ISSN 1948-9358 (online)

World Journal of *Diabetes*

World J Diabetes 2022 August 15; 13(8): 587-667

Published by Baishideng Publishing Group Inc

World Journal of Diabetes

Contents

Monthly Volume 13 Number 8 August 15, 2022

MINIREVIEWS

ORIGINAL ARTICLE

Basic Study

Case Control Study

SYSTEMATIC REVIEWS

LETTER TO THE EDITOR

World Journal of Diabetes

Monthly Volume 13 Number 8 August 15, 2022

Contents

ABOUT COVER

Editorial Board Member of *World Journal of Diabetes*, Wei Wang, MD, PhD, Chief Physician, Professor, Director, Department of Endocrinology, Xiang'an Hospital of Xiamen University, School of Medicine, Xiamen University, Xiamen 361101, Fujian Province, China. wwang@xah.xmu.edu.cn

AIMS AND SCOPE

The primary aim of *World Journal of Diabetes* (*WJD*, *World J Diabetes*) is to provide scholars and readers from various fields of diabetes with a platform to publish high-quality basic and clinical research articles and communicate their research findings online.

WJD mainly publishes articles reporting research results and findings obtained in the field of diabetes and covering a wide range of topics including risk factors for diabetes, diabetes complications, experimental diabetes mellitus, type 1 diabetes mellitus, type 2 diabetes mellitus, gestational diabetes, diabetic angiopathies, diabetic cardiomyopathies, diabetic coma, diabetic ketoacidosis, diabetic nephropathies, diabetic neuropathies, Donohue syndrome, fetal macrosomia, and prediabetic state.

INDEXING/ABSTRACTING

The *WJD* is now abstracted and indexed in Science Citation Index Expanded (SCIE, also known as SciSearch®), Current Contents/Clinical Medicine, Journal Citation Reports/Science Edition, PubMed, PubMed Central, Reference Citation Analysis, China National Knowledge Infrastructure, China Science and Technology Journal Database, and Superstar Journals Database. The 2022 Edition of Journal Citation Reports® cites the 2021 impact factor (IF) for *WJD* as 4.560; IF without journal self cites: 4.450; 5-year IF: 5.370; Journal Citation Indicator: 0.62; Ranking: 62 among 146 journals in endocrinology and metabolism; and Quartile category: Q2.

RESPONSIBLE EDITORS FOR THIS ISSUE

NAME OF JOURNAL
World Journal of Diabetes

ISSN
ISSN 1948-9358 (online)

LAUNCH DATE
June 15, 2010

FREQUENCY
Monthly

EDITORS-IN-CHIEF
Lu Cai, Md. Shahidul Islam, Jian-Bo Xiao, Michael Horowitz

EDITORIAL BOARD MEMBERS
https://www.wjgnet.com/1948-9358/editorialboard.htm

PUBLICATION DATE
August 15, 2022

INSTRUCTIONS TO AUTHORS
https://www.wjgnet.com/bpg/gerinfo/204

GUIDELINES FOR ETHICS DOCUMENTS
https://www.wjgnet.com/bpg/GerInfo/287

GUIDELINES FOR NON-NATIVE SPEAKERS OF ENGLISH
https://www.wjgnet.com/bpg/gerinfo/240

PUBLICATION ETHICS
https://www.wjgnet.com/bpg/GerInfo/288

PUBLICATION MISCONDUCT
https://www.wjgnet.com/bpg/gerinfo/208

ARTICLE PROCESSING CHARGE
https://www.wjgnet.com/bpg/gerinfo/242

STEPS FOR SUBMITTING MANUSCRIPTS
https://www.wjgnet.com/bpg/GerInfo/239

ONLINE SUBMISSION
https://www.f6publishing.com

E-mail: bpgoffice@wjgnet.com https://www.wjgnet.com

World Journal of Diabetes

Submit a Manuscript: https://www.f6publishing.com

World J Diabetes 2022 August 15; 13(8): 587-599

DOI: 10.4239/wjd.v13.i8.587

ISSN 1948-9358 (online)

MINIREVIEWS

Diabetic kidney disease in pediatric patients: A current review

Carmen Muntean, Iuliana Magdalena Starcea, Claudia Banescu

Specialty type: Endocrinology and metabolism

Provenance and peer review: Invited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification

Grade A (Excellent): 0
Grade B (Very good): B, B
Grade C (Good): 0
Grade D (Fair): 0
Grade E (Poor): 0

P-Reviewer: He Z, China; Zavaleta MJC, Peru

Received: March 27, 2022
Peer-review started: March 27, 2022
First decision: May 30, 2022
Revised: June 13, 2022
Accepted: July 11, 2022
Article in press: July 11, 2022
Published online: August 15, 2022

Carmen Muntean, Department of Pediatrics I, "George Emil Palade" University of Medicine, Pharmacy, Sciences and Technology of Târgu Mures, Târgu Mures 540142, Romania

Iuliana Magdalena Starcea, Department of IVth Pediatrics, University of Medicine and Pharmacy "Grigore T. Popa", Iasi 700115, Romania

Claudia Banescu, Center for Advanced Medical and Pharmaceutical Research, University of Medicine, Pharmacy, Sciences and Technology of Târgu Mureş, Mureş, Târgu Mures 540142, Romania

Corresponding author: Carmen Muntean, MD, PhD, Associate Professor, Department of Pediatrics I, "George Emil Palade" University of Medicine, Pharmacy, Sciences and Technology of Târgu Mures, Gheorghe Marinescu No. 38, Târgu Mures 540142, Romania. duicucarmen@yahoo.com

Abstract

In the last decades, a significant increase in the incidence of diabetic kidney disease (DKD) was observed concomitant with rising diabetes mellitus (DM) incidence. Kidney disease associated with DM in children and adolescents is represented by persistent albuminuria, arterial hypertension, progressive decline in estimated glomerular filtration rate to end-stage renal disease and increased cardiovascular and all-cause morbidity and mortality of these conditions. In medical practice, the common and still the "gold standard" marker for prediction and detection of diabetic kidney involvement in pediatric diabetes is represented by microalbuminuria screening even if it has low specificity to detect early stages of DKD. There are some known limitations in albuminuria value as a predictor biomarker for DKD, as not all diabetic children with microalbuminuria or macroalbuminuria will develop end-stage renal disease. As tubular damage occurs before the glomerular injury, tubular biomarkers are superior to the glomerular ones. Therefore, they may serve for early detection of DKD in both type 1 DM and type 2 DM. Conventional and new biomarkers to identify diabetic children and adolescents at risk of renal complications at an early stage as well as renoprotective strategies are necessary to delay the progression of kidney disease to end-stage kidney disease. New biomarkers and therapeutic strategies are discussed as timely diagnosis and therapy are critical in the pediatric diabetic population.

Key Words: Diabetes; Kidney disease; Biomarkers; Microalbuminuria; Therapy; Children

Core Tip: Several reviews in the literature contributed to the pathophysiology, diagnostics and therapeutic options for diabetic kidney disease in pediatric patients. In this review, we reported the latest data regarding novel biomarkers and methods to identify diabetic children and adolescents at risk of renal complications at an early stage as well as renoprotective strategies to delay the progression of kidney disease to end-stage kidney disease.

Citation: Muntean C, Starcea IM, Banescu C. Diabetic kidney disease in pediatric patients: A current review. *World J Diabetes* 2022; 13(8): 587-599
URL: https://www.wjgnet.com/1948-9358/full/v13/i8/587.htm
DOI: https://dx.doi.org/10.4239/wjd.v13.i8.587

INTRODUCTION

Diabetes mellitus (DM), a chronic metabolic condition, is characterized by complete or insufficient insulin production. The main form of DM in childhood and adolescence is type 1 DM (T1DM) compared to type 2 DM (T2DM), which is more frequent in adulthood. Within the last 20 years, DM prevalence increased significantly worldwide. In the last decades, we have also assisted in an ascending trend in the prevalence of T2DM in childhood and youth because of the outbreak in juvenile obesity prevalence [1]. T1DM and T2DM have similar symptoms upon diagnosis, and both include polyuria, polydipsia and polyphagia. While obesity and insulin resistance signs (acanthosis nigricans and polycystic ovarian syndrome) are typical hallmarks of T2DM, loss of weight may be present in both types of DM[1].

Both T1DM and T2DM, with lasting inadequate glycemic control, are associated with long-term vascular complications[2] and a significant increase in mortality, especially in those who develop kidney disease[3]. While DM represents the main worldwide cause of end-stage kidney disease in adults, this is uncommon during childhood[2,3].

Although specific kidney structural changes in DM patients, namely thickening of the glomerular basement membrane and mesangial expansion, appear soon after DM onset (1.5 years to 5.0 years), they are in a clinically silent phase[4]. These structural changes of diabetic kidney injury progress at different rates among T1DM patients, and this is more evident in T2DM cases[4]. Clinical and biological abnormalities (micro/macroalbuminuria) and glomerular filtration rate (GFR) decline will develop over a longer period (10 years to 25 years)[3]. This emphasizes that diabetic kidney disease (DKD) starts early. Therefore, an early diagnosis, intensive monitoring and therapeutic interventions are necessary. Albuminuria and changes in GFR, which are late biomarkers, are the most used tools to assess kidney involvement. Diagnostic strategies for early diagnosis of kidney involvement are necessary.

There are several reviews in the literature that contributed to the pathophysiology, diagnostics and therapeutic options for DKD in pediatric patients. In this work, the state-of-the-art novel biomarkers and methods to identify diabetic children and adolescents at risk of renal complications at an early stage as well as renoprotective strategies to delay the progression of kidney disease to end-stage kidney disease was carried out.

EPIDEMIOLOGY OF DM IN CHILDREN

From 2002 to 2015 the Centers for Disease Control and Prevention reported a 4.8% increase *per* year for T1DM and a 1.9% increase *per* year for T1DM in youths aged < 20 years[5]. A very recent study, comprising six areas of the United States from 2001 to 2017, reported an important increase in estimated prevalence for both T1DM and T2DM (T1DM from 1.48 to 2.15 *per* 1000 youths < 19 years and T2DM from 0.34 to 0.67 *per* 1000 youths among those aged 10-19 years)[6]. Up-to-date research that included a large cohort of Hungarian children and teenagers during the period 2001 to 2016 (covering 16 years), showed that T1DM is still the most common type, and its prevalence is rising, with a significant male predominance (male/female ratio: 1.25). Also, there is a high prevalence of T2DM, affecting more females every year (female/male ratio: 2.86)[7]. A Danish study showed no increase in T2DM prevalence in children and adolescents[8], while in the United Kingdom a rising incidence and prevalence of T2DM have been observed in youths, especially in some ethnicities[9].

Contributing risk factors to this major increase in incidence are obesity, race, ethnicity, exposure to maternal obesity and diabetes as well as exposure to environmental contaminants[6]. There is an increased morbidity and mortality rate, mainly in T1DM and in those with early T2DM onset. According to Rhodes *et al*[10], a considerably lower life expectancy (approximately 15 years) was observed in the diabetic group compared to the general population of children without diabetes[10]. A significantly shorter life expectancy was reported in children developing T1DM before 10 years of age (loss of 17.7 years for females *vs* 14.0 years for males) compared with those diagnosed at 25-30 years

(loss of 10.0 years for females and 9.4 years for males)[11]. There is a double cardiovascular risk in pediatric diabetes that triggers early cardiovascular mortality and a four-fold higher mortality rate for all causes in youth[12]. In a nationwide Swedish study of patients with T1DM, age before 10 years at diabetes onset, was the most important risk factor for survival and cardiovascular disease (coronary heart disease and acute myocardial infarction) in their early adult years, especially in females (2-3-fold higher *vs* males)[13].

DM represents the main cause of end-stage renal disease (ESRD) worldwide in adults[14]. Diabetic nephropathy affects 20% (1 in 5) of adults with diabetes[15]. Within the pediatric population, a significant increase in the incidence of DKD was also observed, the prevalence rate being three times higher in 2013 compared to 2002 (1.16% to 3.44%)[16].

A 4-fold higher risk of kidney failure was found in a large cohort of youth with T2DM *vs* those with T1DM[17]. Also, compared with the control group, those with youth-onset T2DM had a 16-fold higher risk of a kidney disorder, a 23-fold higher risk of severe renal injury and a 39-fold increased risk of ESRD[17]. A multicenter study reported that more than a quarter (28%) of T2DM youth aged under 20 years developed microalbuminuria[18].

PATHOPHYSIOLOGY

Chronic hyperglycemia leads to the occurrence of diabetic nephropathy, retinopathy and neuropathy as well as macrovascular complications (cardiovascular disease: Stroke, coronary artery disease, peripheral vascular disease)[1,19,20]. DKD recognizes four major pathogenic mechanisms: Glomerular damage, tubular injury, inflammation and oxidative stress[21] (Figure 1). In DKD patients there are important alterations in tubules as well as in the interstitium. These findings may pave the way, or they may appear concomitant with glomerular changes[22].

This is sustained by tubular hypertrophy observed in the immediate future of hyperglycemia. Also, an increase in tubular basement membrane thickening was found even among diabetic patients with normoalbuminuria. Tubular basement membrane is one of the location of the earliest structural changes. Therefore, it may represent a better severity marker of DKD than glomerular basement membrane alteration[22]. Pathological glomerular changes in DKD are typical and consist of glomerular basement membrane thickening, podocyte foot process widening, expansion of the mesangial matrix and loss of endothelial fenestrations[23].

There is a greater risk for complication occurrence in youths with T2DM *vs* adults with T1DM and T2DM[1]. The main microvascular complication of diabetes is represented by DKD and later by diabetic nephropathy, which finally leads to ESRD. In time, with diabetes evolution, clinical and biological changes will be observed (Figure 2). DKD, one of the most important and frequent complications of DM, recognizes a wide spectrum of risk factors, some of which are modifiable. Therefore, DKD occurrence or evolution may be considerably influenced by strict control of these factors that are listed in Table 1. Children with T1DM may have damaged renal function at the disease onset as acute complications through acute kidney injury (AKI) and renal tubular damage as well as chronic complications by diabetic nephropathy development[24].

Genetic aspects

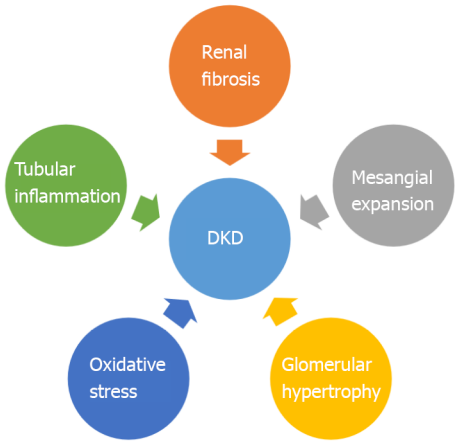
DKD is a multifactorial disorder and is influenced by genetic susceptibility, epigenetics and environmental factors (such as lifestyle, diet and medication). Also, oxidative stress, metabolic disturbance, activation of the renin-angiotensin-aldosterone system and production of inflammatory factors are involved in the development and progression of DKD[25]. Genetic and epigenetic studies were performed to understand the pathogenesis of the DKD and to identify genes that confer susceptibility to disease. Genetic studies of DKD investigated mainly the association between genomic DNA variants (for example, single nucleotide polymorphisms, copy number variants, *etc*) and clinical phenotypes of DKD in both T1DM and T2DM[26]. Epigenetic modifications (histone modifications and DNA methylation) may play a critical role in DKD as it was shown that histone acetylation and methylation are involved in the regulation of inflammation and fibrosis in DKD[27]. Epigenetics studies of DKD investigated the potentially inherited changes in gene expression that occur without changing the DNA nucleotide sequence.

Candidate gene association studies, genome-wide association studies (GWAS) and epigenome-wide association studies were performed in DKD patients[28]. A large meta-analysis study conducted by Mooyaart[29] identified 24 genetic variants in 16 genes (*EPO, APOE, APOC1, ACE, ALR2, eNOS, HSPG2, VEGF, FRMD3, GREM1, ELMO1, CCR5 and CNDP1, CARS, UNC13B* and *CPVL/CHN2*), which are the most likely to be associated with diabetic nephropathy[29]. Recently, Tziastoudi *et al*[30] conducted a systematic review and meta-analysis of genetic association studies in diabetic nephropathy in order to elucidate the contribution of genetic background in the development of this disease and observed an association with the genes revealed by Mooyaart[29] and some additional genes (*ACACB, ADIPOQ, AGT, AGTR1, AKR1B1, ATP1B2, ATP2A3, CGNL1, CNDP1, CYGB-PRCD, EDN1, ENPP1, FLT4, FTO, GLO1, HMGA2, IGF2/INS/TH* cluster, interleukin genes (*IL1B, IL8, IL10*), *KCNQ1, KNG, LOC101927627,*

Table 1 Risk factors for diabetic kidney disease development

Non-modifiable	Modifiable
Small/young age at DM onset	Poor glycemic control
Diabetes duration	Glucose variability: Hypo/hyperglycemia
Puberty	Overweight/obesity
Family history of diabetic complications and insulin resistance	Dyslipidemia
Genetic factors	High blood pressure
Race/ethnicity	Microalbuminuria
	Smoking, alcohol
	Intrauterine exposure (maternal diabetes, obesity)
	Low birth weight

DM: Diabetes mellitus.



DOI: 10.4239/wjd.v13.i8.587

Figure 1 Pathogenesis in diabetic kidney disease. DKD: Diabetic kidney disease.

MTHFR, NOS3, SETD7, SIRT1, SLC2A1, SLC2A2, SLC12A3, SLC19A3, TCF7L2, TGFB1, TIMP1, TTC39C, UNC13B, VEGFA, WTAPP1, WWC1, XYLT1)[30].

Genome-wide association studies identified about 30 genes associated with DKD (for example *ELMO1, CNDP1, FRMD3, MMP9, UMOD, SLC12A3, etc*)[25]. Epigenome-wide association studies identified several genes (for example *TRPM6, AQP9, SLC22A12, HP, HYAL2, AGTX*) that have epigenetic effects on DKD[25]. The data presented above provide further evidence for the contribution of genetic factors in DKD offering new perspectives in the discovery of new therapies for personalized medicine.

DIAGNOSIS

GFR abnormalities

Hyperfiltration, defined as an increase in GFR with more than 2 standard deviations than the mean GFR value, is related to an early increase in renal blood flow and high intraglomerular pressure[31]. In the first phases of DKD, hyperfiltration is observed in up to 40% of diabetic patients[32]. In both T1DM and T2DM, hyperfiltration has been linked to GFR loss[33,34]. Hyperfiltration was noticed more frequently in females *vs* males in both T1DM and T2DM[32,35]. The estimated GFR (eGFR) in children and adolescents with T1DM or T2DM should be screened at diagnosis and then annually[36]. These ongoing changes help us to assess DKD stages, which are presented in Table 2[20,21,37]. Normal GFR values according to child age are listed in Table 3.

Table 2 Diabetic kidney disease stages

Stage	Estimated period	Characteristics	GFR	BP	Biomarker-albuminuria	Biomarker UACR mg/mmoL
1 = hyperfiltration	From diabetes onset to 5 yr	Glomerular hyperfiltration and hypertrophy. No ultrastructure abnormality. A 20% increase in renal size. ↑Renal plasma flow	N/increased	N	Normoalbuminuria < 30 mg/g	< 2
2 = silent	From 2 yr after onset	Mild GBM thickening and interstitial expansion	N	N	Normoalbuminuria < 30 mg/g	< 3
3 = incipient	5–10 yr after onset	More significant changes *vs* stage 2. Moderate tubular and GBM thickening and variable focal mesangial sclerosis	GFR–N or mild decreased	Increasing BP; +/- hypertension	Microalbuminuria appears Albuminuria 30-300 mg/g	2-20
4 = overt	10-15 yr after onset	Marked GBM thickening and variable focal mesangial sclerosis	GFR-decreased < 60 mL/min/1.73 m^2	BP↑	Macroalbuminuria > 300 mg/g	> 20
5 = uremic		Diffuse glomerulosclerosis, ESRD	GFR-marked decreased < 15 mL/min/1.73 m^2	BP↑	Decreasing albuminuria	

UACR: Urinary albumin to creatinine ratio; GBM: Glomerular basement membrane; GFR: Glomerular filtration rate; BP: Blood pressure; ESRD: End-stage renal disease; ↑: Increase; N: Normal.

Table 3 Normal glomerular filtration rate limit at different ages according to KDOQI Guidelines[66] and Hogg *et al*[67]

Age	Gender	Normal GFR
1 wk	Males and females	41 ± 15 mL/min/1.73 m^2
2–8 wk	Males and females	66 ± 25 mL/min/1.73m^2
> 8 wk	Males and females	96 ± 22 mL/min/1.73 m^2
2–12 yr	Males and females	133 ± 27 mL/min/1.73 m^2
13–21 yr	Males	140 ± 30 mL/min/1.73m^2
13–21 yr	Females	126 ± 22 mL/min/1.73m^2

GFR: Glomerular filtration rate.

Seric and urinary biomarkers for DKD

Common markers for kidney injury are creatinine, albuminuria, cystatin C, neutrophil gelatinase-associated lipocalin and alfa-1-microglobulin in plasma and urine. Kidney function in pediatrics is assessed mainly by eGFR according to updated/bedside Schwarts equation eGFR = k × height (cm)/serum creatinine (mg/dL), k = 0.413[38].

In a recent study, 11.5% of Romanian children with T1DM had DKD, manifested as transitory microalbuminuria (7.7%) and incipient diabetic nephropathy (3.8%)[39]. In another research study, T1DM patients were found to have microalbuminuria in 30% of cases, representing the most common microvascular complication. In T1DM children the occurrence of microvascular complications was correlated with metabolic control, higher glycated hemoglobin, albuminuria, systolic blood pressure (SBP), triglycerides and total cholesterol[40].

Microvascular as well as macrovascular complications can lead to serious morbidity and mortality. Nephropathy (which is preceded by microalbuminuria), retinopathy and neuropathy represent diabetic microvascular complications[2,41]. According to the International Society for Pediatric and Adolescent Diabetes guidelines, annual microalbuminuria or urinary protein screening should start from the age of 11 years and after 2 years of diabetes evolution and then annually. It was demonstrated that persistent microalbuminuria predicts the progression to ESRD and is linked with an increased risk of macrovascular complications occurrence[41].

In T1DM pediatric patients, urine microalbumin to creatinine ratio (UACR) monitoring should start at puberty or 10 years of age (whichever is earlier), and when the child has had DM for 5 years this parameter should be checked annually. In T2DM the UACR should be checked at diagnosis and every year thereafter[36].

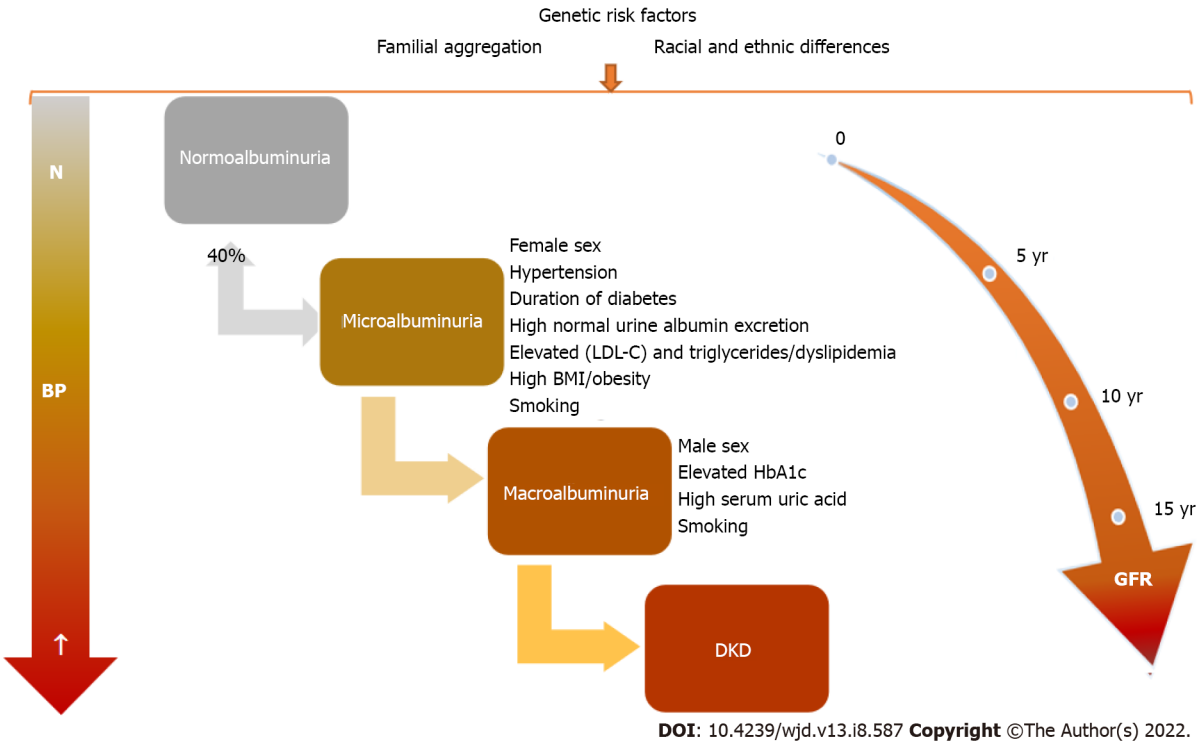


Figure 2 Changes in diabetic kidney disease: Blood pressure evolution and glomerular filtration rate decline along with albuminuria level. Influence of factors involved in diabetic kidney disease occurrence and progression. N: Normal; DKD: Diabetic kidney disease; BP: Blood pressure; GFR: Glomerular filtration rate; LDL-C: Low-density lipoprotein cholesterol; BMI: Body mass index; HbA1c: Glycated hemoglobin.

In medical practice, the common and still the "gold standard" marker for prediction and detection of diabetic kidney involvement in pediatric diabetes is represented by the microalbuminuria screening [21], even if it has a low specificity and sensitivity to detect early stages of DKD. Microalbuminuria screening should be done annually by timed overnight or 24-h urine collections (albumin excretion rate) or first-morning UACR[41].

Definitions of albuminuria and its abnormalities are based on the International Society for Pediatric and Adolescent Diabetes Clinical Practice Consensus Guidelines[37,41]. Normoalbuminuria is defined as a urine albumin level of ≤ 30 mg/L in all first-morning urine specimens, while microalbuminuria is characterized by the presence of an albumin limit of 30–300 mg or 20–200 μg/min in 24-h urine collection or a value of 30-300 mg/L in at least 2 of 3 first-morning urine specimens. Another parameter, namely UACR of 2.5–25.0 mg/mmol in males or 3.5–25.0 mg/mmol in females in at least 2 of 3 first-morning urine specimens quantifies microalbuminuria. Macroalbuminuria is defined as the presence of > 300 mg/L albumins in at least two first-morning urine specimens[37,41].

There are some limitations in albuminuria value as a biomarker for the prediction and detection of DKD, as not all diabetic children with micro- or macroalbuminuria will present a decrease in kidney function. Also, there are a lot of factors that may influence albuminuria level, UACR and eGFR: Fever, infection, diet, hydration status, hemodynamics, stress, physical activity, periods and hyperglycemia. Furthermore, a significant proportion of cases with microalbuminuria (up to 40%) may return to normoalbuminuria with strict glycemic and blood pressure (BP) control. Therefore, microalbuminuria can be transitory[21].

Microalbuminuria incidence in children with T1DM spans between 3% to 30%[37]. A cross-sectional study that involved children with T1DM reported a 25.0% frequency for microalbuminuria, while macroalbuminuria was found in 3.5% of these cases. The results of the cited study revealed a significantly higher (3 times) prevalence of microalbuminuria in T2DM (68%) compared to T1DM (24%) patients[37]. This is of particular interest given that children with T1DM are already at risk for renal complications secondary to DKD over the long term. A recent study reported early occurrence of microalbuminuria within 2 years of diagnosis of DM in 3.5% (7 of 199) of patients, whereas in 2 of those with microalbuminuria it appeared within the 1st year of diagnosis (in 7 mo)[37].

In a recent study, Hursh *et al*[24] showed that more than 64% of children hospitalized for DKD developed AKI. The same authors showed that a decreased serum bicarbonate level (< 10 mEq/L) and an increased heart rate are associated with a higher risk of severe AKI[24]. Higher morbidity and mortality rate is encountered in diabetic children that developed AKI along with a higher risk of chronic kidney disease, a finding that is particularly important in these patients who are already at risk for DKD [24].

It is already known that patients with DM may present with kidney damage (decrease in GFR) but without micro- or macroalbuminuria[42]. Therefore, other biomarkers that precede albuminuria should be considered more reliable to predict renal lesions, especially in the early stages. However, most of these biomarkers still need validation in clinical practice[43].

As tubular damage occurs before the glomerular injury, tubular biomarkers are superior to the glomerular ones, namely microalbuminuria. Therefore, they may serve for early detection of DKD in both T1DM and T2DM[44]. Tubulointerstitial damage may be suggested by the urinary albumin-to-creatinine to total protein-to-creatinine ratio of 0.40, with high sensitivity and specificity[45].

In patients without glomerular involvement, low-molecular-weight (LMW) proteinuria or non-albumin proteinuria represents an adequate marker of tubular dysfunction[46]. Urinary LMW proteins are absorbed in the proximal tubules so healthy individuals excrete up to 20 mg of LMW proteins/d in urine[46]. Alpha1 microglobulin, beta-2 microglobulins, immunoglobulin light chains, retinol-binding protein, cystatin C, neutrophil gelatinase-associated lipocalin (NGAL), N-acetyl-β-D-glucosaminidase, kidney injury molecule 1 and liver-type fatty acid-binding protein, *etc* are included in the LMW protein group[46]. In the early period of diabetes, an increase in urinary tubular biomarkers suggests that kidney injury is present[47].

A recent study showed the association of proximal tubule (alpha-1 microglobulin and kidney injury molecule 1) and podocyte (nephrin, vascular endothelial growth factor) damage biomarkers in T2DM even in the normoalbuminuric stage, indicating they may serve for early DKD diagnosis[47].

Urinary NGAL level increases before the onset of microalbuminuria in the very early phase of the kidney disease[48]. Alongside urinary biomarkers of tubular health (NGAL), the oxidative stress biomarker (pentosidine) may be used in the early detection of diabetic nephropathy before the microalbuminuric phase, as they correlate with albumin excretion and loss of nocturnal dipping of SBP and mean arterial BP[49].

Klotho, a transmembrane protein, is composed of a large extracellular and a small intracellular domain. Klotho is highly expressed in the renal tissue, especially in the distal tubules. The extracellular domain is cleaved by membrane proteases and discharged into the bloodstream, urine and cerebrospinal fluid as soluble klotho (s-klotho)[50,51]. A faster decline in eGFR was observed in DKD patients with low levels of serum s-klotho concentrations[52], which was opposite to the results of another study where s-klotho levels did not correlate with eGFR[50]. Bob *et al*[50] found a direct correlation of s-klotho levels with the rate of eGFR decline and with the serum levels of tubular injury marker kidney injury molecule 1[50]. A recent study found an inverse correlation between the klotho and glycated hemoglobin levels in T1DM children suggesting its possible role in chronic complications of diabetes occurrence[53].

Early stage prediction and recognition of DKD before microalbuminuria occurrence have a pivotal role in providing timely management. In this process, the assessment of more sensitive and specific biomarkers is essential. A new study showed that serum cystatin C may be used as a biomarker for DKD at an early stage in T1DM children with disease duration not exceeding 5 years before albuminuria detection[21]. The same study found a significantly lower eGFR-cystatin C value in diabetic children compared to controls. Also, significantly higher urinary cyclophilin A (CypA) and urinary CypA/creatinine ratios were found in T1DM children with microalbuminuria compared to the control group or normoalbuminuric subjects[21].

Salem *et al*[21] observed a better diagnostic value with the highest sensitivity (93.5%), specificity (71.4%) and accuracy (86.7%) to predict microalbuminuria in T1DM children by the combined use of serum cystatin C and urinary CypA than that of urinary CypA alone[21]. CypA, an endogenous cytosolic protein, is expressed mainly by the proximal tubular epithelial cells. A kidney injury is followed by an increase in urinary CypA concentration[21]. CypA level proved an encouraging biomarker for the early stage of diabetic nephropathy in adults with T2DM, and it correlates with the progression of diabetic nephropathy[54-56]. Novel biomarkers (Table 4) were proposed as early predictors of DKD[21,43].

Urinary biomarkers in DKD are crucial as they can indicate the site of injury within the nephron (structural biomarkers) as well as the loss of/reduced function of the nephron (functional biomarkers) and the main pathophysiological pathways (pathophysiological biomarkers)[57]. The proposed functional and/or structural tubular biomarkers might be valuable in the timely detection of DKD[57].

BP in diabetic children

Another important sign of diabetes-related nephropathy is BP measurement. In pediatric T2DM the guidelines recommend BP and UACR evaluation at diagnosis and annually thereafter[58].

An important and modifiable risk factor for the development of DKD is hypertension[59]. Arterial hypertension is an important and frequent risk factor for the appearance of cardiovascular disease in T1DM patients. High BP triggers the development and progression of microvascular complications, namely nephropathy, and retinopathy.

Ambulatory blood pressure measurement is superior to office BP measurements in predicting future cardiovascular events and targeting organ damage[60]. In their study, Shalaby and Shalaby[60] showed an abnormal BP profile for systolic and diastolic BP, with significant loss of nocturnal dipping. A significantly higher frequency of non-dipping patterns was observed in T1DM patients with microalbu-

Table 4 Renal biomarkers of diabetic kidney injury[21,43]

Biomarkers				
Traditional biomarkers	Traditional biomarkers of glomerular injury	Albumin/creatinine ratio eGFR	Lack of specificity and sensitivity	(1) Predict the late stages of DKD; (2) Daily variation in urine albumin/creatinine ratio; and (3) eGFR values may be affected by the patient's hemodynamics, diet and hydration status
Novel biomarkers	Glomerular biomarkers	NF-α, transferrin, Type IV collagen, L-PGDS, IgG, cerulo-plasmin, laminin, GAGs, fibronectin, podocalyxin, VEGF	Appear before microalbuminuria	Early predictor of DKD
	Tubular biomarkers	α-1-microglobulin CysC; KIM-1; NGAL; nephrin; NAG; L-FABP; VDBP; CypA; s-Klotho	Appear before/precede microalbu-minuria	(1) Are more sensitive *vs* new glomerular biomarkers; (2) Early predictors of DKD; and (3) Predictor of DKD progression
	Biomarkers of inflammation	Cytokines: TNF-α, IL-1β, IL-18, interferon gamma-IP-10, MCP-1, adiponectin, G-CSF, eotaxins, RANTES or CCL-5, orosomucoid	(1) Precede a significantly increased albuminuria; (2) Correlate positively with albumin excretion rate and intima-media thickness; and (3) May trigger direct renal injury	Predictor of DKD progression
	Biomarkers of oxidative stress	Urinary 8oHdG Pentosidine		Predict the development of DKD

L-PGDS: Lipocalin-type prostaglandin D synthase; IgG: Immunoglobulin G; GAGs: Glycosaminoglycans; CysC: Cystatin C; KIM-1: Kidney injury molecule 1; 8oHdG: 8-oxo-7,8-dihydro-2-deoxyguanosine; RANTES: Regulated on activation, normal T cell expressed and secreted; G-CSF: Granulocyte colony-stimulating factor; MCP-1: Monocyte chemoattractant protein 1; IP-10: Induced protein-10; TNF-α: Tumor necrosis factor α; IL: Interleukin; CypA: Cyclophilin A; VDBP: Vitamin D-binding protein; L-FABP: Liver-type fatty-acid binding protein; NAG: N-acetyl-β-D-glucosaminidase; NGAL: Neutrophil gelatinase-associated lipocalin; DKD: Diabetic kidney disease; eGFR: Estimated glomerular filtration rate; VEGF: Vascular endothelial growth factor; CCL-5: Chemokine ligand 5T.

minuria[60].

A recent study that comprises 3529 children and adolescents with T1DM revealed impaired BP regulation with elevated systolic BP, nocturnal diastolic BP, mean arterial pressure and diastolic dipping but lower nocturnal systolic dipping[61]. Lurbe *et al*[62] showed that an increase in nocturnal SBP precedes microalbuminuria occurrence within T1DM children[62].

The non-dipper pattern for SBD is one of the earliest abnormalities in the BP profile detected for children with T1DM. Also, non-dipping status has been associated with kidney damage (renal morphological changes) and hyperfiltration in adolescents with T1DM[63]. Also, the non-dipping status seems to be an early predictor of later nephropathy[63].

Teenagers with T1DM are at risk for hyperfiltration and higher UACR (urinary albumin-to-creatinine ratio), which are biomarkers for early/ incipient nephropathy[35]. A recent meta-analysis found that almost 25% of T2DM patients have arterial hypertension, the male sex being more frequently affected, and that 1 in 4 or 5 children have albuminuria[58].

Mamilly *et al*[49] found an increased urinary NGAL/creatinine (a marker of tubular injury) and pentosidine/creatinine (a marker of oxidative stress) in subjects with T1DM compared to controls even in the absence of microalbuminuria[49]. The same study reported a high incidence of abnormal BP dipping, which is important because dipping abnormalities may serve as a predictor for vascular complications, especially kidney injury in diabetic individuals[49]. The same study proved that urine NGAL correlates with loss of nocturnal dipping of SBP[49].

Based on these data, ambulatory blood pressure measurement represents the gold standard to assess BP regulation and should be used in all diabetic patients for timely therapeutic intervention to prevent renal and cardiovascular diabetic complications later in life.

