Dapagliflozin relieves renal injury in a diabetic nephropathy model by inducing autophagy through regulation of miR-30e-5p/AKT/mTOR pathway

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Abstract

Purpose: To investigate the mechanism of action of dapagliflozin on diabetic nephropathy.
Methods: A rat model of diabetic nephropathy was established by injection of fructose-streptozotocin. Blood glucose and protein levels were measured, while histopathological changes in kidney tissues were determined by hematoxylin & eosin staining (H & E). Serum levels of creatinine (Cr), blood urea nitrogen (BUN), malondialdehyde (MDA), superoxide dismutase (SOD), reduced glutathione (GSH), and lactate dehydrogenase (LDH) were evaluated by enzyme-linked immunosorbent assay (ELISA). Cell apoptosis and autophagy were investigated by evaluating apoptotic and autophagic protein expression by western blot.
Results: Administration of fructose-streptozotocin increased the blood glucose level of the rats (p < 0.001) and induced pathological changes in the kidney tissues, including glomerulosclerosis, renal tubule dilation, and inflammatory cell infiltration of rats. However, long-term treatment with dapagliflozin attenuated the fructose-streptozotocin-induced increases in Cr, BUN, and urinary protein and reversed the fructose-streptozotocin-induced decrease in Bcl-2 expression and increases in Bax and cleaved PARP expression in diabetic rats. Dapagliflozin also reversed the increases in MDA and LDH and decreases in SOD and GSH in diabetic rats. The fructose-streptozotocin-induced increase in p62 expression and decreases in LC3 and Beclin 1 expression were reversed by dapagliflozin. It upregulated miR-30e-5p expression and downregulated phosphorylated AKT and mTOR expression in diabetic rats. MicroRNA-30e-5p targeted AKT and inhibition of miR-30e-5p attenuated the dapagliflozin-induced decrease in p-AKT and p-mTOR expression in diabetic rats.
Conclusion: In fructose-streptozotocin-induced diabetic rats, dapagliflozin ameliorates kidney injury, suppresses cell apoptosis and oxidative stress, and promotes cell autophagy through upregulation of miR-30e-5p and inactivation of the AKT/mTOR pathway. Therefore, dapagliflozin is a potent therapeutic agent for the management of diabetic nephropathy.

Keywords: Dapagliflozin, Apoptosis, Oxidative stress, Autophagy, Diabetic nephropathy, Fructose-streptozotocin
INTRODUCTION

Diabetic nephropathy is a microvascular complication of diabetes mellitus characterized by persistent clinical proteinuria and decreased glomerular filtration rate [1]. The molecular etiology of diabetic nephropathy has not been elucidated completely; however, research has shown that reactive oxygen species-dependent pathways interact with hemodynamic and metabolic abnormalities, which may lead to the functional and structural changes observed in diabetic nephropathies, such as microalbuminuria [2]. Therefore, strategies to ameliorate hemodynamic and metabolic abnormalities by suppressing oxidative stress may prevent diabetic nephropathy [2].

Sodium-glucose co-transporter 2 is responsible for glucose reabsorption in the renal proximal tubule and its activity and expression are enhanced in type 2 diabetes mellitus [3]. Dapagliflozin (DAPA) is an inhibitor of sodium-glucose co-transporter 2 that is used widely to treat chronic kidney disease [4]. Dapagliflozin has also been used clinically to treat diabetic nephropathy [5]. However, the intracellular signaling mechanism involved in dapagliflozin-mediated amelioration of diabetic nephropathy remains unknown.

Previous research has shown that dapagliflozin upregulates expression of circulating miR-30e-5p in patients with type 2 diabetes mellitus [6]. In addition, miR-30e was lower in the plasma of patients with microalbuminuria than in patients with normal albuminuria suggesting that miR-30e could be a diagnostic biomarker for diabetic nephropathy [2]. Therefore, the objective of this study was to investigate the mechanism by which dapagliflozin may arrest the progression of diabetic nephropathy.

