Original Article

Catalpol ameliorates diabetic atherosclerosis in diabetic rabbits

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Abstract: Catalpol, isolated from the roots of Rehmanniaglutinosa, Chinese foxglove, is an iridoid glycoside with antioxidant, anti-inflammatory and anti-hyperglycemic agent. The present study was to investigate the effects of catalpol on diabetic atherosclerosis in alloxan-induced diabetic rabbits. Diabetes was induced in rabbits by a hyperlipidemic diet and intravenous injection of alloxan (100 mg/kg). Rabbits were treated for 12 weeks. The fasting blood glucose, insulin, homeostasis model of insulin resistance, total cholesterol and triglyceride were measured. The thoracic aorta was excised for histology. The plasma and vascular changes including some markers of oxidative stress, inflammatory cytokines and fibrosis factors were examined. Plasma levels of fasting blood glucose, insulin and homeostasis model of insulin resistance were significantly decreased in catalpol group. Catalpol treatment ameliorated diabetic atherosclerosis in diabetic rabbits as demonstrated by significantly inhibited neointimal hyperplasia and macrophages recruitment. Catalpol treatment also enhanced the activities of superoxide dismutase, glutathione peroxidase, and increased the plasma levels of total antioxidant status, meanwhile reduced the levels of malondialdehyde, protein carbonyl groups and advanced glycation end product. Furthermore, catalpol also reduced circulating levels of tumor necrosis factor-α, monocyte chemotactic protein-1 and vascular cell adhesion molecule-1. Catalpol also decreased transforming growth factor-β1 and collagen IV mRNA and protein expressions in the vessels. Catalpol exerts an ameliorative effect on atherosclerotic lesion in alloxan-induced diabetic rabbits. The possible mechanisms may be related to inhibition of oxidative stress inflammatory response and anti-fibrosis and reduced aggregation of extracellular matrix.

Keywords: Catalpol, diabetic atherosclerosis, oxidative stress, inflammatory response

Introduction

Diabetes mellitus (DM) is the most common metabolic disease characterized by chronic hyperglycemia and is associated with long-term complications. The most common and life-threatening complication is cardiovascular diseases (CVD) [1, 2]. Atherosclerosis is accelerated in diabetes, leads to the development of CVD. Although the precise mechanisms of the accelerated atherosclerosis are still unclear, many factors attributed to the development of atherosclerosis in diabetes have been reported, such as hyperglycemia, insulin resistance, dyslipidemia, oxidative stress, inflammation and increased generation of advanced glycation end products (AGEs) [3-5].

Oxidative stress and inflammation play fundamental roles in the pathogenesis of atherosclerosis in DM [6, 7]. It is possible that oxidative stress induced by hyperglycemia and hyperlipidemia, along with products of glycation and lipid peroxidation, plays critical roles in inducing inflammatory cytokines expression [8]. Alongside the increased reactive oxygen species (ROS) generated in diabetes [9], ROS productions are markedly increased along with an obvious decline in the antioxidant defense systems [10, 11]. In conjunction with oxidative stress, the levels of the inflammatory cytokines including TNF-α, MCP-1 and VCAM-1 are elevated in diabetic individuals [7, 12, 13]. Esposito K et al. reported that the release of TNF-α induced by high glucose in vitro experiments may be
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mediated by ROS and oxidative stress be implicated in promoting systemic inflammation in diabetic patients [14]. Therefore, antioxidant and anti-inflammatory therapy may prove to be effective therapeutic tools in preventing diabetic atherosclerosis.

Catalpol, the most abundant bioactive component in the roots of *Rehmanniaglutinosa*, is an iridoid glucoside. It has been reported that catalpol can ameliorate hyperglycemia [15, 16], neurotoxicity [17, 18], and diabetic encephalopathy [19]. In addition, catalpol has anti-oxidative and anti-inflammatory effects [20]. However, there is no report about the effects of catalpol on diabetic atherosclerosis in alloxan-induced diabetic rabbits.

Based on the above background, we aimed to evaluate the protective effects of catalpol in diabetic atherosclerosis in alloxan-induced diabetic rabbits. And also to investigate the possible mechanisms involved in ameliorating oxidative stress and inflammation.