PROPHYLACTIC AND THERAPEUTIC STRATEGIES FOR DKD

The well-known strategies, namely rigorous glycemic control, strict BP control and modulation of obesity, still represent the most important tools to prevent and slow down the progression of diabetic nephropathy/the deterioration of renal function. These therapies proved to be effective mainly by targeting the modifiable risk factors for diabetic nephropathy, which are listed in Table 1.

A recent systematic review confirmed that early high doses of vitamin D supplementation in combination with renin-angiotensin-aldosterone system blockers may slow the onset or progression of

Table 5 Common and new therapeutic strategies in diabetic kidney disease

Therapy	Drug class	Aim	Mechanism of action	DKD result/effect	Dose adjustment to eGFR (mL/min/1.73 m²)
Conventional therapies					
Strict glycemic control (Insulin)	-	HbA1c < 7%	(1) Reduces the risk of microalbuminuria; and (2) Reduces progression of microalbuminuria to macroalbuminuria	Delay DKD progression/risk	GFR = 10–50: Reduce the dose to 75%; GFR < 10: Reduce dose to 50%
Dietary protein/phosphate restriction	-	↓High protein intake	(1) Reduces hyperfiltration; and (2) Slows down/delays the loss of function or progression of diabetic nephropathy in T1DM and T2DM	Lower DKD risk	No restriction. CKD stage 3: 100%-140% of the DRI. CKD stage 4-5: 100%-120% of the DRI
Weight loss, increased physical activity	-		(1) Reduces hyperfiltration; and (2) Reduces albuminuria, especially in moderate/severe obesity	Lower DKD risk	No
Antihypertensive therapy	(1) ACEI/ARB/calcium-channel blockers; and (2) ACEI/ARB + calcium-channel blockers	Control of BP	(1) Reduces albuminuria and delays the onset of DN; (2) Prevents progression of DN in microalbuminuric patients; and (3) Reduces the frequency of microalbuminuria in hypertensive normoalbuminuric cases	Delay DKD progression	ARB, calcium channel blockers: No adjustment ACEI: GFR 30-60: Reduce dose to 50%; GFR < 30: Stop
Treatment of Dyslipidaemia	(1) Atorvastatin; (2) Fluvastatin; and (3) Osuvastatin	Reduce LDL-C	Reduce albuminuria in patients with DKD receiving RAAS blockers	Reduces CV disease/risk	No
Psychological Intervention	(1) Family therapy; (2) Cognitive behavioral therapy; (3) Motivational interviewing; (4) Counselling; (5) Mentoring; and (6) Peer support	Reduce depression	Follow lifestyle adjustment regimens and achieve optimal glucose levels	Delay DKD progression	No
Novel therapies					
Vitamin D analogues	Paricalcitol. Calcitriol		(1) Ameliorates nephropathy by reducing the albuminuria; and (2) Prevent glomerulosclerosis	Delay DKD progression	No
Vitamin D metabolites			Inhibit RAAS and prevent glomerulosclerosis	Delay DKD progression/risk	No
Uric acid antagonist	Allopurinol	Uric acid antagonist/xanthine oxidase inhibitor	(1) Reduces urinary TGF-β1 in diabetic nephropathy; (2) Reduces albuminuria in T2DM; and (3) Improves endothelial dysfunction	Delay DKD risk/progression	GFR > 50: No adjustment. GFR 30-50: Reduce dose by 50%. GFR < 10: Reduce dose to 30%, longer interval
Renin inhibitor	Aliskiren	Block RAAS cascade	Reduces albuminuria and serves as an antihypertensive in T2DM	Delay DKD progression	No
Endothelin antagonist or I inhibitor ETA receptor antagonist	Atransetan, avosentan, sparsentan (irbesartan + ETA)		(1) Reduces residual albuminuria in type 2 diabetic nephropathy; (2) Reduces proteinuria in T2DM patients and nephropathy; and (3) Significant proteinuria reduction	Delay/slow DKD progression	Yes
MRA Mineralocorticoid Receptor Antagonists	Spironolactone = nonselective MRA. Eplerenone	↑Natriuresis	Reduce albuminuria and blood pressure in patients with DN when added to a RAAS inhibitor	Delay DKD risk/progression	GFR > 50: No dose adjustment. GFR 30-50: Reduce dose to 25%, once daily.

					GFR < 10: No use
SGLT2 inhibitors	Empagliflozin, canagliflozin	Glucose-lowering	(1) Improves glycaemic control, reduces fasting blood glucose and HbA1c by increasing urinary glucose excretion; and (2) Reduces the reabsorption of sodium	Delay DKD progression, reduces blood pressure	No
GLP-1 agonist	Liraglutide, semaglutide	Stimulates insulin secretion, ↑satiety	Improves glycaemic control	Delay DKD risk/progression	No
	Exenatide, lixisenatide	Stimulates insulin secretion	Improves glycaemic control	Delay DKD risk/progression	Caution in CrCl < 50 mL/min
DDP-4 inhibitors	Linagliptin, saxagliptin, vildagliptin	Glucose-lowering-preserve the glucagon-like peptide effect	Reduce albuminuria in macroalbuminuric T2DM patients	Delay DKD risk/progression	eGFR < 50 mL/min: Reduce dose by 50%; eGFR < 30 mL/min: Reduce dose by 75%
TZD Thiazolidine-diones	Rosiglitazone. Pioglitazone	↓Hepatic glucose production activate peroxisome proliferator-activated receptor-γ to increase tissue insulin sensitivity	(1) Reduce albuminuria in macroalbuminuric T2DM patients; and (2) Lower microalbuminuria and proteinuria	Delay DKD risk/progression	No
Aldosterone synthase (CYP11B2) inhibition		Decrease in plasma aldosterone levels		Delay DKD risk/progression	NL
Anti-inflammatory Compounds					
CCR2 Antagonists		Emapticap pegol (NOX-E36), CCX-140	Reduces UACR and HbA1c	In T2DM-delay DKD, DN risk/progression	NL
VAP-1 inhibitors	An adhesion molecule for lymphocytes, regulating leukocyte migration into inflamed tissue	ASP-8232	Reduces albuminuria in T2DM in CKD	Delay DKD risk/progression	NL

ETA: Endothelin type A; T2DM: Type 2 diabetes mellitus; DKD: Diabetic kidney disease; UACR: Urine microalbumin to creatinine ratio; HbA1c: Glycated hemoglobin; GFR: Glomerular filtration rate; RAAS: Renin-angiotensin-aldosterone system; eGFR: Estimated glomerular filtration rate; ↓: Decreased; T1DM: Type 1 diabetes mellitus; CKD: Chronic kidney disease; DRI: Dietary reference intake; ACEI: Angiotensin-converting enzyme inhibitor; ARB: Angiotensin II receptor blocker; BP: Blood pressure; DN: Diabetic nephropathy; LDL-C: Low-density lipoprotein cholesterol; CV: Cardiovascular; TGF-1: Transforming growth factor 1; MRA: Mineralocorticoid receptor antagonists; SGLT-2: Sodium-glucose cotransporter-2; GLP-1: Glucagon-like peptide 1; CrCl: Creatinine clearance; DPP-4: Dipeptidyl peptidase 4; TZD: Thiazolidinediones; NL: Not listed; CCR2: Chemokine receptor 2; VAP-1: Vascular adhesion protein 1.

DKD[64]. Standard and some novel proposed therapies in early-stage or late-stage development of diabetic nephropathy are presented in Table 5[20,64,65].

CONCLUSION

DKD, the most significant and frequent burden of this metabolic disorder, is still discovered late as microalbuminuria is the most used biomarker for predicting kidney involvement. Novel biomarkers are valuable tools in the detection of kidney damage in the early phases as well as reliable predictors for DKD progression. Therefore, effective therapies may be proposed. Early stage prediction and recognition of DKD in children and adolescents before microalbuminuria occurrence have a pivotal role in preventing the development of and/or progression to irreversible kidney damage and to provide timely management and appropriate treatment by using conventional and novel therapies that may slow the onset or progression of DKD.

FOOTNOTES

Author contributions: All authors contributed equally to this work; Muntean C and Banescu C contributed to conception and design of the work, interpreting the relevant literature and drafting the manuscript; Muntean C,

Banescu C and Starcea IM performed the research of the literature; Muntean C and Starcea IM made critical revisions of the manuscript; all authors have read and approved the final version of the manuscript.

Conflict-of-interest statement: All the authors report no relevant conflicts of interest for this article.

Country/Territory of origin: Romania

ORCID number: Carmen Muntean 0000-0002-8056-1339.

S-Editor: Fan JR
L-Editor: Filipodia
P-Editor: Fan JR

REFERENCES

1 **Zhao L**, Long T, Hui A, Zhao R, Long S, Peng W. Type 2 Diabetes Mellitus in Children and Adolescents: Early Prevention and Non-Drug Therapy. *J Dia Mell* 2017; **7**: 121-141 [DOI: 10.4236/jdm.2017.73010]
2 **Stoian A**, Bacârea A, Moţăţăianu A, Stoian M, Gliga F, Bacârea V, Duicu C, Bănescu C. Vascular Endothelial Growth Factor Insertion/Deletion gene polymorphism in patients with type 2 diabetes and diabetic peripheral polyneuropathy. *Rev Romana Med Lab* 2014; **22**: 165-172 [DOI: 10.2478/rrlm-2014-0023]
3 **Afkarian M**. Diabetic kidney disease in children and adolescents. *Pediatr Nephrol* 2015; **30**: 65-74; quiz 70 [PMID: 24643739 DOI: 10.1007/s00467-014-2796-5]
4 **Marshall CB**. Rethinking glomerular basement membrane thickening in diabetic nephropathy: adaptive or pathogenic? *Am J Physiol Renal Physiol* 2016; **311**: F831-F843 [PMID: 27582102 DOI: 10.1152/ajprenal.00313.2016]
5 **Divers J**, Mayer-Davis EJ, Lawrence JM, Isom S, Dabelea D, Dolan L, Imperatore G, Marcovina S, Pettitt DJ, Pihoker C, Hamman RF, Saydah S, Wagenknecht LE. Trends in Incidence of Type 1 and Type 2 Diabetes Among Youths - Selected Counties and Indian Reservations, United States, 2002-2015. *MMWR Morb Mortal Wkly Rep* 2020; **69**: 161-165 [PMID: 32053581 DOI: 10.15585/mmwr.mm6906a3]
6 **Lawrence JM**, Divers J, Isom S, Saydah S, Imperatore G, Pihoker C, Marcovina SM, Mayer-Davis EJ, Hamman RF, Dolan L, Dabelea D, Pettitt DJ, Liese AD; SEARCH for Diabetes in Youth Study Group. Trends in Prevalence of Type 1 and Type 2 Diabetes in Children and Adolescents in the US, 2001-2017. *JAMA* 2021; **326**: 717-727 [PMID: 34427600 DOI: 10.1001/jama.2021.11165]
7 **Barkai L**, Kiss Z, Rokszin G, Abonyi-Tóth Z, Jermendy G, Wittmann I, Kempler P. Changes in the incidence and prevalence of type 1 and type 2 diabetes among 2 million children and adolescents in Hungary between 2001 and 2016 - a nationwide population-based study. *Arch Med Sci* 2020; **16**: 34-41 [PMID: 32051703 DOI: 10.5114/aoms.2019.88406]
8 **Oester IM**, Kloppenborg JT, Olsen BS, Johannesen J. Type 2 diabetes mellitus in Danish children and adolescents in 2014. *Pediatr Diabetes* 2016; **17**: 368-373 [PMID: 26111830 DOI: 10.1111/pedi.12291]
9 **Candler TP**, Mahmoud O, Lynn RM, Majbar AA, Barrett TG, Shield JPH. Continuing rise of Type 2 diabetes incidence in children and young people in the UK. *Diabet Med* 2018; **35**: 737-744 [PMID: 29460341 DOI: 10.1111/dme.13609]
10 **Rhodes ET**, Prosser LA, Hoerger TJ, Lieu T, Ludwig DS, Laffel LM. Estimated morbidity and mortality in adolescents and young adults diagnosed with Type 2 diabetes mellitus. *Diabet Med* 2012; **29**: 453-463 [PMID: 22150528 DOI: 10.1111/j.1464-5491.2011.03542.x]
11 **Diabetes Control and Complications Trial Research Group**, Nathan DM, Genuth S, Lachin J, Cleary P, Crofford O, Davis M, Rand L, Siebert C. The effect of intensive treatment of diabetes on the development and progression of long-term complications in insulin-dependent diabetes mellitus. *N Engl J Med* 1993; **329**: 977-986 [PMID: 8366922 DOI: 10.1056/NEJM199309303291401]
12 **Pastore I**, Bolla AM, Montefusco L, Lunati ME, Rossi A, Assi E, Zuccotti GV, Fiorina P. The Impact of Diabetes Mellitus on Cardiovascular Risk Onset in Children and Adolescents. *Int J Mol Sci* 2020; **21** [PMID: 32664699 DOI: 10.3390/ijms21144928]
13 **Rawshani A**, Sattar N, Franzén S, Rawshani A, Hattersley AT, Svensson AM, Eliasson B, Gudbjörnsdottir S. Excess mortality and cardiovascular disease in young adults with type 1 diabetes in relation to age at onset: a nationwide, register-based cohort study. *Lancet* 2018; **392**: 477-486 [PMID: 30129464 DOI: 10.1016/S0140-6736(18)31506-X]
14 **Narres M**, Claessen H, Droste S, Kvitkina T, Koch M, Kuss O, Icks A. The Incidence of End-Stage Renal Disease in the Diabetic (Compared to the Non-Diabetic) Population: A Systematic Review. *PLoS One* 2016; **11**: e0147329 [PMID: 26812415 DOI: 10.1371/journal.pone.0147329]
15 **Murphy D**, McCulloch CE, Lin F, Banerjee T, Bragg-Gresham JL, Eberhardt MS, Morgenstern H, Pavkov ME, Saran R, Powe NR, Hsu CY; Centers for Disease Control and Prevention Chronic Kidney Disease Surveillance Team. Trends in Prevalence of Chronic Kidney Disease in the United States. *Ann Intern Med* 2016; **165**: 473-481 [PMID: 27479614 DOI: 10.7326/M16-0273]
16 **Li L**, Jick S, Breitenstein S, Michel A. Prevalence of Diabetes and Diabetic Nephropathy in a Large U.S. Commercially Insured Pediatric Population, 2002-2013. *Diabetes Care* 2016; **39**: 278-284 [PMID: 26681728 DOI: 10.2337/dc15-1710]

17 **Dart AB**, Sellers EA, Martens PJ, Rigatto C, Brownell MD, Dean HJ. High burden of kidney disease in youth-onset type 2 diabetes. *Diabetes Care* 2012; **35**: 1265-1271 [PMID: 22432116 DOI: 10.2337/dc11-2312]

18 **Rodriguez BL**, Fujimoto WY, Mayer-Davis EJ, Imperatore G, Williams DE, Bell RA, Wadwa RP, Palla SL, Liu LL, Kershnar A, Daniels SR, Linder B. Prevalence of cardiovascular disease risk factors in U.S. children and adolescents with diabetes: the SEARCH for diabetes in youth study. *Diabetes Care* 2006; **29**: 1891-1896 [PMID: 16873798 DOI: 10.2337/dc06-0310]

19 **Lin YC**, Chang YH, Yang SY, Wu KD, Chu TS. Update of pathophysiology and management of diabetic kidney disease. *J Formos Med Assoc* 2018; **117**: 662-675 [PMID: 29486908 DOI: 10.1016/j.jfma.2018.02.007]

20 **Uwaezuoke SN**, Ayuk AC. Diabetic Kidney Disease in Childhood and Adolescence: Conventional and Novel Renoprotective Strategies. *EMJ Nephrol* 2020; **8**: 68-77 [DOI: 10.33590/emjnephrol/20-00077]

21 **Salem NA**, El Helaly RM, Ali IM, Ebrahim HAA, Alayooti MM, El Domiaty HA, Aboelenin HM. Urinary Cyclophilin A and serum Cystatin C as biomarkers for diabetic nephropathy in children with type 1 diabetes. *Pediatr Diabetes* 2020; **21**: 846-855 [PMID: 32304131 DOI: 10.1111/pedi.13019]

22 **Fu H**, Liu S, Bastacky SI, Wang X, Tian XJ, Zhou D. Diabetic kidney diseases revisited: A new perspective for a new era. *Mol Metab* 2019; **30**: 250-263 [PMID: 31767176 DOI: 10.1016/j.molmet.2019.10.005]

23 **Reidy K**, Kang HM, Hostetter T, Susztak K. Molecular mechanisms of diabetic kidney disease. *J Clin Invest* 2014; **124**: 2333-2340 [PMID: 24892707 DOI: 10.1172/JCI72271]

24 **Hursh BE**, Ronsley R, Islam N, Mammen C, Panagiotopoulos C. Acute Kidney Injury in Children With Type 1 Diabetes Hospitalized for Diabetic Ketoacidosis. *JAMA Pediatr* 2017; **171**: e170020 [PMID: 28288246 DOI: 10.1001/jamapediatrics.2017.0020]

25 **Gu HF**. Genetic and Epigenetic Studies in Diabetic Kidney Disease. *Front Genet* 2019; **10**: 507 [PMID: 31231424 DOI: 10.3389/fgene.2019.00507]

26 **Florez JC**. Genetics of Diabetic Kidney Disease. *Semin Nephrol* 2016; **36**: 474-480 [PMID: 27987549 DOI: 10.1016/j.semnephrol.2016.09.012]

27 **Lu HC**, Dai WN, He LY. Epigenetic Histone Modifications in the Pathogenesis of Diabetic Kidney Disease. *Diabetes Metab Syndr Obes* 2021; **14**: 329-344 [PMID: 33519221 DOI: 10.2147/DMSO.S288500]

28 **Liu R**, Lee K, He JC. Genetics and Epigenetics of Diabetic Nephropathy. *Kidney Dis (Basel)* 2015; **1**: 42-51 [PMID: 27536664 DOI: 10.1159/000381796]

29 **Mooyaart AL**. Genetic associations in diabetic nephropathy. *Clin Exp Nephrol* 2014; **18**: 197-200 [PMID: 24129556 DOI: 10.1007/s10157-013-0874-9]

30 **Tziastoudi M**, Stefanidis I, Zintzaras E. The genetic map of diabetic nephropathy: evidence from a systematic review and meta-analysis of genetic association studies. *Clin Kidney J* 2020; **13**: 768-781 [PMID: 33123356 DOI: 10.1093/ckj/sfaa077]

31 **Tonneijck L**, Muskiet MH, Smits MM, van Bommel EJ, Heerspink HJ, van Raalte DH, Joles JA. Glomerular Hyperfiltration in Diabetes: Mechanisms, Clinical Significance, and Treatment. *J Am Soc Nephrol* 2017; **28**: 1023-1039 [PMID: 28143897 DOI: 10.1681/ASN.2016060666]

32 **Bjornstad P**, Nehus E, El Ghormli L, Bacha F, Libman IM, McKay S, Willi SM, Laffel L, Arslanian S, Nadeau KJ; TODAY Study Group. Insulin Sensitivity and Diabetic Kidney Disease in Children and Adolescents With Type 2 Diabetes: An Observational Analysis of Data From the TODAY Clinical Trial. *Am J Kidney Dis* 2018; **71**: 65-74 [PMID: 29157731 DOI: 10.1053/j.ajkd.2017.07.015]

33 **Bjornstad P**, Cherney DZ, Snell-Bergeon JK, Pyle L, Rewers M, Johnson RJ, Maahs DM. Rapid GFR decline is associated with renal hyperfiltration and impaired GFR in adults with Type 1 diabetes. *Nephrol Dial Transplant* 2015; **30**: 1706-1711 [PMID: 26050268 DOI: 10.1093/ndt/gfv121]

34 **Ruggenenti P**, Porrini EL, Gaspari F, Motterlini N, Cannata A, Carrara F, Cella C, Ferrari S, Stucchi N, Parvanova A, Iliev I, Dodesini AR, Trevisan R, Bossi A, Zaletel J, Remuzzi G; GFR Study Investigators. Glomerular hyperfiltration and renal disease progression in type 2 diabetes. *Diabetes Care* 2012; **35**: 2061-2068 [PMID: 22773704 DOI: 10.2337/dc11-2189]

35 **Lovshin JA**, Škrtić M, Bjornstad P, Moineddin R, Daneman D, Dunger D, Reich HN, Mahmud F, Scholey J, Cherney DZI, Sochett E. Hyperfiltration, urinary albumin excretion, and ambulatory blood pressure in adolescents with Type 1 diabetes mellitus. *Am J Physiol Renal Physiol* 2018; **314**: F667-F674 [PMID: 29357443 DOI: 10.1152/ajprenal.00400.2017]

36 **Lopez LN**, Wang W, Loomba L, Afkarian M, Butani L. Diabetic kidney disease in children and adolescents: an update. *Pediatr Nephrol* 2021 [PMID: 34913986 DOI: 10.1007/s00467-021-05347-7]

37 **Zabeen B**, Nahar J, Islam N, Azad K, Donaghue K. Risk Factors Associated with Microalbuminuria in Children and Adolescents with Diabetes in Bangladesh. *Indian J Endocrinol Metab* 2018; **22**: 85-88 [PMID: 29535943 DOI: 10.4103/ijem.IJEM_269_17]

38 **Schwartz GJ**, Muñoz A, Schneider MF, Mak RH, Kaskel F, Warady BA, Furth SL. New equations to estimate GFR in children with CKD. *J Am Soc Nephrol* 2009; **20**: 629-637 [PMID: 19158356 DOI: 10.1681/ASN.2008030287]

39 **Szabo CE**, Man OI, Istrate A, Kiss E, Catana A, Creț V, Șerban RS, Pop IV. Role of Adiponectin and Tumor Necrosis Factor-Alpha in the Pathogenesis and Evolution of Type 1 Diabetes Mellitus in Children and Adolescents. *Diagnostics (Basel)* 2020; **10** [PMID: 33202729 DOI: 10.3390/diagnostics10110945]

40 **El-Samahy MH**, Adly AA, Ismail EA, Salah NY. Regulatory T cells with CD62L or TNFR2 expression in young type 1 diabetic patients: relation to inflammation, glycemic control and micro-vascular complications. *J Diabetes Complications* 2015; **29**: 120-126 [PMID: 25113439 DOI: 10.1016/j.jdiacomp.2014.07.004]

41 **International Diabetes Federation, 2011**. ISPAD. [cited 10 March 2022]. Available from: https://cdn.ymaws.com/www.ispad.org/resource/resmgr/Docs/idf-ispad_guidelines_2011_0.pdf

42 **Currie G**, McKay G, Delles C. Biomarkers in diabetic nephropathy: Present and future. *World J Diabetes* 2014; **5**: 763-776 [PMID: 25512779 DOI: 10.4239/wjd.v5.i6.763]

43 **Uwaezuoke SN**. The role of novel biomarkers in predicting diabetic nephropathy: a review. *Int J Nephrol Renovasc Dis* 2017; **10**: 221-231 [PMID: 28860837 DOI: 10.2147/IJNRD.S143186]

44 **Uwaezuoke SN**, Muoneke VU, Mbanefo NR. Tubular Biomarkers as Diagnostic Tools in Diabetic Kidney Disease: A Review of Published Evidence. *Int J Nephrol Kidney Fail* 2018; 4 [DOI: 10.16966/2380-5498.156]

45 **Smith ER**, Cai MM, McMahon LP, Wright DA, Holt SG. The value of simultaneous measurements of urinary albumin and total protein in proteinuric patients. *Nephrol Dial Transplant* 2012; **27**: 1534-1541 [PMID: 22193048 DOI: 10.1093/ndt/gfr708]

46 **Thethi TK**, Batuman V. Challenging the conventional wisdom on diabetic nephropathy: Is microalbuminuria the earliest event? *J Diabetes Complications* 2019; **33**: 191-192 [PMID: 30651179 DOI: 10.1016/j.jdiacomp.2018.12.006]

47 **Petrica L**, Vlad A, Gluhovschi G, Gadalean F, Dumitrascu V, Gluhovschi C, Velciov S, Bob F, Vlad D, Popescu R, Milas O, Ursoniu S. Proximal tubule dysfunction is associated with podocyte damage biomarkers nephrin and vascular endothelial growth factor in type 2 diabetes mellitus patients: a cross-sectional study. *PLoS One* 2014; **9**: e112538 [PMID: 25397960 DOI: 10.1371/journal.pone.0112538]

48 **Yürük Yıldırım Z**, Nayır A, Yılmaz A, Gedikbaşı A, Bundak R. Neutrophil Gelatinase-Associated Lipocalin as an Early Sign of Diabetic Kidney Injury in Children. *J Clin Res Pediatr Endocrinol* 2015; **7**: 274-279 [PMID: 26777038 DOI: 10.4274/jcrpe.2002]

49 **Mamilly L**, Mastrandrea LD, Mosquera Vasquez C, Klamer B, Kallash M, Aldughiem A. Evidence of Early Diabetic Nephropathy in Pediatric Type 1 Diabetes. *Front Endocrinol (Lausanne)* 2021; **12**: 669954 [PMID: 33995287 DOI: 10.3389/fendo.2021.669954]

50 **Bob F**, Schiller A, Timar R, Lighezan D, Schiller O, Timar B, Bujor CG, Munteanu M, Gadalean F, Mihaescu A, Grosu I, Hategan A, Chisavu L, Pusztai AM, Covic A. Rapid decline of kidney function in diabetic kidney disease is associated with high soluble Klotho levels. *Nefrologia (Engl Ed)* 2019; **39**: 250-257 [PMID: 30396700 DOI: 10.1016/j.nefro.2018.08.004]

51 **Kim JH**, Hwang KH, Park KS, Kong ID, Cha SK. Biological Role of Anti-aging Protein Klotho. *J Lifestyle Med* 2015; **5**: 1-6 [PMID: 26528423 DOI: 10.15280/jlm.2015.5.1.1]

52 **Pavik I**, Jaeger P, Ebner L, Wagner CA, Petzold K, Spichtig D, Poster D, Wüthrich RP, Russmann S, Serra AL. Secreted Klotho and FGF23 in chronic kidney disease Stage 1 to 5: a sequence suggested from a cross-sectional study. *Nephrol Dial Transplant* 2013; **28**: 352-359 [PMID: 23129826 DOI: 10.1093/ndt/gfs460]

53 **Zubkiewicz-Kucharska A**, Wikiera B, Noczyńska A. Soluble Klotho Is Decreased in Children With Type 1 Diabetes and Correlated With Metabolic Control. *Front Endocrinol (Lausanne)* 2021; **12**: 709564 [PMID: 34603200 DOI: 10.3389/fendo.2021.709564]

54 **El-Saeed GK**, El-Deen WAS, Montasr BA, Omar TA, Mohamed DS. Urinary podocalyxin and cyclophilin A: markers for early detection of type 2 diabetic nephropathy. *Menoufia Med J* 2019; **32**: 996-1003 [DOI: 10.4103/mmj.mmj_223_18]

55 **Harun H**, Lunesia R, Azmi S. Correlation between urinary Cyclophilin A and urinary albumin levels on diabetic kidney disease. *Indones J Kidney Hypertension* 2019; **2**: 10-16 [DOI: 10.32867/inakidney.v2i2.29]

56 **Amer HMA**, Sabry IM, Bekhet MMM, Mohammed RNS. The role of urinary cyclophilin A as a new marker for diabetic nephropathy. *Egypt J Hosp Med* 2018; **70**: 1431-1439 [DOI: 10.12816/0044664]

57 **Zeni L**, Norden AGW, Cancarini G, Unwin RJ. A more tubulocentric view of diabetic kidney disease. *J Nephrol* 2017; **30**: 701-717 [PMID: 28840540 DOI: 10.1007/s40620-017-0423-9]

58 **Cioana M**, Deng J, Hou M, Nadarajah A, Qiu Y, Chen SSJ, Rivas A, Banfield L, Chanchlani R, Dart A, Wicklow B, Alfaraidi H, Alotaibi A, Thabane L, Samaan MC. Prevalence of Hypertension and Albuminuria in Pediatric Type 2 Diabetes: A Systematic Review and Meta-analysis. *JAMA Netw Open* 2021; **4**: e216069 [PMID: 33929524 DOI: 10.1001/jamanetworkopen.2021.6069]

59 **Rohani F**, Hooman N, Moradi S, Mobarra M, Najafizadeh M, Tatarpoor P. The Prevalence of Pre-hypertension in Children with Type 1 Diabetes Mellitus. *Int J Prev Med* 2014; **5**: S44-S49 [PMID: 24791191]

60 **Shalaby NM**, Shalaby NM. Study of ambulatory blood pressure in diabetic children: prediction of early renal insult. *Ther Clin Risk Manag* 2015; **11**: 1531-1537 [PMID: 26491340 DOI: 10.2147/TCRM.S87751]

61 **Dost A**, Bechtold-Dalla Pozza S, Bollow E, Kovacic R, Vogel P, Feldhahn L, Schwab KO, Holl RW; Initiative DPV. Blood pressure regulation determined by ambulatory blood pressure profiles in children and adolescents with type 1 diabetes mellitus: Impact on diabetic complications. *Pediatr Diabetes* 2017; **18**: 874-882 [PMID: 28117539 DOI: 10.1111/pedi.12502]

62 **Lurbe E**, Redon J, Kesani A, Pascual JM, Tacons J, Alvarez V, Batlle D. Increase in nocturnal blood pressure and progression to microalbuminuria in type 1 diabetes. *N Engl J Med* 2002; **347**: 797-805 [PMID: 12226150 DOI: 10.1056/NEJMoa013410]

63 **Torbjörnsdotter TB**, Jaremko GA, Berg UB. Nondipping and its relation to glomerulopathy and hyperfiltration in adolescents with type 1 diabetes. *Diabetes Care* 2004; **27**: 510-516 [PMID: 14747237 DOI: 10.2337/diacare.27.2.510]

64 **Uwaezuoke SN**. Vitamin D Analogs Can Retard the Onset or Progression of Diabetic Kidney Disease: A Systematic Review. *Front Clin Dia Heal* 2021; **2**: 763844 [DOI: 10.3389/fcdhc.2021.763844]

65 **Idzerda NMA**, Pena MJ, de Zeeuw D, Heerspink HJL. Future and Novel Compounds in the Treatment of Diabetic Nephropathy. *Springer* 2019 [DOI: 10.1007/978-3-319-93521-8_29]

66 **KDOQI**. KDOQI Clinical Practice Guidelines and Clinical Practice Recommendations for Diabetes and Chronic Kidney Disease. *Am J Kidney Dis* 2007; **49**: S12-154 [PMID: 17276798 DOI: 10.1053/j.ajkd.2006.12.005]

67 **Hogg RJ**, Furth S, Lemley KV, Portman R, Schwartz GJ, Coresh J, Balk E, Lau J, Levin A, Kausz AT, Eknoyan G, Levey AS; National Kidney Foundation's Kidney Disease Outcomes Quality Initiative. National Kidney Foundation's Kidney Disease Outcomes Quality Initiative clinical practice guidelines for chronic kidney disease in children and adolescents: evaluation, classification, and stratification. *Pediatrics* 2003; **111**: 1416-1421 [PMID: 12777562 DOI: 10.1542/peds.111.6.1416]

World Journal of Diabetes

Submit a Manuscript: https://www.f6publishing.com

World J Diabetes 2022 August 15; 13(8): 600-612

DOI: 10.4239/wjd.v13.i8.600

ISSN 1948-9358 (online)

ORIGINAL ARTICLE

Basic Study

Clopidogrel delays and can reverse diabetic nephropathy pathogenesis in type 2 diabetic *db/db* mice

Hong-Qin Li, Nian Liu, Zong-Yu Zheng, Hao-Lin Teng, Jin Pei

Specialty type: Endocrinology and metabolism

Provenance and peer review: Unsolicited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification

Grade A (Excellent): 0
Grade B (Very good): B
Grade C (Good): C, C, C
Grade D (Fair): 0
Grade E (Poor): 0

P-Reviewer: Balbaa ME, Egypt; Saengboonmee C, Thailand

Received: October 18, 2021
Peer-review started: October 18, 2021
First decision: December 27, 2021
Revised: January 26, 2022
Accepted: June 27, 2022
Article in press: June 27, 2022
Published online: August 15, 2022

Hong-Qin Li, Jin Pei, Department of Biopharmacy, Jilin University School of Pharmaceutical Sciences, Changchun 130021, Jilin Province, China

Hong-Qin Li, Nian Liu, Zong-Yu Zheng, Hao-Lin Teng, Department of Urology, The First Hospital of Jilin University, Changchun 130021, Jilin Province, China

Corresponding author: Jin Pei, PhD, Professor, Department of Biopharmacy, Jilin University School of Pharmaceutical Sciences, No. 1163, Xinmin street, Changchun 130021, Jilin Province, China. peijin@jlu.edu.cn

Abstract

BACKGROUND

Diabetic nephropathy (DN) is the principal cause of end-stage renal disease. Previous studies have shown that clopidogrel can prevent the early progression of renal injury.

AIM

To elucidate whether clopidogrel is beneficial against DN by using a *db/db* mouse model.

METHODS

db/db mice with a higher urinary albumin/creatinine ratio (ACR) relative to age- and sex-matched wild-type control mice were randomly allocated to clopidogrel and vehicle treatment groups. Clopidogrel was administered at doses of 5, 10, and 20 mg/kg by gavage for 12 wk. Body mass, blood glucose level, and urinary creatinine and albumin concentrations in each group were measured before and after the intervention. Renal fibrosis was evaluated using periodic acid-Schiff and Masson's trichrome staining. The renal protein expression of tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and F4/80 was assessed using immunohistochemistry. Urinary TNF-α, monocyte chemoattractant protein-1 (MCP-1), and IL-6 levels were analyzed using enzyme-linked immunosorbent assay; *TNF-α* and *IL-1β* mRNA expression was measured using real-time quantitative polymerase chain reaction. The protein expression of fibronectin (FN) and collagen I was assessed using immunohistochemistry.

RESULTS

Clopidogrel treatment did not affect the body mass or blood glucose level of the *db/db* mice; however, it increased bleeding time and reduced urinary ACR in a

dose-dependent manner. Immunohistochemical staining revealed an amelioration of renal fibrosis, significantly lower deposition of FN and collagen I, and significantly lower expression of the proinflammatory cytokines TNF-α and IL-1β and lower levels of urinary TNF-α and MCP-1 in the clopidogrel-treated *db/db* mice ($P < 0.05$). Furthermore, clopidogrel significantly reduced macrophage infiltration into the glomeruli of the *db/db* mice.

CONCLUSION

Clopidogrel significantly reduced renal collagen deposition and fibrosis and prevented renal dysfunction in *db/db* mice, most likely through inhibition of renal macrophage infiltration and the associated inflammation.

Key Words: Diabetes; Clopidogrel; Inflammation; Fibronectin; Diabetic nephropathy

Core Tip: Diabetic nephropathy is the most common microvascular inflammatory disease among the diabetic complications. Previous studies have shown that clopidogrel administration is an effective means of suppressing inflammation in diseases such as acute myocardial infarction, diabetes, and acute ischemic cerebral infarction. In this study, we aimed to determine whether treatment with clopidogrel has a preventive or therapeutic effect in the kidneys of obese, type 2 diabetic *db/db* mice. In this experiment, we demonstrated that clopidogrel significantly reduced renal collagen deposition and fibrosis and prevented renal dysfunction in *db/db* mice, most likely through inhibition of renal macrophage infiltration and the associated inflammation.

Citation: Li HQ, Liu N, Zheng ZY, Teng HL, Pei J. Clopidogrel delays and can reverse diabetic nephropathy pathogenesis in type 2 diabetic *db/db* mice. *World J Diabetes* 2022; 13(8): 600-612
URL: https://www.wjgnet.com/1948-9358/full/v13/i8/600.htm
DOI: https://dx.doi.org/10.4239/wjd.v13.i8.600

INTRODUCTION

Diabetes is a major public health issue, affecting more than 400 million people worldwide[1]. Type 2 diabetes is a highly prevalent condition that is associated with major vascular, renal, and neurologic complications. Diabetic nephropathy (DN) is the most common microvascular inflammatory disease among diabetes complications. Proteinuria is a hallmark of early DN, and the associated morphological abnormalities include glomerular hypertrophy, thickening of the glomerular basement membrane, expansion of the mesangial matrix, and renal tubular injury. Renal changes in the later phases include glomerulosclerosis and tubulointerstitial fibrosis[2-4].

The mechanism of DN is complex. A persistently high glucose level is considered the principal risk factor for DN; however, other factors, such as abnormal renal hemodynamics, may also be involved in the development of DN[5]. Hyperglycemia leads to the expression of proinflammatory mediators (chemokines and cytokines) in injured glomerular and tubular cells, which contributes to renal damage through various mechanisms, including mesangial proliferation, podocyte/tubular damage, and leukocyte infiltration[6,7]. Leukocytic interleukin-6 (IL-6) and tumor necrosis factor (TNF)-α promote the release of vascular endothelial factors, stimulate the proliferation of glomerular mesangial cells, increase the permeability of the endothelium, and promote the synthesis and release of superoxide and proteolytic enzymes, which eventually cause renal structural remodeling and dysfunction[8,9]. An increasing amount of evidence indicates that diabetes-associated vascular and renal inflammation is likely to be associated with high platelet reactivity, which would contribute to high atherothrombotic risk[10,11].

