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Original Article

Catalpol alleviates renal damage by improving lipid metabolism in diabetic db/db mice

Pingping Jiang[1*], Lei Xiang[1*], Zewei Chen[1*], Hanqi Lu[1*], Lin Zhou[2], Lebin Yang[1], Yanzhao Ji[1], Yanyan Liu[1], Xiaomin Sun[1], Yingfeng Deng[3], Xiaoli Nie[1], Ren Luo[1], Xiaoshan Zhao[1]

[1]School of Traditional Chinese Medicine, Southern Medical University, Guangzhou 510515, Guangdong, China; [2]Department of Endocrinology, Nanfang Hospital, Southern Medical University, Guangzhou 510515, Guangdong, China; [3]Touchstone Diabetes Center, Department of Internal Medicine, UT Southwestern Medical Center, Dallas, TX, USA. [*]Equal contributors.

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Abstract: Objective: To evaluate the protective effect of catalpol against diabetic nephropathy in db/db mouse. Methods: 8 week old C57BLKS/J db/db mice (type 2 diabetic mouse model) were divided into three groups to feed for 16 weeks on chow diet with or without catalpol supplementation. Their food intake, water consumption, body weight, and fasting glucose levels were recorded every 4 weeks. At the end of study, urine and blood samples were examined for several metabolic variables, and kidneys were harvested for structural characterization and microarray analysis. Results: Catalpol efficiently lowers the fasting glucose and the 24 h urinary albumin excretion rate. Catalpol significantly lowers serum triglycerides, increases high-density lipoproteins, and improves serum creatinine and urea nitrogen. Catalpol-fed mice preserve their kidney structure and renal function better than chow fed db/db mice. Microarray data indicates that lipid metabolism is a potential target of catalpol in exerting protective effect. Conclusion: Catalpol has a renal protective effect in diabetic db/db mice.

Keywords: Catalpol, db/db diabetic nephropathy, 24 h urinary albumin, gene-chip, lipid metabolic process

Background

Diabetic nephropathy (diabetic kidney diseases, DKD) is a severe microvascular complication of diabetes, which will lead to glomerular sclerosis and end-stage renal disease (ESRD) [1]. Patients with diabetic nephropathy need more frequent dialysis, present more need for kidney transplantation, and have higher rate of mortality [2, 3]. The prevalence of diabetes mellitus in Chinese adults is 11.6%, whereas 114 million are diagnosed with diabetes. Among those with diabetes, the prevalence of developing chronic kidney disease is as high as 63.9% [4]. Patients with DKD have become the second largest group of dialysis recipients in China. The escalated population of DKD patients has put families and government under tremendous pressure to cover the cost for treatment [5]. Due to the complex pathophysiology of diabetic nephropathy, targeted cures are very limited [6]. Instead, in order to alleviate the symptom, patients are usually given customized regimens to improve their control of blood glucose, blood pressure, lipid profile, or proteinuria. These methods, however, have only marginal effects and will not prevent the progression of diabetic nephropathy.

Rehmanniae radix, a traditional Chinese herbal medicine, has long been used by Chinese clinicians in treatment of patient with diabetic disorders. Catalpol, which is isolated from *Rehmanniae glutinosa*, is an iridoid glycoside with the molecular formula of $C_{15}H_{22}O_{10}$. Catapol has been found to have anti-inflammation, anti-aging and anti-apoptosis activities [7-9]. Catalpol exhibits protective effects against oxidative stress, inflammation, and subsequent tissue injuries associated with diabetic complications, including diabetic nephropathy [10, 11]. Catapol can lower blood glucose, prevent extracellular matrix accumulation, and reduce advanced glycation end products (AGEs) [11]

[12]. Mechanistically, Catapol can inhibit the expression of transforming growth factor beta 1 (TGF-β1), connective tissue growth factor (CTGF) and angiotensin II (AngII), and activate the insulin-like growth factor 1 (IGF-1) and insulin-like growth factor receptor (IGF-1R) signaling pathways [13]. However, it remains elusive how catalpol exerts a protective effect against diabetic nephropathy. This study aims to examine the mechanism of catalpol mediated protection on renal function.

Materials and methods

Chemicals and reagents

Catalpol (with a purity ≥98%) was purchased from Chengdu Must Bio-Technology Co., Ltd (Chengdu, Sichuan, China). Mouse urinary albumin enzyme-linked immunoassay kit was purchased from Nanjing Bianzhen Biological Technology Co., Ltd (Nanjing, Jiangsu, China). Periodic Acid-Schiff (PAS) staining kits, hematoxylin-eosin staining solution, Masson staining kits, and electron microscopy fixative were purchased from Wuhan Google Biology Company (Wuhan, Hubei, China).

Animals

C57BLKS/J db/db mice (type 2 diabetic mouse model) and db/+ mice were purchased from Changzhou Covance Experimental Animal Co., Ltd (Changzhou, Jiangsu, China). Mice were fed *ad libitum* in a specific-pathogen-free (SPF) animal room at Guangzhou Institute of Physical Education Experimental Animal Center in a light-controlled room under a 12 h light-dark cycle with temperature of 23 ± 1°C and humidity of 53 ± 2%. All procedures were approved by the Standards for Animal Ethics in Guangzhou Institute of Sport Science.

