose in DM group and DM + A group increased significantly at the end of the trial (P<0.05). Besides, the application of acteoside didn't mitigate the increase of blood glucose in diabetic mice obviously (P>0.05), which indicated that acteoside had no direct effect on decreasing blood glucose (**Figure 2**).

Table 2. Kidney weight/body weight in each group

Group	KW (g)	BW (g)	KW/BW (mg/g)
Control	0.63 ± 0.04	22.35 ± 1.23	27.35 ± 2.76
DM	0.64 ± 0.10	18.23 ± 2.17*	32.76 ± 2.13*
DM + A	0.64 ± 0.13	20.37 ± 1.48*,#	29.49 ± 2.28*,#
A	0.63 ± 0.03	22.19 ± 1.07#,$	27.24 ± 2.84#,$

Note: *P<0.05 compared with control group; #P<0.05 compared with DM group; $P<0.05 compared with DM + A group.

Table 3. Serum creatinine (Scr) and blood urea nitrogen (BUN) in each group

Group	Cr (umol/L)	BUN (mmol/L)
Control	81.23±6.35	5.21±1.19
DM	196.77±48.41*	9.38±1.03*
DM + A	137.56±31.48*,#	6.88±0.93*,#
A	80.23±7.12$	5.11±1.37$

Note: *P<0.05 compared with control group; #P<0.05 compared with DM group; $P<0.05 compared with DM + A group.

KW/BW

The application of acteoside alone had no influence on the kidney weight and body weight of mice. However, compared to the control group, the DM group had no changes in the kidney weight but had dramatic decline in the body weight after the establishment of mice models (P<0.05), thus the ratio of KW/BW was increased markedly (P<0.05). Compared to the DM group, the body weight of mice in DM + A group was slightly increased (P<0.05), but the ratio of KW/BW was decreased significantly and showed distinct differences (P<0.05, **Table 2**).

Scr and BUN

As shown in **Table 3**, compared to the control group, the contents of Scr and BUN were not affected in A group with acteoside only, but they were significantly increased in DM group (P<0.05). The levels of Scr and BUN in DM + A group were higher than those in control group, but obviously lower than those in DM group (P<0.05, **Table 3**), which indicated that the renal injury in diabetic mice was obviously alleviated after being treated with acteoside.

Content of MDA, Y-GCS, SOD in serum and renal injury

Compared to the control group, the levels of MDA, Y-GCS, SOD in serum of mice in A group didn't show significant changes, and the level of MDA in serum of DM group was elevated markedly, indicating that the production of lipid peroxide was increased (P<0.05, **Figure 3A**). However, the level of MDA in serum of DM + A group was significantly decreased, and the content of antioxidative factors (Y-GCS and SOD) was significantly higher than that in DM group (P<0.05, **Figure 3**).

Similarly, the content of MDA in renal tissues in the DM group was significantly increased compared with the control group, while the content of Y-GCS and SOD was significantly decreased (P<0.05). The application of acteoside alone had no obvious effect on the content of MDA, Y-GCS and SOD, but its application on diabetic mice could dramatically reduce the content of MDA and increase the content of Y-GCS and SOD (**Figure 4**).

Expression of inflammatory factors in renal tissue

The expression of renal inflammatory factors (IL-1, IL-6 and TNF-α) in each group was detected by fluorescent quantitative PCR assay, the result showed that the expression of renal inflammatory factors in DM group were markedly increased after the establishment of diabetic mice models, which was significantly different from the control group (P<0.05). Compared to DM group, the application of acteoside could markedly reduce the expression of renal inflammatory factors in diabetic mice, however, the application of acteoside alone didn't show any effect on the expression of renal inflammatory factors (P<0.05, **Figure 5**).

Discussion

Diabetic nephropathy (DN) is one of the severest complications occurring at the advanced stage of diabetes. Its morbidity and mortality are extremely high, which will bring a lot of financial burdens to patients [16, 17]. At present, the understanding of its pathogenesis is still insufficient, and special medications for effective treatment are inadequate. The clinical prevention and treatment of DN are mainly by controlling blood glucose and blood pressure, regulating blood lipid, as well as changing lifestyle. However, the current treatments still can't control and delay the development of this disease very well. Therefore, searching for safe and effective treatments is very necessary.