EXPERIMENTAL

Animal model

Forty-six male Wistar albino rats (6 - 8 weeks old) were acquired from Zhengzhou University, Henan, China and were housed in cages in a controlled temperature and humidity. The rats had free access to the standard diet and water. The study was approved by the Medical Ethics Committee of the University of South China (approval no. 2020-1012) and was in accordance with the National Institutes of Health Laboratory Animal Care and Use Guidelines [8]. The rats were divided into a sham group (n = 6) and an experimental group (n = 40). Rats in the experimental group were given water containing 10 % fructose (PHR1002; Sigma-Aldrich, St Louis, MO, USA) for 2 weeks. Rats in the sham group were given plain water. The experimental rats were then injected intraperitoneally with 40 mg/kg streptozotocin (V900890; Sigma-Aldrich) and the sham rats were injected with distilled water. Three days later, blood glucose levels were measured using Accu-Chek Active (Roche Diagnostics, Mannheim, Germany). Non-fasting blood glucose levels of the diabetic rats were > 300 mg/dL.

The diabetic rats (n = 30) were divided into four groups: diabetic nephropathy (DN; n = 6), DN + 0.5 mg/kg dapagliflozin (DN + 0.5 mg/kg DAPA; n = 6), DN + 1 mg/kg DAPA (n = 6), and DN + 2 mg/kg DAPA (n = 12). Rats in the DAPA groups were administered dapagliflozin orally and daily for 8 weeks. Rats in the DN + 2 mg/kg DAPA group were divided into two groups: DN + 2 mg/kg DAPA + negative control (NC) inhibitor (n = 6) and DN + 2 mg/kg DAPA + miR-30e-5p inhibitor (n = 6). The NC inhibitor or the miR-30e-5p inhibitor (100 nM; Geneharma, Suzhou, China) was injected intravenously twice a week for 8 weeks.

Blood glucose levels were measured every two weeks and urine samples were collected. The rats were euthanized and then blood samples were collected via cardiac puncture and kidney tissues were isolated. The timeline of the treatments is shown in Figure 1.

Histopathological analysis, TUNEL staining, and immunofluorescence staining

Isolated kidney tissues were fixed in 10 % neutral-buffered formalin for 24 h, embedded in paraffin, and then sliced into 5 μm sections using a Reichert-Jung microtome (Vienna, NJ, USA). The sections were dewaxed in xylene, rehydrated in alcohol, and then stained with hematoxylin (MHS1; Sigma-Aldrich) for 5 min and eosin (E4009; Sigma-Aldrich) for 2 min before visualization using a microscope (Olympus, Tokyo, Japan).

The dewaxed and rehydrated sections were incubated with Proteinase K (Sigma-Aldrich) and immersed in 0.5 % H2O2. The sections were then treated using the TUNEL in situ cell death assay kit (Roche, Basel, Switzerland) and DAPI for counterstaining and examined using a microscope (Olympus, Tokyo, Japan).
antibody (Cell Signaling Technology), and the sections were examined using a fluorescence microscope (Olympus, Tokyo, Japan).

Biochemical determinations

Commercially available kits were used to measure serum levels of creatinine (Cr; KA0849; Abnova Corporation, Taipei, Taiwan), blood urea nitrogen (BUN; MAK006; Sigma-Aldrich), and urinary protein in 24 h urine samples (MBS016920; Biocompare, San Mateo, CA, USA). Tissues were lysed in radio-immunoprecipitation assay (RIPA) buffer (89900; Thermo Scientific, Waltham, MA, USA), and protein concentrations were measured using the BCA protein assay kit (23225; Thermo Scientific). ELISA kits (Sigma-Aldrich) were used to measure malondialdehyde (MDA; MAK085), superoxide dismutase (SOD; MAK379), reduced glutathione (GSH; MAK364), and lactate dehydrogenase (LDH; MAK066) levels. The Caspase 3 Assay Kit (CASP3F; Sigma-Aldrich) was used to measure caspase-3 activity.

Quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

RNAs were isolated from kidney tissues using TRIzol (15596026; Invitrogen, Carlsbad, CA, USA) and then reverse-transcribed into cDNAs. The cDNAs and SYBR Green Master Mix (03064760001; Roche, Mannheim, Germany) were used in the qRT-PCR analysis of miR-30e-5p. U6 was used as the endogenous control. The primer sequences used are shown in Table 1.