Materials and methods

Drug preparation

Catalpol (purity >98%, molecular formula: $C_{15}H_{22}O_{10}$, molecular weight: 362.33) was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Catalpol was dissolved in normal saline solution as a vehicle for its administration as previously described [21]. Catalpol was administered 5 mg/kg once a day. The dosage of catalpol used in this study was chosen as indicated by our prior preliminary experiments [20].

Animals and treatment protocols

The Guidelines for the Care and Use of Laboratory Animals published by the Chinese National Institutes of Health were followed while using live animals. The protocol was approved by the Animal Ethics Committee of Weifang Medical University (WYLY 2013-006, Jun 12, 2013). Twenty-eight male 4-month-old New Zealand White rabbits (2.5-2.8 kg), obtained from Experimental Animal Center of Weifang Medical University, were housed in individual cages under controlled temperature and a 12 h light/dark cycles. All rabbits were provided with standard diet and water ad libitum.

After a week of acclimation to the experimental area, the diabetic rabbit model was developed using a hyperlipidemic diet plus alloxan similar to that employed in previous studies [22]. The hyperlipidemic diet consisted of 1% cholesterol and 10% lard. The NC group was given a standard diet. After 4 weeks of dietary intervention, the diabetic rabbit models were injected intravenously with alloxan monohydrate (dissolved in saline solution, 100 mg/kg, Sigma, USA) to induce diabetes. The rabbits with fasting blood glucose levels above 11.1 mmol/l were considered diabetic and were fed with the hyperlipidemic diet continually. All the rabbits were randomly divided into 4 groups with seven in each group: the NC group was treated with saline in a matched volume, the DM group had diabetic rabbits treated with saline in a matched volume, the metformin group had diabetic rabbits administered with metformin (Shi Guibao Co. Ltd, Shanghai, China) 50 mg/kg/d, and the catalpol group had diabetic rabbits administered with catalpol 5 mg/kg/d. All medicines were administered by oral gavage for 12 weeks. Body weight, fasting blood glucose, insulin and HOMA-IR were measured every 4 weeks. At the end of 12 weeks, fasting blood samples were obtained from the marginal ear vein for plasma separation. Plasma samples obtained from centrifugation at 4000×g for 10 min and were stored at -70°C until laboratory analysis. To obtain thoracic aortas, all rabbits were sacrificed humanely with an overdose of pentobarbital intravenously. Thoracic aortas were rapidly excised and fixed in 10% formaldehyde solution for subsequent histological analysis.

Determination of glucose, insulin and lipid levels

The plasma levels of glucose and insulin were determined respectively by glucose oxidase and radioimmunoassay method using commercial diagnostic kits (Nanjing Jiancheng Biotechnology Institute, China). Plasma total cholesterol (TC) and triglyceride (TG) were detected by an automatic blood chemical analyzer (Olympus, AU 600, Japan).

Determination of homeostasis model of insulin resistance

Insulin resistance was evaluated by homeostasis model assessment estimate of insulin resistance (HOMA-IR) [23] as follows: $HOMA-IR = \frac{\text{fasting insulin} (\mu U / ml) \times \text{fasting glucose} (mmol/l)}{405}$. 

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Fasting insulin level (μU/ml)× Fasting blood glucose (mmol)/22.5.

**Oxidative stress status analysis in plasma**

The plasma concentrations of MDA, PCG and AGEs as well as the activities of SOD, GSH-Px and TAS were all determined by using commercially available kits (Nanjing Jiancheng Biotechnology Institute, China). Concentrations of plasma MDA, PCG and AGEs in the present study were used to indicate the oxidation state. MDA content was measured as thiobarbituric acid reactive substances (TBARS) according to the manufacturer’s instructions [24]. PCG concentration was assayed by the modified method of Levine et al. reported [25] according to the manufacturer’s instructions. AGEs concentration was measured by specific ELISA kit according to the manufacturer’s instructions. Plasma SOD, GSH-PX activities and TAS were used to evaluate the antioxidant status in rabbits. SOD activity was assayed using the hydroxylamine oxidation assay method of Kakkar et al. reported [26]. One unit (U) of SOD activity was defined as the amount of enzyme necessary to reduce hydroxylamine by 50%. GHG-PX activity was evaluated by the 5-5’-dithiobis (2-nitrobenzoic acid) (DTNB) method [27]. One U of GSH-PX activity was defined as 1 μmol/l GSH consumption per minute at pH 7.0 and 37°C. TAS was assessed using colorimetry according to the manufacturer’s instructions [28].

