Short Communication

Mechanism of Puerarin Regulating AMPK/mTOR Activated Autophagy Signal Pathway in the Treatment of Diabetic Nephropathy

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To investigate the renal protective effect and mechanism of puerarin in diabetic nephropathy rats. In 18 clean grade male Sprague-Dawley rats aged 4 w to 8 w and 12 other male Sprague-Dawley rats in the normal control group, a tail injection of streptozotocin 100 mg/kg was performed to establish the type 2 diabetes mellitus rat model. After successful modeling, rats were randomly divided into model group and puerarin group, with 6 rats in both group. The dosage of puerarin group was 100 mg/kg/d, and the control group and model group were given the same amount of distilled water by gavage for 8 w. The changes of serum creatinine, blood urea nitrogen, total cholesterol, high density lipoprotein cholesterol, triglycerin in rats were detected. The 24 h urinary micro albumin level was measured. The expression of Beclin 1, light chain 3II/light chain 3I and adenosine-monophosphate activated-protein kinase/mammalian target of rapamycin pathway proteins in rat kidney was detected by Western blot. Compared with the control group, the levels of triglycerin and total cholesterol in the model group were raised ($p<0.05$), and the level of blood lipid in the puerarin group has no difference than that in the model group ($p>0.05$). The levels of blood urea nitrogen, serum creatinine and urinary micro albumin in the model group were raised than those in the control group ($p<0.05$), and the level of urinary micro albumin in the puerarin group was reduced than that in the model group ($p<0.05$), while the other two indicators had no difference compared with the model group. The levels of Beclin 1, light chain 3II/light chain 3I in the renal tissue of the model group were reduced than control group ($p<0.05$); while the expression levels of Beclin1, light chain 3II/light chain 3I in kidney tissue of rats in puerarin group were raised than model group ($p<0.05$). The expression of phospho-mammalian target of rapamycin protein in renal tissue of model group was raised than that of control group, while phospho-adenosine monophosphate activated-protein kinase protein was reduced than that of control group ($p<0.05$). The expression of phospho-mammalian target of rapamycin protein in kidney tissue of rats in puerarin group was reduced than that in model group, but phospho-adenosine-monophosphate activated-protein kinase protein was raised than that in model group ($p<0.05$). Puerarin is helpful to alleviate renal injury induced by diabetes mellitus, and it may be *via* regulating adenosine-monophosphate activated-protein kinase/mammalian target of rapamycin signal pathway mediated podocyte autophagy.

Key words: Puerarin, adenosine-monophosphate activated-protein kinase/mammalian target of rapamycin, autophagy, diabetic nephropathy

An End-Stage Renal Disease (ESRD) can result from Diabetic Nephropathy (DN)[1]. There are many factors involved in DN pathogenesis, including hyperglycemia-mediated intracellular metabolic disorders, autophagy and oxidative stress, leading to increased Minimum Efficient Scale (MES) levels[2]. Neither the underlying mechanism nor an effective prevention or treatment