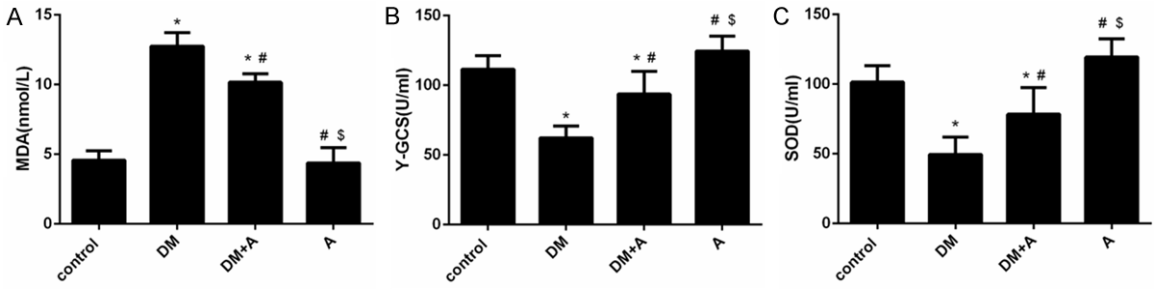

Figure 3. The effect of acteoside on MDA, Y-GCS and SOD in serum of diabetic mice. *P<0.05 compared with control group; #P<0.05 compared with DM group; $P<0.05 compared with DM + A group.

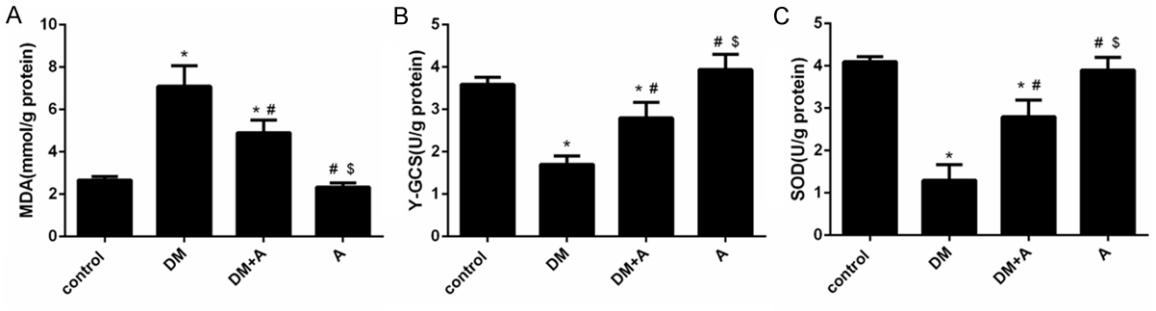

Figure 4. The effect of acteoside on MDA, Y-GCS and SOD in the kidney tissue of diabetic mice. *P<0.05 compared with control group; #P<0.05 compared with DM group; $P<0.05 compared with DM + A group.

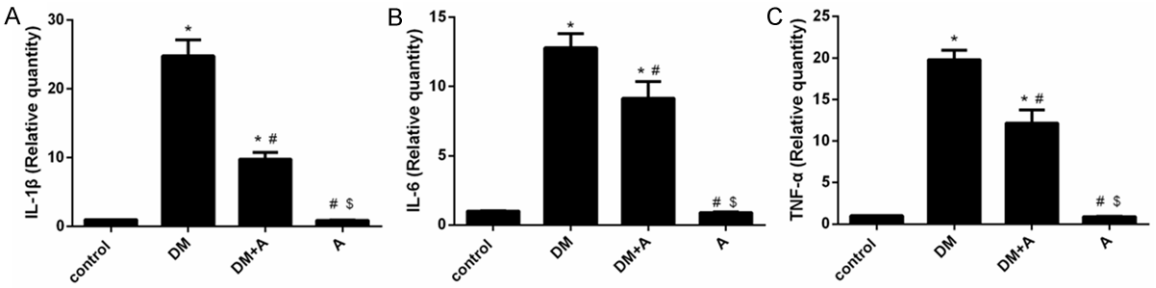

Figure 5. The effect of acteoside on renal inflammatory factors of diabetic mice *P<0.05 compared with control group; #P<0.05 compared with DM group; $P<0.05 compared with DM + A group.

