Renoprotective effect of atorvastatin on STZ-diabetic rats through inhibiting inflammatory factors expression in diabetic rat

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Abstract. – OBJECTIVE: Though increasing evidences showed that statins had potential benefits to diabetic kidney disease (DKD), its mechanism has not been completely elucidated yet. The aim of this study was to investigate the renoprotective effects of atorvastatin on DKD.

MATERIALS AND METHODS: Kidney injury was induced by streptozotocin (STZ) in rats. STZ-diabetic rats were treated with atorvastatin (10 mg/kg/d) for consecutive 8 weeks. Renal functional and morphological changes were evaluated by clinical biochemistry and histological examination. The expression of inflammatory factors in kidney was measured by real-time (RT)-PCR and enzyme-linked immunosorbent assay (ELISA).

RESULTS: Compared with DKD rat, atorvastatin effectively reduced the levels of low-density lipoprotein cholesterol (LDL-C), creatinine (CREA), ratio of urine albumin to creatinine (UACR) (p <0.05). The expression of inflammatory factors TNF-α, MCP-1 and IL-6 in kidney tissue were significantly down-regulated, as well (p <0.05). Atorvastatin improved kidney injury with the reduced morphologic lesions and renal fibrosis and the increasing transforming growth factor-β (TGF-β) and collagen I staining.

CONCLUSIONS: Our results suggested that atorvastatin could ameliorate DKD through inhibiting pro-inflammatory pathways. Atorvastatin may possess a potential anti-inflammatory effect and serve as the therapeutic drug for DKD management.

Key Words: Diabetic kidney disease (DKD), Atorvastatin, Inflammatory response.

Introduction

Epidemiologic studies have shown that diabetes mellitus (DM) is still increasing worldwide and closely correlates with various complications, such as neuropathy, retinopathy, and nephropathy. As one of the major microvascular complications of DM, DKD has been considered as the primary cause of end-stage renal disease and renal failure, which led to high morbidity and mortality and accounted for approximately 20-30% of diabetic patients. To date, the pathogenesis of DKD has not been completely known. Increasing evidences indicate that inflammatory mechanism may play a crucial role in the onset and development of DKD, except that traditional factors, such as, hemodynamic changes, protein kinase C, oxidative stress, polyl pathway and non-enzymatic glycation have been involved in DKD. Previous reports indicated that chronic low-grade inflammation was highly involved in the pathogenesis of DKD. A recent study demonstrated that inflammatory cytokines (for example, TNF-α) may act as a potential therapeutic target for DKD.

Statins are a class of clinical lipids-lowering agents, which could reduce cholesterol biosynthesis through inhibition of 3-hydroxy-3-methyl-glutaryl (HMG)-CoA reductase. In addition, statins had been proved to effectively reduce the risk of cardiovascular events (such as, myocardial infarction and stroke) and exhibit multiple actions. Pitavastatin reduced reactive oxygen species production in high glucose-treated endothelial cells and streptozotocin (STZ)-induced diabetic rats by inhibition of NADPH/NADH oxidase activation. Atorvastatin application ameliorated the joint functional disability in Freund’s Complete Adjuvant (FCA)-induced arthritis rats by inhibition of inflammation and hyperalgesia. A clinical study also illustrated that statin therapy may reduce proteinuria and rates of kidney function loss.
in patients with cardiovascular disease. However, the underlying mechanisms remain unclear.

In the present study, we explored the renoprotective effect of atorvastatin on STZ-induced diabetic rats. The expression levels of inflammatory factors in the kidney were analyzed.

**Materials and Methods**

**Animal and Treatment**

Adult male Sprague-Dawley (SD) rats weighing 200 to 250 g were purchased from the Chengdu Dossy Experimental Animals Co., Ltd (Chengdu, China). Animals were housed in a standard laboratory with the conditions, at room temperature 25 ± 2°C, 12 h light/dark cycle, humidity 60 ± 5% and had free access to water and food. The experimental protocols were approved by the Animal Ethics Committee of Mian Yang Central Hospital.