The mice were acclimated for one week after they arrived in the Institute. The average body weight of 8 week old db/db mice was 40-50 g, and they were divided into two groups: db/db model group and db/db catalpol group (n = 6 per group). The mice were grouped such that the average body weight of two groups were the same at the start of experiment. Age-matched db/+ mice were used as control group (n = 6). The db/db catalpol group was fed catalpol diet which was chow diet supplemented with catalpol at the dose of 1 g/kg. The chow diet was purchased from Experimental Animal Center of Guangdong Province.

Measurement of 24 h urinary albumin excretion rate

Mice were single housed in the metabolic cage for 24 h urine collection. Urine samples were collected 3 days before the mice were sacrificed. Urine was separate from feces during collection in cage. Each urine sample was mixed with 0.5 ml preservative toluene and centrifuged at 5000 rpm for 10 minutes. The supernatants were stored at -80°C until analysis. 24 h urinary proteins were determined by mouse urinary albumin ELISA kit (Nanjing Bianzhen Biological Technology Co., Ltd, China).

Measurement of blood metabolic variables

Blood glucose was measured from mouse tail with glucose meter (Roche). Serum was collected from mice anesthetized with 1% pentobarbital (40 mg/kg). Serum creatinine, urea nitrogen, uric acid, triglyceride, total cholesterol, low-density lipoprotein, and high-density lipoprotein were measured with an automatic biochemistry analyzer (LifeScan Canada Ltd., Burnaby, BC, Canada).

Histopathological examination of tissue

The kidneys from mice were fixed in 4% paraformaldehyde at room temperature and paraffine-embedded for sections. Slides were then processed to H&E staining, PAS staining, and Masson staining. Images under bright light microscope were acquired with a digital camera with magnification ×400 (Nikon, Japan).

Ultrastructural observation with scanning electron microscopy and transmission electron microscopy

The left kidney was rapidly removed and the adipose capsule was separated. The kidney tissue was carefully trimmed to create strips of about 1 mm^3 and then immediately fixed in 2.5% glutaraldehyde in cacodylate buffer for 2 h. Then, tissue was postfixed with 1% osmium tetroxide; dehydrated in a series of graded alcohols; embedded in epoxy resin, and finally polymerized with a Leica automatic microwave electron microscopic tissue processor at 60°C for 24 h. Ultrathin sections (70-80 nm thick)

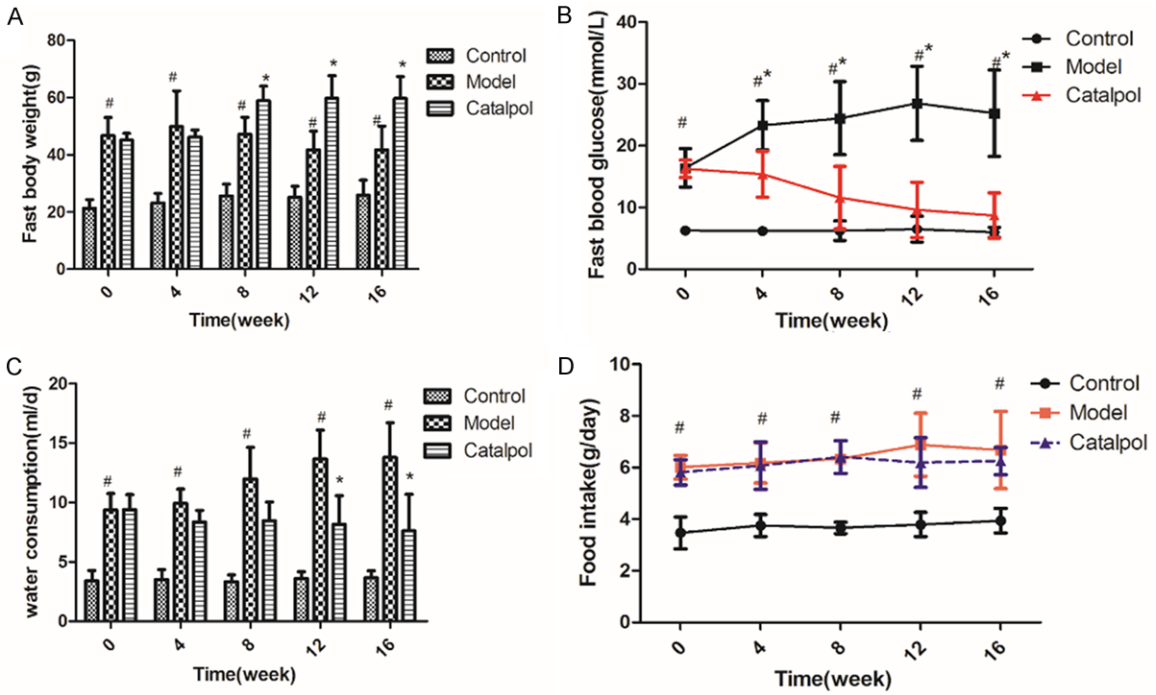

Figure 1. Assessment effect of catalpol on fasting body weight, fasting blood glucose, water consumption, food intake at four-week intervals. A. Fasting body weight. From 8 weeks to 16 weeks, the body weight of mice in the catalpol group was significantly higher than that in the model group. B. Fasting blood glucose levels. Fasting blood glucose levels in the catalpol group were significantly lower than that of the model group from the 4th to 16th week. C. Water consumption. In the 12th week and 16th week, water consumption of the catalpol group was significantly lower than that of the model group. D. Food intake. There was no significant difference in food intake between the model group and the catalpol group. #p<0.05 compared with the control group; *p<0.05 compared with the model group.

were made on an RMC Power Tome XL ultramicrotome (Shanghai Leica Instrument Co., Ltd, Shanghai, China); picked up by copper grids; stained with uranyl acetate and lead citrate, and examined by a U8010 Scanning electron microscopy (HITACHI Co., Japan) and an FE TECNAI transmission electron microscopy (TECNAI, Netherlands). Digital images were captured using a megaview G2 CCD camera (Soft Imaging System GmbH, Münster, Germany) at x8000 magnification.