Clopidogrel is an anti-platelet aggregation drug that with a pyridine-based structure. It can specifically and irreversibly bind to platelet P2RY12 purinergic receptors, which inhibits ADP-mediated platelet activation and aggregation. Clopidogrel not only inhibits platelet aggregation but also inflammatory responses in a platelet activation-dependent or independent manner[12,13]. Previous studies have shown that clopidogrel administration is an effective means of suppressing inflammation in conditions such as acute myocardial infarction, diabetes, and acute ischemic cerebral infarction[14,15]. Lower expression levels of IL-6, TNF-α, and transforming growth factor-β1; lower matrix metalloproteinase (MMP)-2 and MMP-9 activity; and stabilization of the extracellular matrix (ECM) in clopidogrel-treated mice with hyperlipidemia and acute myocardial infarction reflected the protective effect of the drug[16]. We have previously shown that clopidogrel significantly reduced renal collagen and

fibronectin (FN) expression and thus ameliorated diabetes-induced renal fibrosis in a streptozotocin-induced murine model of type 1 diabetes[17]. However, because 80%-90% of the population with diabetes has type 2 disease[1], in the present study, we aimed to determine whether clopidogrel treatment has a preventive or therapeutic effect in the kidneys of obese type 2 diabetic *db*/*db* mice, a widely used mouse model of type 2 diabetes for DN investigations[18].

MATERIALS AND METHODS

Experimental animals, groups, and drug administration

db/db mice used in the present study were leptin receptor (Lepr) knockout mice with the C57BLKS background developed by GemPharmatech Co., Ltd. by using the clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 technique, (https://www.gempharmatech.us/en/strain/detail/?strain_id=3913). This mouse line has features similar to that from the Jackson Laboratory (https://www.jax.org/strain/000642): Significant increases in the body weight starting at 4 wk, hyperglycemia (6-h fasting blood glucose and hemoglobin A1c) at 8 wk, insulinemia (> 3-fold) at 8 wk, and hyperlipidemia (cholesterol, triglyceride, and low-density lipoprotein) along with the early onset of renal dysfunction (indicated by significantly increased microalbumin level in 24-h urine analysis) at 12 wk of age. Therefore, this mouse line has been widely used as a model for studies on type 2 diabetes[19, 20], including for the studies on DN of type 2 diabetes[18,21].

Eight-week-old, specific pathogen-free male C57BLKS *db*/*db* diabetic mice (n = 24) and age-matched C57BLKS [wild-type (WT)] mice (n = 6) were purchased from the Model Animal Research Center of Nanjing University (Cat. No. T002407, GemPharmatech Co., Ltd., Nanjing, China). All animal experiments were approved by the ethics committee of the First Hospital of Jilin University and conformed to the internationally accepted principles for the care and use of laboratory animals. All the mice were adoptively fed until 12 wk of age when these *db/db* mice should show typical DN, defined as the baseline. Then the *db*/*db* mice were randomly assigned to vehicle or low-dose (5 mg/kg), medium-dose (10 mg/kg), or high-dose (20 mg/kg) clopidogrel groups. The mice were administered clopidogrel or vehicle daily by gavage for 3 mo. At the end of the experiment, the animals were intraperitoneally anesthetized with tribromoethanol (350 mg/kg) and sacrificed to collect blood and kidneys for subsequent experiments.

Sample collection

Blood glucose levels were measured at regular intervals by using samples collected from a tail vein. Twenty-four-hour urine collections were performed before and after 3 mo of treatment with the animals in metabolic cages, and the 24-h urine albumin output was measured using a mouse microalbuminuria enzyme-linked immunosorbent assay (ELISA) kit (Beijing XinQuan Tech Company, XQ-EN20536). The urine creatinine output was measured using a creatinine test kit (Nanjing Jiancheng Bioengineering Institute, C011-1-1).

Blood clotting time

Blood clotting time was measured using the mouse tail-vein bleeding assay. Briefly, the mouse's tail was cut 1-2 mm from the tip, where its diameter was approximately 1 mm, and then, it was immediately dipped into a 50-mL tube filled with saline at 37 °C. The bleeding time was recorded over a period of up to 5 min, as in our previous study[17].

Histology and immunohistochemical staining

The collected kidneys were fixed in 10% formalin, dehydrated in a graded series of alcohol, cleaned with xylene, embedded in paraffin, and sectioned at a thickness of 5 μm. Periodic acid-Schiff and Masson staining was performed to facilitate the examination of the glomerular basement membrane and mesangial matrix of the kidneys. Immunohistochemistry was used to assess the expression of TNF-α (Abcam, ab220210), IL-1β (Cell Signaling Technologies, #12242), F4/80 (Abcam, ab100790), FN (Santa Cruz Biotechnology, SC-8422), and collagen I (Abcam, ab253113) in the kidney sections. After 3,3′-diaminobenzidine staining, cells that were positive for target protein expression stained brown-yellow, whereas the cells without the said expression were unstained. The percentage of each area that was stained was quantified using the Image-Pro Plus software.

Measurement of TNF-α, MCP-1, and IL-6 levels in the urine

Urinary TNF-α, MCP-1, and IL-6 levels were measured using a mouse TNF-α precoated ELISA kit (DAKEWE, 1217202), mouse MCP-1 precoated ELISA kit (DAKEWE, 1217392), and mouse IL-6 precoated ELISA kit (DAKEWE, 1210602), respectively. These concentrations were normalized to the urinary creatinine concentrations.

RNA isolation and real-time polymerase chain reaction

RNA was extracted from tissues by using Trizol and reverse-transcribed into cDNA. Real-time polymerase chain reaction (PCR) was performed using SYBR Green chemistry, with *β-actin* as the reference gene. Gene expression was quantified using the $2^{-\Delta\Delta CT}$ method. The primer sequences used were as follows: *TNF-α* (F): TATAAAGCGGCCGTCTGCAC, *TNF-α* (R): TCTTCTGCCAGTTCCACGTC; *IL-1β* (F): TTGACGGACCCCAAAAGATG, *IL-1β* (R): AGAAGGTGCTCATGTCCTCA; and *β-actin* (F): CCCTGTATGCCTCTGGTCGT, *β-actin* (R): CGTGGGGTGAAGCTGTAGCCACG.

Statistical analysis

Data are presented as mean ± SD values for $n = 6$ mice per group. Multiple comparisons of data were performed using one-way analysis of variance, with Tukey's test for *post-hoc* pairwise comparisons. All statistical analyses were conducted using GraphPad Prism 5, and $P < 0.05$ was considered to represent statistical significance.

RESULTS

Effects of the db/db genotype and clopidogrel on the body mass, blood glucose level, and bleeding time of the mice

At the age of 12 wk, the *db/db* mice were significantly heavier and had higher fasting blood glucose levels compared to the WT mice (Figures 1A and B). Their animals' body mass slightly increased with age thereafter, whereas their fasting blood glucose levels did not significantly change during the 3-mo treatment period. Because *db/db* mice start showing typical DN at 12 wk of age, we started to administer clopidogrel from this age with one of three doses (5, 10, or 20 mg/kg), to observe the drug's therapeutic effects or delay DN progression. First, the bleeding times of the WT and diabetic mice that had or had not been administered clopidogrel were measured (Figure 1C). There was no difference between diabetic mice and WT nondiabetic mice at the baseline level, and treatment of the diabetic mice with clopidogrel increased the bleeding time in a dose-dependent manner during the 3-mo treatment. Furthermore, clopidogrel treatment did not affect the body mass or blood glucose levels of the diabetic mice during the experimental period (Figures 1A and B).

Clopidogrel ameliorated diabetes-associated renal dysfunction, glomerular sclerosis, and collagen fiber deposition in the mice

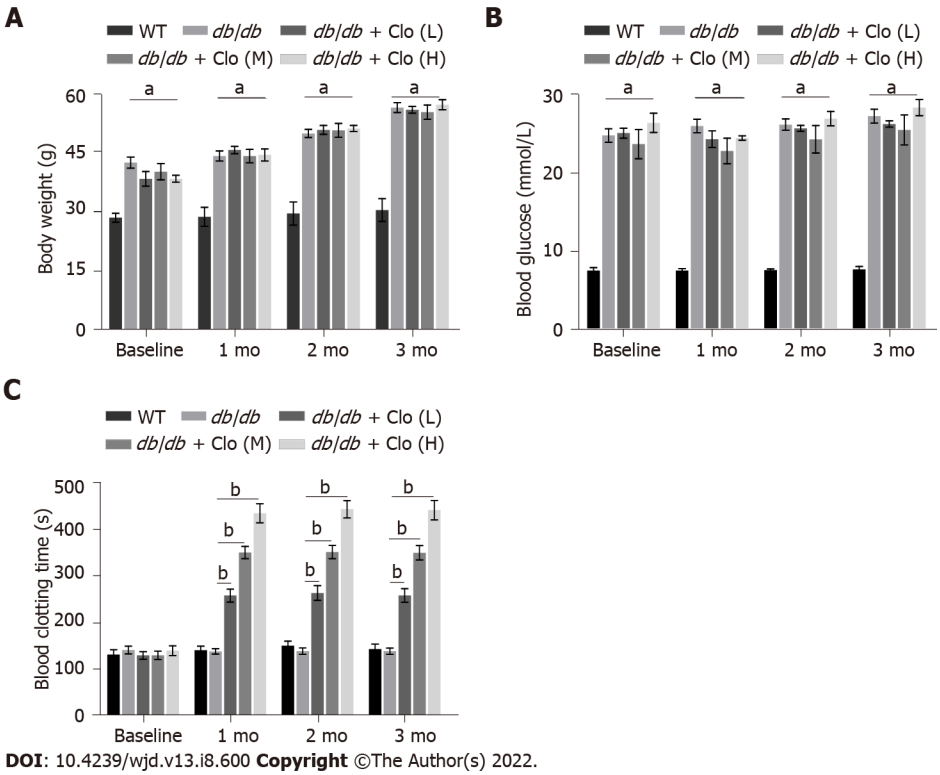
Clopidogrel administration for 3 mo reduced the urinary albumin/creatinine ratio (ACR) of the diabetic mice, especially in the high-dose group, which implies an improvement in the glomerular filtration rate (Figure 2A). The kidneys of the WT mice did not show any obvious pathology, whereas those of the *db/db* mice showed larger renal glomeruli, thickening of the substrate membranes, hyperplasia of mesangial cells, and an expansion of the ECM. These histopathological changes were markedly ameliorated by clopidogrel administration (Figure 2B). Masson's trichrome staining showed greater collagen deposition in the *db/db* mice; however, this was less severe in the mice administered clopidogrel (Figure 2B).

Potential mechanisms for the beneficial effects of clopidogrel: A reduction in the renal accumulation of ECM components responsible for fibrosis

It has been shown that in the early stages of DN, there is a significant increase in the deposition of collagen, FN, and laminin in the glomerular basement membrane and ECM in glomerular mesangial cells[22]. We assessed the protein expression of FN and collagen type I in the kidney cortex of the mice by immunohistochemical staining (Figure 3). No significant FN or collagen I protein expression was identified in the renal glomerulus of WT mice (Figure 3), and there was only a low level of expression around the blood vessels (data not shown). In contrast, there was an excessive deposition of both FN and collagen I in the glomerular membranes and mesangium of the *db/db* mice (Figure 3). However, the accumulation of both proteins was significantly lower in *db/db* mice that had been administered clopidogrel for 3 mo (Figure 3). These data imply that clopidogrel reduced collagen synthesis and ECM deposition and inhibited fibrosis in the kidneys of the *db/db* mice.

Potential mechanisms for the beneficial effects of clopidogrel: Inhibition of the diabetes-associated renal inflammatory response and macrophage infiltration

We next measured the mRNA expression of *TNF-α* and *IL-1β* by using real-time PCR, which showed that both mRNA expression levels were significantly lower in the clopidogrel treatment group (Figure 4A). Furthermore, immunohistochemical staining of kidney sections showed that TNF-α and IL-1β were expressed at low levels in the kidneys of the WT mice. However, there was higher TNF-α expression principally in the renal tubules and higher IL-1β expression in both the glomeruli and renal tubules of the *db/db* mice (Figure 4B). Semi-quantitative analysis showed significantly higher protein



DOI: 10.4239/wjd.v13.i8.600

Figure 1 Effects of clopidogrel on body mass, 6-h fasting blood glucose level in mice, and the bleeding time of mice. A: Body mass; B: Blood glucose; C: Blood clotting time. Data are shown as mean ± SD values of $n = 6$ mice per group. $^{a}P < 0.05$ *vs* wild type mice. $^{b}P < 0.05$ *vs* untreated *db/db* mice. WT: Wild type; *db/db*: *db/db* mice; Clo: Clopidogrel; Clo (L, M, H): Clopidogrel at 5, 10, or 20 mg/kg, respectively.

expression of TNF-α and IL-1β in the kidneys of diabetic mice. Nevertheless, clopidogrel significantly reduced the expression of these renal inflammatory cytokines (Figure 4C).

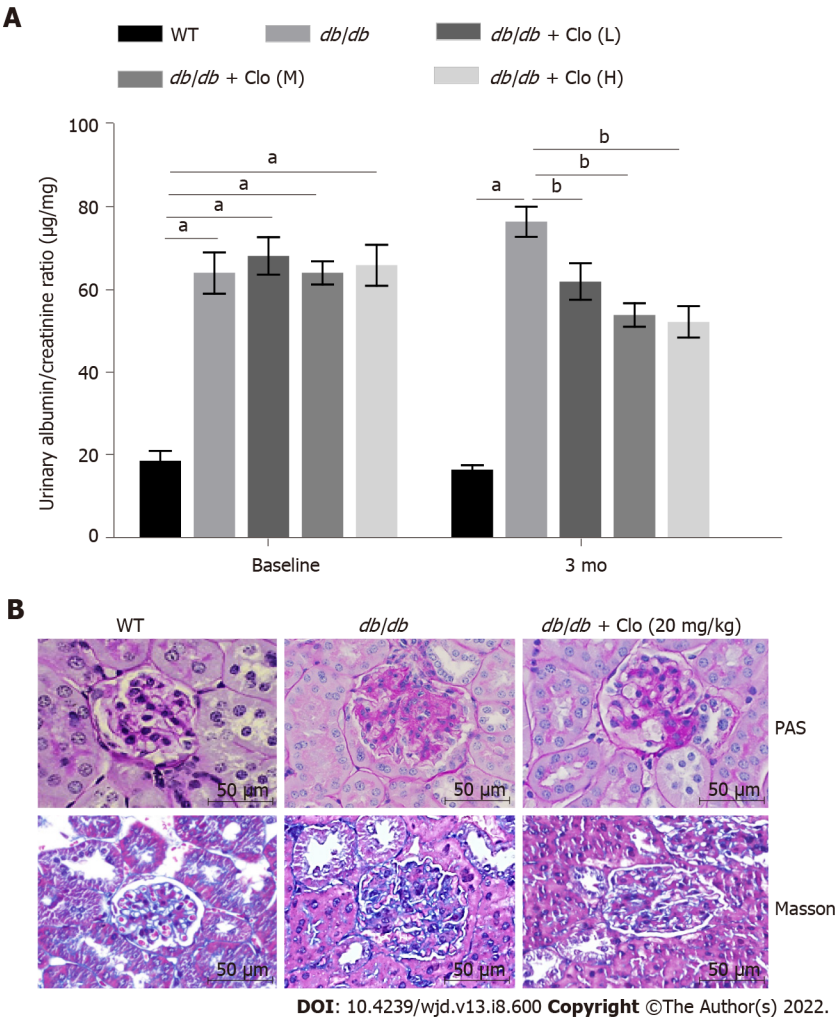
We also examined these inflammatory cytokine levels in the urine, which may be indirect indicators of systemic inflammation. Furthermore, considering that MCP-1 is a potent chemokine in the recruitment of macrophages[23], urinary TNF-α, IL-6, and MCP-1 levels were measured by ELISA (Figure 5). The results showed that the development of diabetes significantly increased urinary TNF-α, IL-6, and MCP-1 levels. However, clopidogrel reduced the levels of urinary TNF-α and MCP-1 (Figures 5A and C), and urinary IL-6 level in *db/db* mice had a slight decrease (Figure 5B). These results indicate that clopidogrel can inhibit the diabetes-associated renal inflammatory response and probably systemic inflammation.

Macrophages, lymphocytes, and mast cells secrete large amounts of proinflammatory mediators and cytokines in the diabetic kidney, which directly or indirectly induce renal damage and accelerate local fibrosis[24,25]. F4/80 is a macrophage marker expressed on the surface of these cells in mice. Consistent with the urinary MCP-1 results, there was no obvious expression of F4/80 in normal mouse kidney tissue (Figure 6). On the other hand, macrophage infiltration was apparent around the glomeruli in diabetic mice. Furthermore, the number of macrophages at this location was significantly high in *db/db* mice; this number was significantly reduced by clopidogrel treatment (Figure 6). These results suggest that clopidogrel reduces macrophage infiltration and inhibits cytokine secretion, thereby reducing damage to the kidneys caused by the inflammatory response.

DISCUSSION

Clopidogrel is an antagonist of the P2RY12 receptor, which is expressed on the surfaces of platelets. It not only inhibits platelet aggregation but also reduces ventricular inflammation and fibrosis in animal models[26-28]. Consistent with this, in this study, we found that clopidogrel administration for 3 mo delayed or prevented the progression of DN in *db/db* diabetic mice. These beneficial effects of clopidogrel in *db/db* mice appear to be mediated by inhibition of the renal deposition of collagen, probably through suppression of macrophage infiltration into the kidney and thus a reduction in proinflammatory cytokine secretion, as illustrated in Figure 7.

We have also shown that clopidogrel administration does not significantly affect the body mass or blood glucose level of *db/db* diabetic mice, which implies that the dose and duration of clopidogrel administration used in these mice were safe, without evidence of potential side-effects. Furthermore,



DOI: 10.4239/wjd.v13.i8.600

Figure 2 Clopidogrel improves the kidney function of *db*/*db* mice. Data are shown as mean ± SD values of $n = 6$ mice per group. [a]$P < 0.05$ *vs* wild type mice; [b]$P < 0.05$ *vs* *db*/*db* mice. A: Urinary albumin/creatinine ratio. Mice at 12 wk of age were defined as the baseline; B: Periodic acid-Schiff staining of kidney sections for glycogen content and Masson's trichrome staining for collagenous connective tissue fibers. Magnification: × 400. WT: Wild type; *db/db*: *db*/*db* mice; Clo: Clopidogrel; Clo (L, M, H): Clopidogrel at 5, 10, or 20 mg/kg, respectively.

these beneficial effects of the drug against DN development in these mice were independent of glycemic control. There have been few previous studies on the effects of clopidogrel on blood glucose. However, in one study, patients with type 2 diabetes were treated daily with one 70 mg clopidogrel tablet for 2 mo, which improved their fasting blood glucose level (from 9.7 ± 0.7 mmol/L to 7.5 ± 0.5 mmol/L). Therefore, the potential hypoglycemic effect of clopidogrel requires further investigation.

In addition, although clopidogrel is an anti-platelet aggregation drug, its preventive or therapeutic effect on the development of DN in *db*/*db* mice does not seem to be predominantly related to its effect on platelet aggregation. We believe this is because the *db/db* mice did not demonstrate shorter bleeding times than those noted in the WT mice before or after 3 mo of clopidogrel administration (Figure 1C). However, clopidogrel increased the bleeding times of the *db/db* mice in a dose-dependent manner (Figure 1C) during the 3-mo treatment period, parallel to the dose-dependent effects of improvement in renal function (Figure 2A).

The urinary albumin concentration is closely related to the progression of glomerular lesions and kidney damage, and this was significantly reduced by clopidogrel administration in *db*/*db* mice to levels that were lower than those prior to treatment (Figure 2A). Inflammation plays an important role in the development and progression of DN, and this involves the production of chemokines and proinflammatory cytokines, infiltration of immune cells into the kidney, formation of immune complexes, and complement activation[29]. Chronic diabetic hyperglycemia causes an increase in the circulating concentration of advanced glycation end-products, which induce macrophage migration through advanced glycation end product receptor-mediated activation of the nuclear factor (NF)-κB inflammatory pathway[30,31]. Furthermore, the production of the proinflammatory cytokines TNF-α and IL-1β is induced by NF-κB activation. TNF-α promotes inflammatory cell aggregation and adhesion, microvascular dilation, vascular permeability, and exacerbation of the inflammatory response, thereby contributing to glomerular tissue damage. Therefore, the serum TNF-α concentration is considered a

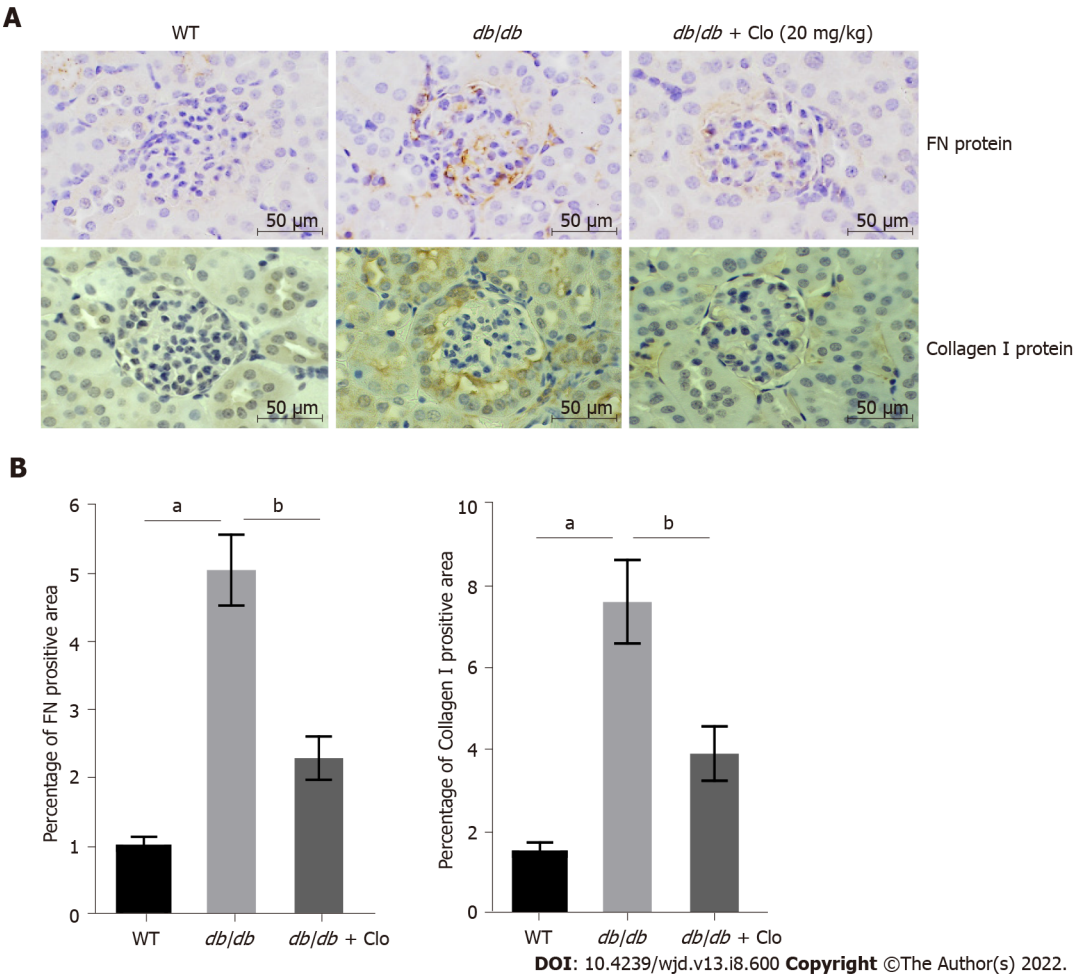
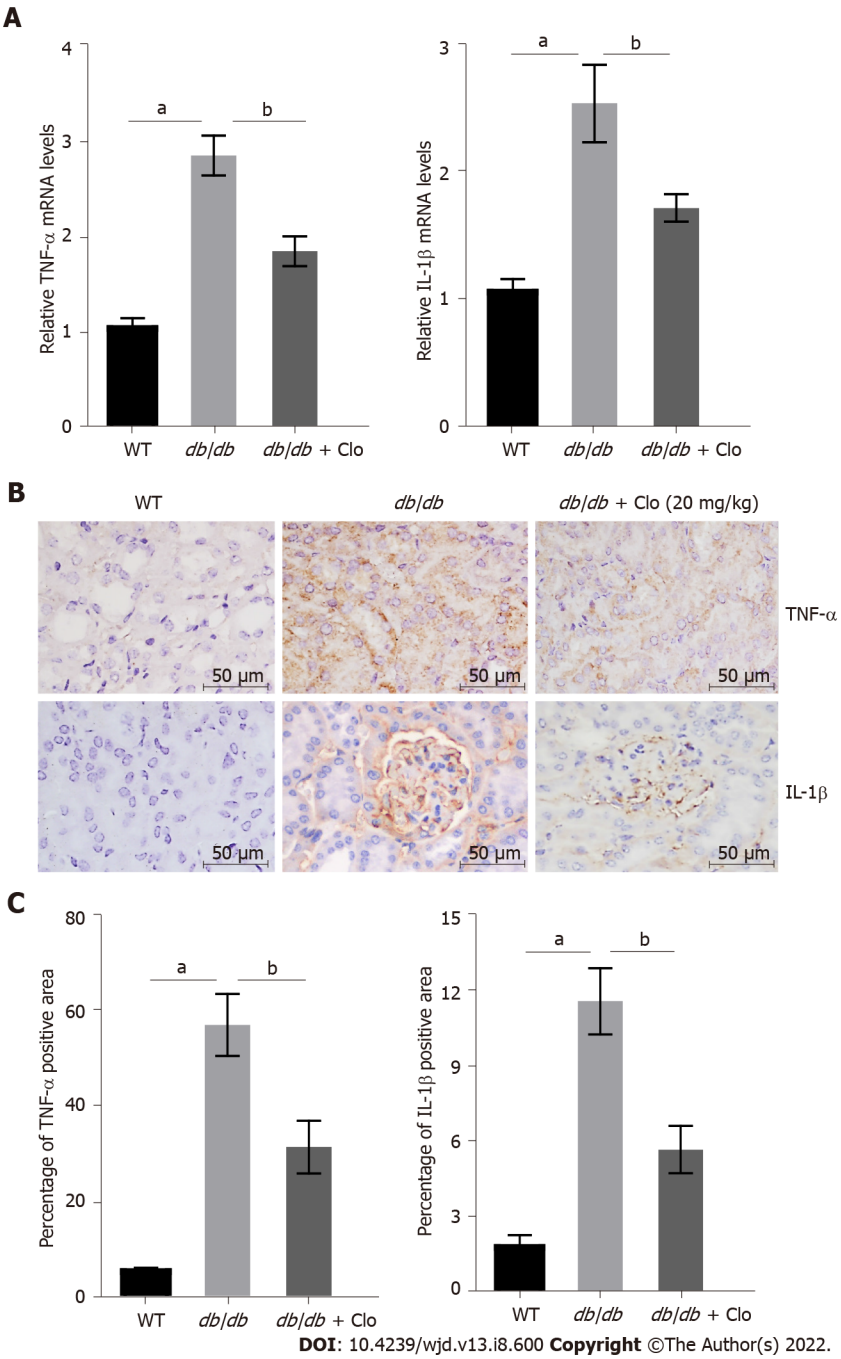


Figure 3 Clopidogrel reduces the production of fibronectin and collagen I in the kidneys of *db/db* mice. A: Representative immunohistochemical staining. Magnification: × 400; B: Quantitative analysis of fibronectin protein and collagen I protein. Data are shown as mean ± SD values of $n = 6$ mice per group. $^{a}P < 0.05$ *vs* wild type mice; $^{b}P < 0.05$ *vs db/db* mice. FN: Fibronectin; WT: Wild type; *db/db*: *db/db* mice; Clo: Clopidogrel.

predictor of disease progression[9,32]. IL-1β stimulates the proliferation of glomerular cells in the kidneys, promotes the production of ECM, and accelerates the process of renal fibrosis[8,33]. Consistent with this, we found significantly higher expression of both TNF-α and IL-1β in the kidneys and urine of *db/db* mice. However, treatment with clopidogrel significantly reduced the expression of both cytokines, which implies that the drug has an anti-inflammatory effect in the kidneys of diabetic mice.

Macrophages are the principal type of immune cells that promote kidney damage in diabetes[34,35]. F4/80 is a macrophage-specific antigen that participates in the maturation and activation of this cell type. Consistent with the increased urinary level of MCP-1, which is a potent chemokine in macrophages, we found a significant increase in macrophage infiltration, mirrored by F4/80-positive staining (Figure 6), in the kidneys of the *db/db* mice. However, the number of macrophages in the kidneys of the clopidogrel-treated mice was significantly lower. Chronic renal inflammation causes glomerular membrane cells to produce large amounts of type I and type IV collagen and FN, which leads to thickening of the glomerular basement membrane and ECM accumulation, ultimately resulting in glomerulosclerosis[36,37]. We also found higher expression of collagen I and FN in the kidneys of the *db/db* mice. However, this was much lower in the kidneys of mice that had been treated with clopidogrel for 3 mo.

Thus, as illustrated in Figure 7, the results of the present study suggest that clopidogrel administration for 3 mo to *db/db* mice, which had significant renal dysfunction (high urinary ACR), delayed the progression of DN and possibly improved renal function. This effect is accompanied by an amelioration of features of the renal pathology of DN, including lower renal accumulation of ECM components, such as collagen and FN. The beneficial effects of clopidogrel on the renal deposition of collagen in the kidneys of *db/db* mice may be explained by its inhibition of diabetes-associated macrophage infiltration and proinflammatory cytokine release. However, further research is required to determine whether there is a genuine cause-and-effect link between these findings. In addition, the lower macrophage infiltration may explain the reduction in ECM accumulation and fibrosis. However, it may be that clopidogrel also directly inhibits renal inflammation and fibrosis. This possibility requires further investigation.

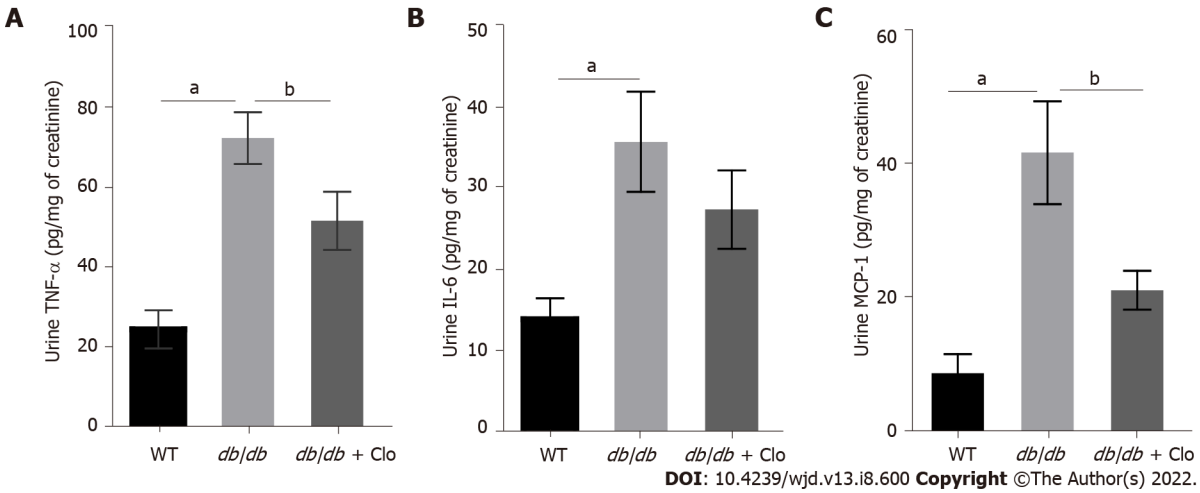


DOI: 10.4239/wjd.v13.i8.600

Figure 4 Clopidogrel inhibits the expression of tumor necrosis factor-α and interleukin-1β in *db/db* mice. Data are shown as mean ± SD values of $n = 6$ per group. [a]$P < 0.05$ *vs* wild-type mice; [b]$P < 0.05$ *vs* *db/db* mice. A: Tumor necrosis factor (TNF)-α and interleukin (IL)-1β gene expression, determined using real-time polymerase chain reaction; B: Immunohistochemical staining; C: Semi-quantitative analysis of TNF-α and IL-1β protein expression. WT: Wild type; *db/db*: *db/db* mice; Clo: Clopidogrel; TNF: Tumor necrosis factor; IL: Interleukin.

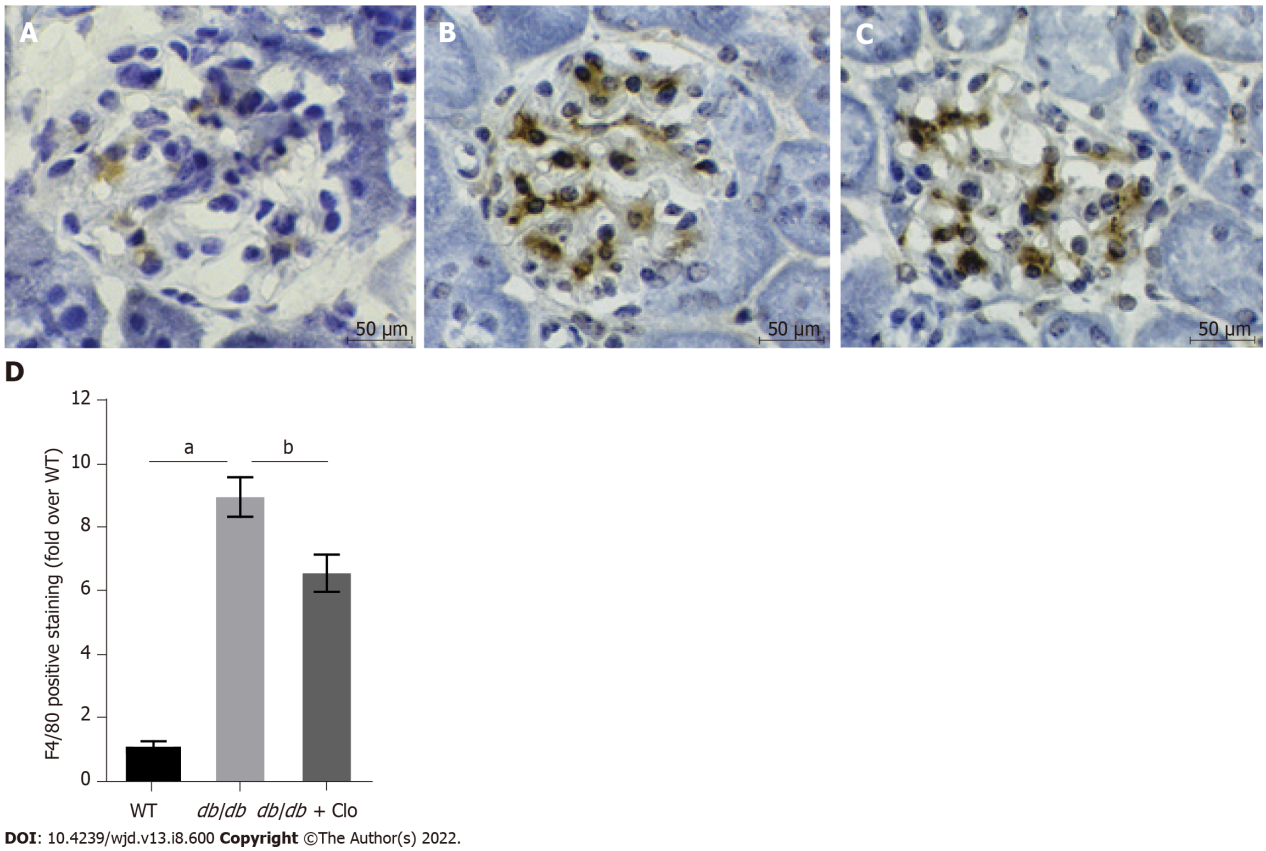
CONCLUSION

We found that clopidogrel significantly reduced renal collagen deposition and fibrosis and prevented renal dysfunction in *db/db* mice. These findings suggest a promising alternative approach to the treatment of diabetes and prevention of DN because clopidogrel is in current use and could be co-administered with other antidiabetic drugs.



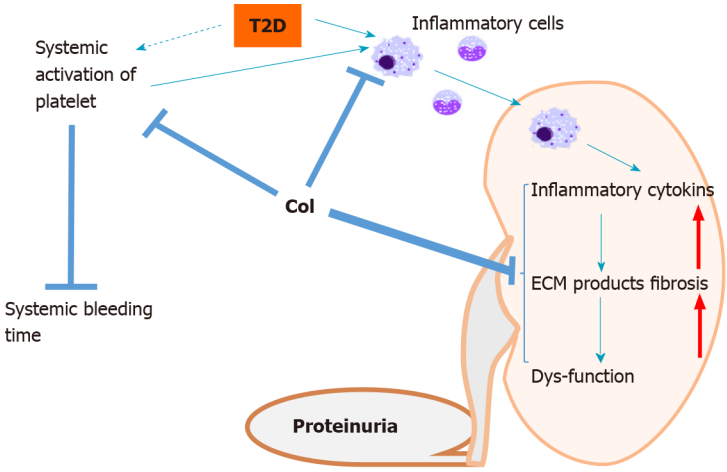
DOI: 10.4239/wjd.v13.i8.600 **Copyright** ©The Author(s) 2022.

Figure 5 Clopidogrel reduces the levels of urinary tumor necrosis factor-α and monocyte chemoattractant protein-1 in *db*/*db* mice. Data are shown as mean ± SD values of $n = 6$ mice per group. [a]$P < 0.05$ *vs* wild type mice; [b]$P < 0.05$ *vs* *db*/*db* mice. A: Tumor necrosis factor-α levels in the urine; B: Interleukin-6 levels in the urine; C: Monocyte chemoattractant protein-1 levels in the urine. WT: Wild type; *db/db*: *db*/*db* mice; Clo: Clopidogrel 20 mg/kg; TNF: Tumor necrosis factor; IL: Interleukin; MCP: Monocyte chemoattractant protein.



DOI: 10.4239/wjd.v13.i8.600 **Copyright** ©The Author(s) 2022.