The rats were fasted for 12h and treated with STZ (60 mg/kg, body weight, Sigma, Wyoming, USA) dissolved in citrate buffer (0.1 M, pH=4.4) by intraperitoneal injection. After 72 h of STZ injection, diabetic rats were identified via assessment of fasting blood glucose level >16.7 mmol/l. Finally, 24 rats were selected and evenly divided into DM + vehicle group and DM + atorvastatin group. Rats in DM + vehicle and DM + atorvastatin group were given orally equal amounts of PBS and atorvastatin (10 mg/kg/day, body weight, Jialin Pharmaceutical Co. Ltd, Beijing, China) for 8 weeks, respectively. Meanwhile, the normal rats (n=12) were selected as control group.

**Sampling and Biochemistry Parameters Measurement**

During the experiment, fasting blood glucose level was measured by a glucometer (Accu-Chek, Roche, Basel, Switzerland). After 8 weeks treatment described above, rats were kept individually in metabolic cages for 24 h to collect urinary samples before sacrificed. Blood samples were collected via the retro-orbital venous plexus and centrifuged at 4°C at 12,000 × g for 10 min. Then serum was stored in -80°C for further use. The renal tissue samples were taken from anesthetized rats for further analysis. Biochemistry parameters, including serum glucose, creatinine (CREA), blood urea nitrogen (BUN), low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C), urine albumin, creatinine were measured by automatic biochemistry analyzer (Model Cobas® c311, Roche, Basel, Switzerland). The ratio of urine albumin to creatinine (UACR) was calculated.

**RT-PCR Analysis**

Total RNA was isolated from renal tissues by using Trizol reagent (Invitrogen, Carlsbad, CA, USA). First-strand cDNA was synthesized by using a Superscript-II kit (Invitrogen, Carlsbad, CA, USA). RT-PCR was performed on the IQ™5 RT-PCR Detection System (Bio-Rad, Hercules, CA, USA) with SYBR green supermix (Bio-Rad, Hercules, CA, USA). Primers used in the experiment are as follows: IL-6F: 5'-TATGTTGCTATGCTTAAG-3’, IL-6R: 5'-ATTGGCGATTTCTCTGTA-3’; TNF-αF: 5'-AATCTGTTGCTTCTAACT TA-3’, TNF-αR: 5’-TTCTGAGCATCTGATGGT-3’; MCP-1F: 5'-GGTTCCAGAGAGTAGATTA-3’, MCP-1R: 5'-GGTCAGGTCCATTCAAA3’; β-actinF: 5'-CTAGCACCATGAAGATCAAGAT-3’, β-actinR: 5'-CCAGGATAGCAAGCTCAATGAT-3’. β-actin gene was used as internal control. The relative expression of mRNA levels was calculated using the 2 ^ (−ΔΔCt) analysis method.

**ELISA Assay**

Renal tissue samples were homogenized in PBS buffer on the ice and centrifuged at 4°C at 12,000 × g for 5 min. Then the supernate was collected. The concentrations of inflammatory factors, including IL-6, TNF-α, MCP-1 in renal tissues were measured by using commercial ELISA kit (R&D, Santa Monica, CA, USA) according to the manufacturer’s instruction.

**Histological Examination**

Renal tissue were fixed in 10% formalin and made into 5 μm paraffin sections. Partial of sections were stained with HE staining and PAS staining. The other sections were stained by IHC staining, as follows: Renal sections were deparaffinized, antigen retrieval with citrate buffer, inactivation of endogenous peroxidase with 10% H2O2, blocked with 1% BSA, and then incubated with diluted primary rabbit anti-TGF-β1 antibody (Santa Cruz, CA, USA) and mouse anti-collagen I antibody (Abcam, MA, USA) overnight at 4°C. After washed with PBS, the sections were incubated with secondary antibody against rabbit or mouse IgG labeled with horseradish peroxidase (HRP) (Santa Cruz, CA, USA) and then visualized with diaminobenzidine (DAB).