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strategy has been identified to date. A podocyte is a highly differentiated epithelial cell that surrounds glomerular capillaries and maintains the glomerular filtration barrier[3]. It is essential that podocytes maintain their structural integrity in order to prevent leakage of albumin and micro albuminuria[4]. The mechanism of podocyte dysfunction in chronic kidney disease is increasingly being studied. Puerarin is an active compound of traditional Chinese medicine, which plays a renal protective role in Streptozotocin-induced endothelial Nitric Oxide Synthase (eNOS) deficiency in diabetic mouse model[5]. However, the detailed mechanism of puerarin's renal protective effect in DN has not been explored. In this study, puerarin was tested for its renal protective effect in DN rats, as well as its mechanism of reducing oxidative stress and podocyte injury in diabetic rats. Eighteen male Sprague-Dawley (SD) rats aged 4 w-8 w, weighing (190±15) g (purchased from Beijing Hufukang Science and Technology Co., Ltd). The animals were divided into cages (7-9 animals) and housed in Specific-Pathogen-Free (SPF) animal rooms with room temperature of 22°-25° and humidity of 50 %-60 %. The light and dark hours were 12 h, and the animals were fed and watered freely. The normal Control Group (CG) was fed with conventional diet, and the other rats were fed with high sugar and fat diet. Puerarin (Sigma Company, United States of America (USA)); Streptozotocin (Sigma, USA); rat Enzyme-Linked Immunoassay (ELISA) kit (Tianjin Haosi Biotechnology Co., Ltd.); protease inhibitor and Bicinchoninic Acid (BCA) protein concentration assay kit (Solarbio, Beijing); Rat primary antibody (CST, USA); Beta (β)-actin antibody (Tianjin Sanjian Biotechnology Co., Ltd.); immunohistochemically staining concentrated DAB kit (Beijing Zhongshan Jinqiao Biotechnology Co., Ltd.); light cycle (Roche, Switzerland); high-speed refrigerated centrifuge (Herseus, Germany); BA110S electronic balance (Sartorius, Germany); vertical electrophoresis apparatus, electro transfer device (Bio-Rad, USA); Automatic gel imaging analysis system (BD, USA) and optical microscope and micro camera (OLYMPUS, Japan). After 8 w of feeding, the blood glucose, lipid and fasting islet levels were measured by taking epicanthus veins after 12 h of fasting. After 3 d of stability, the weight of rats in the high-glucose and high-fat diet group was measured after 12 h of fasting without water, and streptozotocin 100 mg/kg was injected into the tail vein to establish a Type 2 Diabetes Mellitus (T2DM) rat model. The CG was given 0.1 mol/l citrate and sodium citrate buffer. After successful modeling, 12 rats were randomly divided into Model Group (MG) and Puerarin Group (PG), with 6 rats in both groups. Puerarin was administered at a dose of 100 mg/kg/d by intragastric administration for 8 w, then the samples were sacrificed and the CG and MG were given the same amount of distilled water by intragastric administration. The dosage of the drug was adjusted according to the change of the body weight of the rats, and the general condition of the rats was monitored. After the rats were executed, 2 ml arterial blood at the root of the legs was collected and placed in Ethylenediaminetetraacetic Acid (EDTA) anticoagulant tube. After centrifugation, the supernatant was collected. Serum Creatinine (SCR), blood lipids Total Cholesterol (TC), Blood Urea Nitrogen (BUN), High Density Lipoprotein Cholesterol (HDL-C) and Triglycerin (TG) of the rats were detected by automatic biochemical analyzer. Urine samples were collected for 24 h, and the Urine Micro Albumin (U-MALB) level was measured by Roche analyzer. The expressions of Beclin1, Light Chain (LC) 3II/LC3I and Adenosine-Monophosphate Activated-Protein Kinase (AMPK)/mammalian Target of Rapamycin (mTOR) proteins in rat kidney tissues were detected by Western blotting. Measurement data were expressed as standard deviation ($\bar{x}\pm s$). One-way variance was used for comparison and analysis between multiple groups. Least Significant Difference (LSD)-t test was used for pairwise comparison, $^{*}p<0.05$ was considered statistically significant, $^{\#}p<0.05$ denotes comparison with control group and $p<0.05$ indicates comparison with model group. The levels of TG and TC in the MG were raised than CG ($p<0.05$), while HDL-C level has no difference ($p>0.05$), and the serum lipid level of rats in PG also has no difference compared with MG ($p>0.05$) as shown in Table 1. The levels of BUN, SCr and U-MALB in mg were raised than those in CG ($p<0.05$), the level of U-MALB in PG was reduced than that in MG ($p<0.05$), while the other two indexes have no difference compared with the MG as shown in Table 2. The levels of Beclin 1 and LC3II/LC3I in renal tissue of the MG were reduced