Figure 6 Clopidogrel reduces renal macrophage infiltration in *db*/*db* mice. [a]$P < 0.05$ *vs* wild type mice; [b]$P < 0.05$ *vs* *db*/*db* mice. Immunohistochemical staining, magnification: × 400. A: Wild type; B: *db/db* mice; C: *db/db* + clopidogrel (20 mg/kg); D: Immunohistochemical analysis of F4/80 expression. WT: Wild type; *db/db*: *db*/*db* mice; Clo: Clopidogrel.



DOI: 10.4239/wjd.v13.i8.600 **Copyright** ©The Author(s) 2022.

Figure 7 Outline of the potential mechanisms whereby clopidogrel ameliorates defects in renal structure and function in *db*/*db* mice. Diabetes is associated with the activation of platelets, predisposing toward systemic thrombosis, which is reflected in a shorter bleeding time. Diabetes is also characterized by the activation of inflammatory cells, including macrophages, which infiltrate the kidney and release proinflammatory cytokines, causing systemic and renal inflammation, which is followed by renal damage, remodeling, and dysfunction. The treatment of *db*/*db* mice with clopidogrel may exert its effects in three main ways: (1) Direct inhibition of the infiltration of circulating inflammatory cells into the kidney; (2) Inactivation of activated platelets, preventing their promotion of inflammatory cell infiltration; and (3) Direct prevention of the diabetes-related renal inflammatory response and associated downstream pathways. The dashed arrow indicates that the systemic activation of platelet aggregation in diabetes requires further confirmation in longer-term studies of diabetes models. T2D: Type 2 diabetes; ECM: Extracellular matrix.

ARTICLE HIGHLIGHTS

Research background

Diabetic nephropathy (DN) is the leading cause of end-stage kidney disease in the United States and most developed countries. New strategies are required to delay the development and the progression of DN.

Research motivation

Previous studies have shown that clopidogrel administration is an effective means of suppressing inflammation in diabetes. Moreover, clopidogrel can ameliorate diabetes-induced renal fibrosis in a streptozotocin-induced murine model of type 1 diabetes.

Research objectives

We aimed to determine whether treatment with clopidogrel has a preventive or therapeutic effect in the kidneys of obese type 2 diabetic *db/db* mice.

Research methods

Clopidogrel at doses of 5, 10, or 20 mg/kg was administered by gavage for 12 wk. The body mass, blood glucose, and urinary creatinine and albumin concentrations were measured. Immunohistochemistry, enzyme-linked immunosorbent assay and real-time quantitative polymerase chain reaction were used to evaluate the expression of cytokines. Fibronectin (FN), and collagen I was assessed using immunohistochemistry.

Research results

Clopidogrel treatment reduced urinary albumin/creatinine ratio. Immunohistochemical staining revealed an amelioration of renal fibrosis, significantly less deposition of FN and collagen I. Lower expression of the proinflammatory cytokines tumor necrosis factor-α (TNF-α) and interleukin-1β and lower levels of urinary TNF-α, monocyte chemoattractant protein-1 and significantly reduced macrophage infiltration of the *db/db* mice.

Research conclusions

Clopidogrel prevented renal dysfunction in *db/db* mice, most likely through inhibition of renal macrophage infiltration and the associated inflammation.

Research perspectives

The present findings suggest a promising alternative approach to the treatment of patients with diabetes

and the prevention of DN because clopidogrel is in current use and could be co-administered with other antidiabetic drugs.

FOOTNOTES

Author contributions: Pei J and Li HQ contributed to conception and design of the study; Li HQ and Liu N performed the experiment; Zheng ZY organized the database; Teng HL performed the statistical analysis; Li HQ and Liu N wrote the draft of the manuscript; and all authors contributed to manuscript revision, read, and approved the submitted version.

Institutional animal care and use committee statement: All animal experiments conformed to the internationally accepted principles for the care and use of laboratory animals.

Conflict-of-interest statement: All the authors report no relevant conflicts of interest for this article.

Data sharing statement: No additional data are available.

ARRIVE guidelines statement: The authors have read the ARRIVE guidelines, and the manuscript was prepared and revised according to the ARRIVE guidelines.

Country/Territory of origin: China

ORCID number: Hong-Qin Li 0000-0002-3260-922X; Nian Liu 0000-0003-4518-4045; Zong-Yu Zheng 0000-0001-5523-1891; Hao-Lin Teng 0000-0001-8222-2536; Jin Pei 0000-0001-8735-1994.

S-Editor: Wang JJ
L-Editor: A
P-Editor: Wang JJ

REFERENCES

1 **Khursheed R**, Singh SK, Wadhwa S, Kapoor B, Gulati M, Kumar R, Ramanunny AK, Awasthi A, Dua K. Treatment strategies against diabetes: Success so far and challenges ahead. *Eur J Pharmacol* 2019; **862**: 172625 [PMID: 31449807 DOI: 10.1016/j.ejphar.2019.172625]
2 **Soldatos G**, Cooper ME. Diabetic nephropathy: important pathophysiologic mechanisms. *Diabetes Res Clin Pract* 2008; **82** Suppl 1: S75-S79 [PMID: 18994672 DOI: 10.1016/j.diabres.2008.09.042]
3 **Lehmann R**, Schleicher ED. Molecular mechanism of diabetic nephropathy. *Clin Chim Acta* 2000; **297**: 135-144 [PMID: 10841915 DOI: 10.1016/s0009-8981(00)00240-0]
4 **Zeng LF**, Xiao Y, Sun L. A Glimpse of the Mechanisms Related to Renal Fibrosis in Diabetic Nephropathy. *Adv Exp Med Biol* 2019; **1165**: 49-79 [PMID: 31399961 DOI: 10.1007/978-981-13-8871-2_4]
5 **de Zeeuw D**, Coll B, Andress D, Brennan JJ, Tang H, Houser M, Correa-Rotter R, Kohan D, Lambers Heerspink HJ, Makino H, Perkovic V, Pritchett Y, Remuzzi G, Tobe SW, Toto R, Viberti G, Parving HH. The endothelin antagonist atrasentan lowers residual albuminuria in patients with type 2 diabetic nephropathy. *J Am Soc Nephrol* 2014; **25**: 1083-1093 [PMID: 24722445 DOI: 10.1681/ASN.2013080830]
6 **Wada J**, Makino H. Innate immunity in diabetes and diabetic nephropathy. *Nat Rev Nephrol* 2016; **12**: 13-26 [PMID: 26568190 DOI: 10.1038/nrneph.2015.175]
7 **Dalla Vestra M**, Masiero A, Roiter AM, Saller A, Crepaldi G, Fioretto P. Is podocyte injury relevant in diabetic nephropathy? *Diabetes* 2003; **52**: 1031-1035 [PMID: 12663476 DOI: 10.2337/diabetes.52.4.1031]
8 **Hasegawa G**, Nakano K, Sawada M, Uno K, Shibayama Y, Ienaga K, Kondo M. Possible role of tumor necrosis factor and interleukin-1 in the development of diabetic nephropathy. *Kidney Int* 1991; **40**: 1007-1012 [PMID: 1762301 DOI: 10.1038/ki.1991.308]
9 **Yeo ES**, Hwang JY, Park JE, Choi YJ, Huh KB, Kim WY. Tumor necrosis factor (TNF-alpha) and C-reactive protein (CRP) are positively associated with the risk of chronic kidney disease in patients with type 2 diabetes. *Yonsei Med J* 2010; **51**: 519-525 [PMID: 20499416 DOI: 10.3349/ymj.2010.51.4.519]
10 **Chen G**, Huang W, Hong S, Li X, Su B, Tang S. Effectiveness of antiplatelet drugs for the prevention of diabetic nephropathy: A meta-analysis of randomized controlled trials. *Clin Nephrol* 2018; **90**: 419-426 [PMID: 30378535 DOI: 10.5414/CN109588]
11 **Westein E**, Hoefer T, Calkin AC. Thrombosis in diabetes: a shear flow effect? *Clin Sci (Lond)* 2017; **131**: 1245-1260 [PMID: 28592700 DOI: 10.1042/CS20160391]

12 **Jansen MP**, Emal D, Teske GJ, Dessing MC, Florquin S, Roelofs JJ. Release of extracellular DNA influences renal ischemia reperfusion injury by platelet activation and formation of neutrophil extracellular traps. *Kidney Int* 2017; **91**: 352-364 [PMID: 27692564 DOI: 10.1016/j.kint.2016.08.006]

13 **Cerda A**, Pavez M, Manriquez V, Luchessi AD, Leal P, Benavente F, Fajardo CM, Salazar L, Hirata MH, Hirata RDC. Effects of clopidogrel on inflammatory cytokines and adhesion molecules in human endothelial cells: Role of nitric oxide mediating pleiotropic effects. *Cardiovasc Ther* 2017; **35** [PMID: 28371087 DOI: 10.1111/1755-5922.12261]

14 **Woodward M**, Lowe GD, Francis LM, Rumley A, Cobbe SM; CADET Study Investigators. A randomized comparison of the effects of aspirin and clopidogrel on thrombotic risk factors and C-reactive protein following myocardial infarction: the CADET trial. *J Thromb Haemost* 2004; **2**: 1934-1940 [PMID: 15550024 DOI: 10.1111/j.1538-7836.2004.01017.x]

15 **Ramadan R**, Dhawan SS, Syed H, Pohlel FK, Binongo JN, Ghazzal ZB, Quyyumi AA. Effects of clopidogrel therapy on oxidative stress, inflammation, vascular function, and progenitor cells in stable coronary artery disease. *J Cardiovasc Pharmacol* 2014; **63**: 369-374 [PMID: 24336012 DOI: 10.1097/FJC.0000000000000057]

16 **Korish AA**. Clopidogrel Prophylaxis Abates Myocardial Ischemic Injury and Inhibits the Hyperlipidemia-Inflammation Loop in Hypercholestrolemic Mice. *Arch Med Res* 2020; **51**: 515-523 [PMID: 32487324 DOI: 10.1016/j.arcmed.2020.05.003]

17 **Zheng Z**, Ma T, Lian X, Gao J, Wang W, Weng W, Lu X, Sun W, Cheng Y, Fu Y, Rane MJ, Gozal E, Cai L. Clopidogrel Reduces Fibronectin Accumulation and Improves Diabetes-Induced Renal Fibrosis. *Int J Biol Sci* 2019; **15**: 239-252 [PMID: 30662363 DOI: 10.7150/ijbs.29063]

18 **Wang Y**, Niu A, Pan Y, Cao S, Terker AS, Wang S, Fan X, Toth CL, Ramirez Solano MA, Michell DL, Contreras D, Allen RM, Zhu W, Sheng Q, Fogo AB, Vickers KC, Zhang MZ, Harris RC. Profile of Podocyte Translatome During Development of Type 2 and Type 1 Diabetic Nephropathy Using Podocyte-Specific TRAP mRNA RNA-seq. *Diabetes* 2021; **70**: 2377-2390 [PMID: 34233930 DOI: 10.2337/db21-0110]

19 **Kuang S**, He F, Liu G, Sun X, Dai J, Chi A, Tang Y, Li Z, Gao Y, Deng C, Lin Z, Xiao H, Zhang M. CCR2-engineered mesenchymal stromal cells accelerate diabetic wound healing by restoring immunological homeostasis. *Biomaterials* 2021; **275**: 120963 [PMID: 34153785 DOI: 10.1016/j.biomaterials.2021.120963]

20 **Wu QR**, Zheng DL, Liu PM, Yang H, Li LA, Kuang SJ, Lai YY, Rao F, Xue YM, Lin JJ, Liu SX, Chen CB, Deng CY. High glucose induces Drp1-mediated mitochondrial fission *via* the Orai1 calcium channel to participate in diabetic cardiomyocyte hypertrophy. *Cell Death Dis* 2021; **12**: 216 [PMID: 33637715 DOI: 10.1038/s41419-021-03502-4]

21 **Wang S**, Yang Y, He X, Yang L, Wang J, Xia S, Liu D, Liu S, Liu W, Duan H. Cdk5-Mediated Phosphorylation of Sirt1 Contributes to Podocyte Mitochondrial Dysfunction in Diabetic Nephropathy. *Antioxid Redox Signal* 2021; **34**: 171-190 [PMID: 32660255 DOI: 10.1089/ars.2020.8038]

22 **Giralt-López A**, Molina-Van den Bosch M, Vergara A, García-Carro C, Seron D, Jacobs-Cachá C, Soler MJ. Revisiting Experimental Models of Diabetic Nephropathy. *Int J Mol Sci* 2020; **21** [PMID: 32438732 DOI: 10.3390/ijms21103587]

23 **Navarro-González JF**, Mora-Fernández C, Muros de Fuentes M, García-Pérez J. Inflammatory molecules and pathways in the pathogenesis of diabetic nephropathy. *Nat Rev Nephrol* 2011; **7**: 327-340 [PMID: 21537349 DOI: 10.1038/nrneph.2011.51]

24 **Klessens CQF**, Zandbergen M, Wolterbeek R, Bruijn JA, Rabelink TJ, Bajema IM, IJpelaar DHT. Macrophages in diabetic nephropathy in patients with type 2 diabetes. *Nephrol Dial Transplant* 2017; **32**: 1322-1329 [PMID: 27416772 DOI: 10.1093/ndt/gfw260]

25 **Chow F**, Ozols E, Nikolic-Paterson DJ, Atkins RC, Tesch GH. Macrophages in mouse type 2 diabetic nephropathy: correlation with diabetic state and progressive renal injury. *Kidney Int* 2004; **65**: 116-128 [PMID: 14675042 DOI: 10.1111/j.1523-1755.2004.00367.x]

26 **Jia LX**, Qi GM, Liu O, Li TT, Yang M, Cui W, Zhang WM, Qi YF, Du J. Inhibition of platelet activation by clopidogrel prevents hypertension-induced cardiac inflammation and fibrosis. *Cardiovasc Drugs Ther* 2013; **27**: 521-530 [PMID: 23887740 DOI: 10.1007/s10557-013-6471-z]

27 **Wu L**, Zhao F, Dai M, Li H, Chen C, Nie J, Wang P, Wang DW. P2y12 Receptor Promotes Pressure Overload-Induced Cardiac Remodeling *via* Platelet-Driven Inflammation in Mice. *Hypertension* 2017; **70**: 759-769 [PMID: 28827474 DOI: 10.1161/HYPERTENSIONAHA.117.09262]

28 **An X**, Jiang G, Cheng C, Lv Z, Liu Y, Wang F. Inhibition of Platelets by Clopidogrel Suppressed Ang II-Induced Vascular Inflammation, Oxidative Stress, and Remodeling. *J Am Heart Assoc* 2018; **7**: e009600 [PMID: 30608200 DOI: 10.1161/JAHA.118.009600]

29 **Hanefeld M**, Appelt D, Engelmann K, Sandner D, Bornstein SR, Ganz X, Henkel E, Haase R, Birkenfeld AL. Serum and Plasma Levels of Vascular Endothelial Growth Factors in Relation to Quality of Glucose Control, Biomarkers of Inflammation, and Diabetic Nephropathy. *Horm Metab Res* 2016; **48**: 620 [PMID: 27756080 DOI: 10.1055/s-0036-1585504]

30 **Zhou G**, Li C, Cai L. Advanced glycation end-products induce connective tissue growth factor-mediated renal fibrosis predominantly through transforming growth factor beta-independent pathway. *Am J Pathol* 2004; **165**: 2033-2043 [PMID: 15579446 DOI: 10.1016/s0002-9440(10)63254-3]

31 **Sanajou D**, Ghorbani Haghjo A, Argani H, Aslani S. AGE-RAGE axis blockade in diabetic nephropathy: Current status and future directions. *Eur J Pharmacol* 2018; **833**: 158-164 [PMID: 29883668 DOI: 10.1016/j.ejphar.2018.06.001]

32 **Sartain SE**, Turner NA, Moake JL. TNF Regulates Essential Alternative Complement Pathway Components and Impairs Activation of Protein C in Human Glomerular Endothelial Cells. *J Immunol* 2016; **196**: 832-845 [PMID: 26673143 DOI: 10.4049/jimmunol.1500960]

33 **Manchanda PK**, Kumar A, Bid HK, Mittal RD. Interleukin-1beta and receptor antagonist (IL-1Ra) gene polymorphisms and the prediction of the risk of end-stage renal disease. *Biomarkers* 2006; **11**: 164-173 [PMID: 16766392 DOI: 10.1080/13547500500525383]

34 **Li T**, Shen K, Li J, Leung SWS, Zhu T, Shi Y. Glomerular Endothelial Cells Are the Coordinator in the Development of Diabetic Nephropathy. *Front Med (Lausanne)* 2021; **8**: 655639 [PMID: 34222276 DOI: 10.3389/fmed.2021.655639]

35 **Wei TT**, Yang LT, Guo F, Tao SB, Cheng L, Huang RS, Ma L, Fu P. Activation of GPR120 in podocytes ameliorates

kidney fibrosis and inflammation in diabetic nephropathy. *Acta Pharmacol Sin* 2021; **42**: 252-263 [PMID: 32948825 DOI: 10.1038/s41401-020-00520-4]

36 **Mason RM**, Wahab NA. Extracellular matrix metabolism in diabetic nephropathy. *J Am Soc Nephrol* 2003; **14**: 1358-1373 [PMID: 12707406 DOI: 10.1097/01.asn.0000065640.77499.d7]

37 **Gruden G**, Perin PC, Camussi G. Insight on the pathogenesis of diabetic nephropathy from the study of podocyte and mesangial cell biology. *Curr Diabetes Rev* 2005; **1**: 27-40 [PMID: 18220580 DOI: 10.2174/1573399052952622]

World Journal of Diabetes

Submit a Manuscript: https://www.f6publishing.com

World J Diabetes 2022 August 15; 13(8): 613-621

DOI: 10.4239/wjd.v13.i8.613

ISSN 1948-9358 (online)

ORIGINAL ARTICLE

Basic Study

Improved systemic half-life of glucagon-like peptide-1-loaded carbonate apatite nanoparticles in rats

Nabilah Ibnat, Rahela Zaman, Mohammad Borhan Uddin, Ezharul Chowdhury, Chooi Yeng Lee

Specialty type: Endocrinology and metabolism

Provenance and peer review: Invited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification

Grade A (Excellent): 0
Grade B (Very good): 0
Grade C (Good): C, C
Grade D (Fair): 0
Grade E (Poor): 0

P-Reviewer: Arslan M, Turkey; Dziegielewska-Gesiak S, Poland

Received: December 15, 2021
Peer-review started: December 15, 2021
First decision: January 12, 2022
Revised: January 24, 2022
Accepted: July 16, 2022
Article in press: July 16, 2022
Published online: August 15, 2022

Nabilah Ibnat, Rahela Zaman, Mohammad Borhan Uddin, Ezharul Chowdhury, School of Medicine and Health Sciences, Monash University Malaysia, Subang Jaya 47500, Selangor, Malaysia

Chooi Yeng Lee, School of Pharmacy, Monash University Malaysia, Subang Jaya 47500, Selangor, Malaysia

Corresponding author: Chooi Yeng Lee, PhD, Senior Lecturer, School of Pharmacy, Monash University Malaysia, Jalan Lagoon Selatan, Subang Jaya 47500, Selangor, Malaysia.
chooi.yeng.lee@monash.edu

Abstract

BACKGROUND

Glucagon-like peptide-1 (GLP1) is an endogenous peptide that regulates blood glucose level. But its susceptibility to rapid metabolic degradation limits its therapeutic use.

AIM

To prepare GLP1-encapsulated nanosize particle with controlled release property to improve the systemic half-life of GLP1.

METHODS

GLP1 nanoparticles were prepared by complexation of GLP1 with carbonate apatite nanoparticles (CA NPs). The physicochemical properties of the CA NPs, the effects of GLP1-loaded CA NPs on cell viability, and the systemic bioavailability of GLP1 after CA NPs administration were determined.

RESULTS

The GLP1-loaded CA NPs was within 200 nm in size and stable in fetal bovine serum. The formulation did not affect the viability of human cell lines suggesting that the accumulation of CA NPs in target tissues is safe. In Sprague Dawley rats, the plasma GLP1 Levels as measured from the GLP1-loaded CA NPs-treated rats, were significantly higher than that of the control rats and free GLP1-treated rats at 1 h post-treatment ($P < 0.05$), and the level remained higher than the other two groups for at least 4 h.

CONCLUSION

The GLP1-loaded CA NPs improved the plasma half-life of GLP1. The systemic bioavailability of GLP1 is longer than other GLP1 nanoparticles reported to date.

Key Words: Glucagon-like peptide-1; Metabolic syndrome; Nanoparticles; Plasma half-life; Rat

Core Tip: Glucagon-like peptide-1 (GLP1), owing to its physiological properties, is a promising peptide in the treatment of obesity and diabetes. Due to the short half-life of GLP1 and in order to improve GLP1 therapeutic use, GLP1 receptor agonists (GLP1-RAs) have been widely synthesised and encapsulated into nanocarriers for targeted delivery. But the use of GLP1-RAs is associated with unwanted side effects and risks. In the present study, we synthesised a new nanocarrier for native GLP1 - the GLP1 carbonate apatite nanoparticles. The nanocarrier appears comparable if not significantly better than other GLP1 nanoparticles, which have shown promising features as therapeutic agents.

Citation: Ibnat N, Zaman R, Uddin MB, Chowdhury E, Lee CY. Improved systemic half-life of glucagon-like peptide-1-loaded carbonate apatite nanoparticles in rats. *World J Diabetes* 2022; 13(8): 613-621
URL: https://www.wjgnet.com/1948-9358/full/v13/i8/613.htm
DOI: https://dx.doi.org/10.4239/wjd.v13.i8.613

INTRODUCTION

The incretin hormone glucagon-like peptide-1 (GLP1) is a peptide secreted from the L cells of the distal ileum and colon in response to nutrient to stimulate insulin secretion. However, as with other peptides, GLP1 is rapidly broken down by circulating enzyme dipeptidyl peptidase-IV (DPP-IV). The poor bioavailability of peptide-based therapeutics is the main challenge of achieving maximum benefit from the drugs.

The metabolic instability of GLP1 has led to the development of GLP1 receptor agonists (GLP1-RAs), which are now widely used in diabetic treatments. GLP1-RAs are generally delivered as payload of drug carriers in injectable formulation. Besides retaining the physiological functions of GLP1, GLP1-RAs have shown additional effects such as regulation of body weight, blood pressure, and cholesterol level [1,2]. But the use of GLP1-RAs was accompanied by side effects such as nausea, vomiting, adverse injection-site reaction, and has posed the risks for pancreatitis and thyroid cell carcinomas[2]. Another aspect of the GLP1-RAs preparations that is worth considering is their pharmacokinetics profile, which essentially is determined by the properties of the drug carriers. Systemic bioavailability of the same drug varies depending on the route of administration that the drug carriers are designed for.

Frequent parenteral administration of therapeutic peptides may lead to a lower patient compliance as compared to oral administration, and also increase the chance of side effects. Moreover, systemically administered peptide drugs have very short half-lives owing to renal clearance and their interaction with the host immune system[3], thus necessitating multiple administration over the course of treatment, which may in turn causes systemic toxicity and undesirable off-target effects. The drawback of GLP1 has also led to the common strategies of conjugating GLP1 or its analogues to polyethylene-glycol (PEG)[4,5] and albumin[6,7] with the aim to extend the peptide's half-life. However, the efficiency and long-term safety of these conjugates have limited their use[6]. Of note, most of these chemical conjugations was done on GLP1-RAs.

Controlled release formulation is a promising approach as it releases the peptide molecule depending on the needs. Such formulation is therefore able to maintain the circulating level of the peptide and prolong therapeutic activities[8-10]. Previously, we demonstrated that pH sensitive inorganic carbonate apatite nanoparticles (CA NPs) were excellent carriers for intracellular delivery of deoxyribonucleic acid (DNA). We have reported the properties of the CA NPs including their sizes, distribution and zeta potential, measurements from the fourier transform-infrared spectroscopy (FT-IR) and x-ray diffraction and dissolution studies, and effects on crystal growth kinetics[11].

In this study, GLP1-loaded CA NPs was formulated, and the *in vitro* and *in vivo* properties of the NPs, specifically their ability to improve the systemic half-life of GLP1 were assessed. We asked the following questions: (1) Does GLP1-CA NPs increase the systemic bioavailability of GLP1 as compared to free GLP1? And (2) Will GLP1-CA NPs with controlled release properties improve the systemic half-life of GLP1 to a similar extent to that of GLP1-RAs?

MATERIALS AND METHODS

Fabrication of GLP-1-loaded CA NPs

The CA NPs was prepared by dissolving 44 mmol/L of sodium bicarbonate and Dulbecco's Modified Eagle Medium (DMEM) powder in mili Q water (pH adjusted to 7.4). The DMEM solution was then mixed with 7 mmol/L concentration of calcium chloride ($CaCl_2$), followed by 30 min incubation at 37°C. For the complexation of GLP1 with CA NPs, a series of GLP1 concentrations ranging from 10 µg to 2 mg, was added to the DMEM solution prior to the addition of 7 mmol/L $CaCl_2$, and the preparations were incubated at 37°C for 30 min.

Turbidity measurement of GLP1-loaded CA NPs

Turbidity measurement was carried out to determine the growth of the particles. The CA NPs and the GLP1-loaded CA NPs were formulated, as described above. The turbidity of the particle suspensions was assessed using ultraviolet (UV) spectrophotometer at 320 nm absorbance wavelength (UV 1800 Spectrophotometer, Shimadzu, Japan).

Visualisation of GLP1-loaded CA NPs under a Field Emission-Scanning Electron Microscope

The field emission-scanning electron microscope (FE-SEM) was used to observe the morphology of the NPs. The GLP1-CA NPs samples were prepared by adding GLP1 (1 and 10 µg) along with 4 mmol/L $CaCl_2$ to 1 mL bicarbonate-buffered medium containing inorganic phosphate. One drop of the complex particle suspension was dried on a glass slide at 37°C for 1 h. The slide was placed onto a carbon tape-coated sample holder. The dried samples underwent platinum sputtering with 30 mA sputter current at 2.30 tooling factor for 70 s, and the sputtered particles visualised at 5.00 kV (Hitachi/SU8010, Tokyo, Japan).

Binding affinity of GLP1 to CA NPs

Fetal bovine serum (FBS, 1%) was used as a source of serum protein to test the binding affinity of GLP1 to CA NPs. The CA NPs containing 5 mmol/L $CaCl_2$ was prepared under the same condition as mentioned above. The CA NPs was then added with 1% FBS and incubated for 10 min. The NPs, coupled with different concentrations of GLP1 - 500 µg, 1 mg and 2 mg were incubated for 30 min at 37°C. These samples together with the free GLP1 were centrifuged at 13000 rpm for 10 min, where the supernatant was discarded. The pellet was washed with 1 mL DMEM before being dissolved with ethylenediaminetetraacetic acid (EDTA) in phosphate-buffered saline (50 mmol/L). The samples were then subjected to sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) in 1% agarose gel. The gel was fixed in a fixing solution for 1 h, stained with Coomassie Blue for 20 min with gentle agitation, and de-stained in a de-staining solution. The image of the de-stained gel was captured using the gel documentation system from Bio-Rad (The United States of America, United States).

Effect of GLP1-CA NPs on the viability of human cell line

The human Michigan Cancer Foundation-7 (MCF-7) cell line, which is a breast cancer cell line, was grown in DMEM supplemented with 10% FBS and 1% penicillin and streptomycin antibiotic in a 25 mm^3 culture flask. One day before the treatment, the exponentially growing cells were trypsinised, centrifuged and re-suspended using DMEM. Cells were counted under the optical microscope using a haemocytometer and seeded on a 24-well plate with cell density of 50000 cells per well. The cells were allowed to attach overnight at 37°C with 5% carbon dioxide (CO_2). Cells were then treated with either free GLP1 or GLP1-CA NPs in the presence or absence of 10% FBS, and for different length of time prior to the cytotoxicity study using 3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide (MTT). In the MTT assay, 50 µL MTT (5 mg/mL in phosphate buffered saline) was added to each well and incubated for 4 h. The formazan products were dissolved with 300 µL dimethyl sulfoxide, and the absorbance measured at 595 nm wavelength with reference to 630 nm on a microplate reader (Dynex Opsys MR, United States).

Measurement of plasma GLP1 Levels following intravenous injection in rats

The experiment was approved by the Monash University Animal Ethics Committee (MARP/2016/008). Male Sprague Dawley rats (n = 18, 6 wk old) were obtained from the Monash Animal Facility and handled according to the appropriate animal care guidelines. Each rat was housed in an individually ventilated cage, in a temperature- and humidity-controlled room with a 12 h light-dark cycle (lights on 06:00-18:00), and was allowed free access to control diet (Gold Coin Sdn. Bhd.) and water. After 7 d of acclimatisation period, the rats were divided into three groups (n = 6 per group), and administered *via* the tail vein, one of the following preparations: CA NPs only, 1 mg/kg free GLP1, and GLP1-loaded CA NPs containing 1 mg/kg GLP1. Approximately 300 µL of blood sample was collected from the tail vein and transferred to a sterile 1.5 mL EDTA-coated tube. Samples were collected at 0 h (pre-treatment), and 1, 2, 4, and 24 h post-treatment, and the blood was centrifuged at 3000 rpm for 15 min at 4°C to separate out the plasma, which was then stored at -20°C. The plasma levels of GLP1 were measured using a commercially available GLP1 enzyme-linked immunosorbent assay (ELISA) kit (Millipore, United

States).

Statistical analysis

Results were presented as mean ± standard error of the mean (SEM). The statistical significance of the treatment groups compared to the control was analysed using the Student's *t* test. Student's *t* test is a well-established parametric statistical method that compares the means of two independent groups and is more appropriate to be used when sample size is small. A *P* value of less than 0.05 was considered statistically significant[12].

RESULTS

The growth profile of GLP1 nanoparticles

As illustrated in Figure 1, increasing the concentration of free GLP1 in the DMEM solution did not change the absorbance intensity of GLP1. An inverse relationship between GLP1 concentrations (ranged from 10 μg to 2 mg) and the formation of CA NPs was found. This indicates that GLP1 might interact with the growing CA NPs and modulate the NPs growth kinetics.

Size and elemental analysis

As shown in Figure 2A, CA NPs was approximate 200 nm in sizes. The presence of GLP1 (1 and 10 μg) along with 4 mmol/L $CaCl_2$ yielded particles of heterogeneous sizes. Accordingly, 1 μg GLP1 gave rise to particle sizes ranging from approximate 15 to 200 nm (Figure 2B), while 10 μg GLP1 produced particles with intermediate sizes ranging from approximate 60 to 70 nm (Figure 2C). Energy Dispersive X-Ray Analysis-based elemental analysis showed that particles formed with 10 μg GLP1 contained more carbon than particles formed with 1 μg GLP1, which may be explained by the presence of higher amount of hydrocarbon in the particles formed with 10 μg GLP1. In addition, the calcium/phosphate (Ca^{2+}/P) ratio was found to be much higher in particles fabricated with higher amount of GLP1, probably as a result of phase transformation (data not shown).

Binding affinity of GLP1 towards CA NPs

In SDS-PAGE, a GLP1 band along with a FBS band was observed for CA NPs prepared with 1 mg and 2 mg GLP1. A more prominent GLP1 band was seen from the latter (Figure 3). Results confirmed that there was sufficiently stable complex formation between the NPs and GLP1. Serum proteins did not trigger the dissociation of GLP1 from the CA NPs.

Effect of GLP1-CA NPs on the viability of human cell line

In the MTT assay, both the free GLP1 and CA NPs-bound GLP1 did not exert noticeable effects on the viability of the human cell line (Figure 4). This suggests that the GLP1-loaded CA NPs are likely to be safe for clinical application. More pre-clinical studies are needed to confirm this.

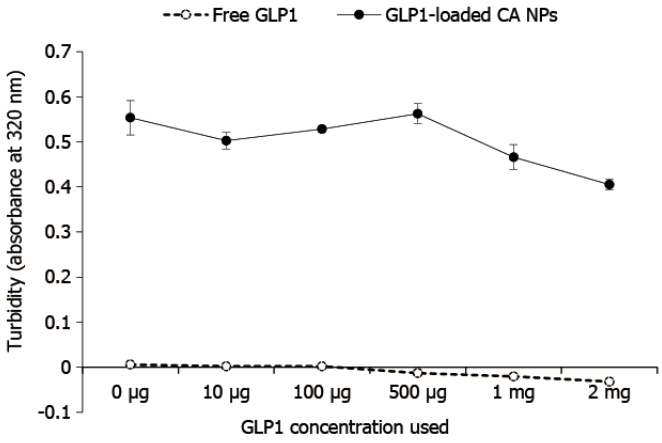
Plasma half-life of GLP1

The plasma levels of GLP1 in rats treated with GLP1-CA NPs were significantly higher than control rats at 1 h (49.61 ± 9.24 picomolar (pM), $P < 0.05$), 2 h (20.22 ± 5.20 pM, $P < 0.05$), and 4 h (16.32 ± 4.01 pM, $P < 0.05$) (Figure 5). The increased plasma GLP1 Level at 1 h in GLP1-CA NPs treated rats was also significantly higher than the levels measured from rats administered with free GLP1 (15.68 ± 4.34 pM, $P < 0.05$).

DISCUSSION

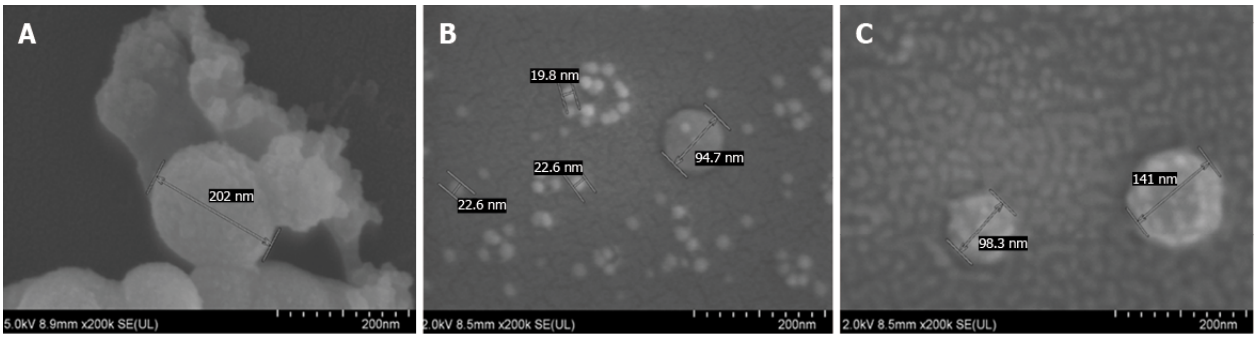
Therapeutic drug with a high tendency of reaching its target site may have increased efficiency and limited side effects. Nanosize particle has the advantage in this aspect as it allows targeted drug delivery. In the present study, a new GLP1-loaded nanosize particle with controlled release property was successfully developed. The formulation adds to the list of GLP1 nanoparticles reported to date, which has made little progress since our review of clinically available GLP1 NPs for diabetic treatment 5 years ago[13].

The slow progress or lack of interest in developing particles that encapsulate native GLP1 could be attributed to the rapid metabolism of GLP1, and as such, making GLP1-RAs a potentially more viable option. The only NPs preparation for intravenous GLP1 administration, a study design closest to the present study, was reported more than a decade ago[14]. The preparation, which used liposome as the drug carrier, produced a 3.6-fold higher serum GLP1 Level than that of free GLP1 at 15 min post-treatment. But the elevated GLP1 Level decreased rapidly thereafter. Another preparation, which was comparable to the present study in terms of the administrative dose and test subject, and involved a



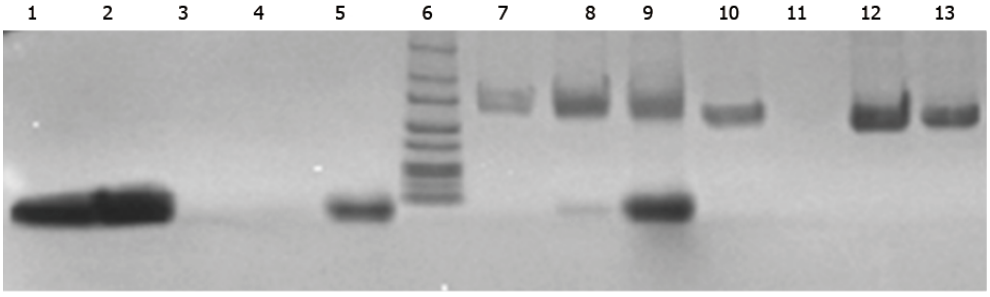
DOI: 10.4239/wjd.v13.i8.613 **Copyright** ©The Author(s) 2022.

Figure 1 Turbidity measurement. The turbidity of samples measured at absorbance 320 nm was an indicator of carbonate apatite nanoparticles (CA NPs) particles growth. The growth of glucagon-like peptide-1 (GLP1)-loaded CA NPs declined in inverse proportion as the concentration of GLP1 increased.



DOI: 10.4239/wjd.v13.i8.613 **Copyright** ©The Author(s) 2022.

Figure 2 The field emission-scanning electron microscope images of the carbonate apatite nanoparticles. Particles approximate 200 nm in size were formed when carbonate apatite nanoparticles (CA NPs) were fabricated without glucagon-like peptide-1 (GLP1) (A). When the CA NPs were fabricated with 1 μg GLP1 (B) and 10 μg GLP1 (C), various sizes of particles were observed with the latter preparation gave a greater size heterogeneity.



DOI: 10.4239/wjd.v13.i8.613 **Copyright** ©The Author(s) 2022.