RNA isolation and gene microarray analysis

Kidneys were removed, washed in several volumes of RNase-free water, de-capsulated, and stored overnight at 4°C in 5 volumes of RNA protective solution (Wuhan Google Biotechnology Co., Ltd, Wuhan, Hubei, China). Samples were preserved at -80°C. Total RNA was extracted using Takara RNAiso Plus Kit (Cat#9109, Takara, Dalian, Liaoning, China) following manufacturer's instructions. Samples were checked for RNA integrity by an Agilent Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA). Qualified total RNA was further purified by RNeasy mini kit (Cat#74106, QIAGEN, GmBH, Germany) and RNase-Free DNase Set (Cat# 79254, QIAGEN, GmBH, Germany).

The Affymetrix gene-chip MTA1.0 array (Affymetrix, Inc., Santa Clara, CA, USA), which contains more than 23,000 mouse genes, was employed for the purposes of this study (Genminix Informatics Co., Ltd, Shanghai, China). Total RNA (1 μg) from each sample was amplified, labeled and purified by Affymetrix WT PLUS Reagent Kit (Cat#902280, Affymetrix, Santa Clara, CA, USA) according to the manufacturer's instructions in order to obtain biotin labeled cDNA. Labeled cDNA was hybridized to the array for 16 h at 48°C using a plate shaker at 60 rpm. The gene-chip was scanned using the Gene-chip® Scanner 3000 (Cat#00-002-13, Affymetrix, Santa Clara, CA, USA). Expression data were analyzed using the Command

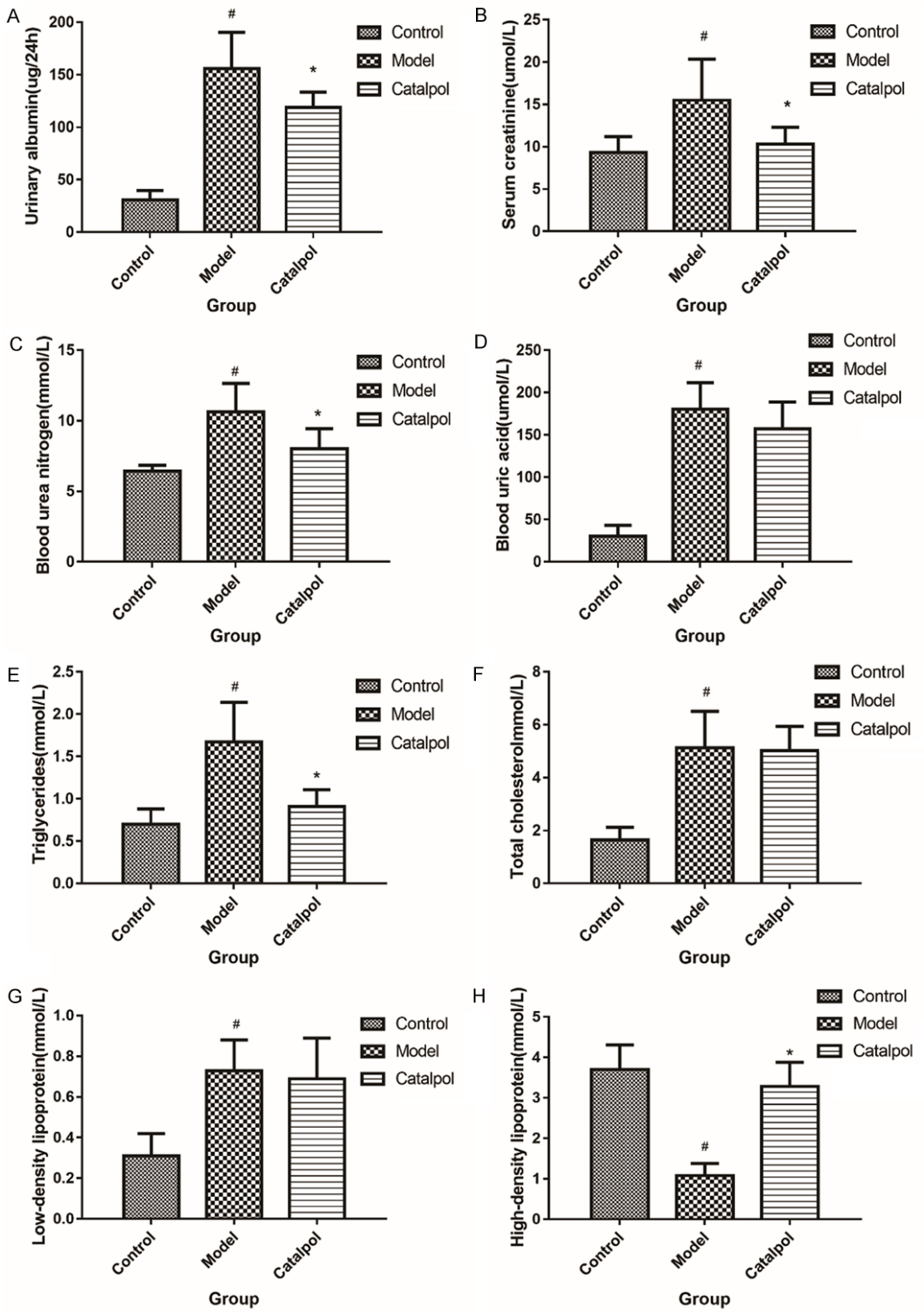

Figure 2. Assessment effect of catalpol on renal function (A-D) and triglycerides, total cholesterol, low-density lipoproteins and high-density lipoproteins (E-H). (A) Catalpol could reduce 24 h urinary albumin. (B) Catalpol could