than CG ($p<0.05$); while those in renal tissue of rats in PG were raised than MG ($p<0.05$) as shown in Table 3. The expression of p-mTOR protein in MG kidney tissue was raised than that of CG, while p-AMPK protein was reduced than that of CG ($p<0.05$); The expression of p-mTOR protein in PG rats renal tissue was reduced than that in MG, but p-AMPK protein was raised than that in MG ($p<0.05$) as shown in Table 4. Studies have shown that puerarin can inhibit microalbuminuria in early DN patients[6]. The combination of puerarin and Angiotensin Converting Enzyme Inhibitor (ACEI) in the treatment of DN patients could further reduce albuminuria[7]. Meanwhile, studies have shown that puerarin can improve albuminuria in diabetic rodent model[8], which suggesting that puerarin has a good effect on DN. In addition, puerarin exhibits antioxidant activity both *in vitro* and *in vivo*[9], and it inhibits apoptosis of proximal renal tubular cells by restoring mitochondrial function[10]. The mechanism of puerarin remains unknown. It has been found that the antioxidant effect of puerarin on renal protection is mediated in part by inhibiting Nicotinamide Adenine Dinucleotide Phosphate Hydrogen Oxidase 4 (NOX4) expression in podocytes. DN is attenuated by NOX4 knockdown in podocytes, indicating that NOX4 plays an important role in DN regulation[11]. Other studies have shown that puerarin upregulates Sirtuin1 (SIRT1) and plays an anti-inflammatory role in Nuclear Factor Kappa B (NF-κB) mediated deacetylation by SIRT1 in DN. Furthermore, puerarin inhibits NF-κB activity through SIRT1 upregulation to reduce NOX4 expression, this confirms that NOX4 binds to NF-κB[12]. Further, previous studies have shown that NF-κB directly regulates NOX4[13]. Additionally, puerarin may have renal protective effects that go beyond antioxidants. Apoptosis of proximal renal tubular cells can be attenuated by puerarin *via* restoring mitochondrial function[14]. Moreover, puerarin improves DN by regulating Matrix Metalloproteinase 9 (MMP9) in podocytes[15]. By transferring damaged organelles and misfolded proteins to lysosomes, autophagy regulates cell differentiation, nutrient metabolism and cell environment[16]. In DN, podocyte loss and extensive proteinuria are caused by autophagy[17]. In this study, the corresponding changes of autophagy-related proteins were detached in the DN model. The expression levels of Beclin 1 and LC3II/LC3I in renal tissue of the MG were raised than CG ($p<0.05$) and those in renal tissue of rats in PG were raised than MG ($p<0.05$). The results showed that puerarin activated autophagy by increasing LC3-II/LC3-I ratio and Beclin 1 level, suggesting the restoration of autophagy by puerarin may play a protective role. The AMPK/mTOR signaling pathway regulates autophagy in podocytes, a complex process regulated by numerous signaling pathways. The serine/threonine protein kinases mTOR and AMPK play an important role in maintaining blood glucose and energy balance[18]. Increased mTOR activity can inhibit downstream signaling proteins in the autophagy pathway, since mTOR is an upstream regulator of the autophagy pathway[19]. In podocytes, excess mTOR activity leads to autophagy dysregulation, which results in autophagy cell death, which plays a major role in the development of DN[20]. Autophagy is known to be inhibited by the mTOR pathway, which protects podocyte function[21]. The results showed that the expression of p-mTOR protein in renal tissue of the MG was raised than that of the CG, while p-AMPK protein was reduced than that of the CG ($p<0.05$); the expression of p-mTOR protein in renal tissue of rats in PG was reduced than that in MG, but p-AMPK protein was raised than that in MG ($p<0.05$). p-AMPK decreased while p-mTOR increased in the DN model, indicating autophagy inhibition. Puerarin may activate autophagy by regulating the AMPK/mTOR pathway, preventing podocyte proliferation. As a result of its anti-oxidative stress properties, puerarin regulates autophagy. Puerarin also plays a significant role in the treatment of many other diseases. It protects against osteoporosis, neurological diseases and cardiovascular diseases[22]. When lipopolysaccharide-D-galactosamine causes liver injury in mice, puerarin improves survival, reduces Alanine Transaminase (ALT) and Aspartate Transaminase (AST) levels, and reduces proinflammatory cytokines by inhibiting apoptosis[23]. By promoting autophagy and reducing apoptosis, puerarin attenuates hypoxia/reoxygenation injury in primary cardiomyocytes[24]. Meanwhile, puerarin inhibits autophagy and activates AMPK-mTOR-ULK1 signaling to protect the brain against ischemia/reperfusion injury[25]. DN exerts their kidney protective effects through several mechanisms, including autophagy,

antioxidants and anti-apoptotic agents. Further studies are needed to elucidate the mechanism in mediating the nephroprotective effects of puerarin. In conclusion, puerarin can alleviate renal injury caused by diabetes, and it may be *via* regulating AMPK/mTOR pathway and the mediation of podocyte autophagy.