Figure 3 The binding affinity of glucagon-like peptide-1 towards carbonate apatite nanoparticles. Lane 1. Free glucagon-like peptide-1 (GLP1) 50 μg; 2. Free GLP1 100 μg; 3. GLP1 500 μg + Ca^{2+}; 4. GLP1 1 mg + Ca^{2+}; 5. GLP1 2 mg + Ca^{2+}; 6. protein ladder; 7. GLP1 500 μg + Ca^{2+} +1% fetal bovine serum (FBS); 8. GLP1 1 mg + Ca^{2+} + 1% FBS; 9. GLP1 2 mg + Ca^{2+} + 1% FBS; 10. Dulbecco's Modified Eagle Medium (DMEM) + Ca^{2+} + 1% FBS; 11. Blank; 12. 1% FBS; 13. Ca^{2+} + 1% FBS. GLP1 band was observed from carbonate apatite nanoparticles prepared with 1 mg and 2 mg GLP1, as shown in Lanes 8 and 9, along with the FBS band.

silica-based pH sensitive nanomatrix system, showed a burst release of GLP1 during the first hour. The plasma level of GLP1 however, returned to basal level at 4 h[15].

The GLP1-CA NPs have improved the systemic bioavailability of GLP1, showing better sustained release properties than the liposomal carrier and pH sensitive nanomatrix system. The plasma GLP1 Level of the CA NPs-treated rats was 3.2-fold higher compared to the free GLP1-treated rats at 1 h, and

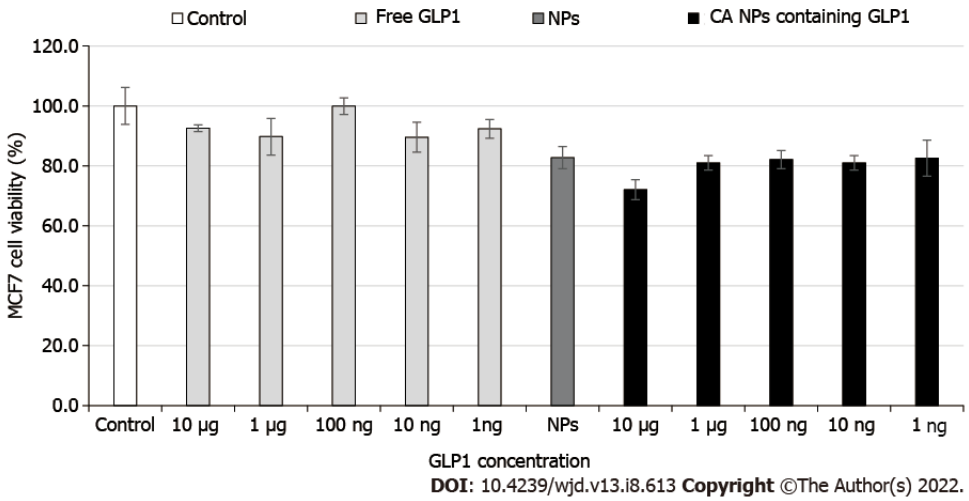


Figure 4 The effect of glucagon-like peptide-1 nanoparticles on the viability of Michigan Cancer Foundation-7 cells. The carbonate apatite nanoparticles with increasing glucagon-like peptide-1 concentrations did not affect the viability of the cells.

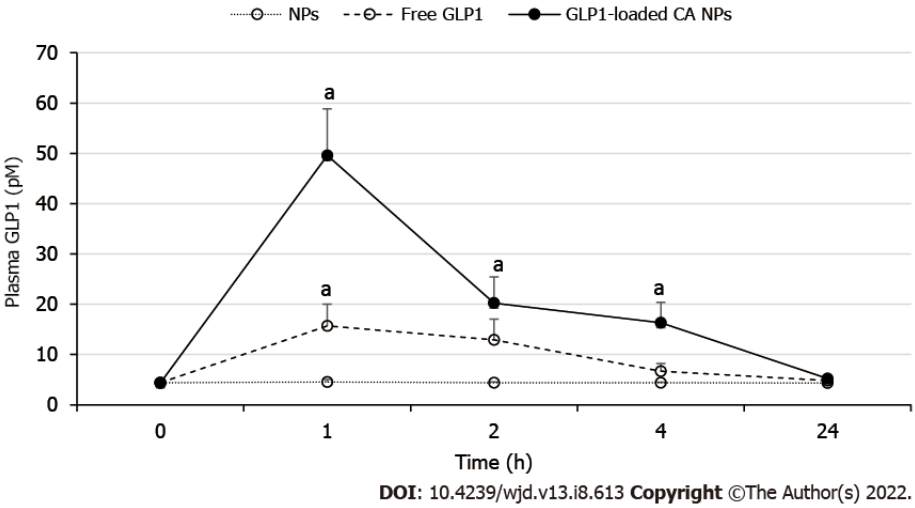


Figure 5 Plasma-time profile of glucagon-like peptide-1. At 1 h post-treatment, Sprague Dawley rats (n = 6) administered with carbonate apatite nanoparticles containing 1 mg/kg of glucagon-like peptide-1 (GLP1) showed significantly higher plasma GLP1 level than the control rats and 1 mg/kg of free GLP1-treated rats ($^aP < 0.05$). The increased plasma GLP1, which was continued to be seen at 4 h, showed that GLP1 was released from the particles in a controlled manner.

the level was sustained for at least 4 h post-treatment. Although carbonate apatite was reported to have strong affinity toward bovine serum albumin at physiological pH[16], the observed high binding affinity of GLP1 towards CA NPs may have negated the potential interaction between CA NPs and various blood proteins.

The liposomal preparation[14], and the silica-based pH sensitive nanomatrix system[15] mentioned above reported significant reduction in the glucose level, and the liposomal GLP1 also significantly increased insulin secretion. Given that CA NPs showed superior plasma GLP1 profile to these preparations, it is reasonable to predict that GLP1-loaded CA NPs will exert similar insulinotropic and hypoglycaemic effects. Nonetheless, further studies are necessary to confirm the therapeutic effects of GLP1-CA NPs.

Despite having a short plasma half-life, GLP1 may still be preferred to GLP1 agonists for therapeutic use. This is evidenced in several studies which used nanomaterials without GLP1 or GLP1-RAs as payload to stimulate GLP1 secretion[5,17,18]. The surface-modified lipid-based nanocarriers not only increased GLP1 secretion significantly, but normalised plasma glucose levels, and reduced insulin resistance in obese/diabetic mice following a 4 wk treatment[5].

Liraglutide, exenatide and exendin-4 are GLP1-RAs widely used to improve the therapeutic effects of GLP1. It is therefore useful to understand the pharmacokinetic/pharmacodynamic profiles of GLP1-RA-loaded carrier systems so as to gain a better understanding of how significant these preparations had in

improving GLP1 bioavailability when comparing with GLP1 nanoparticles, and this was reviewed recently[19]. In order to make comparison between GLP1-CA NPs and GLP1-RAs preparations for their systemic bioavailability and usefulness, only studies that used rats as test subjects and preparations meant for oral administration were discussed below.

Exendin-4 released from enteric-coated capsule containing pH responsive NPs showed a maximum plasma concentration at 5 h after treatment[20]. Another preparation that involved poly(lactic-co-glycolic acid) NPs conjugated with dextran and a C-terminal Src kinase peptide showed peak plasma exenatide level at 6 h post-administration. However, the elevated plasma exenatide level was not significantly different from rats receiving subcutaneous injection of exenatide solution, which was used as a positive control[21]. Zhang *et al*[22,23] who used functionalised NPs for oral exenatide delivery, reported maximum plasma exenatide level at 4 h and 6 h, respectively after administration. But similar to the study by Song *et al*[21], the exenatide level was not significantly different from that seen in rats administered subcutaneously with an exenatide solution. Overall, the sustainability of the plasma concentration of GLP1-RAs encapsulated in various types of nanomaterials was between 4-6 h, only slightly higher than that of GLP1-encapsulated CA NPs, although an oral formulation may produce better patient compliance than parenteral administration.

On this note, it is necessary to highlight that the GLP1 systemic bioavailability and/or the biological effects of the different nanomaterial preparations discussed above were cited from and compared between animal subjects. Preparations which are under pre-clinical testing stages, as per the present study, provide useful information on the potential usability and practicality of the preparations in human, and are instructive for future investigation on other animal species and subsequently, human subjects.

Liraglutide and semaglutide have been approved by the United States Food and Drug Administration as weight management agents, and semaglutide in obese or overweight adults with at least one weight-related condition including diabetes. Emerging evidences support the implementation of pharmacotherapy along with behavioural therapy and dietary intervention in optimising obesity treatment. The same approach may apply to diabetes management because consumption of food with antioxidant and anti-inflammatory properties could synergise the effects of GLP1 and GLP1-RAs[24]. If combination therapy is the recommendation for the treatment of metabolic syndrome, the overall cost of management should be taken into consideration.

This study was limited by the lack of chronic and sub-chronic *in vivo* study involving obese or diabetic animals. A longer duration of study and measurement of metabolic parameters will provide evidence of the effectiveness of GLP1 CA NPs in the long-term treatment of metabolic syndrome. Nevertheless, we showed that this new preparation has therapeutic potential. A second limitation is on the discussion of the data itself. In order to make valid and comparable comparison of the pharmacokinetics profiles between the different GLP1 nanocarriers, only studies that used the same animal species, and native GLP1 were included in the discussion. This means that not all GLP1 and GLP1 RAs nanocarriers that are reported to date have been included. The literature search has nevertheless implied that there is higher interest in GLP1-RAs than native GLP1 despite the high cost of producing GLP1-RAs.

CONCLUSION

In summary, we have developed a new nanocarrier for GLP1. *In vitro*, the GLP1-CA NPs are safe on human cell lines, and stable against dissociation from interaction with plasma proteins. *In vivo*, GLP1-CA NPs demonstrated sustained release properties by significantly improving the plasma half-life of GLP1. The preparation has resulted in a better GLP1 systemic bioavailability than previously reported GLP1-loaded nanocarriers, making it a potentially promising treatment option for metabolic syndrome.

ARTICLE HIGHLIGHTS

Research background

Apart from Glucagon-like peptide-1 (GLP1) receptor agonists that are being widely used and studied, more effort should also be channeled to designing carrier with sustained release properties for native GLP1 because both approaches may be equally effective in improving the systemic half-life of GLP1.

Research motivation

The GLP1-carbonate apatite nanoparticles (CA NPs) overcome the short half-life of GLP1. The nanoparticles could be a potential therapeutic option for metabolic syndrome and warrant further investigation.

Research results

A stable GLP1-CA NPs was successfully fabricated. The NPs improved the systemic half-life of GLP1 as compared with free GLP1-treated rats. The increased plasma GLP1 Level was maintained for at least 4 h post-treatment.

Research methods

The nanoparticles were fabricated through complexation between GLP1 and CA NPs. The GLP1-CA NPs was then evaluated for physicochemical properties, tested for their potential cytotoxic effects on human cell line, and finally measured for systemic bioavailability in rats through intravenous administration.

Research objectives

To fabricate GLP1-loaded carbonate apatite nanoparticles (GLP1-CA NPs), and improve the systemic half-life of GLP1 through GLP1-CA NPs.

Research conclusions

pH sensitive inorganic carbonate apatite nanoparticles, which we have successfully formulated previously may be a potential carrier for GLP1.

Research perspectives

GLP1 is an endogenous peptide with established glucose lowering property. Its therapeutic use however is limited due to it being rapidly degraded in the systemic circulation. Nanosize particles with sustained release property may protect as well as extend the plasma half-life of GLP1.

ACKNOWLEDGEMENTS

The authors would also like to thank Md. Karim E for his assistance in the *in vivo* study.

FOOTNOTES

Author contributions: Lee CY and Chowdhury E contributed to the study concept and design; Ibnat N, Zaman R and Uddin B acquired the data; Ibnat N, Uddin B, Lee CY and Chowdhury E analysed the data; Ibnat N drafted the manuscript; Lee CY reviewed and edited the manuscript; all authors have read and approved the final manuscript.

Supported by an FRGS grant from the Ministry of Education, Malaysia, No. FRGS/2/2014/SG05/MUSM/03/1.

Institutional animal care and use committee statement: The study was reviewed and approved by the Monash University Animal Ethics Committee (Approval No. MARP/2016/008).

Conflict-of-interest statement: There is no conflict-of-interest.

Data sharing statement: No additional data are available.

ARRIVE guidelines statement: The authors have read the ARRIVE guidelines, and the manuscript was prepared and revised according to the ARRIVE guidelines.

Country/Territory of origin: Malaysia

ORCID number: Nabilah Ibnat 0000-0002-7018-4867; Rahela Zaman 0000-0002-8249-7668; Mohammad Borhan Uddin 0000-0003-4384-4900; Ezharul Chowdhury 0000-0002-5705-841X; Chooi Yeng Lee 0000-0001-5847-0329.

S-Editor: Wu YXJ
L-Editor: Filipodia
P-Editor: Wu YXJ

REFERENCES

1 **Chatterjee S**, Ghosal S, Chatterjee S. Glucagon-like peptide-1 receptor agonists favorably address all components of metabolic syndrome. *World J Diabetes* 2016; **7**: 441-448 [PMID: 27795818 DOI: 10.4239/wjd.v7.i18.441]
2 **Prasad-Reddy L**, Isaacs D. A clinical review of GLP-1 receptor agonists: efficacy and safety in diabetes and beyond. *Drugs Context* 2015; **4**: 212283 [PMID: 26213556 DOI: 10.7573/dic.212283]
3 **Patel A**, Cholkar K, Mitra AK. Recent developments in protein and peptide parenteral delivery approaches. *Ther Deliv* 2014; **5**: 337-365 [PMID: 24592957 DOI: 10.4155/tde.14.5]
4 **van Witteloostuijn SB**, Pedersen SL, Jensen KJ. Half-Life Extension of Biopharmaceuticals using Chemical Methods: Alternatives to PEGylation. *ChemMedChem* 2016; **11**: 2474-2495 [PMID: 27775236 DOI: 10.1002/cmdc.201600374]
5 **Xu Y**, De Keersmaecker H, Braeckmans K, De Smedt S, Cani PD, Préat V, Beloqui A. Targeted nanoparticles towards increased L cell stimulation as a strategy to improve oral peptide delivery in incretin-based diabetes treatment. *Biomaterials* 2020; **255**: 120209 [PMID: 32580098 DOI: 10.1016/j.biomaterials.2020.120209]
6 **Bech EM**, Pedersen SL, Jensen KJ. Chemical Strategies for Half-Life Extension of Biopharmaceuticals: Lipidation and Its Alternatives. *ACS Med Chem Lett* 2018; **9**: 577-580 [PMID: 30034579 DOI: 10.1021/acsmedchemlett.8b00226]
7 **Park J**, Bak M, Min K, Kim HW, Cho JH, Tae G, Kwon I. Effect of C-terminus Conjugation *via* Different Conjugation Chemistries on In Vivo Activity of Albumin-Conjugated Recombinant GLP-1. *Pharmaceutics* 2021; **13** [PMID: 33672039 DOI: 10.3390/pharmaceutics13020263]
8 **Patil S**, Vhora I, Amrutiya J, Lalani R, Misra A. Role of Nanotechnology in Delivery of Protein and Peptide Drugs. *Curr Pharm Des* 2015; **21**: 4155-4173 [PMID: 26323432 DOI: 10.2174/1381612821666150901095722]
9 **Lee PW**, Pokorski JK. Poly(lactic-co-glycolic acid) devices: Production and applications for sustained protein delivery. *Interdiscip Rev Nanomed Nanobiotechnol* 2018; **10**: e1516 [DOI: 10.1002/wnan.1516]
10 **Raza F**, Zafar H, Zhu Y, Ren Y, -Ullah A, Khan AU, He X, Han H, Aquib M, Boakye-Yiadom KO, Ge L. A Review on Recent Advances in Stabilizing Peptides/Proteins upon Fabrication in Hydrogels from Biodegradable Polymers. *Pharmaceutics* 2018; **10** [PMID: 29346275 DOI: 10.3390/pharmaceutics10010016]
11 **Chowdhury EH**, Maruyama A, Kano A, Nagaoka M, Kotaka M, Hirose S, Kunou M, Akaike T. pH-sensing nano-crystals of carbonate apatite: effects on intracellular delivery and release of DNA for efficient expression into mammalian cells. *Gene* 2006; **376**: 87-94 [PMID: 16723196 DOI: 10.1016/j.gene.2006.02.028]
12 **Kim TK**. T test as a parametric statistic. *Korean J Anesthesiol* 2015; **68**: 540-546 [PMID: 26634076 DOI: 10.4097/kjae.2015.68.6.540]
13 **Lee CY**. Glucagon-Like Peptide-1 Formulation--the Present and Future Development in Diabetes Treatment. *Basic Clin Pharmacol Toxicol* 2016; **118**: 173-180 [PMID: 26551045 DOI: 10.1111/bcpt.12524]
14 **Hanato J**, Kuriyama K, Mizumoto T, Debari K, Hatanaka J, Onoue S, Yamada S. Liposomal formulations of glucagon-like peptide-1: improved bioavailability and anti-diabetic effect. *Int J Pharm* 2009; **382**: 111-116 [PMID: 19698772 DOI: 10.1016/j.ijpharm.2009.08.013]
15 **Qu W**, Li Y, Hovgaard L, Li S, Dai W, Wang J, Zhang X, Zhang Q. A silica-based pH-sensitive nanomatrix system improves the oral absorption and efficacy of incretin hormone glucagon-like peptide-1. *Int J Nanomedicine* 2012; **7**: 4983-4994 [PMID: 23028226 DOI: 10.2147/IJN.S30013]
16 **Hossain S**, Stanislaus A, Chua MJ, Tada S, Tagawa Y, Chowdhury EH, Akaike T. Carbonate apatite-facilitated intracellularly delivered siRNA for efficient knockdown of functional genes. *J Control Release* 2010; **147**: 101-108 [PMID: 20620182 DOI: 10.1016/j.jconrel.2010.06.024]
17 **Beloqui A**, Alhouayek M, Carradori D, Vanvarenberg K, Muccioli GG, Cani PD, Préat V. A Mechanistic Study on Nanoparticle-Mediated Glucagon-Like Peptide-1 (GLP-1) Secretion from Enteroendocrine L Cells. *Mol Pharm* 2016; **13**: 4222-4230 [PMID: 27934480 DOI: 10.1021/acs.molpharmaceut.6b00871]
18 **Xu Y**, Carradori D, Alhouayek M, Muccioli GG, Cani PD, Préat V, Beloqui A. Size Effect on Lipid Nanocapsule-Mediated GLP-1 Secretion from Enteroendocrine L Cells. *Mol Pharm* 2018; **15**: 108-115 [PMID: 29226685 DOI: 10.1021/acs.molpharmaceut.7b00742]
19 **Eissa NG**, Elsabahy M, Allam A. Engineering of smart nanoconstructs for delivery of glucagon-like peptide-1 analogs. *Int J Pharm* 2021; **597**: 120317 [PMID: 33540005 DOI: 10.1016/j.ijpharm.2021.120317]
20 **Nguyen HN**, Wey SP, Juang JH, Sonaje K, Ho YC, Chuang EY, Hsu CW, Yen TC, Lin KJ, Sung HW. The glucose-lowering potential of exendin-4 orally delivered *via* a pH-sensitive nanoparticle vehicle and effects on subsequent insulin secretion in vivo. *Biomaterials* 2011; **32**: 2673-2682 [PMID: 21256586 DOI: 10.1016/j.biomaterials.2010.12.044]
21 **Song Y**, Shi Y, Zhang L, Hu H, Zhang C, Yin M, Chu L, Yan X, Zhao M, Zhang X, Mu H, Sun K. Synthesis of CSK-DEX-PLGA Nanoparticles for the Oral Delivery of Exenatide to Improve Its Mucus Penetration and Intestinal Absorption. *Mol Pharm* 2019; **16**: 518-532 [PMID: 30601014 DOI: 10.1021/acs.molpharmaceut.8b00809]
22 **Zhang L**, Shi Y, Song Y, Duan D, Zhang X, Sun K, Li Y. Tf ligand-receptor-mediated exenatide-Zn^{2+} complex oral-delivery system for penetration enhancement of exenatide. *J Drug Target* 2018; **26**: 931-940 [PMID: 29619854 DOI: 10.1080/1061186X.2018.1455839]
23 **Zhang L**, Shi Y, Song Y, Sun X, Zhang X, Sun K, Li Y. The use of low molecular weight protamine to enhance oral absorption of exenatide. *Int J Pharm* 2018; **547**: 265-273 [PMID: 29800739 DOI: 10.1016/j.ijpharm.2018.05.055]
24 **Lee CY**. A Combination of Glucagon-Like Peptide-1 Receptor Agonist and Dietary Intervention Could Be a Promising Approach for Obesity Treatment. *Front Endocrinol (Lausanne)* 2021; **12**: 748477 [PMID: 34616367 DOI: 10.3389/fendo.2021.748477]

World Journal of Diabetes

Submit a Manuscript: https://www.f6publishing.com

World J Diabetes 2022 August 15; 13(8): 622-642

DOI: 10.4239/wjd.v13.i8.622

ISSN 1948-9358 (online)

ORIGINAL ARTICLE

Basic Study

In vivo evaluation and mechanism prediction of anti-diabetic foot ulcer based on component analysis of Ruyi Jinhuang powder

Xiu-Yan Li, Xiao-Tong Zhang, Yi-Cheng Jiao, Hang Chi, Ting-Ting Xiong, Wen-Jing Zhang, Mi-Nan Li, Yan-Hong Wang

Specialty type: Endocrinology and metabolism

Provenance and peer review: Unsolicited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification

Grade A (Excellent): A
Grade B (Very good): B, B
Grade C (Good): 0
Grade D (Fair): 0
Grade E (Poor): 0

P-Reviewer: Bahrmann A, Germany; Elnakib AA, Egypt; Lee AS, Australia

Received: May 6, 2022
Peer-review started: May 6, 2022
First decision: May 30, 2022
Revised: June 10, 2022
Accepted: July 6, 2022
Article in press: July 6, 2022
Published online: August 15, 2022

Xiu-Yan Li, Yi-Cheng Jiao, Hang Chi, Ting-Ting Xiong, Wen-Jing Zhang, Mi-Nan Li, Yan-Hong Wang, Key Laboratory of Basic and Application Research of Beiyao, Heilongjiang University of Chinese Medicine, Harbin 150040, Heilongjiang Province, China

Xiu-Yan Li, College of Pharmacy, Harbin Medical University, Harbin 150040, Heilongjiang Province, China

Xiao-Tong Zhang, Key Laboratory of Basic and Application Research of Beiyao, Heilongjiang University of Chinese Medicine, Ministry of Education, College of Pharmacy, Harbin 150040, Heilongjiang Province, China

Corresponding author: Yan-Hong Wang, MD, Teacher, Key Laboratory of Basic and Application Research of Beiyao, Heilongjiang University of Chinese Medicine, No. 24 Heping Road, Xiangfang District, Harbin 150040, Heilongjiang Province, China. 799378826@qq.com

Abstract

BACKGROUND

Diabetes is a metabolic disease with a high complication rate. Diabetic foot ulcers (DFUs) seriously affect the quality of life of patients. A total of 15%-20% of diabetic patients develop DFUs, which heal with difficulty over a long time and can result in amputation and disability. Traditional Chinese medicine has a unique effect in the treatment of skin ulcerative diseases. Ruyi Jinhuang powder (RHP) is one of the classic prescriptions in traditional Chinese medicine and is widely used in clinical practice.

AIM

To verify the ability of RHP to promote wound healing by electron microscopy analysis in animal models and hematoxylin-eosin (HE) staining. The effective components of RHP were extracted and identified by gas chromatography-mass spectrometry (GC-MS), and the obtained chemical components were analyzed by network pharmacology methods to predict its therapeutic mechanism.

METHODS

Sprague Dawley rats were injected with streptozotocin to establish the DFU model. HE staining was used to observe the wound tissue under an electron microscope. The chemical constituents of RHP were extracted first by supercritical fluid extraction and alcohol extraction, and then, GC-MS and ultra-performance

liquid chromatography–MS were used to separately identify the chemical constituents. In addition, the "herb-component-target" link was established through the Traditional Chinese Medicine Systems Pharmacology database to obtain the target information, and the molecular docking of important components and key targets was performed in Discovery Studio software. Cytoscape software was used to visualize and analyze the relationship between the chemical composition, targets and Traditional Chinese Medicine network.

RESULTS

RHP promoted DFU healing in rats by affecting fibroblasts and nerve cells. A total of 89 chemical components were obtained by GC-MS. Network pharmacological analysis revealed that RHP was associated with 36 targets and 27 pathways in the treatment of DFU, of which the important components were luteolin, trans caryophyllene, ar-turmerone, palmitic acid, methyl palmitate, gallic acid, demethoxycurcumin, berberine, and rheic acid. The key targets were posttranscriptional silencing, topoisomerase II alpha, muscarinic acetylcholine receptor M2, interleukin 6, tumor necrosis factor and retinoic X receptor alpha, and the key pathways were the phosphoinositide 3-kinase-protein kinase B signaling pathway, neuroactive ligand–receptor interactions, and the forkhead box O signaling pathway.

CONCLUSION

Our results indicated that RHP may play a role in the treatment of DFU through these target pathways by affecting insulin resistance, altering the nervous system and immune system, participating in inflammatory responses and regulating cell proliferation, differentiation and apoptosis through other specific mechanisms.

Key Words: Ruyi Jinhuang powder; Diabetic foot ulcer; Mass spectrometry-chromatography; Network pharmacology; Hematoxylin-eosin staining; Components analysis

Core Tip: Although some studies have suggested that Ruyi Jinhuang powder (RHP) has a therapeutic effect on diabetic foot ulcers (DFUs), few have used component analysis, investigated the mechanism of action, and utilized wound-healing experiments. The components of RHP were used to predict the mechanism of action, and wound healing was observed by establishing a DFU rat model to further prove the therapeutic effect of RHP on DFU to finally determine a possible mechanism of action.

Citation: Li XY, Zhang XT, Jiao YC, Chi H, Xiong TT, Zhang WJ, Li MN, Wang YH. *In vivo* evaluation and mechanism prediction of anti-diabetic foot ulcer based on component analysis of Ruyi Jinhuang powder. *World J Diabetes* 2022; 13(8): 622-642

URL: https://www.wjgnet.com/1948-9358/full/v13/i8/622.htm

DOI: https://dx.doi.org/10.4239/wjd.v13.i8.622

INTRODUCTION

Ruyi Jinhuang powder (RHP) is included in "Authenticity of Surgery" written by Chen Shigong in the Ming Dynasty, which includes "*THF* [*Trichosanthin* (*Tian Hua Fen*)], *DH* [*Rhubarb* (*Da Huang*)], *HB* [*Phellodendron* (*Huang Bai*)], *JH* [*Turmeric* (*Jiang Huang*)], *BZ* [*Angelica dahurica* (*Bai Zhi*)], *TNX* [*Arisaema* (*Tian Nan Xing*)], *CZ* [*Atractylodes lancea* (*Cang Zhu*)], *HP* [*Magnolia officinalis* (*Hou Po*)], *CP* [*Pericarpium Citri Reticulatae* (*Chen Pi*)] and *GC* [*Glycyrrhiza uralensis* (*Gan Cao*)]"[1] and is now included in the Chinese Pharmacopoeia. This prescription is used for swelling relief and pain relief. In this prescription, *Trichosanthes* is the monarch medicine, and the minister medicine *rhubarb* with the same cold and bitter taste is used to purge fire detumescence; *angelica dahurica* and *turmeric* are the minister pungent medicines with compatibility to discharge pus pain; *pericarpium citri reticulatae*, *Magnolia officinalis*, *atractylodes lancea and glycyrrhiza uralensis* are combined to remove dampness and regulate Qi; and *arisaema* alleviates swelling pain. The above five medicines act together as adjuvants with *glycyrrhiza uralensis* to reconcile and detoxify the medicine. Modern clinical applications mainly include cutaneous vasculitis, gouty arthritis, herpes zoster and diabetic foot ulcer (DFU). After many studies on its pharmacological effects, it was found that RHP can inhibit bacterial infection, increase lysosomal content, enhance immune defenses and inhibit inflammation. The traditional preparation is through the addition of honey to the powder, which is then directly applied to the affected area to treat diseases;

however, the formulation has been innovated using the original preparation through the continuous implementation of modern technology and made into creams, cataplasms, films, and sponges[2,3].

DFU is a diabetic complication. Its pathogenic causes are often vascular and nerve lesions, resulting in lower limb infection and the formation of foot ulcers[4]. The clinical manifestations are ischemic necrosis or damage to skin tissue, incomplete skin, wound exudation, abscess generation, *etc*. From the perspective of traditional Chinese medicine, DFUs are caused by deficiency of Qi and Yin, weakness of pulse, and blockage of dampness-heat and blood stasis toxin. Traditional chinese medicine also has shown promising effects with regard to safety and renoprotection in some prospective, multicenter, randomized, controlled clinical trial conducted[5]. And studies have found that RHP has a good effect in the treatment of DFUs in traditional Chinese medicine. Shao *et al*[6] found that its combination with antibiotics can quickly reduce the swelling of patients' ulcers to shorten the course of treatment. Liu *et al* [7] treated 40 patients with diabetic skin abscesses with the external application of RHP and found that the rate of effective treatment in the treatment group was higher than the control group. This result shows that RHP has a therapeutic effect on DFU. Zhang[8] treated patients with RHP and Simiao Tongluo decoction and found that this prescription can promote wound healing to promote improvement and play a therapeutic role.

Gas chromatography-mass spectrometry (GC–MS) and ultra-performance liquid chromatography-mass spectrometry (UPLC–MS) are widely used in the separation and identification of complex components, of which GC–MS is mainly used to separate the sample into volatile products in the instrument by pyrolysis of the sample at high temperature, with the advantages of high sensitivity, large information content, high efficiency, and small sample requirements. UPLC–MS technology allows the sample to be separated in the mobile phase after the ionization process through fragment ion mass number analysis and identification. This technology can compensate for the disadvantages of GC–MS, which cannot analyze components with features such as strong polarity, thermal instability, and difficult volatilization; UPLC-MS has the advantages of low detection limit, high automation, wide analysis range, and short analysis time. Network pharmacology research is mainly based on databases and software to obtain important target information for drug treatment of diseases and establish network connections to predict its mechanism of action. Because network pharmacology analysis shows synergy and compatibility with traditional Chinese medicine in the treatment of diseases and the therapeutic principle of syndrome differentiation and treatment, it is widely used in traditional Chinese medicine to explore the mechanism of action in the treatment of diseases from multiple targets. Wound healing is caused by a variety of molecular proteins that affect cells and remodel the tissue. Hematoxylin-eosin (HE) staining electron microscope observation is a more intuitive way to observe the healing of the wound surface. Fibroblasts can be clearly observed to evaluate drug treatment.

Although it has been proven that RHP has a therapeutic effect on DFU, the specific mechanism of action is not clear. In this study, the chemical components of RHP were extracted by supercritical extraction and alcohol extraction and separated and identified by GC–MS and UPLC–MS techniques, respectively, to obtain the active ingredients of the RHP formula, and the target pathway for the treatment of DFU was studied using network pharmacological analysis. In addition, a rat model of DFU was established to verify the therapeutic effect of RHP on wound healing, providing a direction for further clinical research.

MATERIALS AND METHODS

Materials

RHP (201202056, Jilin Shuangshi Pharmaceutical Co., Ltd) was purchased from Harbin Shiyitang Chinese Herbal Medicine Co., Ltd. (Harbin, China). Methanol (purity) and acetonitrile (purity) were all obtained from Merck Co., Inc. (Germany). Formic acid (purity) was obtained from West Asia Chemical Technology Co., Ltd. All other chemicals and solvents were of analytical grade.

Animals and drug administration

Thirty healthy male Sprague Dawley rats (weights, 200 ± 20 g) were purchased from Jinan Pengyue Experimental Animal Breeding Co., Ltd. (Jinan, China) and housed for adaptive feeding for 2 d. The animal facilities and protocols were approved by the Animal Ethics Committee of Heilongjiang University of Chinese Medicine (Heilongjiang, China).

Thirty rats were divided into 3 groups with 10 rats in each group, including the blank, model and RHP groups. The model and RHP groups were injected with streptozotocin/0.1 mol·L^{-1} citrate buffer solution (1/100, g/v). After 72 h, fasting blood glucose was measured with an electronic blood glucose meter (Sannuo Biosensor Co., Ltd, China). The modeling standard was that the random blood glucose level was greater than 12.0 mmol/L, accompanied by the typical symptoms of diabetes mellitus. In the establishment of the ulcer model, the rat hindfoot skin in each group was cut with scissors (the depth of the wound that would reach the fascia). The wound was cleaned before each treatment every day. The wound area was measured on the 5th, 7th, and 14th days, and the wound-healing rate was calculated. The formula for the healing rate was as follows: Healing rate (%) = (original wound area-unhealed

area)/original wound area × 100%.

After 14 d, the rats were sacrificed, and the skin around the wound was cut into 3 cm × 3 cm areas. The cut tissue was fixed with a 2.5% pentanediol solution and stored at low temperature (-80 °C).

The sample tissues were placed in liquid paraffin, freeze-fixed and sliced. Dewaxing, hydration and dyeing with hematoxylin staining solution and eosin dye solution were performed. The samples were air dried, sealed and observed with transmission electron microscopy (HT7700, Hitachi, Japan).

Composition analysis of RHP by GC–MS

Supercritical fluid extraction (SFE) equipment (HA220-40-11, Nantong Huaan Supercritical Extraction Co., Ltd, China) was used to extract volatile oil. First, an appropriate amount of RHP sample was crushed and sifted through a 40 μm mesh; the medicinal material was placed into supercritical extraction equipment, and the pressure was boosted to the set parameters for extraction. The pressure of the extraction kettle was 25 MPa, and its temperature was 45 °C. The pressure of the separating kettle was 8 MPa, and its temperature was 60 °C. The pressure of the other separating kettle was 4.5 MPa, and its temperature was 37 °C. The pump frequency of the SFE was 18 Hz, and the flow rate was 60 L/h. After extraction, the materials were removed to obtain the SFE.

GC–MS (5975B, Agilent Technologies, Inc., United States) was used to analyze the chemical composition of the SFE extraction. The prepared SFE extract was vortexed for 2 min, extracted with a solid-phase microextraction needle for 20 min, centrifuged for 20 min, and filtered with a membrane. Each of the samples was injected into GC–MS equipment equipped with an HP-INNOWAX (25 m × 0.20 mm, 0.40 μm) column (Agilent, United States) at 100 °C for 5 min. Then, the temperature was raised to 150 °C at 5 °C/min and then to 280 °C at 30 °C/min. The inlet temperature was 240 °C, and the flow rate of the carrier gas was 1.0 mL/min. The ion source temperature of MS with an EI source was 200 °C, and its transmission line temperature was 250 °C. The bombardment voltage was 70 eV.

Composition analysis of RHP by UPLC–MS

UPLC–MS (Ultimate 3000LC, Q Exactive HF, Thermo Fisher, United States) was used to analyze the chemical composition of RHP. First, the sample was pulverized, passed through a 40-μm mesh sieve, accurately weighed and placed in a stoppered conical flask. Hydrochloric acid/ethanol solution (1/100, v/v) was accurately added and ultrasonically treated for 40 min. The filtrate was shaken well, filtered and diluted into a 50-mL volumetric flask to obtain a sample solution. The reference substance was precisely weighed and placed in a 10-mL volumetric flask; methanol solution was added, and the solution was diluted to volume after ultrasonic treatment and shaken well to obtain the reference substance solution. Each of the samples was injected into UPLC–MS equipment equipped with a C18 chromatographic (2.1 mm × 100 mm, 1.8 μm) column (Zorbax Eclipse, Agilent, United States) at 30 °C. The flow rate was 0.3 mL/min. The mobile phase was water/formic acid (0.1/100, v/v) (A)/acetonitrile (B). The injection volume was 2 μL.

Mass spectrometry conditions utilized positive and negative modes for UPLC–MS. Electrospray ionization was used in ionization mode with a sheath gas flow rate of 45 arb. The auxiliary gas flow rate was 15 arb. The purge gas flow rate was 1 arb. The electrospray voltage was 3.5 KV. The capillary temperature was 330 °C. The S-Lens RF level was 55%. The scan mode was full scan/dd-MS2 (TopN = 10) with a scanning range of 100-1500 m/z and a resolution of 120000/60000. The collision mode was high energy collision dissociation.

Building a drug-component-target-disease network relationship graph

Cytoscape (v3.7.2.) is an analysis software that shows the complex corresponding relationship of "drug-component-target-disease" in the form of a network graph. It can conveniently visualize a network relationship, perform network topology analysis, analyze the degree of connection between each node according to the relevant parameters, and thus enable researchers to draw the corresponding conclusions.

Determining the "Chemical Composition-Target" correspondence

The Traditional Chinese Medicine Systems Pharmacology (TCMSP) database (http://Lsp.nwu.edu.cn/) is a platform for the analysis and study of traditional Chinese medicines in many aspects, and it synthesizes the chemical composition and drug target data of traditional Chinese medicines[9], closely links diseases with targets and components, and explains the mechanism of action of traditional Chinese medicines[10]. The chemical components extracted from ten traditional Chinese medicines of the RHP formula were searched by entering the CAS number of chemical components in the TCMSP database, and the information related to the components could be obtained, of which the "Relatedtarget" column contained the targets corresponding to the component, and the obtained targets were integrated to determine the corresponding relationship between the components and the target.