**Statistical Analysis**

Statistical analysis was performed using SPSS (version 11.5, SPSS Inc., Chicago, IL, USA). All
data were expressed as means ± SD. Comparisons among groups were conducted using one-way analysis of variance (ANOVA) followed by LSD test. A value of p < 0.05 or p < 0.01 was considered as statistically significant.

Results

Effect of atorvastatin on biochemistry Variables

As shown in Table I, the diabetic rats had significantly higher glucose, LDL-C, BUN, CREA and UACR, and HDL-C than the normal rats (p <0.05). The diabetic rats showed clear hyperlipidemia and impaired renal function. In contrast, atorvastatin application significantly reduced LDL-C, CREA and UACR levels, and increased HDL-C level in the rats of DM + atorvastatin group than DM + vehicle group (p <0.05). Whereas, glucose and BUN levels were not significantly affect in the diabetic rats (p >0.05). The results indicated that atorvastatin had a protective effect on renal function in the diabetic rats.

Measurement of Inflammatory Factors Expression in Renal Tissue

To further figure out the effect of atorvastatin on renal function, the mRNA and protein levels of inflammatory factors in renal tissue were analyzed by RT-PCR and ELISA, respectively (Figure 1).

Table I. General and biochemical parameters in experimental groups.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control</th>
<th>DM + vehicle</th>
<th>DM + atorvastatin</th>
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<tbody>
<tr>
<td>Glucose (mmol/L)</td>
<td>4.2 ± 0.5</td>
<td>26.8± 3.2a</td>
<td>27.1±4.8b</td>
</tr>
<tr>
<td>LDL-C (mmol/L)</td>
<td>0.5± 0.1</td>
<td>2.2±0.4a</td>
<td>1.5±0.2 b</td>
</tr>
<tr>
<td>HDL-C (mmol/L)</td>
<td>1.3±0.2</td>
<td>0.4±0.2a</td>
<td>0.8±0.2 b</td>
</tr>
<tr>
<td>BUN (mmol/L)</td>
<td>6.4±1.1</td>
<td>16.3±3.5a</td>
<td>13.2±2.7a</td>
</tr>
<tr>
<td>CREA (mmol/L)</td>
<td>22.6±3.3</td>
<td>55.8±5.4a</td>
<td>41.7±4.1a</td>
</tr>
<tr>
<td>UACR (mg/mmol)</td>
<td>0.5±0.1</td>
<td>8.4±1.2a</td>
<td>5.6±1.7a</td>
</tr>
</tbody>
</table>

Note: Low density lipoprotein cholesterol (LDL-C), High density lipoprotein cholesterol (HDL-C), Blood urea nitrogen (BUN), Creatinine (CREA), Ratio of urine albumin to creatinine (UACR). The superscript letters indicated significantly different. "a", p <0.05 (DM + vehicle group vs control group); "b", p <0.05 (DM + atorvastatin group vs control group); "c", p <0.05 (DM + atorvastatin group vs DM + vehicle group).
Compared with the normal rats in the control group, the diabetic rats showed increasing mRNA levels of TNF-α, MCP-1 and IL-6 in other two groups (p < 0.05). Meanwhile, atorvastatin treatment significantly decreased the mRNA levels of these inflammatory factors in the rats of DM + atorvastatin group than in DM + vehicle group (p < 0.05). ELISA results showed that the expression of corresponding proteins had the similar patterns with mRNA expression. The diabetic rats had higher protein levels of TNF-α, MCP-1 and IL-6 than the normal rats. The MCP-1 and IL-6 protein levels in DM + vehicle group were significantly higher than in control group (p <0.01). Moreover, compared with DM + vehicle group, the protein levels of TNF-α, MCP-1 and IL-6 were significantly decreased in DM + atorvastatin group (p <0.05). The results indicated that atorvastatin ameliorated renal injury in the diabetic rat may through inhibiting the expression of inflammatory factors.