TABLE 1: COMPARISON OF SERUM LIPID LEVELS IN EACH GROUP (x̄±s)

Group	n	TG (mmol/l)	TC (mmol/l)	HDL-C (mmol/l)
Control	6	1.73±1.35	2.32±1.18	0.63±0.24
Model	6	4.38±1.72*	6.41±2.25*	0.50±0.13
Puerarin	6	3.64±1.22*	6.28±2.14*	0.52±0.12

Note: *p<0.05

TABLE 2: COMPARISON OF RENAL FUNCTION AND U-MALB LEVEL IN EACH GROUP (x̄±s)

Group	n	BUN (mmol/l)	SCr (μmol/l)	U-mAlb (μg/24 h)
Control	6	5.35±2.01	15.84±1.42	43.49±11.38
Model	6	11.52±2.43*	31.95±4.53*	247.61±23.67*
Puerarin	6	10.98±1.68	31.16±4.08	156.68±13.56*#

Note: *p<0.05 and #p<0.05

TABLE 3: CHANGES OF AUTOPHAGY-RELATED PROTEINS IN RENAL TISSUE OF RATS (x̄±s)

Group	n	Beclin 1	LC3II/LC3I
Control	6	1.18±0.19	1.64±0.18
Model	6	0.38±0.09*	0.45±0.09*
Puerarin	6	0.72±0.08*#	0.76±0.12*#

Note: *p<0.05 and #p<0.05

TABLE 4: AMPK /mTOR PATHWAY PROTEINS EXPRESSION IN RAT KIDNEY TISSUES

Group	n	p-AMPK	p-mTOR
Control	6	0.95±0.10	0.64±0.15
Model	6	0.34±0.06*	1.28±0.24*
Puerarin	6	0.68±0.10*#	0.93±0.21*#

Note: *p<0.05 and #p<0.05

Author's contributions:

Hongxia Su and Yuan Jang have contributed equally to this work.

Conflict of interests:

The authors declared no conflict of interests.