Screening targets of RHP

Comparative Toxicogenomics Database (CTD) (http://ctdbase.org/) is a database that brings together detailed information on the intersection between genes, proteins and diseases[11], and the combination

Table 1 Statistical analysis of wound healing rate at different times

Time	0 d (%)	3 d (%)	7 d (%)	14 d (%)
Control group	0	10.41 ± 0.24	17.43 ± 0.13	33.73 ± 0.28
Experimental group	0	32.78 ± 0.46	38.14 ± 0.28	53.11 ± 0.07
Homogeneity of variance	–	√	√	√
P value	–	< 0.05	< 0.05	< 0.05

of these data with that of their pathways and functions can further elucidate the mechanism of diseases [12]. A component usually corresponds to one or more targets, and information unrelated to the treatment of DFU in the above integrated "component-target" dataset needs to be screened out. In the TCMSP database, the information related to each target in the "Relatedtarget" column above was viewed, the relevant target with the keywords "diabetes-related diseases", "pain" and "bacterial infection" was selected according to the disease type in "Relateddiseases", and its "GenecardID" was recorded. To prevent the limitations of the TCMSP database on the target and disease correspondence and make the study more accurate, the targets without keywords were searched in the CTD database to further determine whether they were related to DFU in the "Diseases" category. Two databases, TCMSP and CTD, were used to screen for and obtain the chemical compositions of targets related to DFU.

Molecular docking of ingredients and targets

DiscoveryStudio (DS v19.1.0) software is a life science molecular simulation software that can be used to establish molecular docking models. Cytoscape was used to visualize the targets and components, analyze the key targets and important components, perform molecular docking, and observe the binding effect of chemical components to target proteins. First, the structural model of the component was downloaded from the TCMSP database; the human protein number corresponding to the target from the UniProt database was queried, and the number from the PDB database was the input to obtain the three-dimensional structural model of the target protein. The component structure and protein structure were opened in DiscoveryStudio software; H_2O and ligand were removed, the protein was hydrogenated, and the relationship between the molecule and the protein was established. In the end, the binding sites can be identified.

Pathway enrichment analysis

The STRING database (https://cn.string-db.org/) and the DAVID database (https://david.ncifcrf.gov) are mainly used to provide information on the individual target genes and proteins or the interaction between multiple histones and to perform GO or KEGG pathway enrichment analysis on them. The STRING database was used to observe the correlation between target proteins of RHP formula herbs, and the DAVID database was used to enrich KEGG pathways for target components and targets to obtain possible pathways for the treatment of DFU.

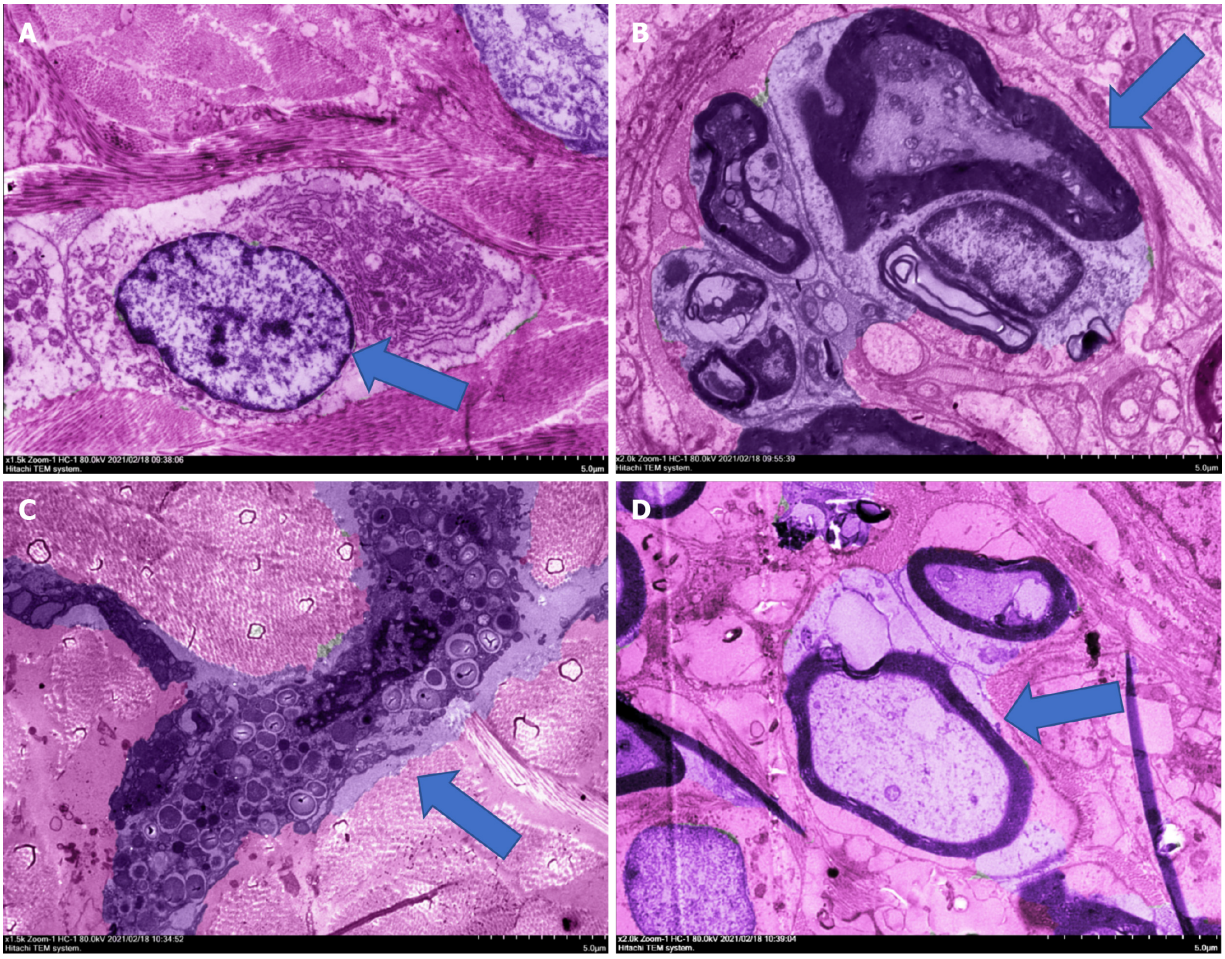
Statistical analyses

SPSS software (Version 24.0.0, Chicago, IL, United States) was used to analyze the wound-healing rate results of rats at different times, and all data are expressed as the mean ± SD. The differences were considered significant at $P < 0.05$.

RESULTS

In vivo wound healing effect

During continuous culture for 14 d, the wound areas of the rats on the first, third, seventh, and fourteenth days were recorded, and the cure rate was calculated. The results are shown in Table 1; the difference was significant. The results of the three groups of experiments showed that the cure rate of the RHP group was increased and reached 53.11%, indicating that RHP has a good therapeutic effect on the healing of DFU wounds in rats. The HE staining electron microscope results showed that after 14 d of uninterrupted administration, the ulcer tissues of 10 rats in the model group showed varying degrees of demyelination changes, unlike rats in the blank group, which showed symptoms of neuritis, as shown in Figure 1. In addition, neutrophil infiltration and granulation tissue loosening were observed. The mice treated with powder had good fibroblast function, mature granulation tissue, and restored nerve cells. The thickness of the stratum corneum and the integrity of the epidermis were good, showing a good state of recovery, and intact lymphocytes were observed, but there were a few mast cells. RHP promoted the healing of DFUs in rats by affecting fibroblasts and nerve cells.



DOI: 10.4239/wjd.v13.i8.622

Figure 1 Hematoxylin-eosin staining electron micrograph of rat diabetic foot ulcer. A: The fibroblasts of blank group; B: The neuritis of model group; C: Mast cells of powder group; D: Nerve cells of powder group. The mice treated with powder had good fibroblasts function, mature granulation tissue, and restored nerve cells. The thickness of the stratum corneum and the integrity of the epidermis were good, showing a good state of recovery, and intact lymphocytes would be observed, but there were a few mast cells. Ruyi Jinhuang powder promoted the healing of diabetic foot ulcers in rats by affecting fibroblasts and nerve cells.

Composition analysis of RHP by GC–MS

The GC–MS full spectrum identification results of RHP are shown in Table 2, and a total of 43 components were detected to obtain the total ion flow diagram (Figure 2), which mainly included alcohols, enes, esters, phenols, and other components, accounting for 27.91%, 20.93%, 20.93%, 16.28%, 4.65%, and 9.30% of the total, respectively. Among them, the higher components were ar-turmerone 24.33% (No. 23), tigerone 20.74% (No. 17), agarospirol 13.80% (No. 18), β-eudesmol 9.06% (No. 22), palmitic acid 2.31% (No. 43), and (E)-allylantone 2.23% (No. 29).

Composition analysis of RHP by UPLC–MS

The UPLC–MS full spectrum identification results of RHP are shown in Table 3. A total of 46 components were detected. The main components include flavonoids, organic nitrogens, isopentenol esters, carboxylic acids and their derivatives, benzene and its substituted derivatives, diarylheptans, fatty acyls, anthracyclines and other components, accounting for 23.92%, 15.23%, 10.88%, 8.71%, 6.52%, 6.52%, 6.52%, 4.26% and 17.44% of the total, respectively. The positive and negative ion flow diagrams are shown in Figure 3. Among them, the components with the highest levels were α,α-trehalose (409.04%; No. 6), curcumin (307.20%; No. 37), berberine (232.25%; No. 24), (3R,5R)-1,3,5-trihydroxy-4-{[(2E)-3-(4-hydroxy-3-methoxyphenyl)-2-propenoyl]oxy}cyclohexanecarboxylic acid (201.34%; No. 16), bisdemethoxycurcumin (153.92%; No. 33), genistein (153.83%; No. 39), demethoxycurcumin (151.99%; No. 35), and citricol acid (145.29%; No. 9).

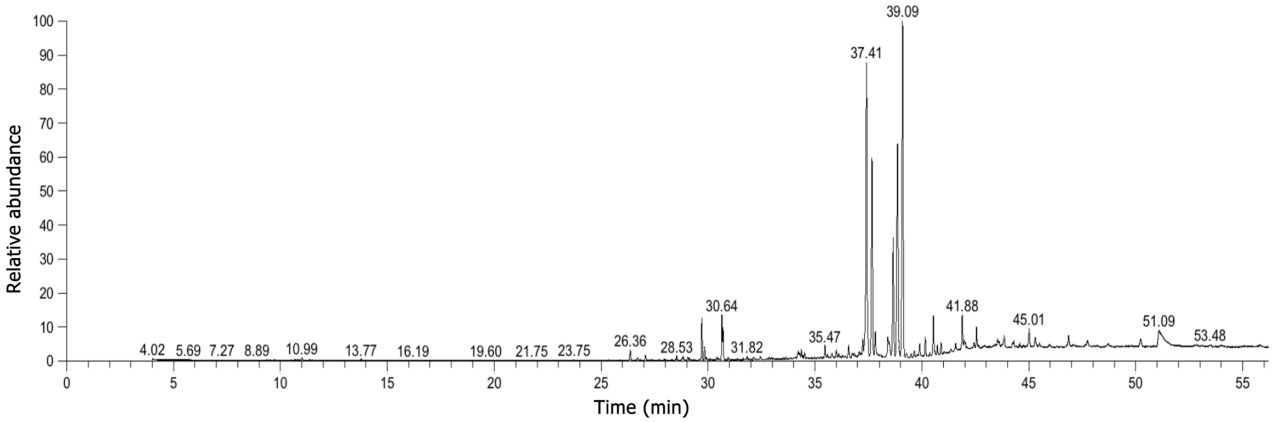
Establishment and analysis of the network relationship of "Traditional Chinese Medicine-Ingredients-Target"

Eighty-nine components were obtained by GC–MS and UPLC–MS. According to the "target-disease" relationship in the database, 43 relevant components for the treatment of DFU were finally screened

Table 2 Gas chromatography–mass spectrometry full spectrum identification results

No.	RT (min)	Relative content (%)	Qualitative	CAS	Name
1	26.36	0.59	51.56	512-61-8	(-)-α-Santalene
2	27.07	0.26	25.81	87-44-5	Caryophyllene
3	29.71	1.86	65.77	495-60-3	α-Zingiberene
4	29.83	0.64	14.58	495-61-4	6-methyl-2-(4-methylcyclohex-3-enyl)hept-1,5-diene
5	30.64	1.95	60.85	20307-83-9	β-Sesquiphellandrene
6	30.7	1.33	80.1	644-30-4	α-Curcumene
7	34.21	0.38	26.47	56192-70-2	(Z)-a-Atlantone
8	34.27	0.23	61.92	1139-30-6	Caryophyllene oxide
9	34.36	0.30	19.52	83173-76-6	2-Methyl-1-(4-methylphenyl)-3-buten-1-ol
10	34.52	0.25	58.19	108549-47-9	(6Z)-2-Methyl-6-(4-methyl-3-cyclohexen-1-ylidene)-2-hepten-4-one
11	35.47	0.58	29.36	639-99-6	Elemol
12	35.78	0.28	58.71	145512-84-1	Trans-Sesquisabinene hydrate
13	36	0.37	62.02	58334-55-7	Zingiberenol
14	36.57	0.74	86.3	6989-21-5	Atractylon
15	36.83	0.25	12.5	82508-14-3	2-methyl-6-(4-methylidenecyclohex-2-en-1-yl)hept-2-en-4-one
16	37.23	0.77	22.65	1209-71-8	γ-Eudesmol
17	37.41	20.74	91.81	180315-67-7	Tumerone
18	37.67	13.80	22.04	1460-73-7	Agarospirol
19	37.81	1.75	38	112-39-0	Methyl palmitate
20	38.41	1.26	41.2	473-16-5	a-Eudesmol
21	38.48	0.72	9.97	515-20-8	Costol
22	38.65	9.06	76.48	473-15-4	β-Eudesmol
23	39.1	24.33	97.75	532-65-0	ar-Turmerone
24	39.28	0.24	11.67	19888-00-7	4,8-Cycloundecadien-1-ol, 6,6,9-trimethyl-2-methylene-, (1R,4E,8E)-
25	39.47	0.23	4.42	65646-68-6	N-(4-hydroxyphenyl)retinamide
26	39.64	0.39	8.22	19912-67-5	(+)-Epicubenol
27	39.9	0.85	49.47	30666-87-6	2-Methyl-6-(4-methylphenyl)-4-heptanone
28	40.16	1.05	49.07	72441-71-5	(6R,7R)-Bisabolone
29	40.53	2.23	89.2	108645-54-1	(E)-Atlantone
30	40.89	0.65	82.79	120681-80-3	2-Cyclohexene-1-propanol,g-methyl-4-methylene-a-(2-methyl-1-propen-1-yl)
31	41.58	0.45	75.15	112-61-8	Octadecanoic acid, methyl ester
32	41.88	1.87	16.03	112-62-9	Methyl oleate
33	41.98	0.65	7.88	1937-62-8	trans-octadec-9-enoicacidmethylester
34	42.55	1.03	26.03	112-63-0	methyl linoleate
35	43.54	0.42	42.85	301-00-8	9,12,15-Octadecatrienoic acid, methyl ester
36	43.61	0.22	4.63	29550-55-8	Teresantalol(7CI)
37	44.58	0.24	88.5	69301-27-5	2-(1,5-Dimethyl-4-Hexenyl)-5-Methyl-Phenol
38	43.85	0.84	6.23	38142-57-3	2-Methyl-6-(p-tolyl)hept-2-en-4-ol
39	45.01	1.11	28.14	3218-36-8	4-Biphenylcarboxaldehyde
40	46.86	0.84	75.64	4666-84-6	Proximadiol
41	47.75	0.46	98.07	949081-10-1	(S)-3-Methyl-6-((S)-6-methyl-4-oxohept-5-en-2-yl)cyclohex-2-enone

42	48.71	0.24	9.54	2566-90-7	Methyl 4,7,10,13,16,19-cis-docosahexanenoate
43	51.08	2.31%	83.12	1957-10-3	Palmitic acid



DOI: 10.4239/wjd.v13.i8.622 **Copyright** ©The Author(s) 2022.

Figure 2 The total ion flow diagram of gas chromatography–mass spectrometry. Among them, the higher components were ar-turmerone 24.33%, tumerone 20.74%, agarospirol 13.80%, β-eudesmol 9.06%, palmitic acid 2.31%, (E) atlantone 2.23%, β-quadruphellandrene 1.95%, methyloleate 1.87%, α-zingiberene 1.86%, and methylmitate 1.75%.

from the ten medicines. There were 36 related targets. The results are shown in Table 4. Cytoscape software was used to visualize the network relationship of "TCM-Ingredient-Target", which was constructed by the parameters of "Degree", "Betweenness" and "Closeness" of each node, as shown in Figure 4. The important medicinal materials of RHP for the treatment of DFU are *turmeric, Magnolia officinalis*, and *rhubarb*, and the important components are luteolin, naringin, demethoxycurcumin, gallic acid, methyl linoleate, caryophyllene, arylcurcumin, methyl palmitate, berberine and rheic acid. The important targets are posttranscriptional silencing (PTGS2), muscarinic acetylcholine receptor M2 (CHRM2), Dipeptidyl peptidase 4, topoisomerase II alpha (TOP2A), retinoic X receptor alpha (RXRA), Protein tyrosine phosphatase nonreceptor type 1, tumor necrosis factor (TNF), interleukin 6 (IL-6), *etc.*

Analysis of the docking results of important components and key target molecules

Molecular docking was performed between important components of luteolin, methyl palmitate, ar-turmeric, methyl linoleate, palmitic acid, demethoxycurcumin, and naringin and the key targets PTGS2, TOP2A, CHRM2, and RXRA. The results are shown in Table 5, in which LibDockScore indicates the docking effect, and the value indicates the docking effect. Seven components (luteolin, methyl palmitate, ar-turmeric, methyl linoleate, palmitic acid, demethoxycurcumin and naringin) had a large LibDockScore, indicating that the binding effect between these components and key targets was good. The component docking conformations shown in Figure 5 indicate the following: The complex naringin-PTGS2 was stabilized by five conventional hydrogen bonds, eight carbon-hydrogen bonds, and two bonds to the alkyl with residues including GLY, ANS, ARG, PHE, ASN, HIS, GLY, GLN, LEU; the complex methyl linoleate-RXRA was stabilized by three carbon-hydrogen bonds, two Pi bonds to the alkyl with residues including DA and DG; the complex luteolin-TOP2A was stabilized by four conventional hydrogen bonds, two carbon-hydrogen bonds, two Pi bonds to the alkyl, six Pi bonds to the benzene ring with residues including LYS, GLU, SER, and ASP; the complex naringin-TOP2A was stabilized by three conventional hydrogen bonds, four carbon-hydrogen bonds, one Pi bond to the alkyl, four Pi bonds to the benzene ring with residues including ASP, ARG, ASN, and LYS; the complex methyl palmitate-PTGS2 was stabilized by two conventional hydrogen bonds, one Pi bond to the alkyl with residues including VAL, ASN, and TRP; the complex ar-turmeric-CHRM2 was stabilized by nine Pi bonds to the alkyl, two Pi bonds to the benzene ring and Van der Waals interactions with residues including PHE, LYS, ILE, VAL, LEU, ASP, ARG, ASN, and THR; the complex methyl linoleate-PTGS2 two carbon-hydrogen bonds, one Pi bond and one bond to the alkyl with residues including VAL, ASN, ILE, and TRP; the complex ar-turmeric-PTGS2 was stabilized by one conventional hydrogen bond, one Pi bond and three bonds to the alkyl, two Pi bonds to the benzene ring with residues including GLN, PHE, VAL, PRO, and ASN; the complex palmitic acid-PTGS2 was stabilized by two conventional hydrogen bonds with residues including VAL and GLY; the complex demethoxycurcumin-PTGS2 was stabilized by four conventional hydrogen bonds, one carbon-hydrogen bond, three Pi bonds to the benzene ring with residues including GLY, VAL, ASN, and PHE.

Table 3 Ultra-performance liquid chromatography full spectrum identification results

No.	Name	CAS	Molecular weight	RT (min)	Relative concentration (μg/mL)
1	DL-Arginine	7200-25-1	174.11175	0.783	24.44253696
2	Nitrosobis(2-oxopropyl)amine	60599-38-4	158.06914	0.817	15.74503993
3	Gluconic acid	526-95-4	196.05765	0.821	82.48616363
4	D-(+)-Proline	344-25-2	115.06357	0.846	15.35704499
5	Cabotegravir	1051375-10-0	405.11182	0.847	20.08010135
6	α,α-Trehalose	99-20-7	342.11623	0.847	409.0449144
7	D-(-)-Quinic acid	77-95-2	192.06275	0.85	56.2670819
8	Isocitric acid	320-77-4	192.02637	0.939	63.29259087
9	Citric acid	77-92-9	192.02637	1.169	145.2856943
10	Gallic acid	149-91-7	170.02078	1.899	23.57871448
11	Chlorogenic acid	327-97-9	354.09527	5.332	44.58986172
12	Catechin	88191-48-4	290.07917	5.431	98.07463825
13	methyl chlorogenate	123483-19-2	368.11081	5.501	18.15074983
14	6-Acetylcodeine	6703-27-1	341.16237	5.886	12.3548527
15	2-(3,4-Dihydroxyphenyl)ethyl 3-O-(6-deoxy-β-L-mannopyranosyl)-6-O-[(2E)-3-(3,4-dihydroxyphenyl)-2-propenoyl]-β-D-glucopyranoside	61303-13-7	624.20617	6.092	20.78761602
16	(3R,5R)-1,3,5-Trihydroxy-4-{[(2E)-3-(4-hydroxy-3-methoxyphenyl)-2-propenoyl]oxy}cyclohexanecarboxylic acid	2613-86-7	368.11084	6.196	201.3429867
17	Emodin-8-Beta-D-Glucoside	23313-21-5	432.10571	6.579	7.280885459
18	2-[4-[3-[3,4-dihydroxy-4-(hydroxymethyl)oxolan-2-yl]oxy-4,5-dihydroxy-6-(hydroxymethyl)oxan-2-yl]oxyphenyl]-7-hydroxy-2,3-dihydrochromen-4-one	74639-14-8	550.16902	6.625	13.98565399
19	Baicalin	21967-41-9	446.0852	6.663	16.85569046
20	Liquiritin	551-15-5	418.12676	6.737	22.91985259
21	Naringin	10236-47-2	580.17964	6.959	26.36327029
22	Hesperidin	520-26-3	610.19038	7.191	85.82076776
23	Azelaic acid	123-99-9	188.1042	7.548	4.773123466
24	Berberine	2086-83-1	335.11525	8.242	232.2489146
25	isosakuranetin-7-O-rutinoside	14259-47-3	594.19556	8.493	4.34803523
26	Tinnevellin glucoside	80358-06-1	408.14228	8.786	13.59498227
27	Daidzein	486-66-8	254.05778	9.009	13.05981793
28	Chrysophanol 8-O-β-D-glucoside	13241-28-6	416.11099	9.01	17.88080565
29	Licoricesaponin G2	118441-84-2	838.39998	9.824	7.195669531
30	Luteolin	491-70-3	286.04776	9.909	1.034661042
31	(15Z)-9,12,13-Trihydroxy-15-octadecenoic acid	95341-44-9	330.24071	9.938	19.41925634

32	Rheic acid	478-43-3	284.03211	11.029	81.8044913
33	Bisdemethoxycurcumin	24939-16-0	308.10496	11.328	153.9247824
34	6-Hydroxy-2-naphthoic acid	16712-64-4	188.04652	11.328	7.047135466
35	Demethoxycurcumin	22608-11-3	338.11567	11.473	151.9906609
36	5,7-dihydroxy-2-(4-hydroxy-3-methoxyphenyl)-6-(3-methylbut-2-enyl)-2,3-dihydro-chromen-4-one	76735-58-5	370.14168	11.541	55.08087646
37	Curcumin	458-37-7	368.12606	11.619	307.1972754
38	Isoimperatorin	482-45-1	270.08918	11.847	1.025466018
39	Genistein	446-72-0	270.05279	12.256	153.8276373
40	(+/-)9-HODE	98524-19-7	296.23511	13.146	23.43829567
41	(+)-ar-Turmerone	532-65-0	216.15135	13.605	23.84749189
42	Indane	496-11-7	118.07843	13.605	27.63249372
43	Prespatane	100387-71-8	204.18777	13.956	2.010509748
44	(+)-Nootkatone	4674-50-4	218.16696	14.497	16.6606139
45	Aristolone	6831-17-0	218.16696	14.588	45.88543598
46	2,2'-Methylenebis(4-methyl-6-tert-butylphenol)	119-47-1	340.24015	16.788	25.70628216

RT: Radiotherapy.

Analysis of KEGG pathway enrichment results

The interaction between each target protein was preliminarily obtained by entering the target protein into the STRING database, as shown in Figure 6. Among the 36 targets, except for CHRM2, CHRM4, PCYT1A and Sodium voltage-gated channel alpha subunit 5, the remaining 32 target proteins are closely related to each other and may participate in multiple pathways. The target list was uploaded to the DAVID database to establish the "pathway-target" link. A total of 27 pathways related to DFU treatment were retrieved. The number of targets contained in each pathway is shown in Figure 7A. Cytoscape was used to construct the "pathway-target" network relationship diagram, which is shown in Figure 7B; the "pathway-target" relationship is shown in Figure 7C. Correspondence between channel categories is shown in Figure 7D. Most of the 27 pathway diagrams belong to the immune system and participate in the processes of inflammatory and infectious diseases, which demonstrate signal transduction effects. This finding shows that RHP can affect the body's immune system, regulate inflammation through signal transduction, and exert curative effects in DFU. Further analysis of the literature shows that RHP is mainly related to the phosphoinositide 3-kinase (PI3K)-protein kinase B (Akt) signaling pathway, neuroactive ligand–receptor interaction and the forkhead box O (FoxO) signaling pathway in the treatment of DFU.

We established the network relationship between the three pathways of the PI3K-Akt signaling pathway, neuroactive ligand–receptor interaction and FoxO signaling pathway and the target and chemical components and analyzed the topological parameters of each node. The network diagram (Figure 8) showed that CHRM2 and RXRA are the common targets of the three pathways.

The prediction of anti-DFU mechanism

According to the literature, wound healing mainly involves the three processes of hemostasis and inflammation, proliferation, and remodeling. The target pathways involved are diverse and complex, as shown in Figure 9. RHP can act on leukocytes by regulating IL-6 and TNF, regulating the inflammatory response, and then participating in hemostasis to participate in inflammation and remodeling stages. RHP can also affect platelets, macrophages and fibroblasts by acting on the PI3K-Akt pathway to promote angiogenesis participation in the proliferation stage and facilitate wound healing in DFUs.

Further analysis of the effects of the three pathways in the human body found that if the PI3K-Akt signaling pathway is inhibited in diabetic neurons, neuronal apoptosis is increased, and diabetic neuropathic pain is induced; thus, activating this pathway can alleviate painful diabetic peripheral neuropathy. The abnormal expression or loss of genes related to the neuroactive ligand–receptor interaction pathway can cause neuropathy, such as with the downregulation of glial gene expression, which impairs nerve impulse conduction and increases the potential nerve involvement of systemic

Table 4 Ruyi Jinhuang powder "Chinese medicine-ingredients-target" correspondence

CAS	Name	GenecardID	Attribution
512-61-8	(-)-α-Santalene	PTGS2, CHRM2	JH
87-44-5	Caryophyllene	PTGS2, CHRM2	BZ, JH, CZ, ZHP, TNX
495-60-3	α-Zingiberene	PTGS2, DPP4	JH
20307-83-9	β-Sesquiphellandrene	PTGS2	JH
644-30-4	α-Curcumene	PTGS2, TOP2A, DPP4	JH
1139-30-6	Caryophyllene oxide	CHRM2, DPP4	ZHP, TNX
639-99-6	Elemol	CHRM2	ZHP, JH
58334-55-7	Zingiberenol	CHRM2	JH
6989-21-5	Atractylon	SCN5A, CHRM4, HTR2A, CHRM2, OPRM1	CZ
82508-14-3	2-methyl-6-(4-methylidenecyclohex-2-en-1-yl)hept-2-en-4-one	PTGS2, CHRM2	JH, ZHP, CZ
112-39-0	Methyl palmitate	PTGS2, IL-10, TNF, IL-6	JH, ZHP, BZ, HB, THF
473-15-4	beta-Eudesmol	CHRM2	ZHP, JH, CZ
532-65-0	(6S)-2-methyl-6-(4-methylphenyl)hept-2-en-4-one	DPP4, PTGS2, CHRM2x, ampC	JH
112-62-9	Methyl oleate	RXRA	ZHP, THF
1937-62-8	trans-octadec-9-enoicacidmethylester	RXRA	ZHP, THF
112-63-0	methyl linoleate	PTGS2, RXRA	BZ, THF, ZHP, TNX
301-00-8	9,12,15-Octadecatrienoic acid, methyl ester, (Z,Z,Z)-	PTGS2, RXRA	THF
38142-57-3	2-Methyl-6-(p-tolyl)hept-2-en-4-ol	PTGS2, CHRM2, DPP4, ampC, RXRA	THF, ZHP, TNX
1957—10—3	Palmitic acid	PTGS2, IL-10, TNF, PCYT1A	DH, ZHP, THF
149-91-7	Gallic acid	PTGS2, PTPN1, TOP2A	DH, TNX, CP, GC, JH
23313-21-5	Emodin-8-Beta-D-Glucoside	TOP2A	DH
458-37-7	Curcumin	PTGS2, PTPN1	JH
22608-11-3	Demethoxycurcumin	PTGS2, PTPN1, AKR1B1, ABCB1, SCN5A	JH
520-26-3	Hesperidin	PTGS2	CP
14259-47-3	isosakuranetin-7-O-rutinoside	TOP2A	CP
10236-47-2	Naringin	TOP2A, CDKN1A, TNF, RASGRF2, RAF, PTGS2, ampC, MTTP, APOB, mvaA, PPARA	CP, GC
21967-41-9	Baicalin	PTPN1, TOP2A	DH
88191-48-4	Catechin	CHRM2	DH, CP
491-70-3	Luteolin	PTGS2, DPP4, EGFR, IL10, CDK4, TNF, IL6, NFKBIA, psdA1, IL2, TOP2A, SLC2A4, INSR, MET	CP, ZHP
551-15-5	Liquiritin	PTGS2	GC
478-43-3	Rheic acid	PTGS2, AKR1B1	DH
2086-83-1	Berberine	PTGS2, RXRA	HB
77-92-9	Citric acid	PTGS2, AKR1B1, GRIN2A, GRIA2, PTPN1	DH
320-77-4	Isocitric acid	PTGS2	DH
7200-25-1	DL-Arginine	PTGS2	THF

446-72-0	Genistein	PTGS2, EGFR, TNF, SELE, IL8	GC
482-45-1	Isoimperatorin	PTGS2	BZ
486-66-8	Daidzein	PTGS2, RXRA	GC
6831-17-0	Aristolone	CHRM2, PTGS2	BZ
532-65-0	(+) -ar-Turmerone	PTGS2, RXRA, CHRM2, ampC, DPP4	JH
327-97-9	Chlorogenic acid	PTGS2	HB
77-95-2	D-(-)-Quinic acid	PTGS2	HB
1460-73-7	Agarospirol	CHRM2	ZHP

TNF: Tumor necrosis factor; IL: Interleukin; PTGS2: Posttranscriptional silencing; TOP2A: Topoisomerase II alpha; CHRM2: Muscarinic acetylcholine receptor M2; RXRA: Retinoic X receptor alpha; DPP4: Dipeptidyl peptidase 4; SCN5A: Sodium voltage-gated channel alpha subunit 5; HTR2A: 5-hydroxytryptamine (serotonin) receptor 2A; OPRM1: Opioid Receptor Mu 1; EGFR: Epidermal Growth Factor Recepto; PTPN1: Protein tyrosine phosphatase nonreceptor type 1; THF: Trichosanthin (Tian Hua); DH: Rhubarb (Da Huang); HB: Phellodendron (Huang Bai); JH: Turmeric (Jiang Huang); BZ: Angelica dahurica (Bai Zhi); TNX: Arisaema (Tian Nan Xing); CZ: Atractylodes lancea (Cang Zhu); HP: Magnolia officinalis (Hou Po); CP: Pericarpium Citri Reticulatae (Chen Pi); GC: Glycyrrhiza uralensis (Gan Cao).

Table 5 Docking parameters of chemical components and target protein molecules

Name	Target	LibDockScore
Luteolin	TOP2A	104.333
Methyl palmitate	PTGS2	114.221
ar-Turmeric	PTGS2	91.0625
	CHRM2	88.3765
Methyl Linoleate	RXRA	138.583
	PTGS2	130.838
Palmitic acid	PTGS2	114.349
Demethoxycurcumin	PTGS2	135.479
Naringin	TOP2A	141.197
	PTGS2	164.305

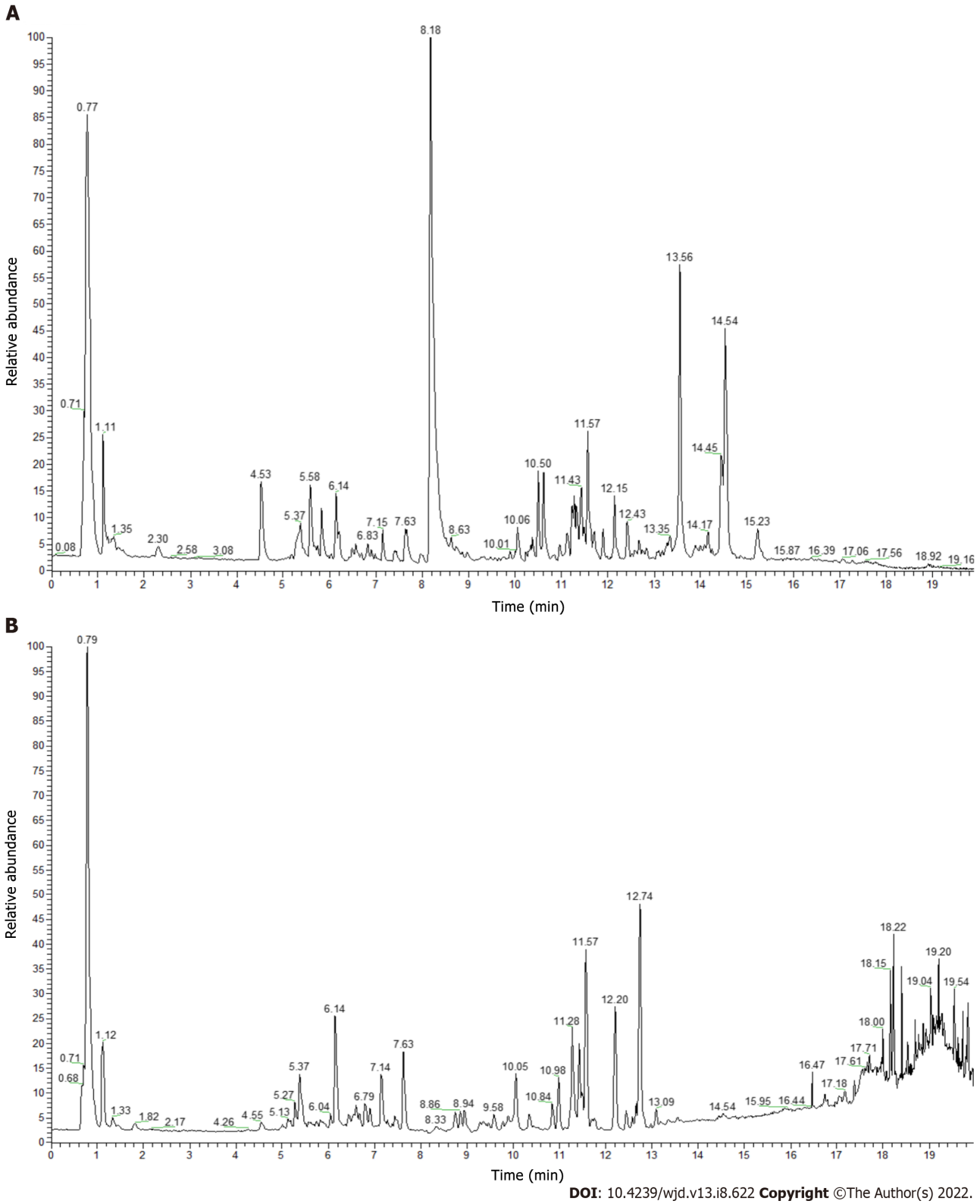
PTGS2: Posttranscriptional silencing; TOP2A: Topoisomerase II alpha; CHRM2: Muscarinic acetylcholine receptor M2; RXRA: Retinoic X receptor alpha.

diseases. The link between the FoxO signaling pathway and diabetes is that type II diabetes mellitus causes abnormal tissue signaling due to hyperglycemia or insulin resistance. The identified important targets, such as IL-6 and TNF, participate in the abovementioned pathways and play a role in hemostasis and tissue remodeling so that the DFU wound can heal. The mechanism of action is shown in Figure 10.

DISCUSSION

The results of the three groups of experiments showed indicated that RHP has a good therapeutic effect on the healing of DFU wounds in rats. The HE staining electron microscopy results showed neutrophil infiltration and granulation tissue loosening in the model group and blank group. The fibroblasts of the RHP group had good function, the granulation tissue was mature, and nerve cells were restored. The thickness of the stratum corneum and the integrity of the epidermis were good, showing a good state of recovery, and intact lymphocytes could be observed, but there were a few mast cells. Thus, RHP promoted the healing of DFUs in rats by affecting fibroblasts and nerve cells.