improve serum creatinine. (C) Catalpol could ameliorate blood urea nitrogen, and blood uric acid (D). The catalpol group showed lower triglycerides and higher high-density lipoproteins than the model group after administration of catalpol. (E) Catalpol could reduce the plasma triglyceride. (F) Catalpol and model group has no difference on total cholesterol and (G) low-density lipoproteins. (H) Catalpol could elevate high-density lipoproteins. #p<0.05 compared with the control group; *p<0.05 compared with the model group.

Console Software 4.0 (Affymetrix, Santa Clara, CA, USA) and normalized with the robust multi-array averaging method. The differentially expressed genes were selected according to the fold change (linear) ≤0.66 or fold change (linear) ≥1.5, and *t- test* p<0.05.

Gene ontology (GO) and Kyoto encyclopedia of genes and genomes (KEGG) analysis

GO terms (www.geneontology.org), and the Kyoto Encyclopedia of Genes and Genomes (KEGG; www.genome.jp/kegg/) pathway annotation databases, were used to identify potential function and provide an insight into the signal pathways of differentially expressed genes. GO analysis was conducted to organize genes into hierarchical categories and to determine the differential expression gene regulatory network. In addition, pathway analysis was utilized to identify potential pathways of the differentially expressed genes using the KEGG database. GO terms and potential pathways were selected as p<0.05.

Statistical analysis

SPSS 20.0 is used as the statistical analysis software, and the measurement data shall use $\bar{x} \pm s$ to express. T-test is adopted for the comparison between each two groups. And groups were analyzed by one-way ANOVA and subsequent Dunnett testing for multiple comparisons. When P<0.05 difference was statistically significant.

Results

Effects of catalpol on metabolic variables in db/db mice during a 16-week study

8-week-old db/db mice were switched to catalpol chow diet and were monitored for bodyweight every 4 weeks. The catalpol-fed db/db mice increased their bodyweight at a rate faster than control db/db mice. By 8 weeks, the bodyweight of catalpol-fed db/db mice were significantly higher than the control db/db mice, and the catalpol fed group maintained this difference through to the end of the study (**Figure 1A**).

At week 0, the fasting blood glucose levels in db/db mice were significantly higher than db/+ group. By 8 weeks, the catalpol-fed mice had their fasting glucose levels lowered such that their glucose levels were similar to the db/+ mice (**Figure 1B**). The effect of catalpol on lowering blood glucose was maintained in the db/db mice through the rest of the study.

Although the bodyweight gain was higher in catalpol-fed db/db mice, the average food intake was not altered by caltapol (**Figure 1D**). In contrast, the catalpol-fed db/db mice trended towards reduced water consumption, and their daily water consumption was significantly lower than control db/db mice by the end of 12 weeks (**Figure 1C**).

Effects of catalpol on metabolic variables after 16-week food supplementation

At the end of the 16-week study, urinary albumin, serum creatinine, serum urea nitrogen in catalpol-fed db/db mice were significantly lower than the control db/db mice, suggesting an improvement of renal function by catalpol (**Figure 2A-D**). The triglyceride levels in db/db mice were also lowered by catalpol (**Figure 2E**). Although, catalpol did not change total cholesterol or LDL levels in the db/db mice, it restored the HDL levels in db/db mice (**Figure 2F-H**). The data above indicates that catalpol has a complicated effect on lipid metabolism.

Effect of catalpol on renal function after 16-week food supplementation

Compared with the control group, the number of glomerular endothelial cells and mesangial cells in the model group was significantly reduced, and the glomerular capillary loops were not clear. Still, the glomerular mesangial interstitium was increased and Bowman's sac was irregular. Compared with the model group, the number of glomerular endothelial cells and