**Inflammatory cytokines in plasma and transforming growth factor-β1 in vascular**

Plasma concentrations of TNF-α, MCP-1 and VCAM-1 and transforming growth factor-β1 (TGF-β1) in vascular were measured by specific ELISA kits (Boster Biotechnology Co., Ltd, Wuhan, China) according to the manufacturer’s instructions. Optical density was read at 450 nm using THERMO microplate reader. Standard curve was generated by correlating the known concentration and the corresponding optical densities.

**Histological and immunohistochemical staining**

To determine the effect of catalpol on diabetic atherosclerosis, hematoxylin-eosin (HE) and immunohistochemical staining were performed on 5 mm-thin paraffin sections from thoracic aortas. Intima-media thickness was evaluated by HE staining according to the method of Zhang et al. [29]. The expression of macrophages and vascular smooth muscle cells (VSMCs) was evaluated by immunohistochemical method. After routine deparaffinization and rehydration, 5 mm-thin paraffin sections were incubated with 3% hydrogen peroxide for 10 minutes, and then with 5% BSA serum for 20 minutes. Subsequently, they were immunostained with macrophages, monoclonal antibody (mAb) RAM-11 (DAKO, dilution 1:200) and smooth muscle cells, mAb HHF-35 (DAKO, dilution 1:200) overnight at 4°C, and then with horse-radish peroxidase complex system (1:200, Santa Cruz, USA) as a secondary antibody for 30 minutes at 37°C. Visualisation of a positive reaction was developed with the use of a DAB Peroxidase Substrate Kit (Vector Laboratories) to display the reaction product with a brown color and the sections were then counterstained with hematoxylin. All the photographs were analyzed using Image-Pro Plus6.0 (Media Cybernetics, USA). Incubation with PBS instead of the primary antibody was used as a negative control.

**Quantification of mRNA levels**

Vascular tissues were lysed in TRIZOL reagent and total RNA was isolated. The protocol of synthesis of cDNA is previously described [30]. For quantification of TGF-β1 and Collagen IV transcripts, conventional real-time RT-PCR was carried out with total RNA samples extracted from the blood vessel.

**Western blot analyses**

The protein samples were extracted from aorta, following essentially the same procedure as described in detail elsewhere [30]. Protein samples (50 μg) were fractionated by SDS-PAGE (7.5-10% polyacrylamide gels). The primary antibodies against Collagen IV (Abcam), with β-actin (Abcam) used as an internal control.

**Statistical analysis**

All the data were analysed using SPSS (version 18.0) for Windows. Data was expressed as mean ± SE. Statistical analysis was performed using one-way analysis of variance (ANOVA).
Bartlett’s box-test was used to test the homogeneity of variance. Individual differences among groups were analyzed by Dunnett-t test. A significance level of $P < 0.05$ was required for all tests.

**Results**

**Effects of catalpol on body weight, blood glucose, insulin, HOMA-IR and lipid**

All the rabbits completed the experimental process. There were no significant differences in mean body weight at baseline. With the progress of the experiment, the plasma levels of blood glucose, HOMA-IR, TC and TG were significantly increased, but the plasma insulin and body weight were significantly reduced in diabetic rabbits compared with normal rabbits. Catalpol and metformin treatment could both significantly improved plasma insulin level and body weight, and decreased the blood glucose and HOMA-IR in diabetic rabbits. Catalpol and metformin could both reduced plasma levels of TG and TC compared to the diabetic rabbits, however the result for catalpol was not statistically significant (Figure 1). These findings suggest that catalpol improved hyperglycemia and hypoinsulinemia, but had no effect on plasma lipids profiles in diabetic rabbits.

**Effects of catalpol on oxidative stress**

MDA, PCG and AGEs are widely recognized as presumptive biomarkers of enhanced oxidative
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stress. The generations of MDA, PCG and AGEs were increased significantly in the DM group compared with the NC group (P < 0.05) (Figure 2). Catalpol treatment markedly decreased the plasma concentration of MDA, PCG and AGEs compared with the DM and metformin group (P < 0.05) (Figure 2). The activities of SOD, GSH-Px and plasma level of TAS were used to evaluate the antioxidant status in rabbits. The reduction of SOD, GSH-Px activity and TAS level in the DM group were remarkably preserved after catalpol and metformin treatment. There were significant differences between catalpol and metformin group (Figure 2). These data suggest that catalpol attenuates oxidative stress in alloxan-induced diabetic rabbits by inhibiting oxidation and promoting the antioxidant capacity.