Dual-luciferase reporter assay

The wild-type and mutant 3'UTR AKT1 sequences were sub cloned individually into the pmirGLO luciferase reporter vector (E1330; Promega, Madison, Wisconsin, USA) and named pmirGLO-AKT1-WT and pmirGLO-AKT1-MUT, respectively. HEK-293 cells were co-transfected with a pmirGLO luciferase reporter vector and the miR-30e-5p mimic or the NC mimic (GenePharma). After two days, luciferase activities were measured using the Lucifer Reporter Assay System (E1483; Promega) and the Sirius Luminometer (Berthold Detection Systems, Huntsville, AL, USA). The ratio of the average activity (firefly luciferase activity/renilla luciferase activity) was calculated.

Western blot assay

Proteins were extracted from kidney tissues using RIPA buffer (Beyotime, Beijing, China), separated by SDS-PAGE, and transferred onto polyvinylidene difluoride membranes. The membranes were blocked in 5 % bovine serum albumin and probed with the primary antibodies anti-Bax and anti-Bcl-2 (1:2000; Abcam, Cambridge, MA, USA), anti-cleaved PARP and anti-β-actin (1:2500; Abcam), anti-LC3, anti-Bec-l1, and anti-p62 (1:3000; Abcam), anti-AKT and anti-p-AKT (1:3500; Abcam), anti-mTOR and anti-p-mTOR (1:4000; Abcam), and anti-S6K and anti-p-S6K (1:4500; Abcam). Then, the membranes were incubated with the corresponding horseradish peroxidase-conjugated secondary antibody (1:5000; Abcam) and ECL reagent (345818; Millipore, Billerica, MA, United States) for visualization and analysis by Image J. Protein levels were normalized to β-actin.

Statistical analysis

All data were analyzed using the student’s t-test or one-way analysis of variance and were expressed as mean ± standard deviation (SD). P-values < 0.05 were considered statistically significant.

RESULTS

Dapagliflozin attenuated the pathological changes

To establish an in vivo diabetic nephropathy model, rats were treated with fructose-streptozotocin and this treatment increased blood glucose levels (Figure 1 A). Hematoxylin and eosin staining showed that the structures of the renal corpuscles in the sham group rats were normal (Figure 1 B), whereas glomerulosclerosis, renal tubule dilation, and inflammatory cell infiltration were observed in the kidneys of the rats treated with fructose-streptozotocin (Figure 1 B). Fructose-streptozotocin induced glomerulosclerosis, renal tubule dilation, and inflammatory cell infiltration in kidneys, whereas dapagliflozin attenuated these histopathological changes.

Table 1: Primer sequences

| Gene   | Forward (5’-3’)                  | Reverse (5’-3’)                  |
|--------|----------------------------------|----------------------------------|
| miR-30e-5p | TGTAAAGCATCCCTTGACTGGAAGG         | CCAGTGCGAATACCTCGGAC             |
| U6     | CTCGGTCTGGCAGGACA                 | AACGGTTCCAGAATTTGCCT             |
At least five fields of stained tissue were evaluated. G indicates glomerular areas, green arrows indicate vacuolation of renal cells, black arrows indicate glomerular fragmentation with increased Bowman space, and blue arrows indicate diffuse interstitial inflammation. (Figure 1 B).

In addition, levels of the renal function markers Cr and BUN increased in diabetic rats (Figure 1 C); however, dapagliflozin reduced the Cr and BUN levels in diabetic rats (Figure 1 C). The urinary protein levels also increased in diabetic rats but decreased with dapagliflozin (Figure 1 D). Furthermore, dapagliflozin reduced blood glucose levels in diabetic rats in a dose-dependent manner (Figure 1 E) suggesting that dapagliflozin has an anti-diabetic effect and is effective against diabetic nephropathy.