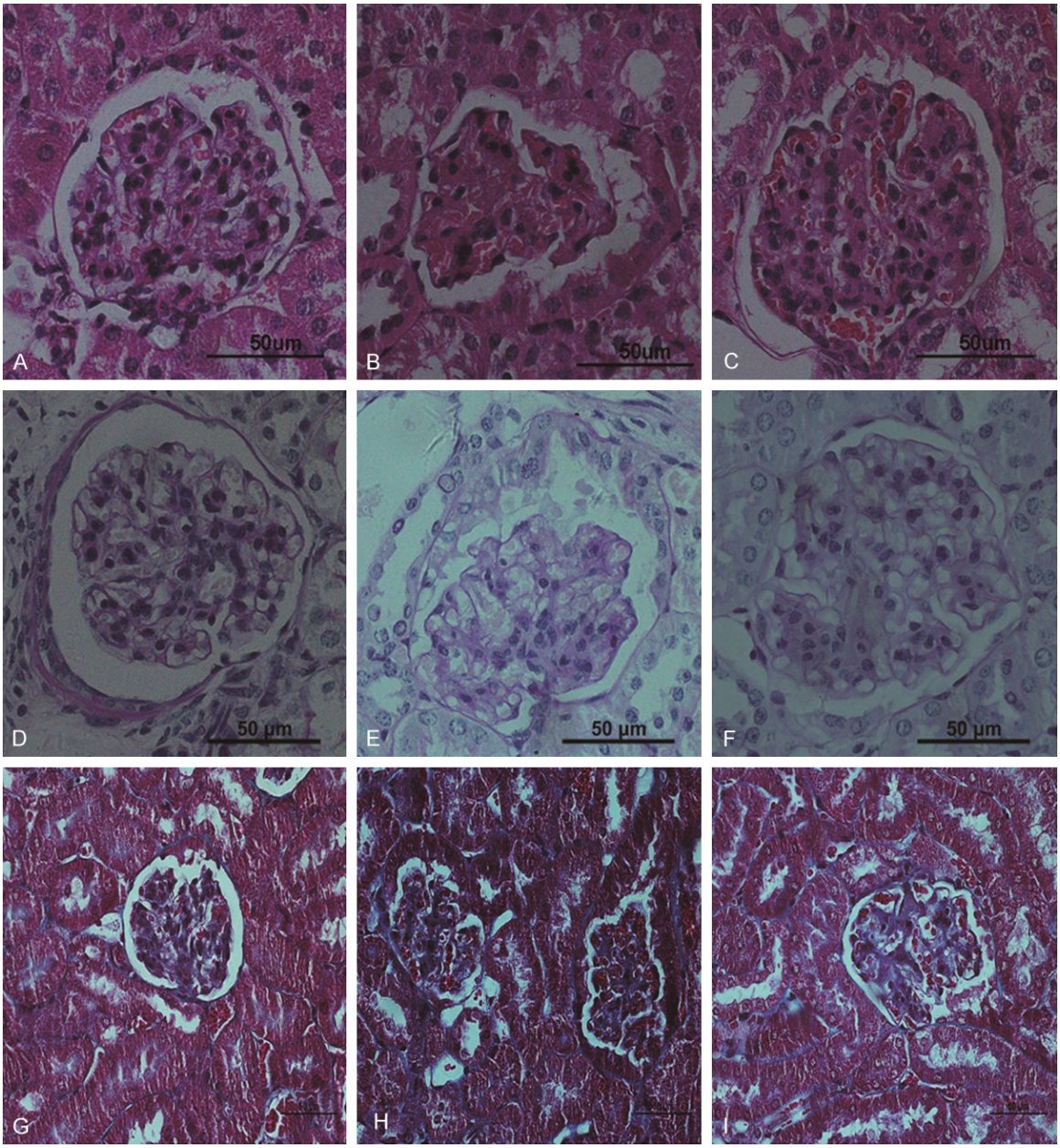

Figure 3. Histopathological changes of the kidney in different groups. A-C. Hematoxylin and eosin staining of kidney tissue thick sections from each group. Kidney tissue mounts were stained with H&E. B. The number of glomerular endothelial cells and mesangial cells was significantly reduced and the glomerular capillary loops were not clear indicating severe renal damage. C. Catalpol alleviated the renal damage by increasing the number of glomerular endothelial and mesangial cells. D-F. Periodic Acid-Schiff (PAS) staining of kidney tissues in each group. Kidney tissue mounts were stained with PAS. Cells with a positive reaction stained purple. E. Fuchsia positive cells were more abundant than in the other two groups. F. Catalpol alleviated the renal damage by lowering glomerular regional glycogen deposition. G-I. Masson staining of kidney tissue mounts in each group. Mounts of kidney tissue sections were stained with ponceau red and aniline blue. Positive reactions are represented by blue staining. H. Blue stained areas were more abundant than that of the other two groups, indicating more severe fibrosis. I. Catalpol alleviated the renal damage by reducing collagen fibers. A, D and G, control; B, E and H, model group; C, F and I, catalpol group.

mesangial cells in the catalpol-fed group was increased, and the glomerular capillary loop became clear. Additionally, the glomerular mesangial interstitium was not obvious and Bowman's sac was observed to be more regular (**Figure 3A-C**).