Effects of catalpol on inflammatory cytokines

Inflammation plays a pivotal role in atherosclerosis. Therefore, we assessed inflammatory cytokines expression in plasma. Plasma levels of TNF-α, MCP-1 and VCAM-1 were significantly increased in the DM group compared with the
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NC group as the experiment time progressed (P < 0.05). In contrast, catalpol treatment markedly reduced the levels of TNF-α, MCP-1 and VCAM-1 compared with DM group and metformin group (P < 0.05). There were significant differences between the DM and metformin groups (Figure 3). These data suggested that catalpol might suppress inflammation in alloxan-induced diabetic rabbits through reducing the over-expression of TNF-α, MCP-1 and VCAM-1 in circulation.

Effects of catalpol on atherosclerotic lesions

The lipid content of the plaques was determined by HE staining of the thoracic aorta sections. Intima-media thickness was significantly increased in the DM group compared with the NC group (P < 0.05). However, catalpol treatment markedly inhibited neointimal hyperplasia compared with the DM and metformin group (P < 0.05). There was significant difference between the DM and metformin groups (Figure 4). Plaque content of the VSMCs and macrophage infiltration was determined by immunohistochemical staining of the thoracic aorta sections, as shown in Figure 4. Our results showed that the content of VSMCs in plaque was higher in the DM group than that of the NC group, whereas it was lower in catalpol and metformin groups. Meanwhile, rabbits in the...
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DM group showed extensive macrophage infiltration into the wall of aorta. However, the macrophage content was lower in catalpol and metformin groups. Significant difference between the catalpol and metformin groups was observed. These findings suggest that catalpol can attenuate atherosclerotic lesions and delay atherosclerosis progression in alloxan-induced diabetic rabbits.

Effects of catalpol on TGF-β1 and collagen IV

Fibrosis and aggregation of extracellular matrix can also be seen in atherosclerosis. Therefore, we assessed TGF-β1 and collagen IV expression in vessel with and without atherosclerotic plaques. Vascular expressions of TGF-β1 and collagen IV mRNA and protein were significantly increased in the DM group compared with the NC group (P < 0.05, Figure 5). In contrast, catalpol treatment significantly decreased the expressions of TGF-β1 and collagen IV mRNA and protein compared with DM group and metformin group (Figure 5). These data suggested that catalpol might suppress fibrosis and aggregation of extracellular matrix through reducing the over-expression of TGF-β1 and collagen IV in vessel.

Discussion

The present study, observed for the first time, that catalpol attenuates atherosclerotic lesion and delays progression of atherosclerosis progression in alloxan-induced diabetic rabbits. The protective effects of catalpol were associated with the reduction of blood glucose and inhibition of oxidative stress and inflammation, limiting the progression of diabetic atherosclerosis.

The loss of body weight is one of the threats associated with DM. We observed that alloxan-induced diabetic rabbits also tend to lose weight. Catalpol and metformin treatments reversed weight loss in the test rabbits. Hyperglycemia and insulin resistance generates adverse metabolic events in endothelial cells.

Figure 5. Effects of catalpol on TGF-β1 and collagen IV. A: Effects of catalpol on vascular TGF-β1 mRNA. B: Effects of catalpol on vascular TGF-β1 protein. C: Effects of catalpol on vascular collagen IV mRNA. D: Effects of catalpol on vascular collagen IV protein. *P < 0.05 and **P < 0.01 vs NC; #P < 0.05 and ##P < 0.01 vs DM; &P < 0.05 vs catalpol.
leading to endothelial dysfunction, oxidative stress augmented, inflammation, and thrombosis [31, 32]. Elevated TG and TC levels also increase the risk of cardiovascular diseases in diabetes [33].