Figure 1: Dapagliflozin attenuated the pathological changes in the kidneys of rats with diabetic nephropathy. (A) Blood glucose levels increased in rats upon fructose-streptozotocin injection. (B) Fructose-streptozotocin induced glomerulosclerosis, renal tubule dilatation, and inflammatory cell infiltration in kidneys, whereas dapagliflozin attenuated these histopathological changes. Scale bar: 200 μm. (C) Dapagliflozin attenuated the fructose-streptozotocin-induced increase in Cr and BUN in rats. (D) Dapagliflozin attenuated the fructose-streptozotocin-induced increase in urinary protein. (E) Dapagliflozin decreased the blood glucose levels. @@@ @ @ @P < 0.001 vs. sham; ** @ @ @P < 0.01, *** @ @ @P < 0.001 vs. DN

Dapagliflozin attenuated renal cell apoptosis

The number of TUNEL-positive renal cells increased in rats with diabetic nephropathy (Figure 2 A) suggesting that apoptosis of renal cells was promoted in diabetic rats. Dapagliflozin decreased the number of TUNEL-positive cells in diabetic rats (Figure 2 A). Dapagliflozin also attenuated the fructose-streptozotocin-induced increase in caspase-3 activity in rats (Figure 2 B). Moreover, the decrease in Bcl-2 protein expression and the increases in Bax and cleaved PARP protein expression in diabetic rats were reversed upon dapagliflozin (Figures 2 C and D). These data revealed the anti-apoptotic effect of dapagliflozin against diabetic nephropathy.

Figure 2: Dapagliflozin attenuated renal cell apoptosis in rats with diabetic nephropathy. (A) Dapagliflozin attenuated the fructose-streptozotocin-induced increase in TUNEL-positive renal cells. Scale bar: 200 μm. (B) Dapagliflozin attenuated the fructose-streptozotocin-induced increase in caspase-3 activity. (C) Dapagliflozin attenuated the fructose-streptozotocin-induced decrease in Bcl-2 expression and increases in Bax and cleaved PARP expression. (D) Relative expression of Bcl-2, Bax, and cleaved PARP. @@@ @ @ @P < 0.001 vs. sham; * @ @ @P < 0.05, *** @ @ @P < 0.001 vs. DN

Dapagliflozin attenuated renal oxidative stress

In addition to its anti-apoptotic effect, dapagliflozin attenuated the fructose-streptozotocin-induced increase in MDA (Figure 3 a), decrease in SOD (Figure 3 b), decrease in GSH (Figure 3 c), and increase in LDH (Figure 3 d) in rats. These data reveal the anti-oxidant effect of dapagliflozin in diabetic nephropathy.

Dapagliflozin promoted renal autophagy

Protein expression of p62 increased and protein expression of LC3 and Beclin1 decreased in diabetic rats suggesting that fructose-streptozotocin suppressed autophagy in rats (Figure 4 A). However, dapagliflozin reversed the effects of fructose-streptozotocin on LC3, Beclin1, and p62 protein expressions in rats (Figure 4 A). Dapagliflozin also increased expression of LC3II in the kidney tissues of rats.
with diabetic nephropathy (Figure 4 B) demonstrating the pro-autophagic role of dapagliflozin in diabetic nephropathy.

**Figure 3:** Dapagliflozin attenuated renal oxidative stress in rats with diabetic nephropathy. (A) Dapagliflozin attenuated the fructose-streptozotocin-induced increase in MDA. (B) Dapagliflozin attenuated the fructose-streptozotocin-induced decrease in SOD. (C) Dapagliflozin attenuated the fructose-streptozotocin-induced decrease in GSH. (D) Dapagliflozin attenuated the fructose-streptozotocin-induced decrease in LDH. @@@P < 0.001 vs. sham; *p < 0.05, **P < 0.01, ***P < 0.001 vs. DN

**Figure 4:** Dapagliflozin promoted renal autophagy in rats with diabetic nephropathy. (A) Dapagliflozin attenuated the fructose-streptozotocin-induced increase in p62 expression and decreases in LC3 and Beclin1 expression. (B) Immunofluorescence showed that dapagliflozin increased LC3II expression in the kidneys of rats with diabetic nephropathy. @@@P < 0.001 vs. sham; *p < 0.05, **p < 0.001 vs. DN

**Dapagliflozin increased miR-30e-5p expression and suppressed activation of AKT/mTOR**

Expression of miR-30e-5p was downregulated in diabetic rats and upregulated with dapagliflozin treatment (Figure 5 A). Protein expression of AKT, p-AKT, p-mTOR, and p-S6K were upregulated in diabetic rats and downregulated upon dapagliflozin treatment (Figure 5 B and C). These results demonstrated that dapagliflozin increased miR-30e-5p expression and suppressed activation of AKT/mTOR in diabetic rats.