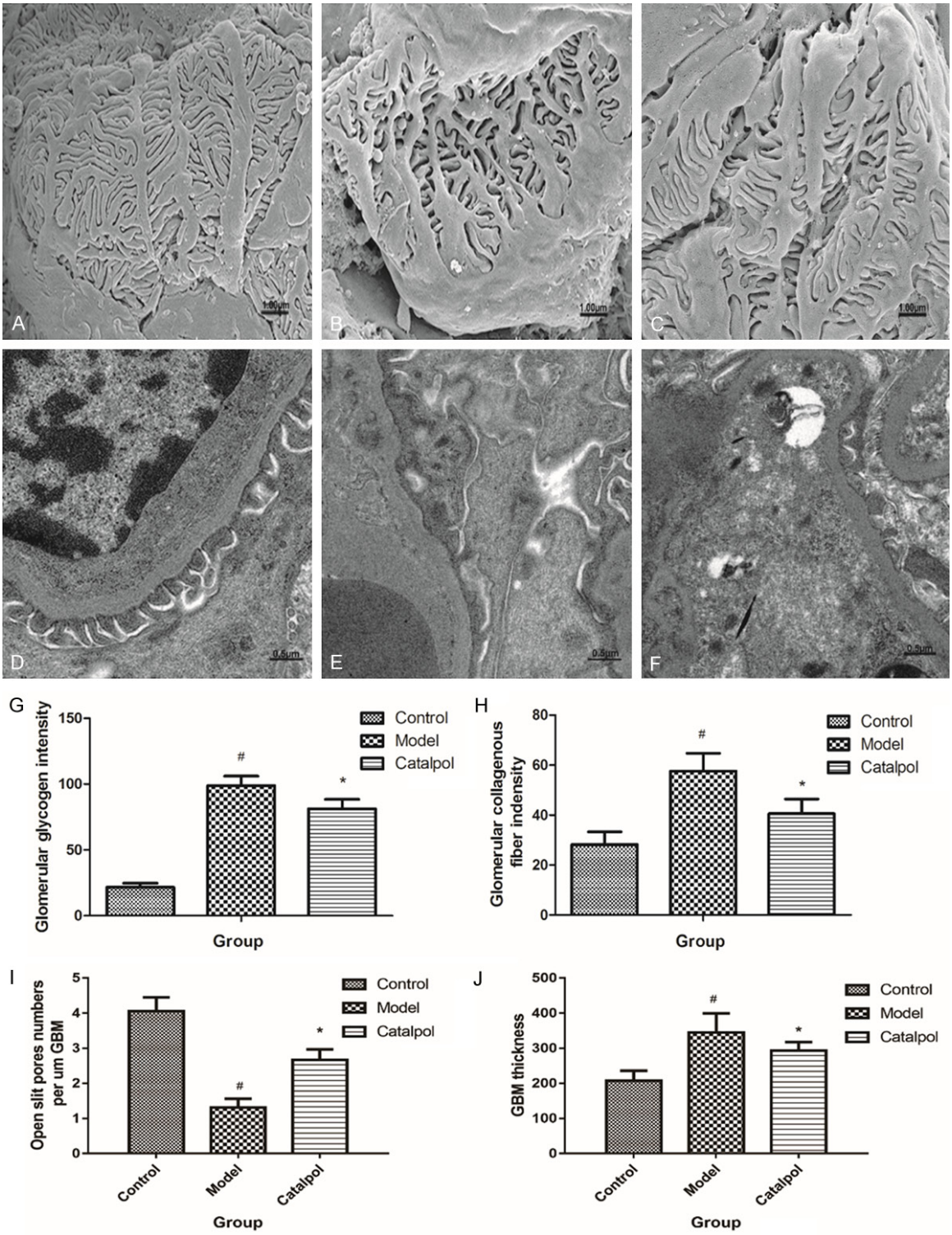

Figure 4. The ultrastructure of the kidney in different groups. (A-C) Scanning electron microscopy of renal tissue (A control, B model, C catalpol). (B) The gap between podocyte processes in the model group was bigger, while the density of podocyte process was smaller. (C) The catalpol group showed an amelioration in both podocyte processes gaps and podocyte process density. (D-F) Transmission electron microscopy of renal tissues (D control, E model, F catalpol). (E) The number of glomerular podocyte processes of db/db mice was significantly decreased, and the glomerular basement membrane was thickened. (F) The glomerular basement membrane of the catalpol group showed a markedly reduced thickness. (G) PAS semi-quantitative analysis. (H) Masson semi-quantitative analysis. (I, J) TEM quantitative analysis. #p<0.05 compared with the control group; *p<0.05 compared with the model group.

Table 1. Genes that were differentially expressed among control, model, catalpol groups (A control group, B model group, C catalpol group)

Gene symbol	A/B	C/B
Scd2	1.87	1.54
Gm20706	1.87	6.45
Map7	0.64	0.65
Slc5a8	0.622	0.51
Gm26160	0.55	0.47
Ugt2b5	0.50	0.58
Acsm5	0.50	0.44
Insig1	0.48	0.54
Slc16a1	0.40	0.61
Hist1h1c	0.29	0.37
Mansc4	0.29	0.63
Acmsd	0.29	0.26

By PAS staining, compared with the control group, the content of glomerular glycogen in db/db mice increased significantly. Semi-quantitative analysis using a positive optical microscope, NIKON ECLIPSE CI (Nikon Corporation of Japan), showed that the content of glomerular glycogen in the db/db mice was significantly higher than in the control group ($p<0.05$). Compared with the model group, glycogen deposition in the glomerular area of mice in the catalpol-fed group was significantly reduced (**Figures 3D-F**, **4G**, $p<0.05$).

By Masson staining, compared with the control group, the glomerular collagen content in the model group was significantly increased. Semi-quantitative analysis showed that the content of glomerular collagen fibers in the model group was higher. The content of collagen fibers in the glomerular area decreased in the catalpol-fed group (**Figures 3G-I**, **4H**, $p<0.05$).

Ultrastructural changes of renal tissue after 16-week catalpol-fed supplementation

Compared with the control group, the gap between podocyte processes in the model group was bigger, while the density of podocyte process was shown to be smaller. The catalpol group showed an amelioration in both podocyte processes gaps and podocyte process density (**Figure 4A-C**). Compared with the control group, the number of glomerular podocyte processes of db/db mice was significantly decreased, and the glomerular basement membrane was thickened ($p<0.05$). Compared with the model group, the glomerular basement membrane of the catalpol group showed a markedly reduced thickness (**Figure 4D-F**, **4I**, **4J**, $p<0.05$).