In early stage, DN is characterized by the high glomerular filtration rate while normal content of urinary albumin. However, with the development of the disease, a small amount of proteinuria and even massive proteinuria can be detected [18]. In this trial, the content of urinary albumin was normal at the beginning of modeling, and it was obviously increased after 3 weeks of modeling. The tissue injuries became increasingly severe and the content of urinary albumin was increased gradually with the proceeding of establishment of the diabetic mice models. However, the application of acteoside significantly reduced the excretion of urinary albumin, the ratio of KW/BW and the level of Scr and BUN, indicating that acteoside played a protective effect on diabetic renal injuries and delayed the progression of DN. But compared to the DM group, the blood glucose in DM + A group was not decreased markedly, indicating that acteoside had no direct effects on decreasing blood glucose, and its protective effects on the kidney of diabetic mice was not achieved by decreasing blood glucose.

Currently, it is believed that oxidative stress and inflammatory reaction of kidney play an important role in the occurrence and develop-

ment of DN [5]. The increase of oxidative stress can promote the generation of inflammatory factors. In return, the release of inflammatory factors can facilitate the emergence of free radicals, and then promote the occurrence of oxidative stress. Both of them are promoted mutually and then jointly facilitate the development of DN. Nowadays, most of the scholars think that the oxidative stress is the initial factor of diabetic renal injuries [19]. A large amount of glucose anto-oxidation and overloaded mitochondria in diabetic patients can result in the increase of reactive oxygen species (ROS) [20]; meanwhile, the decrease of body's antioxidant capacity can lead to a large accumulation of ROS. Consequently, the excessive ROS can induce the production of damaged media through a variety of signaling molecules, such as NF-κB, JNK/SARK, ERK and so on, and then aggravate the renal injury. In DM group, the content of MDA, which represented the degree of lipid peroxidation, was significantly increased both in the detection of serum and renal tissues, while the degree of lipid peroxidation was markedly lessened in DM + A group. What's more, the content of antioxidant factors (Y-GCS and SOD) in DM + A group was significantly increased compared to the DM group, which indicated that acteoside could mitigate the injuries of cells caused by oxidative stress by alleviating the lipid peroxidation and improving the level of antioxidant factors. In this way, progression of diabetic nephropathy could be delayed.

Many inflammatory factors are involved in the progression of DN, they will eventually cause renal injuries and dysfunctions through complex inflammatory signaling pathways. TNF-α plays a critical role in the development of diabetes, and can be produced by many types of cells, such as vascular endothelial cells, glomerular mesangial cells and infiltrating mononuclear macrophages. It also can stimulate the release of various vasoactive substances, leading to the long-term imbalance of vasomotoricity and eventually causing renal injuries [21]. Studies have shown that IL-1 can enhance the permeability of vascular endothelial cells, stimulate the proliferation of mesangial cells and the production of extracellular matrix, thus leading to renal injuries [22]. IL-6 can promote the proliferation of glomerular mesangial cells, increase the production of fibronectin, and boost the expression of adhesion molecules in endothelial cells [23]. In this trial, the application of acteoside significantly reduced the expression of renal inflammatory factors in diabetic mice, suggesting that it might protect the diabetic mice by reducing the renal inflammatory reactions, which could further mitigate the renal injuries.

In conclusion, this study shows that acteoside has no direct effects on reducing blood glucose, but it can mitigate the level of oxidative stress, alleviate the renal inflammatory reactions and delay the progression of diabetic renal injuries at the same time in mice with DN. The result indicates that acteoside has an important value in clinical researches and applications. And further investigation is necessary to delineate the exact effects of acteoside on the mice, though its obvious toxic and side effects are not found in this study.

Disclosure of conflict of interest

None.

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