**Figure 5:** Dapagliflozin increased miR-30e-5p expression and suppressed activation of AKT/mTOR in rats with diabetic nephropathy. (A) Dapagliflozin attenuated the fructose-streptozotocin-induced decrease in miR-30e-5p expression. (B) Dapagliflozin attenuated the fructose-streptozotocin-induced increase in AKT, p-AKT, p-mTOR, and p-S6K expression (C) Ratios of p-AKT/AKT, p-mTOR/mTOR, and p-S6K/S6K expression. @@@P < 0.001 vs. sham; *p < 0.05, **P < 0.001 vs. DN

**MiR-30e-5p targeted AKT/mTOR**

It was predicted that AKT1 contains the potential binding site of miR-30e-5p (Figure 6 A). Overexpression of miR-30e-5p reduced pmirGLO-AKT1-WT luciferase activity (Figure 6 B) indicating that miR-30e-5p binds to the 3’UTR of AKT1. Inhibition of miR-30e-5p by injection with the miR-30e-5p inhibitor attenuated the dapagliflozin-induced decrease in AKT, p-AKT, p-mTOR, and p-S6K expression in diabetic rats (Figure 6 C) indicating that dapagliflozin suppresses AKT/mTOR activation by upregulating miR-30e-5p, thereby ameliorating diabetic nephropathy.
Inhibition of miR-30e-5p reversed the protective effect of dapagliflozin

Inhibition of miR-30e-5p attenuated dapagliflozin-induced decreases in Cr and BUN in rats with diabetic nephropathy (Figure 7 A). Urinary protein (Figure 7 B) and caspase-3 activity (Figure 7 C) increased in rats with diabetic nephropathy who were treated with the miR-30e-5p inhibitor. In addition, knockdown of miR-30e-5p weakened the dapagliflozin-induced decrease in MDA and increased SOD and GSH (Figure 7 D) in rats with diabetic nephropathy. miR-30e-5p inhibition reversed the dapagliflozin-induced decrease in p62 protein expression and the increases in LC3 and Beclin 1 protein expression in rats with diabetic nephropathy (Figure 7 E). These results showed that inhibition of miR-30e-5p reversed the protective effect of dapagliflozin on the renal system.

DISCUSSION

The anti-diabetic effect of sodium/glucose co-transporter 2 (SGLT2) inhibitors against diabetic nephropathy has been investigated widely [5]. Dapagliflozin was shown to exert anti-apoptotic, anti-inflammatory, and anti-angiogenic renoprotective effects on diabetic nephropathy in rats [9]. This study found that dapagliflozin conferred a renoprotective effect on diabetic nephropathy in rats via the miR-30e-5p/AKT/mTOR pathway.

Figure 6: miR-30e-5p targeted AKT/mTOR. (A) Potential binding between the 3’ UTR of AKT1 and miR-30e-5p. (B) Overexpression of miR-30e-5p reduced pmirGLO-AKT1-WT luciferase activity. (C) Inhibition of miR-30e-5p attenuated the dapagliflozin-induced decreases in AKT, p-AKT, p-mTOR, and p-S6K expression in diabetic rats. &P < 0.05, ##P < 0.01, ###P < 0.001 vs. sham; **P < 0.001 vs. DN; ###P < 0.001 vs. DN + 2 mg/kg DAPA

Figure 7: Inhibition of miR-30e-5p reversed the protective effect of dapagliflozin against diabetic nephropathy. (A) Inhibition of miR-30e-5p attenuated the dapagliflozin-induced decreases in Cr and BUN. (B) Inhibition of miR-30e-5p attenuated the dapagliflozin-induced decrease in caspase-3 activity. (D) Inhibition of miR-30e-5p attenuated the dapagliflozin-induced decrease in MDA and increases in SOD and GSH. (E) Inhibition of miR-30e-5p attenuated the dapagliflozin-induced decrease in p62 expression and increases in LC3 and Beclin 1 expression. ###P < 0.001 vs. sham; ***P < 0.001 vs. DN; ###P < 0.001 vs. DN + 2 mg/kg DAPA

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The fructose-streptozotocin injection has been shown to induce renal tubular injury, renal interstitial fibrosis, glomerulosclerosis, and renal histopathological changes, and has been used widely to establish an animal model of diabetic nephropathy [10]. In this study, blood glucose, Cr, BUN, and urinary protein increased in rats upon fructose-streptozotocin injection confirming the successful establishment of diabetic nephropathy. In addition, dapagliflozin decreased blood glucose, Cr, BUN, and urinary protein levels and ameliorated the renal histopathological changes induced by fructose-streptozotocin, thus dapagliflozin provided significant renal protection against early diabetic nephropathy in rats. This study confirmed the anti-diabetic role of dapagliflozin in diabetic nephropathy.