REFERENCES

1. Saran R, Robinson B, Abbott KC, Agodoa LY, Albertus P, Ayanian J, *et al*. US renal data system 2016 annual data report: Epidemiology of kidney disease in the United States. Am J Kidney Dis 2017;69(3):A7-8.
2. Lin CL, Hsu YC, Huang YT, Shih YH, Wang CJ, Chiang WC, *et al*. A KDM6A–KLF10 reinforcing feedback mechanism aggravates diabetic podocyte dysfunction. EMBO Mol Med 2019;11(5):e9828.
3. Bae DH, Lane DJ, Jansson PJ, Richardson DR. The old and new biochemistry of polyamines. Biochim Biophys Acta Gen Subj 2018;1862(9):2053-68.
4. Michael AJ. Polyamines in eukaryotes, bacteria and archaea. J Biol Chem 2016;291(29):14896-903.
5. Li X, Cai W, Lee K, Liu B, Deng Y, Chen Y, *et al*. Puerarin attenuates diabetic kidney injury through the suppression of NOX4 expression in podocytes. Sci Rep 2017;7(1):14603.
6. Lian S. Clinical investigation of stage-IV diabetic nephropathy with treatment of Chen's Tangshen decoction and conventional Western medicine. Shanghai J Tradit Chin Med 2010;44:3.
7. Wang B, Chen S, Yan X, Li M, Li D, Lv P, et al. The therapeutic effect and possible harm of puerarin for treatment of stage III diabetic nephropathy: A meta-analysis. Altern Ther Health Med 2015;21(1):36-44.
8. Xu X, Zheng N, Chen Z, Huang W, Liang T, Kuang H. Puerarin, isolated from *Pueraria lobata* (Willd.), protects against diabetic nephropathy by attenuating oxidative stress. Gene 2016;591(2):411-6.
9. Ma JQ, Ding J, Xiao ZH, Liu CM. Puerarin ameliorates carbon tetrachloride-induced oxidative DNA damage and inflammation in mouse kidney through ERK/Nrf2/ARE pathway. Food Chem Toxicol 2014;71:264-71.
10. Song XB, Liu G, Wang ZY, Wang L. Puerarin protects against cadmium-induced proximal tubular cell apoptosis by restoring mitochondrial function. Chem Biol Interact 2016;260:219-31.
11. Jha JC, Thallas-Bonke V, Banal C, Gray SP, Chow BS, Ramm G, *et al*. Podocyte-specific Nox4 deletion affords renoprotection in a mouse model of diabetic nephropathy. Diabetologia 2016;59(2):379-89.
12. Liu R, Zhong Y, Li X, Chen H, Jim B, Zhou MM, *et al*. Role of transcription factor acetylation in diabetic kidney disease. Diabetes 2014;63(7):2440-53.
13. Zhao QD, Viswanadhapalli S, Williams P, Shi Q, Tan C, Yi X, *et al*. NADPH oxidase 4 induces cardiac fibrosis and hypertrophy through activating Akt/mTOR and NFκB signaling pathways. Circulation 2015;131(7):643-55.
14. Song X, Li Z, Liu F, Wang Z, Wang L. Restoration of autophagy by puerarin in lead-exposed primary rat proximal tubular cells *via* regulating AMPK–mTOR signaling. J Biochem Mol Toxicol 2017;31(3):e21869.
15. Zhong Y, Zhang X, Cai X, Wang K, Chen Y, Deng Y. Puerarin attenuated early diabetic kidney injury through down-regulation of matrix metalloproteinase 9 in streptozotocin-induced diabetic rats. PloS One 2014;9(1):e85690.
16. Turkmen K. Inflammation, oxidative stress, apoptosis and autophagy in diabetes mellitus and diabetic kidney disease: The four horsemen of the apocalypse. Int Urol Nephrol 2017;49(5):837-44.
17. Yasuda-Yamahara M, Kume S, Tagawa A, Maegawa H, Uzu T. Emerging role of podocyte autophagy in the progression of diabetic nephropathy. Autophagy 2015;11(12):2385-6.
18. Szrejder M, Piwkowska A. AMPK signalling: Implications for podocyte biology in diabetic nephropathy. Biol Cell 2019;111(5):109-20.
19. Li W, Zhu L, Ruan ZB, Wang MX, Ren Y, Lu W. Nicotinamide protects chronic hypoxic myocardial cells through regulating mTOR pathway and inducing autophagy. Eur Rev Med Pharmacol Sci 2019;23(12).
20. Li Z, Li Q, Lv W, Jiang L, Geng C, Yao X, *et al*. The interaction of Atg4B and Bcl-2 plays an important role in Cd-induced crosstalk between apoptosis and autophagy through disassociation of Bcl-2-Beclin1 in A549 cells. Free Radic Biol Med 2019;130:576-91.
21. Bork T, Liang W, Yamahara K, Lee P, Tian Z, Liu S, *et al*. Podocytes maintain high basal levels of autophagy independent of mTOR signaling. Autophagy 2020;16(11):1932-48.
22. Hou B, Zhao Y, Qiang G, Yang X, Xu C, Chen X, *et al*. Puerarin mitigates diabetic hepatic steatosis and fibrosis by inhibiting TGF-β signaling pathway activation in type 2 diabetic rats. Oxid Med Cell Longev 2018;2018:4545321.
23. Li L, Yin H, Zhao Y, Zhang X, Duan C, Liu J, *et al*. Protective role of puerarin on LPS/D-Gal induced acute liver injury *via* restoring autophagy. Am J Transl Res 2018;10(3):957.
24. Ma Y, Gai Y, Yan J, Jian J, Zhang Y. Puerarin attenuates anoxia/reoxygenation injury through enhancing Bcl-2 associated athanogene 3 expression, a modulator of apoptosis and autophagy. Med Sci Monit 2016;22:977.
25. Wang JF, Mei ZG, Fu Y, Yang SB, Zhang SZ, Huang WF, *et al*. Puerarin protects rat brain against ischemia/reperfusion injury by suppressing autophagy *via* the AMPK-mTOR-ULK1 signaling pathway. Neural Regen Res 2018;13(6):989.