In this study, GC–MS and UPLC–MS were used to collect and identify the chemical components of RHP. Eighty-nine compounds were detected and analyzed, including flavonoids, terpenes, and coumarins. The components obtained by GC–MS are mainly volatile substances, and the components obtained by UPLC–MS are mostly high boiling point, nonvolatile, high molecular weight substances. The network pharmacology analysis of these compounds shows that RHP plays a role in the treatment

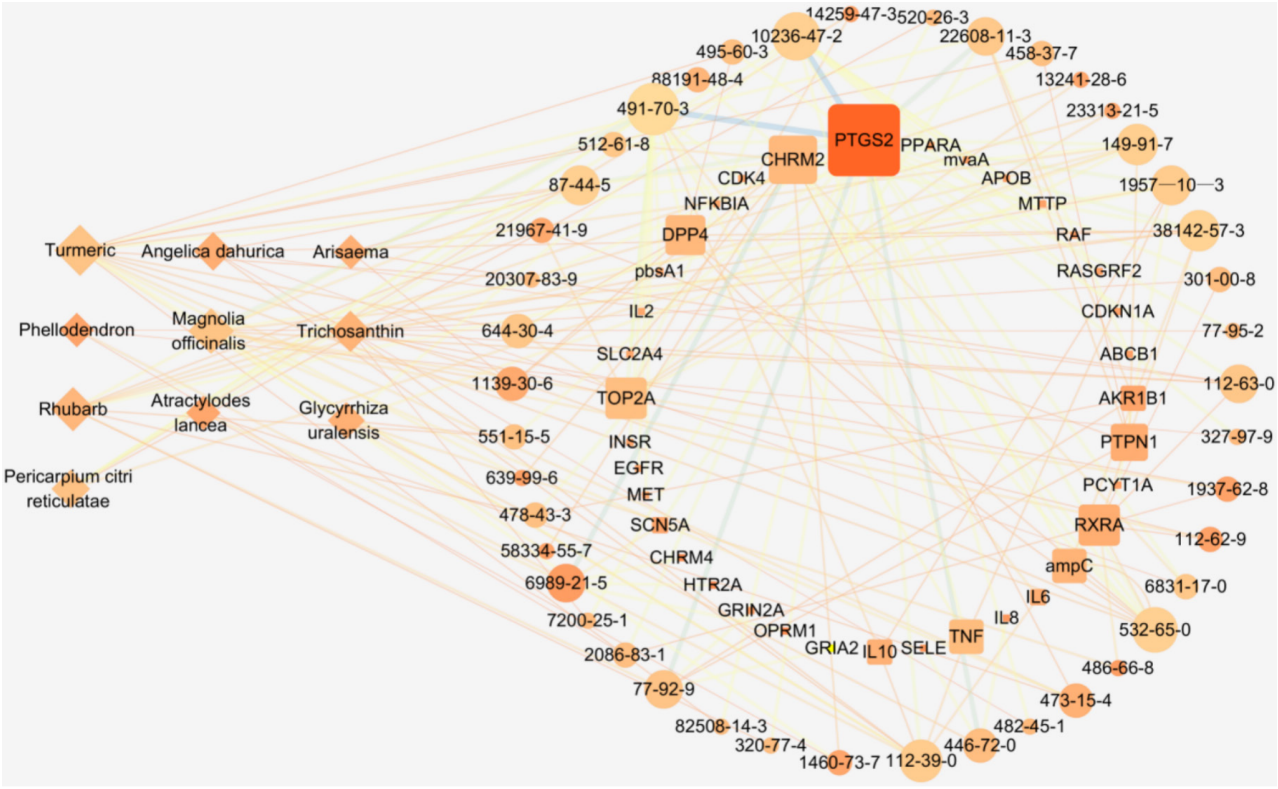


DOI: 10.4239/wjd.v13.i8.622 **Copyright** ©The Author(s) 2022.

Figure 3 Ultra-performance liquid chromatography ion flow diagram of Ruyi Jinhuang powder. A: Positive ion flow diagram; B: Negative ion flow diagram. Among them, the components with high levels were α,α-trehalose 409.04% (No. 6), curcumin 307.20% (No. 37), berberine 232.25% (No. 24), (3R,5R)-1,3,5-trihydroxy-4-{[(2E)-3-(4-hydroxy-3-methoxyphenyl)-2-propenoyl]oxy}cyclohexanecarboxylic acid 201.34% (No. 16), bisdemethoxycurcumin 153.92% (No. 33), genistein 153.83% (No. 39), demethoxycurcumin 151.99% (No. 35), and citricol acid 145.29% (No. 9).

of DFUs through multiple targets and channels. However, the manner in which specific components are combined with target proteins needs to be further explored.

DFU is one of the main complications of diabetes. Current studies have found that its main causes are neuropathy, vascular disease and impaired immune function[13,14]. The specific pathogenesis is that when persistent hyperglycemia occurs, nerve cells accumulate a large number of toxic components,

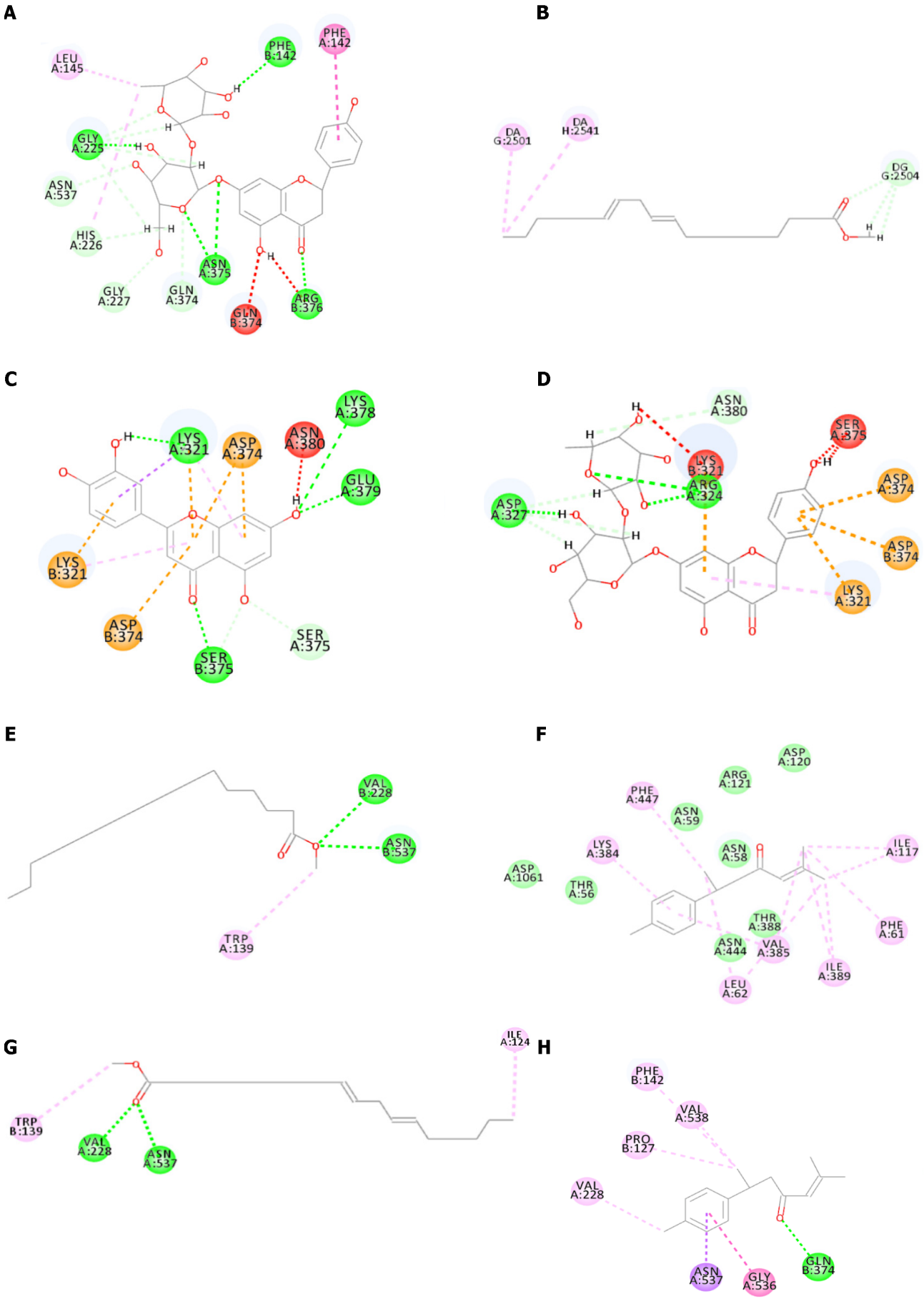


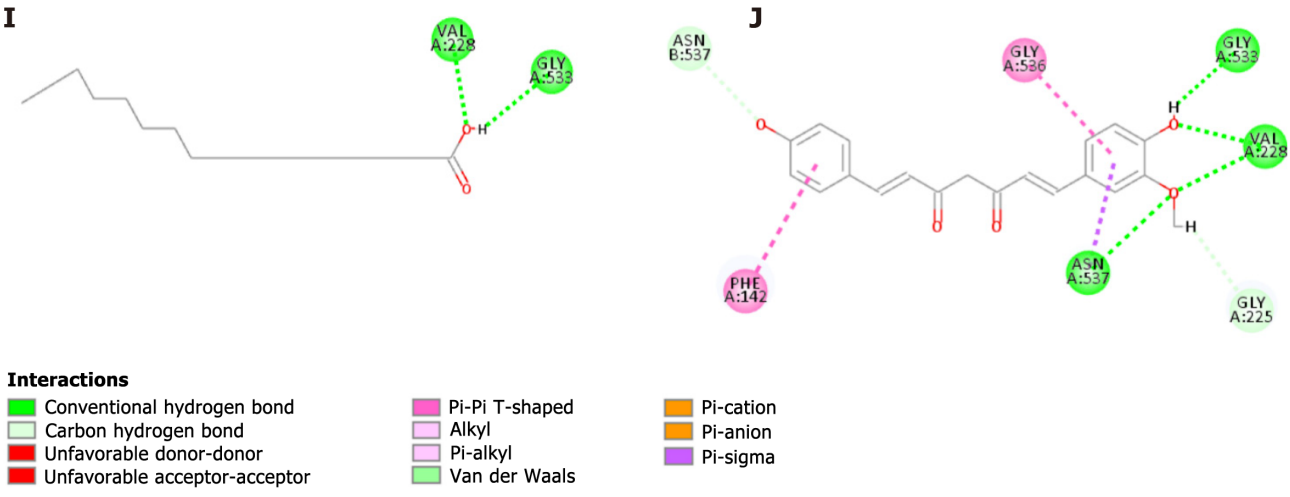
DOI: 10.4239/wjd.v13.i8.622

Figure 4 The "traditional Chinese medicine-ingredient-target" network of Ruyi Jinhuang powder topology diagram. There are a total of 89 nodes and 186 edges; diamonds represent ten herbs of Ruyi Jinhuang powder, circles represent 43 related chemical components (represented in the form of CAS number), squares represent 36 target targets (represented in the form of GenecardID), nodes indicate that the greater Degree value, the darker color indicates the greater Closness value, and the thicker lines indicate the greater Betweenness value.

damage to endothelial cell function occurs, vascular tension is decreased, and nerve ischemia occurs; thus, nerve and vascular lesions develop. In addition, persistent hyperglycemia affects the inflammatory response of the wound by acting on cells and the immune system, and the wound is persistently infected and difficult to heal[15,16].

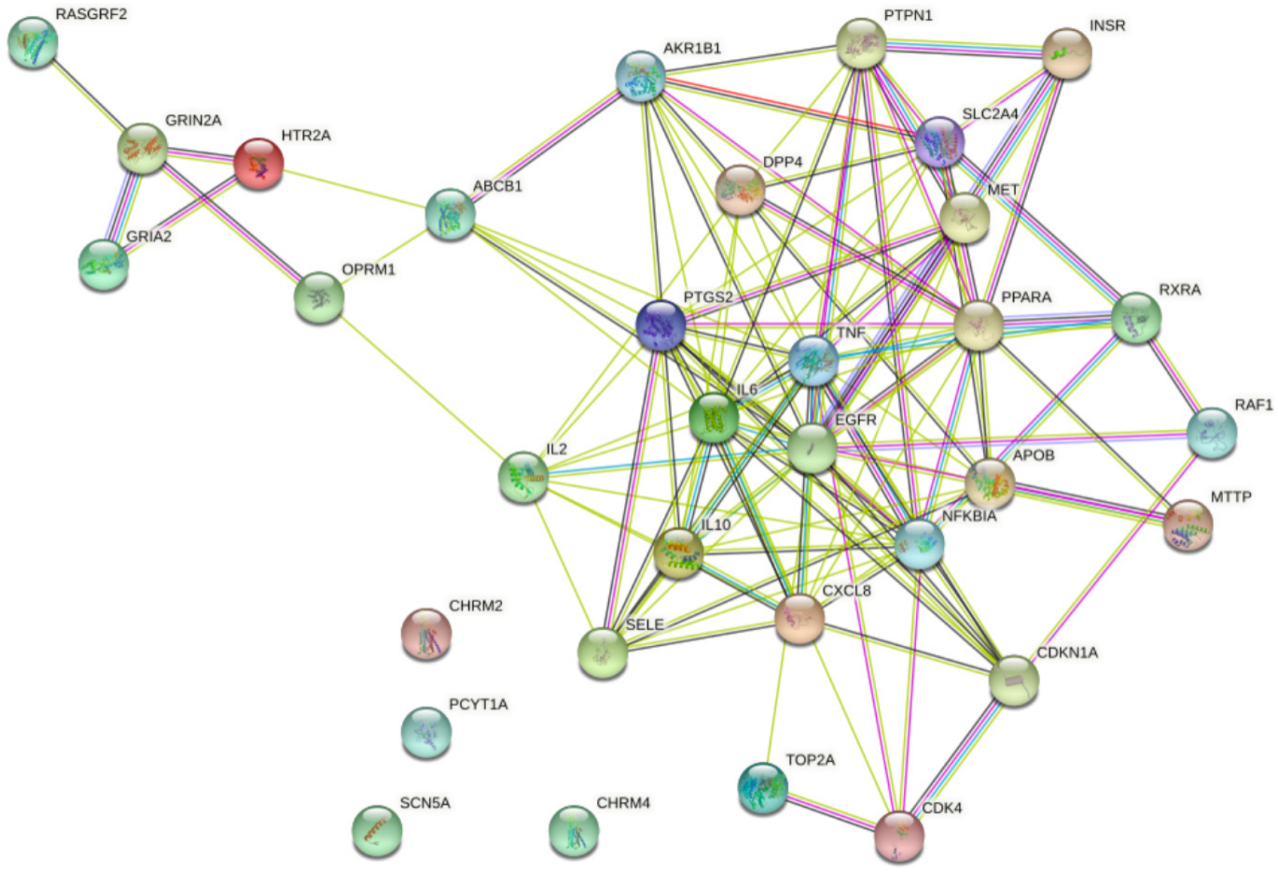
The active ingredients were extracted from the ten herbs of RHP, and network pharmacological analysis of "herb-component-target pathway-disease" was performed to identify three key pathways: The PI3K-Akt signaling pathway; neuroactivated-receptor interactions; and the FoxO signaling pathway. These pathways are important targets for the treatment of DFU. Studies have found that RXRA has a negative regulatory effect on glucose-stimulated insulin secretion[17], while CHRM2 mainly plays a role in the central nervous system and peripheral nervous system and can activate endothelial cell NO lyase to relax vascular smooth muscle[18,19], which is a major cause of foot ulcers caused by diabetic vascular disease and is a key target for the treatment of vascular disease[20]. The mechanism of the PI3K-Akt signaling pathway in the treatment of DFU is reflected in the following two aspects. On the one hand, insulin initiates this signaling pathway by upregulating PI3K expression with Akt molecules and inhibits FoxO1 expression, thereby allowing normal glucose uptake by cells and improving abnormal lipid metabolism[21-23]. On the other hand, if the PI3K-Akt signaling pathway is inhibited in diabetic neurons, neuronal apoptosis is increased, and diabetic neuropathic pain is induced; thus, activation of this pathway can relieve painful diabetic peripheral neuropathy[24]. In addition, diabetic patients have difficulty healing repeated wound infections due to a prolonged wound inflammatory response due to metabolic disorders, and the PI3K-Akt signaling pathway can promote the expression of hypoxia-inducible Factor 1, an important factor in wound healing, to accelerate wound cell proliferation to promote healing[25]. The neuroligand-interreceptor interaction pathway is a collection of intracellular and extracellular related receptor ligands, and abnormal expression or deletion of related genes in this pathway can cause neuropathic lesions; for example, downregulation of Glral gene expression impairs nerve impulse conduction and increases the potential incidence of neurological diseases[26,27]. The link between the FoxO signaling pathway and diabetes is that type 2 diabetes induces skeletal muscle atrophy due to abnormal tissue signaling and protein disorders caused by hyperglycemia or insulin resistance, and insulin phosphorylates mediators of the FoxO signaling pathway and inhibits autophagy-related gene expression. Luteolin can reduce neuropathic pain by decreasing FoxO1 acetylation, regulating this pathway, and inhibiting the expression of inflammation- and pain-related genes[28]. In addition to these three pathways, the PI3K-Akt signaling pathway,


A
LEU A:145
PHE B:142
PHE A:142
GLY A:225
ASN A:537
HIS A:226
ASN A:375
GLY A:227
GLN A:374
GLN B:374
ARG B:376
B
DA G:2501
DA H:2541
DG G:2504
C
LYS A:378
LYS A:321
ASP A:374
ASN A:380
GLU A:379
LYS B:321
ASP B:374
SER B:375
SER A:375
D
ASN A:380
SER A:375
LYS B:321
ARG A:324
ASP A:374
ASP A:327
ASP B:374
LYS A:321
E
VAL B:228
ASN B:537
TRP A:139
F
ASP A:120
ARG A:121
PHE A:447
ASN A:59
LYS A:384
ASN A:58
ILE A:117
ASP A:1061
THR A:56
THR A:388
PHE A:61
ASN A:444
VAL A:385
ILE A:389
LEU A:62
G
ILE A:124
TRP B:139
VAL A:228
ASN A:537
H
PHE B:142
VAL A:538
PRO B:127
VAL A:228
GLN B:374
ASN A:537
GLY A:536



DOI: 10.4239/wjd.v13.i8.622 **Copyright** ©The Author(s) 2022.

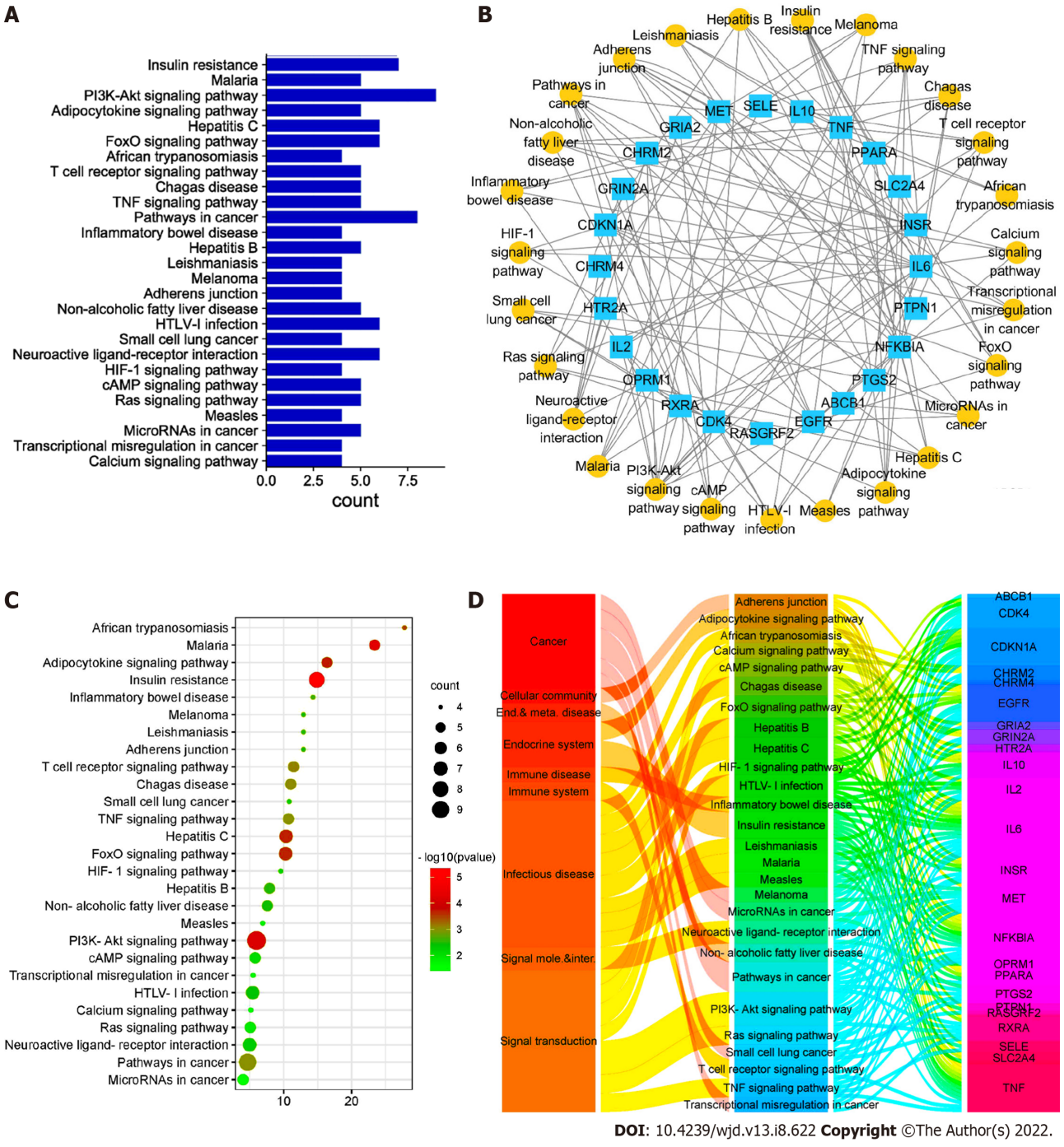
Figure 5 The virtual docking of bioactive ingredients from Ruyi Jinhuang powder for diabetic foot ulcer targets. A and D: The virtual docking of naringin with post-transcriptionalgenesilencing (PTGS2) and Topoisomerase II alpha (TOP2A); B and G: The virtual docking of methyl linoleate with Retinoid X Receptor alpha and PTGS2; C: The docking of Luteolin and TOP2A; E: The docking of Methyl palmitate and PTGS2; F and H: The virtual docking of ar-Turmeric with Muscarinic Acetylcholine receptor M2 and PTGS2; I: The virtual docking of palmitic acid and PTGS2. J represents the virtual docking of demethoxycurcumin and PTGS2.



DOI: 10.4239/wjd.v13.i8.622 **Copyright** ©The Author(s) 2022.

Figure 6 Interaction diagram of target proteins. The edges with different colors represent different association relationships. Among the 36 targets, except for Muscarinic Acetylcholine receptor M2 (CHRM2), CHRM4, Choline-phosphate cytidylyltransferase A and Sodium channel protein type 5 subunit alpha, the remaining 32 target proteins are closely related to each other and may participate in multiple pathways.

neuroactivated-receptor interactions, and the FoxO signaling pathway, the network topological properties of the human T-cell leukemia virus-infection signaling pathway were also found to be prominent in the RHP network pharmacology analysis; however, there is no specific research result on



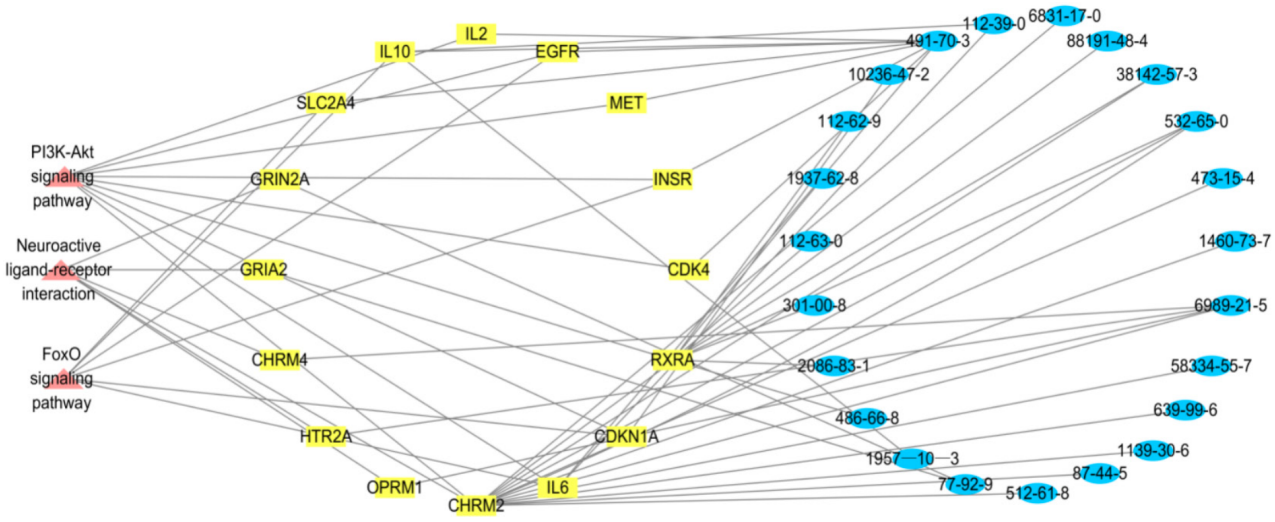
DOI: 10.4239/wjd.v13.i8.622 **Copyright** ©The Author(s) 2022.

Figure 7 Target-pathway diagram of Ruyi Jinhuang powder-diabetic foot ulcer. A: A bar graph of Kyoto Encyclopedia of Genes and Genomes (KEGG) signal pathway enrichment. The left side is the pathway name, and the right side is the number of targets contained; B: The target-path network relationship diagram. The yellow circle represents 27 pathways, and the blue square represents 36 targets; C: An enriched bubble diagram of the KEGG signaling pathway. The left side is the pathway name, and the abscissa is the fold enrichment. The bubble size corresponds to the number of targets contained, and the color category corresponds to the *P* value; D: The correspondence diagram of pathway categories. The first column is the ownership of the pathway, the second column is the pathway, and the third column is the target.

how this pathway affects DFU and needs further study.

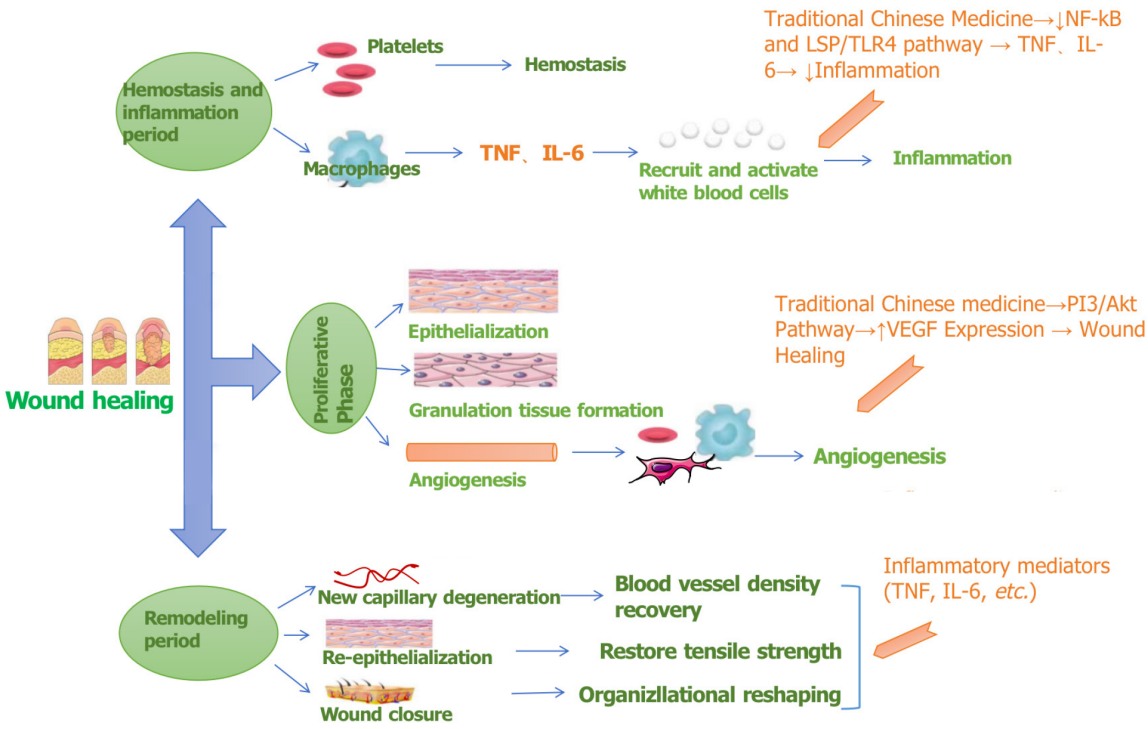
CONCLUSION

RHP, a traditional Chinese medicine formula, may play a role in the treatment of DFU through these target pathways by affecting insulin resistance, altering the nervous system and immune system, participating in inflammatory responses, and regulating cell proliferation, differentiation and apoptosis through other specific mechanisms. The key active components were luteolin, methyl palmitate, ar-



DOI: 10.4239/wjd.v13.i8.622 **Copyright** ©The Author(s) 2022.

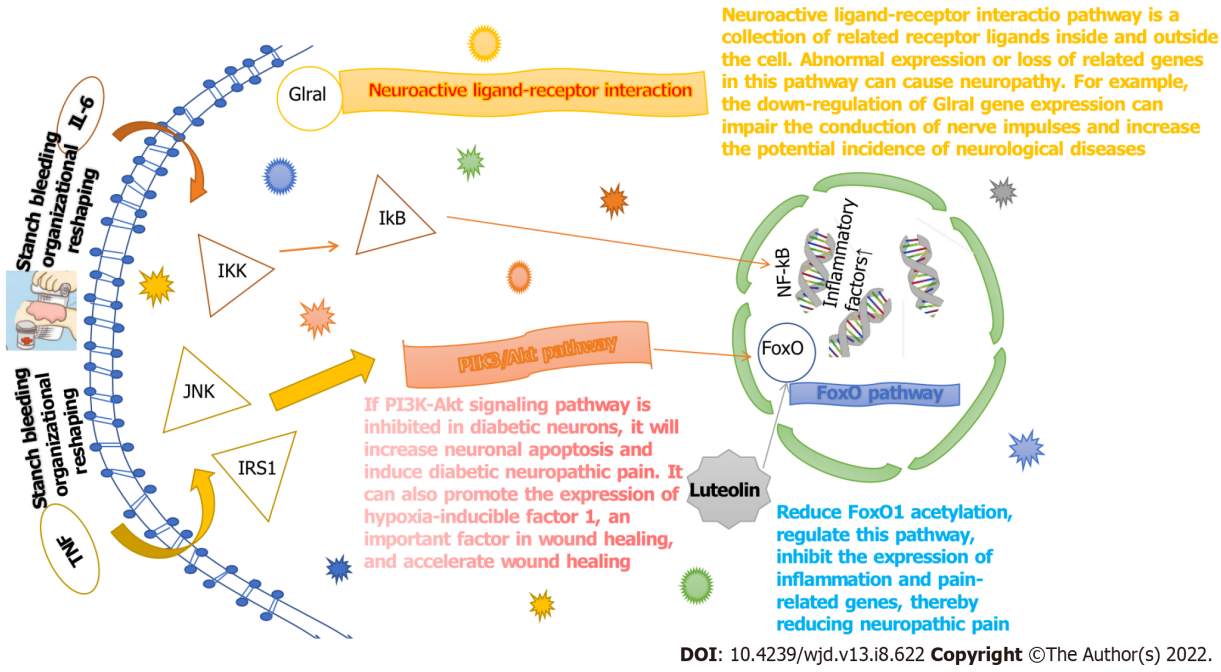
Figure 8 Critical pathway-target-component network relationship diagram of Ruyi Jinhuang powder. Pink represents the pathway, yellow represents the target and blue represents the components. It was shown that Muscarinic Acetylcholine receptor M2 and Retinoid X Receptor alpha are common targets of the three pathways. Luteolin, atractylenone and arylodone are the key components in the three pathways.



DOI: 10.4239/wjd.v13.i8.622 **Copyright** ©The Author(s) 2022.

Figure 9 Wound healing mechanism diagram. TNF: Tumor necrosis factor; IL: Interleukin; VEGF: Vascular endothelial growth factor; NF-kB: Nuclear factor-kappaB.

turmeric, methyl linoleate, palmitic acid, demethoxycurcumin, and naringin.



DOI: 10.4239/wjd.v13.i8.622

Figure 10 Target pathway mechanism of Ruyi Jinhuang powder. TNF: Tumor necrosis factor; IL: Interleukin; NF-kB: Nuclear factor-kappaB; IKK: IkappaB kinase; JNK: c-Jun N-terminal kinase; IkB: Inhibitor kB; IRS1: Insulin receptor substrate-1.

ARTICLE HIGHLIGHTS

Research background

Diabetic foot ulcer (DFU) seriously affects the quality of life of patients. Traditional Chinese medicine has a unique effect in the treatment of skin ulcerative diseases. Ruyi Jinhuang powder (RHP) is one of the classic prescriptions in traditional Chinese medicine and is widely used in clinical practice.

Research motivation

Although there have been studies suggesting that RHP has a therapeutic effect on DFU, there is a lack of research that further verify the mechanism of RHP to promote wound healing.

Research objectives

The effective components of RHP were extracted and identified by chromatography-mass spectrometry, and the obtained chemical components were analyzed by network pharmacology methods to predict its therapeutic mechanism. Gas chromatography–mass spectrometry (MS) and ultra-performance liquid chromatography-MS were used to separately identify the chemical constituents.

Research methods

Sprague Dawley rats were injected with streptozotocin to establish the DFU model. Hematoxylin-eosin staining was used to observe the wound tissue under an electron microscope. Medicine Systems Pharmacology database to obtain the target information, and the molecular docking of important components and key targets was performed in Discovery Studio software. Cytoscape software was used to visualize and analyze the relationship between the chemical composition, targets and Traditional Chinese Medicine network.

Research results

RHP was shown to promote the healing of diabetic foot ulcers in rats by affecting fibroblasts and nerve cells. A total of 89 chemical components were obtained by chromatography-mass spectrometry. Network pharmacological analysis revealed that RHP was associated with 36 targets and 27 pathways in the treatment of DFU.

Research conclusions

Our results indicated that RHP may play a role in the treatment of DFU through these target pathways by affecting insulin resistance, altering the nervous system and immune system, participating in inflammatory responses and regulating cell proliferation, differentiation and apoptosis and through other specific mechanisms.

Research perspectives

We found that RHP plays a role in the treatment of diabetic foot ulcers through multiple targets and channels. However, the way in which specific components are combined with target proteins needs to be further explored.

FOOTNOTES

Author contributions: Li XY, Zhang XT, Jiao YC, Chi H, Xiong TT and Zhang WJ, Li MN performed the experiments and acquired and analyzed the data; Li XY, Zhang XT and Wang YH wrote the manuscript; all authors approved the final version of the article.

Supported by National Natural Science Foundation of China, No. 82074025; Scientific Research Project of Heilongjiang Health Committee, No. 2020-293; and Scientific and Technological Innovation Project for College Students of Heilongjiang University of Chinese Medicine, No. 2021-13.

Institutional review board statement: This study does not involve human.

Institutional animal care and use committee statement: This study was approved by Ethics Committee of Heilongjiang University of Chinese Medicine, Harbin, China.

Conflict-of-interest statement: All the authors report no relevant conflicts of interest for this article.

Data sharing statement: No additional data are available.

ARRIVE guidelines statement: The authors have read the ARRIVE guidelines, and the manuscript was prepared and revised according to the ARRIVE guidelines.

Country/Territory of origin: China

ORCID number: Yan-Hong Wang 0000-0003-2092-2400.