**Histological Analysis**

HE and PAS staining results revealed that the diabetic rats had a clear renal injury with glomer-
ular hypertrophy, glomerulosclerosis, swelling and vacuolation of proximal tubular cells, compared with the normal rats in the control group. In contrast, atorvastatin ameliorated these lesions in the rats in DM + atorvastatin group. The degree of renal injury was also assessed by IHC staining. Moreover, the diabetic rats showed the higher intensity of TGF-β and collagen I staining in both renal tubules and glomeruli than the rats in the control group. Whereas atorvastatin treatment reduced the intensity of TGF-β and collagen I staining in the diabetic rats (Figure 2). The result indicated that atorvastatin was able to reduce renal fibrosis in diabetic rats.

**Discussion**

As we described previously, DN is the primary cause of end-stage renal failure in the world and leads to high morbidity and mortality in the patients with DM. Although increasing evidences indicated the benefits of statins to chronic kidney disease, its effects on DKD remain uncertain. In this study, we evaluated the potential renoprotective effects of atorvastatin in STZ-induced diabetic rats.

Due to its presentation of similar characteristics to human patients, the STZ-induced diabetic animal model is widely used in the study of the pathogenesis and therapeutic drugs development related to DKD. In the present study, the diabetic rats showed obvious renal histological abnormalities and increasing CREA, BUN and UCAR levels, which reflected seriously impaired renal function. Statins are the most commonly used lipid-lowering drugs, which have been proved to be effective in regulating lipid metabolism and decreasing the risks of cardiovascular diseases. Meanwhile, recent studies showed that statins can also help to slow the rate of kidney function decline, and reduce the morbidity and mortality of chronic kidney disease. Our findings showed that atorvastatin treatment effectively reduced renal histological injury and improved renal function in the diabetic rats, which was consistent with the previous study. Moreover, renal fibrosis was characterized by an excessive accumulation of extracellular matrix (ECM) such as collagen I and fibronectin, which ultimately resulted in DKD. TGF-β1 has been considered as a major mediator of renal fibrosis, which involved in multiple fibrotic signaling pathways. In this study, we observed higher intensity of TGF-β and collagen I staining in the diabetic rat kidney tissues, and atorvastatin application remarkably reduced their levels. Our results indicated the role of atorvastatin on inhibiting the ECM deposition and fibrosis.

Although the effects of statins in preventing cardiovascular diseases may derive from the lipid-lowering activity, the mechanism of renoprotective effects on DKD is less clear. Inflammatory factors have been proved to play an important role in the onset and progression of DKD. Cytokines (such as, TNF-α) can induce renal cells apoptosis and necrotic death, and even disturb cell-cell junction, leading to endothelial dysfunction. Albuminuria positively correlated with IL-6 in diabetic patients and animals, and this may be related to mesangial cells proliferation and ECM molecules expression. In addition, statins have been reported to ameliorate albuminuria and renal mesangial expansion in db/db mice and reduce oxidative damages in STZ-induced diabetic rats. Some study showed also that statins could ameliorate such inflammatory diseases as experimental arthritis, which manifested the potential for treatment of DKD. However, the effects of statins on inflammatory factors in kidney are less addressed. In the present study, our findings demonstrated the anti-inflammatory effects of atorvastatin on the kidney of diabetic rats, with the expression levels of inflammatory factors TNF-α, MCP-1 and IL-6 significantly decreased. Undoubtedly, statins such as atorvastatin are promising to serve as a potential therapeutic drug for DKD and more DKD patients will benefit from it.

**Conclusions**

This work demonstrated the renoprotective effects of atorvastatin on diabetic rats, which may be mediated by inhibition of inflammatory factors expression. Atorvastatin could ameliorate the renal morphologic lesions and reduce renal fibrosis with concomitant decrease of albuminuria, LDL-C, CREA and UACE and the decreasing expression of inflammatory factors, TNF-α, MCP-1 and IL-6.

**Conflicts of interest**

The authors declare no conflicts of interest.
References