A previous study showed that hyperglycemia and hypertension induced renal cell apoptosis and caused renal damage leading to the development of diabetic nephropathy [11]. Hyperglycemia and an increase in advanced glycation end-products led to increased accumulation of reactive oxygen species and decreased expression of the anti-oxidant enzyme SOD and GSH, thereby contributing to the progression of diabetic nephropathy [12]. The anti-apoptotic and anti-oxidant effects of dapagliflozin on diabetic nephropathy have been investigated previously [9,13].

This study showed that dapagliflozin attenuated the fructose-streptozotocin-induced decrease in Bcl-2 expression and increased caspase-3 activity and Bax and cleaved PARP expression. Dapagliflozin also attenuated the fructose-streptozotocin-induced increases in MDA and LDH and decreases in SOD and GSH in diabetic rats. Thus, these results confirmed the anti-apoptotic and anti-oxidant effects of dapagliflozin on diabetic nephropathy.

Overactivation or inhibition of autophagy causes podocyte injury during the development of diabetic nephropathy, and a deficiency in podocyte autophagy was observed in patients with diabetes and macroalbuninuria [14]. Impaired autophagy leads to the onset of diabetic nephropathy suggesting that restoration of autophagy may be a promising strategy for the treatment of diabetic nephropathy [14]. It was shown that dapagliflozin attenuated the palmitic acid-induced increase in p62 expression and decreases in LC3B and Beclin1 expression to restore autophagy [15].

This study also showed that dapagliflozin decreased p62 expression and increased expression of the autophagy-related markers LC3 and Beclin1 in diabetic rats indicating a pro-autophagic effect of dapagliflozin in diabetic nephropathy.

In accordance with previous research that showed that dapagliflozin upregulated expression of circulating miR-30e-5p in patients with type 2 diabetes mellitus [6], expression of miR-30e-5p was also upregulated by dapagliflozin in diabetic rats. Overexpression of miR-30e was shown to promote autophagy in cardiomyocytes and ameliorate ischemia/hypoxia-induced injury [16]. Therefore, dapagliflozin may promote autophagy and ameliorate diabetic nephropathy by increasing miR-30e-5p expression. It has also been shown that suppression of AKT/mTOR alleviated glomerular pathological changes during the progression of diabetic nephropathy [17]. Dapagliflozin was found to regulate the AKT/mTOR pathway and attenuate myocardial ischemia/reperfusion injury [18].

This study showed that dapagliflozin reduced protein expression of AKT, p-AKT, and p-mTOR, thereby suppressing activation of the AKT/mTOR pathway in diabetic rats. Moreover, AKT1 was identified as the binding target of miR-30e-5p in this study, and inhibition of miR-30e-5p attenuated the dapagliflozin-induced decreases in AKT, p-AKT, and p-mTOR expression in diabetic rats. Therefore, dapagliflozin may increase miR-30e-5p expression to suppress activation of the AKT/mTOR pathway to attenuate diabetic nephropathy.

CONCLUSION

The findings of this study show that dapagliflozin exerts anti-apoptotic, anti-oxidant, and pro-autophagic effects against fructose-streptozotocin-induced diabetic nephropathy in rats through miR-30e-5p-mediated inactivation of AKT/mTOR pathway. This finding provides a potential strategy for the treatment of diabetic nephropathy.

DECLARATIONS

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Ethical approval

None provided.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Jun Zhang and Ting Ding designed the study and supervised the data collection. XIMEi Zhang analyzed and interpreted the data. Dongxing Tang and Jianping Wang prepared the manuscript for publication and reviewed the draft of the manuscript. All authors read and approved the manuscript.

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