Effect of catalpol on gene expression by microarray analysis after 16-week food supplementation

To examine the effect of catalpol on gene expression in kidney, we analyzed the mRNA extracted from renal tissues with microarray, and identified that a total of twelve genes were differentially expressed among the three groups: db/+ control group, db/db model group and catalpol-fed db/db group (**Table 1**). As shown in the hierarchical cluster analysis (**Figure 5A**), two genes were upregulated by catalpol with fold change ≥1.5, and 10 genes were downregulated with fold change ≤0.66.

Using GO and pathway analysis with threshold of significance defined as $p<0.05$, we found these 12 genes were annotated with several functions by the DAVID database. Among them, the top five metabolic pathways associated are: lipid metabolic process, fatty acid metabolic process, transmembrane transport, metabolic process, and transport, which are important for distinct biological processes (**Figure 5B**). Our data suggests that catalpol has a potent effect on renal gene expression, which might cause systemic change in metabolism.

Discussion

The db/db mice harbor a gene mutation on leptin receptor, which makes mice unable to respond to satiety, leading to hyperphage and obesity. The db/db mice of C57BLKs/J strain are featured with hyperglycemia and used as an animal model for type 2 diabetes. Male mice show more severe hyperglycemia than female mice and the male db/db mice develop hyperglycemia at the age of 6 weeks [14]. They start proteinuria at 8 weeks old [15], and their renal functions decline when the mice are about 15 weeks old [16, 17]. Therefore, the db/db mice have also been widely used for the study of diabetic nephropathy. The db/db mice with strain background of C57BL/6J and FVB/NJ exhibit symptoms similar to C57BLKs/J strain [18-21]. Considering the time-course of hyperglycemia

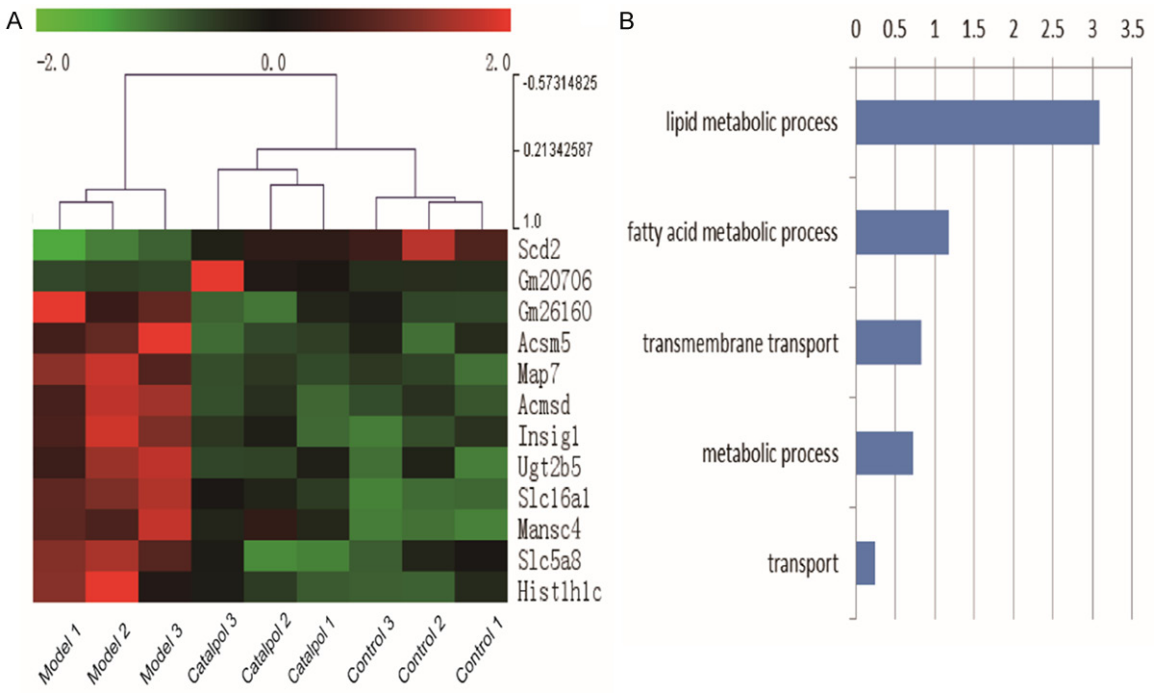

Figure 5. Gene expression profiles in renal tissue and pathway analysis. A. Hierarchical cluster heat map of differentially expressed genes in mouse renal tissue from control, model and catalpol groups. Red signals indicate upregulated expression and green signals indicate downregulated expression (n = 3). B. Biological processes of differentially expressed genes. Twelve differentially expressed genes were associated with the following five metabolic pathways: lipid metabolic process, fatty acid metabolic process, transmembrane transport, metabolic process, and transport.