In the present study, rabbits in the DM group exhibit hyperglycemia, insulin resistance and hyperlipidemia, the hallmarks of diabetic atherosclerosis, setting stage for a more sensitive evaluation on the effects of catalpol on diabetic atherosclerosis. Our study showed that oral supplementation of catalpol significantly decreased the blood glucose level and ameliorated insulin resistance, as well as, reduced intima-media thickness. These results suggested that catalpol has an excellent anti-atherosclerotic effect in diabetic rabbits. Hyperplasia of VSMCs and the infiltration of macrophages in the arteries were early events in the development of atherosclerosis [34]. We also noted a reduction in the expression of α-SM actin and RAM-11. These observations could explain the reduction of plaque extension and retardation of atherosclerosis in the aorta of diabetic rabbits treated with catalpol.

Diabetes, insulin resistance and hyperlipidemia accelerate atherosclerosis by inducing vascular and endothelial dysfunction, enhancing oxidative stress and increasing the inflammatory response [6, 35]. Elevated serum glucose level stimulates the formation and accumulation of AGEs, which induce a continuous oxidative stress as well as an increased synthesis of ROS [36]. MDA, as a product of lipid peroxidation, is considered to be a strong predictor of CVD and found to be increased in diabetic rats [37]. Among various oxidative modifications of proteins, formation of PCG perhaps is an early marker of protein oxidation and was reported to be increased in diabetic rabbits [38].

Antioxidants such as SOD, GSH-Px and TAS were reported to be reduced in diabetic rabbits [39, 40]. Consistent with previous reports, the activities of SOD, GSH-Px and TAS significantly reduced coupling with a marked increase in the level of MDA, AGEs and PCG in alloxan-induced diabetic rabbits. Treatment with catalpol markedly inhibited the concentration of MDA, AGEs, PCG, and promoted the activity of SOD, GSH-Px and TAS in the serum of diabetic rabbits with atherosclerosis. The effects of catalpol on oxidative stress were obvious, consistent with previous studies [19, 41-43]. These effects demonstrated that catalpol confers antioxidant effects via inhibiting the oxidation and enhancing the antioxidant capacity, which is an essential to promote the anti-atherosclerosis. It’s interesting to note that, the antioxidant effects of catalpol were better than metformin.

Atherosclerosis is an inflammatory process, initiated by the adhesion of monocytes to arterial endothelial cells [34], followed by migration into the sub-endothelial space triggered by chemotactic activation processes [44]. Subsequently, monocytes differentiate into intimal macrophages, taking up lipids and become foam cells, leading to the formation of fatty streak lesions, fibrosis and aggregation of extracellular matrix in vascular walls [45]. Type 2 DM is increasingly being recognized as resulting from chronic low grade inflammation [46]. Numerous researchers reported that the circulating TNF-α, MCP-1, VCAM-1 are usually elevated in established type 2 DM [30, 47]. Inflammatory cytokines are associated with enhanced macrophage adhesion to vascular endothelium, resulting in neointimal hyperplasia and endothelial dysfunction. Levels of TNF-α, MCP-1, VCAM-1, TGF-β1 and collagen IV were significantly increased in alloxan-induced diabetic rabbits in the present study, consistent with the above reports. Treatment with catalpol drastically decreased the circulating levels of these inflammatory cytokines and vascular pro-fibrotic factors and reduced aggregation of extracellular matrix compared with DM group and metformin group. The anti-inflammatory, anti-fibrosis and reducing extracellular matrix aggregation effects of catalpol were better if not similar to metformin.

In conclusion, our findings demonstrate that catalpol has protective effects in alloxan-induced diabetic rabbits, attenuated oxidative stress, suppressed inflammatory responses, has anti-fibrosis effects and reduced aggregation of extracellular matrix in vascular walls. We for the first time report catalpol as an alternative anti-hyperglycemic and anti-atherosclerotic drug in DM with promising effects. Despite the limitations in our study, the extant results provided supporting evidence and important novel pharmacological effect of catalpol, which may have potential therapeutic value for the treatment and/or prevention of atherosclerosis in diabetic patients.
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Disclosure of conflict of interest

None.

Authors’ contribution

All authors actively participated in the study and in the review and approval of the manuscript. JYL, CZZ, XPH, DJZ and PY contributed to drafting of the manuscript and to study concept and design; JYL, CZZ, XPH and DJZ contributed to data acquisition; JYL, CZZ, XPH, AWM and DJZ contributed to data analysis and interpretation; AWM and PY contributed to critical revision of the manuscript for important intellectual content; JYL, DJZ and PY contributed to study supervision; and JYL and PY contributed to acquisition of funding.

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