development and renal injury, we chose 8-week-old C57BLKs/J male db/db mice to examine the effect of catalpol on diabetic nephropathy so that the potential of catapol in protecting mice from renal injury can be evaluated. Using age-matched db/+ mice as a control group, we verified that the db/db mice in the study had all developed diabetic nephropathy based on analysis of fasting blood glucose, 24 h urinary albumin, and lipid profiles. Consistent with the data from metabolic variables, db/db mice had altered kidney morphology, suggesting regnal injury at cellular level which includes decreased glomerular endothelial cells and mesangial cells, increased glomerular mesangial interstitial, increased irregular Bowman's capsule sac, and increased glomerular glycogen and collagen fiber. Moreover, scanning and transmission electron microscopy demonstrated the db/db mice had reduced number of podocyte, thickened glomerular basement membrane, and widened podocyte gap. The renal morphological and structural changes observed in the db/db mice mimic the kidney manifestations from patients with diabetic nephropathy. Lacking an efficient cure for renal injury, patients with diabetic nephropathy were given customized regimens that aim to improve their metabolic variables associated with diabetes or dyslipidemia. For patients with hyperglycemia, metformin and insulin are prescribed to lower their blood glucose. For patients with hypertension, losartan or benazepril are prescribed as anti-hypertension medicine. For patients with dysregulated lipid metabolism, simvastatin or rosuvastatin are considered as the treatment. For patients with microcirculation dilation, pancreatic kininogenase is frequently administrated. If no improvement can be achieved through the regimens described above, the last hope for the patients are renal replacement therapy, dialysis, or kidney transplantation in the extreme cases of diabetic nephropathy [22]. Recently, some patients with diabetic nephropathy are treated with drugs of targeted biological functions, such as, pyridorin with anti-AGE activity [23], suloexide with anti-fibrosis effects [24], and atrasentan that antagonizes endothelin receptor signaling [25]. Although several positive effects have been reported for these drugs,

their long-term efficacy remains questionable and their side effects are strong [26, 27].

Using db/db mice as a model for diabetic nephropathy, we demonstrated that catalpol can effectively preserve kidney structure and renal function, prevent glomerular regional deposition of glycogen and collagen fibers, and increase number of podocytes to protect mice from diabetec kidney disease. This is consistent with the finding that oral catalpol administration (120 mg/kg) improved diabetic nephropathy induced by streptozotocin (STZ) in rats [11]. It remains elusive how catalpol exerts the protective effects. It has been suggested that catalpol downregulates TGF-β1 levels in renal tissue through the down-regulation of renal TGF-β1 gene expression. It is also reported that catalpol increases CTGF expression, and increased CTGF can protect renal tissue through prevention of extracellular matrix accumulation [11-13]. Interestingly, catalpol can protect mice from STZ-induced diabetes even when they were on high fat and high sugar diet. This protection may result from a restored balance between oxidative and anti-oxidative enzymes in pancreas [28]. Consistent with its antidiabetic effect, catalpol has been found of multiple roles in regulating cell signaling and metabolism. Catalpol can improve liver insulin resistance in db/db mice through downregulation of hepatic gene expression of acetyl-CoA carboxylase (ACC) and hydroxymethyl glutaric acid acyl CoA reductase (HMGCR), and increase phosphorylated AMPKα1/2 [29]. It can also attenuate adipose tissue inflammation by suppression of the c-Jun N-terminal kinase (JNK) and nuclear factor-kappa B (NF-κB) pathways [30].

In our study, we found catalpol altered renal gene expression in db/db mice. Using stringent criteria, we screened out twelve genes as potential targets of catalpol: Scd2, Slc5a8, Ugt2b5, Acsm5, Insig1, Gm20706, Map7, Gm-26160, Slc16a1, Hist1h1c, Mansc4, and Acmsd. Although two of them were downregulated, and the other ten were upregulated by catalpol, all the twelve genes were restored to the levels similar to those observed in db/+ mice, indicating catalpol helps correct the gene expressions which result from leptin receptor deficiency. Interestingly, the twelve genes identified are critical for several metabolic pathways that involves lipid turnover, which indicates that lipid homeostasis in kidney is a potential target for catalpol to exert its antidiabetic effect. In fact, type 2 diabetes mellitus is characterized by dyslipidemia with elevated free fatty acids (FFAs) in circulation. To understand the pathology of diabetic nephropathy and the mechanism of catalpol's protective action, we examined the relevance of changes in the twelve gene expression to renal structure and function. Podocytes are highly susceptible to saturated FFAs, and loss of podocytes is a hallmark of diabetic nephropathy [31]. In contrast to saturated FFAs, monounsaturated FFAs are not toxic to podocytes [32]. In order to reduce the toxicity of saturated FFAs, synthesis of monosaturated FFA from saturated FFAs is a possible strategy. Insig1 and Scd have been shown to be important for saturated-to-monounsaturated FFA conversion [33]. Both Insig1 and Scd2 are identified in our study as ones changed in db/db mice, but restored by catalpol. Slc5a8, a novel transporter of mono-carboxylates located in mouse intestine and kidney [34], plays an important role in mucosal immune system [35]. Its expression is downregulated in db/db mice, but restored by catalpol.

Conclusion

In this study, we confirmed the protective effect of catalpol against diabetic nephropathy in db/db mice. We showed that catalpol effectively lowers fasting blood glucose, water consumption, and 24 h urinary albumin excretion in diabetic animals. In addition to improving several metabolic variables associated with diabetes and dyslipidemia, catalpol protects kidney from structural injury and preserves renal function in db/db mice. Microarray analysis revealed that catalpol alters gene expression in kidney, and highlighted metabolic pathways for lipid turnover as a potential mechanism of catalpol-mediated protection against diabetic nephropathy.

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Disclosure of conflict of interest

None.

Address correspondence to: Xiaoshan Zhao and Ren Luo, School of Traditional Chinese Medicine, Southern Medical University, Guangzhou 510515, Guangdong, China. E-mail: zhaoxs0609@163.com (XSZ); E-mail: luoren2014@126.com (RL)

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