S-Editor: Fan JR
L-Editor: A
P-Editor: Chen YX

REFERENCES

1 **Chen SG**. Surgery Zhengzong. Beijing: People's Medical Publishing House, 2007: 345
2 **Zhang SQ**, Zeng CH, Chen C, Wei L. Modern research progress of Ruyi Jinhuang Powder. *Zhongchengyao* 2018; **40**: 411-415 [DOI: 10.3969/j.issn.1001-1528.2018.02.032]
3 **Sun D**, Zhao YD, Hu MY, Li WB, Yan XN. The application of Ruyi Jinhuang Powder in dermatology. *Linchuangyixue Yanjiu Yu Shijian* 2020; **17**: 194-196 [DOI: 10.19347/j.cnki.2096-1413.202017072]
4 **Bandyk DF**. The diabetic foot: Pathophysiology, evaluation, and treatment. *Semin Vasc Surg* 2018; **31**: 43-48 [PMID: 30876640 DOI: 10.1053/j.semvascsurg.2019.02.001]
5 **Wang XH**, Lang R, Liang Y, Zeng Q, Chen N, Yu RH. Traditional Chinese Medicine in Treating IgA Nephropathy: From Basic Science to Clinical Research. *J Transl Int Med* 2021; **9**: 161-167 [PMID: 34900626 DOI: 10.2478/jtim-2021-0021]
6 **Shao Y**, Han XL, Wang JJ. External application of Ruyi Jinhuang Powder combined with antibiotics in the treatment of 30 cases of diabetes with erysipelas in lower limbs. *Neimenggu Zhongyiyao* 2015; **9**: 68-69 [DOI: 10.16040/j.cnki.cn15-1101.2015.09.083]
7 **Liu XY**, Li YQ, Wang XZ, Zhao PT, Liu J, Zhang ZH, Li J, Gao LH, Jia N, Zhang CL. External application of Ruyi Jinhuang Powder to treat 20 cases of diabetes with skin abscess. *Henan Zhongyi* 2014; **34**: 1996-1997 [DOI: 10.16367/j.issn.1003-5028.2014.10.059]
8 **Zhang D**. Observation on the clinical efficacy of oral administration of Simiao Tongluo Decoction combined with external application of Ruyi Jinhuang Powder in the treatment of diabetic foot with damp-heat injection. *Huabei Ligong Daxue* 2020 [DOI: 10.27108/d.cnki.ghelu.2020.000812]
9 **Kibble M**, Saarinen N, Tang J, Wennerberg K, Mäkelä S, Aittokallio T. Network pharmacology applications to map the unexplored target space and therapeutic potential of natural products. *Nat Prod Rep* 2015; **32**: 1249-1266 [PMID: 26030402 DOI: 10.1039/c5np00005j]

10 **Zhang YF**, Huang Y, Ni YH, Xu ZM. Systematic elucidation of the mechanism of geraniol *via* network pharmacology. *Drug Des Devel Ther* 2019; **13**: 1069-1075 [PMID: 31040644 DOI: 10.2147/DDDT.S189088]

11 **Davis AP**, Grondin CJ, Johnson RJ, Sciaky D, Wiegers J, Wiegers TC, Mattingly CJ. Comparative Toxicogenomics Database (CTD): update 2021. *Nucleic Acids Res* 2021; **49**: D1138-D1143 [PMID: 33068428 DOI: 10.1093/nar/gkaa891]

12 **Calvier FÉ**, Bousquet C. Integrating the Comparative Toxicogenomic Database in a Human Pharmacogenomic Resource. *Stud Health Technol Inform* 2020; **270**: 267-271 [PMID: 32570388 DOI: 10.3233/SHTI200164]

13 **Tang HY**, Jiang AJ, Ma JL, Wang FJ, Shen GM. Understanding the Signaling Pathways Related to the Mechanism and Treatment of Diabetic Peripheral Neuropathy. *Endocrinology* 2019; **160**: 2119-2127 [PMID: 31318414 DOI: 10.1210/en.2019-00311]

14 **Dixon D**, Edmonds M. Managing Diabetic Foot Ulcers: Pharmacotherapy for Wound Healing. *Drugs* 2021; **81**: 29-56 [PMID: 33382445 DOI: 10.1007/s40265-020-01415-8]

15 **Feng J**, Dong C, Long Y, Mai L, Ren M, Li L, Zhou T, Yang Z, Ma J, Yan L, Yang X, Gao G, Qi W. Elevated Kallikrein-binding protein in diabetes impairs wound healing through inducing macrophage M1 polarization. *Cell Commun Signal* 2019; **17**: 60 [PMID: 31182110 DOI: 10.1186/s12964-019-0376-9]

16 **Theocharidis G**, Baltzis D, Roustit M, Tellechea A, Dangwal S, Khetani RS, Shu B, Zhao W, Fu J, Bhasin S, Kafanas A, Hui D, Sui SH, Patsopoulos NA, Bhasin M, Veves A. Integrated Skin Transcriptomics and Serum Multiplex Assays Reveal Novel Mechanisms of Wound Healing in Diabetic Foot Ulcers. *Diabetes* 2020; **69**: 2157-2169 [PMID: 32763913 DOI: 10.2337/db20-0188]

17 **Sun HS**. RXRA has a negative regulatory effect on glucose-stimulated insulin secretion Study on the regulation of pancreatic β-cell function by nuclear receptor RXRα. In: CNKI.net [Internet]. [cited 10 March 2022]. Available from: https://kns.cnki.net/kcms/detail/detail.aspx?FileName=1019055238.nh&DbName=CMFD2020

18 **Bradley SJ**, Tobin AB, Prihandoko R. The use of chemogenetic approaches to study the physiological roles of muscarinic acetylcholine receptors in the central nervous system. *Neuropharmacology* 2018; **136**: 421-426 [PMID: 29191752 DOI: 10.1016/j.neuropharm.2017.11.043]

19 **Chen J**, Cheuk IWY, Shin VY, Kwong A. Acetylcholine receptors: Key players in cancer development. *Surg Oncol* 2019; **31**: 46-53 [PMID: 31536927 DOI: 10.1016/j.suronc.2019.09.003]

20 **Yeh YH**, Hsiao HF, Yeh YC, Chen TW, Li TK. Inflammatory interferon activates HIF-1α-mediated epithelial-to-mesenchymal transition *via* PI3K/AKT/mTOR pathway. *J Exp Clin Cancer Res* 2018; **37**: 70 [PMID: 29587825 DOI: 10.1186/s13046-018-0730-6]

21 **Zheng XD**, Huang Y, Li H. Regulatory role of Apelin-13-mediated PI3K/AKT signaling pathway in the glucose and lipid metabolism of mouse with gestational diabetes mellitus. *Immunobiology* 2021; **226**: 152135 [PMID: 34521048 DOI: 10.1016/j.imbio.2021.152135]

22 **Cai SY**, Li YY. Study on the mechanism of mulberry leaf extract regulating IRS-1/PI3K/GLUT4 pathway and affecting insulin resistance in type 2 diabetes. *Xinzhongyi* 2020; **52**: 1-6 [DOI: 10.13457/j.cnki.jncm.2020.01.001]

23 **Bathina S**, Das UN. Dysregulation of PI3K-Akt-mTOR pathway in brain of streptozotocin-induced type 2 diabetes mellitus in Wistar rats. *Lipids Health Dis* 2018; **17**: 168 [PMID: 30041644 DOI: 10.1186/s12944-018-0809-2]

24 **Kim JH**, Bae HC, Kim J, Lee H, Ryu WI, Son ED, Lee TR, Jeong SH, Son SW. HIF-1α-mediated BMP6 down-regulation leads to hyperproliferation and abnormal differentiation of keratinocytes in vitro. *Exp Dermatol* 2018; **27**: 1287-1293 [PMID: 30230035 DOI: 10.1111/exd.13785]

25 **Lauss M**, Kriegner A, Vierlinger K, Noehammer C. Characterization of the drugged human genome. *Pharmacogenomics* 2007; **8**: 1063-1073 [PMID: 17716238 DOI: 10.2217/14622416.8.8.1063]

26 **Yang XY**. Changes in gene expression of the neural activity ligand-receptor interaction pathway in the brain tissue of IVM mice can lead to abnormal gene expression in the second generation of paternal offspring. *Zhejiang University* 2016; 1-67

27 **Liang MD**, Yang XY, Du GH. Research progress in the mechanism of type 2 diabetes inducing skeletal muscle atrophy and the effect of commonly used hypoglycemic drugs. *Yaoxue Xuebao* 2021; **57**: 568-575 [DOI: 10.16438/j.0513-4870.2021-1217]

28 **Hua GF**, Jin HF, Lu C, Guo XW. The effect of intraperitoneal injection of luteolin to regulate the Sirt1/FOXO1 pathway on pain sensitization in rats with chronic sciatic nerve ligation. *Zhejiang Yixue* 2021; **43**: 1489-1512 [DOI: 10.12056/j.issn.1006-2785.2021.43.14.2021-971]

World Journal of Diabetes

Submit a Manuscript: https://www.f6publishing.com

World J Diabetes 2022 August 15; 13(8): 643-653

DOI: 10.4239/wjd.v13.i8.643

ISSN 1948-9358 (online)

ORIGINAL ARTICLE

Case Control Study

Association of rs1137101 with hypertension and type 2 diabetes mellitus of Mongolian and Han Chinese

Ke-Yu Zhao, Meng-Lu Yuan, Yun-Na Wu, Hong-Wei Cui, Wen-Yan Han, Jing Wang, Xiu-Lan Su

Specialty type: Endocrinology and metabolism

Provenance and peer review: Invited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification

Grade A (Excellent): 0
Grade B (Very good): B
Grade C (Good): C
Grade D (Fair): D
Grade E (Poor): 0

P-Reviewer: Javor E, Croatia; Mahmoud MZ, Saudi Arabia; Mrzljak A, Croatia

Received: February 17, 2022
Peer-review started: February 17, 2022
First decision: May 30, 2022
Revised: June 14, 2022
Accepted: July 26, 2022
Article in press: July 26, 2022
Published online: August 15, 2022

Ke-Yu Zhao, Xiu-Lan Su, Clinical Medical Research Center of The Affiliated Hospital, Inner Mongolia Key Laboratory of Medical Cellular Biology, Inner Mongolia Medical University, Hohhot 010050, Inner Mongolia Autonomous Region, China

Meng-Lu Yuan, School of Public Health, Inner Mongolia Medical University, Huhhot 010050, Inner Mongolia Autonomous Region, China

Yun-Na Wu, Medical Clinical Laboratory, Huhhot First Hospital, Huhhot 010050, Inner Mongolia Autonomous Region, China

Hong-Wei Cui, Department of Scientific Research, Inner Mongolia Autonomous Region Cancer Hospital/The Affiliated People's Hospital of Inner Mongolia Medical University, Huhhot 010050, Inner Mongolia Autonomous Region, China

Wen-Yan Han, Clinical Medical Laboratory Center, The Second Affiliated Hospital of Inner Mongolia Medical University, Huhhot 010050, Inner Mongolia Autonomous Region, China

Jing Wang, Graduate School, Inner Mongolia Medical University, Huhhot 010050, Inner Mongolia Autonomous Region, China

Corresponding author: Xiu-Lan Su, MMed, Professor, Clinical Medical Research Center of The Affiliated Hospital, Inner Mongolia Key Laboratory of Medical Cellular Biology, Inner Mongolia Medical University, No. 1 North Tongdao Road, Hohhot, 010050, Inner Mongolia Autonomous Region, China. xlsu@sina.com

Abstract

BACKGROUND

Hypertension (HTN) and type 2 diabetes mellitus (T2DM) are often coincident, and each condition is considered a risk factor for the other. Both occur frequently in the Inner Mongolia region of China. The reasons for differences in risk between Han and Mongolian ethnic groups are not known. The *LEPR* gene and its polymorphism, rs1137101 (Gln223Arg), are both considered risk factors for HTN and T2DM, but any role of rs1137101 in the occurrence of HTN + T2DM remains unclear for Mongolian and Han populations in the Inner Mongolia region.

AIM

To investigate the relationship between rs1137101 and the occurrence of HTN with T2DM in Mongolian and Han populations in Inner Mongolia.

METHODS
A total of 2652 subjects of Han and Mongolian ethnic origins were enrolled in the current study, including 908 healthy controls, 1061 HTN patients and 683 HTN patients with T2DM.

RESULTS
The association between the rs1137101 polymorphism and HTN with T2DM was analyzed, and differences between Han and Mongolian individuals assessed. There was a significant correlation between rs1137101 and HTN (co-dominant, dominant, over-dominant and log-additive models) and HTN + T2DM (co-dominant, dominant, over-dominant and log-additive models) after adjustment for sex and age in individuals of Mongolian origin. rs1137101 was significantly associated with HTN (co-dominant, recessive and log-additive models) and HTN + T2DM (co-dominant, dominant, over-dominant and log-additive models) in the Han Chinese population.

CONCLUSION
Mongolian and Han subjects from Inner Mongolia with HTN who had rs1137101 were protected against the development of T2DM. Allele A has the opposite impact on the occurrence of HTN in Mongolian and Han Chinese populations.

Key Words: rs1137101; Mongolian; Han Chinese; Hypertension; Type 2 diabetes mellitus; Associate study

Core Tip: Hypertension and type 2 diabetes mellitus are often coincident, and each condition is a risk factor for the other. It is unknown why there are differences in risk between Han and Mongolian ethnic groups. The *LEPR* gene and its polymorphism, rs1137101 (Gln223Arg), are considered risk factors for the occurrence of hypertension and type 2 diabetes mellitus. The current study investigated the relationship between rs1137101 and the occurrence of hypertension with type 2 diabetes mellitus in Mongolian and Han populations in Inner Mongolia. Differences between the two populations were analyzed. The aim was to inform further research on advanced metabolic disease.

Citation: Zhao KY, Yuan ML, Wu YN, Cui HW, Han WY, Wang J, Su XL. Association of rs1137101 with hypertension and type 2 diabetes mellitus of Mongolian and Han Chinese. *World J Diabetes* 2022; 13(8): 643-653
URL: https://www.wjgnet.com/1948-9358/full/v13/i8/643.htm
DOI: https://dx.doi.org/10.4239/wjd.v13.i8.643

INTRODUCTION

The causes of hypertension (HTN) are multifactorial, and the condition is in turn a risk factor for cardiovascular disease and nephropathy[1]. Current estimates put a global figure of 1.3 billion[2,3] on the number of people with high blood pressure, an estimate that is set to rise to 1.6 billion by 2025[2,4]. Advanced age, gender, obesity and genotype are all risk factors for HTN[2]. Diabetes mellitus (DM) is another public health problem that has increased rapidly over recent years with 80%-90% patients having type 2 DM (T2DM)[5,6]. Epidemiological studies have shown that HTN is a major risk factor for T2DM[7]. One-third of HTN patients also have T2DM and are at an increased risk of cardiovascular disease and mortality[8,9].

The leptin (LEP) receptor (LEPR) is a transmembrane protein encoded by the *LEPR* gene. Several variants have been characterized, and there is widespread expression throughout the body's tissues[10]. The LEP hormone is known to have roles in the regulation of hunger, energy balance, metabolism, reproduction and insulin secretion mediated by binding to LEPR[11,12]. Binding of LEP to its hypothalamic receptor has been shown to raise blood pressure in mice, and blockade of LEPR resulted in lower values[13,14]. LEPR has roles in insulin secretion, and its activity is relevant to the development of insulin resistance[12,15]. Indeed, a recent study has correlated *LEPR* polymorphisms with DM and HTN[16,17]. Among the Han Chinese population, the *LEPR* gene polymorphism, rs13306519, has been associated with DM and rs12037879 with HTN[5]. Moreover, rs1137100 (Arg109Lys) and rs8179183 (Lys656Asn) have been associated with both DM and HTN[15,18].

The *LEPR* gene polymorphism, rs1137101, is located on chromosome 1p31 and involves a substitution of the 223rd amino acid residue, gln (Q) for Arg (R). This mutation affects the ObRIg domain, according to the PFAM database (http://pfam.xfam.org/protein/P48357; Figure 1A and Table 1). Construction of a 3D model of the region including amino acids 126 to 533 using Swiss-model software (https://swissmodel.expasy.org/) revealed a consequent change in protein structure (Figure 1B). These

Table 1 Domain boundaries and score for each of the domains

Source	Domain	Start	End	Gathering threshold (bits)		Score (bits)		E-value	
				Sequence	Domain	Sequence	Domain	Sequence	Domain
Pfam	ObR_Ig	126	233	25.8	25.8	170.5	61.1	3.50E-47	3.9E-13
Pfam	Lep_receptor_Ig	329	420	28.8	28.8	84.8	84.8	1.00E-20	1.00E-20
Pfam	ObR_Ig	431	533	25.8	25.8	170.5	112.7	3.50E-47	3.40E-29
Transmembrane	NA	840	862	NA	NA	NA	NA	NA	NA
Low_complexity	NA	849	863	NA	NA	NA	NA	NA	NA
Disorder	NA	924	927	NA	NA	NA	NA	NA	NA
Low_complexity	NA	937	946	NA	NA	NA	NA	NA	NA
Disorder	NA	966	967	NA	NA	NA	NA	NA	NA
Disorder	NA	970	973	NA	NA	NA	NA	NA	NA
Disorder	NA	975	976	NA	NA	NA	NA	NA	NA
Disorder	NA	997	1001	NA	NA	NA	NA	NA	NA
Disorder	NA	1064	1065	NA	NA	NA	NA	NA	NA

NA: No adoption.

predictions imply that the rs1137101 mutation may influence protein structure and have an impact on protein function. Previous studies have associated rs1137101 (Gln223Arg) with obesity, cancer, HTN and DM[9,19,20]. It also has been shown to be a risk factor for HTN and T2DM in the Chinese population[21,22]. The current study investigated the relationship between rs1137101 and the occurrence of HTN with T2DM in Mongolian and Han populations in Inner Mongolia.

MATERIALS AND METHODS

Study subjects

A total of 2652 subjects, including 908 healthy controls, 1061 HTN patients and 683 patients with HTN + T2DM, were randomly selected from adult residents of Mongolia (Hohhot, Wuhai, Xilinhot) and enrolled in the study. Study participants were unrelated, and the ethnic composition was 1347 Han and 1305 Mongolian. All participants provided written informed consent. The study was performed in accordance with the declaration of Helsinki and approved by the ethical committee of the affiliated hospital of Inner Mongolia Medical University.

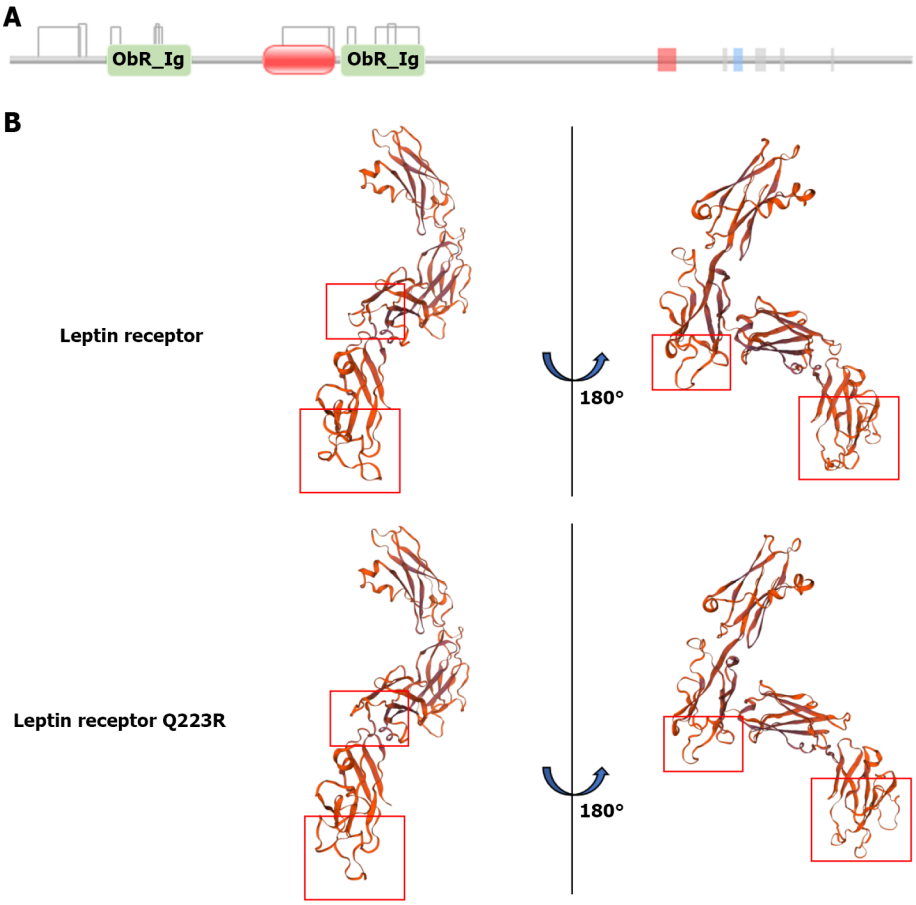
T2DM and HTN were diagnosed according to the following criteria established by the World Health Organization: HTN: Systolic blood pressure ≥ 140 mmHg and/or diastolic blood pressure ≥ 90 mmHg or current prescription for antihypertensive medication[23]. Participants with chronic renal disease, renal artery stenosis, primary hyperaldosteronism, thyroid disease, Cushing syndrome, phaeochromocytoma or other diseases known to cause HTN were excluded; T2DM: Fasting blood sugar (FBS) ≥ 7.0 mmol /L or postprandial blood glucose ≥ 11.1 mmol/L or current definitive diagnosis of T2DM[24]. Participants with T1DM, cancer or other severe metabolic disease were excluded.

Data collection

Age, weight and medical history were collected by questionnaire. Body mass index was calculated according to the formula: Mass (kg)/height^2 (m^2). Blood pressure was measured on the right arm using a mercury sphygmomanometer. Blood samples of HTN, T2DM and HTN + T2DM groups were collected after an 8 h fast. Genomic DNA was isolated from whole blood using a Maga bio plus whole blood genomic DNA purification Kit II (Hangzhou Bioer Technology co. Ltd, China) according to the manufacturer's instructions. FBS, triglyceride, cholesterol, high density lipoprotein and low density lipoprotein were measured after plasmapheresis.

Genotyping

rs1137101 (Gln223Arg) polymorphisms were assessed by PCR amplification. The primers used were forward: 5'-TTCCCCAAAAAGGCAGTTTTCA-3' and reverse: 5'-AGAAGCCACTCTTAATAC-CCCCAGT-3'. The target DNA sequences were amplified using a multiplex PCR method. Thermal



DOI: 10.4239/wjd.v13.i8.643

Figure 1 Leptin receptor domains and 3D structure. A: The PFAM database obtains the domains of the leptin receptor (LEPR) protein; B: Swiss-model was used to construct the 3D model of the leptin receptor and the leptin receptor (Q223R) protein fragment 126 to 533. The red frame represented the differences between two models.

cycling was performed for the rs1137101 loci in Gene Amp PCR system 9600 (PerkinElmer, Waltham, MA, United States) fluorescent products of ligase detection reaction differentiated by 3130xl genetic analyzer (Applied Biosystems, CA, United States).

Statistical analysis

Statistical analysis was performed using SPSS 22.0 (IBM Corp., Armonk, NY, United States) and SNPStats (https://www.snpstats.net/start.htm)[25] software. Categorical variables were presented as frequencies. Continuous data were reported as the mean ± standard deviation. Student's *t* test was used to compare age, weight, height, body mass index, FBS, systolic blood pressure, diastolic blood pressure, triglyceride, cholesterol, high density lipoprotein and low density lipoprotein and statistical hypotheses were tested using the 2-tailed *t* test. The χ^2 test was used to analyze ethnic and gender differences. Logistic regression was used to compute the odds ratio (OR) by adjusting for age and sex and the adjusted OR is presented with 95% confidence interval. Logistic regression, Hardy Weinberg Equilibrium and five genetic models (co-dominant, dominant, recessive, over-dominant and log-additive) were calculated using SNPStats software. A value of $P < 0.05$ was considered to be significant.

RESULTS

Baseline demographic characteristics

Baseline demographic characteristics of the study population are summarized in Table 2. Significant differences were found in ethnicity, gender, age, weight, height, FBS, Systolic blood pressure, diastolic blood pressure and high density lipoprotein between cases with HTN, those with both HTN + T2DM and controls. No significant deviation from the Hardy Weinberg Equilibrium was detected (Table 3). Allele frequency was not significant in the Han population, but significant differences between Mongolian groups were observed (Table 4).

Table 2 Baseline characteristics

		Control, *n* = 908	HTN, *n* = 1061	HTN with T2DM, *n* = 683	*P* value		
					Control *vs* HTN	Control *vs* HTN + T2DM	HTN *vs* HTN + T2DM
Ethnic	Han	455	406	486	< 0.0001	< 0.0001	< 0.0001
	Mongolian	453	655	197			
Gender	Male	357	601	397	< 0.0001	< 0.0001	0.542
	Female	551	460	286			
Age		48.11 ± 15.06	54.49 ± 15.67	63.89 ± 11.17	< 0.0001	< 0.0001	< 0.0001
Weight (kg)		66.14 ± 11.06	72.32 ± 12.06	73.35 ± 12.48	< 0.0001	< 0.0001	0.2156
Height (cm)		163 ± 0.09	168 ± 0.08	161.26 ± 0.10	< 0.0001	0.6110	0.0032
BMI (kg/m^2)		25.27 ± 8.50	25.56 ± 3.63	26.48 ± 5.12	0.4067	0.7588	0.8723
FBS (mmol/L)		5.06 ± 0.49	5.73 ± 0.77	8.59 ± 3.37	0.0721	< 0.0001	< 0.0001
SBP (mm Hg)		117.21 ± 14.27	151.10 ± 18.94	166.47 ± 17.53	< 0.0001	< 0.0001	< 0.0001
DBP (mm Hg)		77.20 ± 7.95	88.59 ± 12.74	100.84 ± 13.31	< 0.0001	< 0.0001	< 0.0001
TG (mmol/L)		1.63 ± 1.06	2.24 ± 1.52	2.59 ± 12.14	0.0626	0.0051	0.4768
CHO (mmol/L)		4.53 ± 1.30	4.47 ± 3.59	4.52 ± 1.26	0.8913	0.9984	0.8885
HDL (mmol/L)		1.44 ± 0.54	1.75 ± 0.95	1.27 ± 0.35	< 0.0001	< 0.0001	< 0.0001
LDL (mmol/L)		2.84 ± 1.00	2.87 ± 1.38	2.94 ± 11.1	0.9921	0.9225	0.9586

Data presented as mean ± SD and percentages. *P* value of < 0.05 was considered significant. BMI: Body mass index; FBS: Fasting blood sugar; SBP: Systolic blood pressure; DBP: Diastolic blood pressure; TG: Triglyceride; CHO: Cholesterol; HDL: High density lipoprotein; LDL: Low density lipoprotein; HTN: Hypertension; T2DM: Type 2 diabetes mellitus.

Table 3 Hardy Weinberg equilibrium analysis

	Group	G/G	G/A	A/A	G	A	*P* value
Han, *n* = 1347	Control, *n* = 455	351	94	10	796	114	0.2
	HTN, *n* = 406	312	91	3	715	97	0.24
	HTN + T2DM, *n* = 486	394	84	8	872	100	0.21
Mongolian, *n* = 1305	Control, *n* = 453	343	101	9	787	119	0.68
	HTN, *n* = 655	436	202	17	1074	236	0.29
	HTN + T2DM, *n* = 197	151	42	4	344	50	0.53

P value of < 0.05 was considered significant. HTN: Hypertension; T2DM: Type 2 diabetes mellitus.

LEPR gene polymorphisms and HTN in ethnic Han and Mongolian Chinese

The correlation between the *LEPR* gene polymorphism, rs1137101, and HTN in ethnic Han and Mongolian Chinese subjects was analyzed. A total of 861 subjects of Han origin (control = 455; HTN = 406) and 1108 subjects of Mongolian origin (control = 453; HTN = 655) were assessed. Logistic regression analysis was used to evaluate whether rs1137101 was independently associated with HTN after adjusting for sex and age (Table 5). Use of five inheritance models, codominant, dominant, recessive, over-dominant and log-additive, gave the following results: Co-dominant (A/G) model: OR = 0.88 (0.62-1.27); co-dominant (A/A) model: OR = 0.21 (0.05-0.80); and recessive (A/A) model: OR = 0.21 (0.05-0.82) for hypertensive Han subjects compared with controls. Results for Mongolian subjects were: Co-dominant (A/G) model: OR = 1.49 (1.12-1.97); co-dominant (A/A) model: OR = 1.47 (0.64-3.34); dominant (A/G-A/A) model: OR = 1.49 (1.13-1.95); over-dominant (A/A) model: OR = 1.47 (1.11-1.95); and log-additive model: OR = 1.40 (1.10-1.79). An association between rs1137101 and HTN was established for subjects of Mongolian ethnic origin.

Table 4 Statistics of allele and genotype frequencies

Population	Allele	All subjects count (%)	Control count (%)	HTN count (%)	HTN + T2DM count (%)	*P* value
Han, *n* = 1347	G	2383 (88)	796 (87)	715 (88)	872 (90)	0.288
	A	311 (12)	114 (13)	97 (12)	100 (10)	
	A/A	21 (2)	10 (2)	3 (1)	8 (2)	0.153
	G/A	269 (20)	94 (21)	91 (22)	84 (17)	
	G/G	1057 (78)	351 (77)	312 (77)	394 (81)	
Mongolian, *n* = 1305	G	2205 (84)	787 (87)	1074 (82)	344 (87)	0.002
	A	405 (16)	119 (13)	236 (18)	50 (13)	
	A/A	30 (2)	9 (2)	17 (3)	4 (2)	0.006
	G/A	345 (26)	101 (22)	202 (31)	42 (21)	
	G/G	930 (71)	343 (76)	436 (67)	151 (77)	

P value of < 0.05 was considered significant. HTN: Hypertension; T2DM: Type 2 diabetes mellitus.

Table 5 Association of the control and hypertension groups for Han and Mongolian Chinese

Model	Genotype	Han, *n* = 861				Mongolian, *n* = 1108			
		Control, *n* (%)	HTN, *n* (%)	OR (95%CI)	*P* value	Control, *n* (%)	HTN, *n* (%)	OR (95%CI)	*P* value
Co-dominant	G/G	351 (77.1)	312 (76.8)	1	0.041	343 (75.7)	436 (66.6)	1	0.016
	A/G	94 (20.7)	91 (22.4)	0.88 (0.62-1.27)		101 (22.3)	202 (30.8)	1.49 (1.12-1.97)	
	A/A	10 (2.2)	3 (0.7)	0.21 (0.05-0.80)		9 (2.0)	17 (2.6)	1.47 (0.64-3.34)	
Dominant	G/G	351 (77.1)	312 (76.8)	1	0.23	343 (75.7)	436 (66.6)	1	0.004
	A/G-A/A	104 (22.9)	94 (23.1)	0.81 (0.57-1.15)		110 (24.3)	219 (33.4)	1.49 (1.13-1.95)	
Recessive	G/G-A/G	445 (97.8)	403 (99.3)	1	0.015	444 (98)	638 (97.4)	1	0.51
	A/A	10 (2.2)	3 (0.7)	0.21 (0.05-0.82)		9 (2.0)	17 (2.6)	1.32 (0.58-2.99)	
Over-dominant	G/G-A/A	361 (79.3)	315 (77.6)	1	0.63	352 (77.7)	453 (69.2)	1	0.0064
	A/G	94 (20.7)	91 (22.4)	0.91 (0.64-1.31)		101 (22.3)	202 (30.8)	1.47 (1.11-1.95)	
Log-additive	-	-	-	0.75 (0.55-1.04)	0.082	-	-	1.40 (1.10-1.79)	0.0059

Adjusted for sex and age. *P* value of < 0.05 was considered significant. HTN: Hypertension; OR: Odd ratio; CI: Confidence interval.

The correlation between rs1137101 and HTN with T2DM in Han and Mongolian subjects

The association of rs1137101 with HTN + T2DM was analyzed. A total of 683 subjects, composed of 197 Mongolian and 486 Han, were included. The same five genetic models, codominant, dominant, recessive, over-dominant and log-additive, were used to analyze associations between HTN + T2DM as described above for HTN. OR (adjusted for sex and age) for the five genetic models in Mongolian subjects were: Co-dominant (A/G): 0.70 (0.44-1.11); co-dominant (A/A): 1.06 (0.27-4.25); dominant (A/G-A/A): 0.72 (0.46-1.13); recessive (G/G-A/G):1.15 (0.29-4.57); over-dominant (A/G): 0.70 (0.44-1.11); and log-additive: 0.78 (0.52-1.16). OR (adjusted for sex and age) for the five genetic models in Han subjects were: Co-dominant (A/G): 0.59 (0.40-0.87); co-dominant (A/A): 0.38 (0.14-1.08); dominant (A/G-A/A): 0.56 (0.39-0.82); recessive (G/G-A/G): 0.43 (0.15-1.21); over-dominant (A/G): 0.61 (0.41-0.89); and log-additive: 0.60 (0.43-0.83). No significant differences were found in Mongolian subjects, but the genotypes GA and AA significantly decreased the risk of HTN + T2DM in Han subjects (Table 6). Thus, the LEPR polymorphism is associated with the occurrence of HTN + T2DM in Han Chinese populations but not in Mongolian Chinese.

A comparison was made between patients with HTN and those with HTN + T2DM to analyze the correlation between the *LEPR* polymorphism and the occurrence of these disorders in Mongolian and Han populations. OR (95% confidence interval) (adjusted for sex and age) for Han subjects for the same five genetic models were: Co-dominant (A/G): 0.65 (0.46-0.92); co-dominant (A/A): 1.61 (0.41-6.28); dominant (A/G-A/A): 0.68 (0.49-0.96); recessive (A/A): 1.77 (0.46-6.87); over-dominant (A/G): 0.65

Table 6 Association of rs1137101 with hypertension + type 2 diabetes mellitus (control *vs* hypertension + type 2 diabetes mellitus)

Model	Genotype	Han, *n* = 941				Mongolian, *n* = 650			
		Control, *n* (%)	HTN with T2DM, *n* (%)	OR (95%CI)	*P* value	Control, *n* (%)	HTN with T2DM, *n* (%)	OR (95%CI)	*P* value
Co-dominant	G/G	351 (77.1)	394 (81.1)	1	0.0075	343 (75.7)	151 (76.7)	1	0.3
	A/G	94 (20.7)	84 (17.3)	0.59 (0.40-0.87)		101 (22.3)	42 (21.3)	0.70 (0.44-1.11)	
	A/A	10 (2.2)	8 (1.6)	0.38 (0.14-1.08)		9 (2.0)	4 (2.0)	1.06 (0.27-4.25)	
Dominant	G/G	351 (77.1)	394 (81.1)	1	0.0024	343 (75.7)	151 (76.7)	1	0.15
	A/G-A/A	104 (22.9)	92 (18.9)	0.56 (0.39-0.82)		110 (24.3)	46 (23.4)	0.72 (0.46-1.13)	
Recessive	G/G-A/G	445 (97.8)	478 (98.3)	1	0.11	444 (98.0)	193 (98.0)	1	0.84
	A/A	10 (2.2)	8 (1.6)	0.43 (0.15-1.21)		9 (2.0)	4 (2.0)	1.15 (0.29-4.57)	
Over-dominant	G/G-A/A	361 (79.3)	402 (82.7)	1	0.01	352 (77.7)	155 (78.7)	1	0.12
	A/G	94 (20.7)	84 (17.3)	0.61 (0.41-0.89)		101 (22.3)	42 (21.3)	0.70 (0.44-1.11)	
Log-additive	-	-	-	0.60 (0.43-0.83)	0.0018	-	-	0.78 (0.52-1.16)	0.22

Adjusted for sex and age. *P* value of < 0.05 was considered significant. OR: Odd ratio; HTN: Hypertension; T2DM: Type 2 diabetes mellitus; CI: Confidence interval.

(0.46-0.91); and log-additive: 0.75 (0.55-1.02). All values were non-significant. For Mongolian subjects, OR (adjusted for sex and age) were: Co-dominant (A/G): 0.54 (0.36-0.81); co-dominant (A/A): 0.55 (0.17-1.79); dominant (A/G-A/A): 0.54 (0.36-0.80); recessive (A/A): 0.65 (0.20-2.11); over-dominant (A/G): 0.55 (0.37-0.82); and log-additive: 0.59 (0.41-0.84). The co-dominant A/G model, dominant A/G-A/A model, over-dominant A/G model and log-additive model were all associated with a significantly decreased risk of HTN + T2DM in Mongolian and Han patients (Table 7).

DISCUSSION

HTN and T2DM are major risk factors for cardiovascular and cerebrovascular diseases, and both conditions are known to result from interactions between genetics and environment[26,27]. The *LEPR* gene has been widely studied with respect to T2DM and HTN. We have previously demonstrated an association between rs1137101 and HTN in Han subjects and an association between rs7555955 and HTN in Mongolian subjects[28]. No association was found between rs1137101 and HTN or other metabolic traits in Mexican children[29] nor with HTN or cardiovascular disease in Iranian subjects[17]. A meta-analysis did show an association between rs1137101 and T2DM[30], and a Brazilian study suggested a relationship between T2DM and being overweight[31]. Furthermore, rs1137101 was correlated with T2DM, insulin change and being overweight among the Punjabi population of North India[32]. These findings indicate that associations are very dependent on the origins of the population under study. Inner Mongolia is a vast territory with demarcation of urban, agricultural, pastoral and part-farming/part-pastoral areas. Each region has a unique lifestyle with specific eating habits, all of which have an impact on rates of HTN. Overlain on these variations are traditional risk factors, such as smoking, drinking and salt intake[33,34] plus environmental factors[35,36]. Results of the current study were not in accord with those of previous studies and discrepancies may be due to population and lifestyle differences.

The current study focused on the conditions of HTN and HTN + T2DM in ethnic Han and Mongolian populations in Inner Mongolia. There was a significant association between rs1137101 and HTN and HTN + T2DM in Han Chinese subjects. The genotypes, AA and GA, may decrease risk of HTN and HTN + T2DM for control and HTN groups. Whereas rs1137101 was associated with a significantly increased risk of HTN for control subjects, it was associated with a decreased risk of developing T2DM for HTN patients. Further investigations involving larger study populations with further data relating to environmental and lifestyle factors are required to substantiate interactions between genetics and the environment.

Table 7 Association of rs1137101 with hypertension and type 2 diabetes mellitus (hypertension and hypertension + type 2 diabetes mellitus)

Model	Genotype	Han, *n* = 892				Mongolian, *n* = 852			
		Control, *n* (%)	HTN with T2DM, *n* (%)	OR (95%CI)	*P* value	Control, *n* (%)	HTN with T2DM, *n* (%)	OR (95%CI)	*P* value
Co-dominant	G/G	312 (76.8)	394 (81.1)	1	0.034	436 (66.6)	151 (76.7)	1	0.0067
	A/G	91 (22.4)	84 (17.3)	0.65 (0.46-0.92)		202 (30.8)	42 (21.3)	0.54 (0.36-0.81)	
	A/A	3 (0.7)	8 (1.6)	1.61 (0.41-6.28)		17 (2.6)	4 (2.0)	0.55 (0.17-1.79)	
Dominant	G/G	312 (76.8)	394 (81.1)	1	0.026	436 (66.6)	151 (76.7)	1	0.0016
	A/G-A/A	94 (23.1)	92 (18.9)	0.68 (0.49-0.96)		219 (33.4)	46 (23.4)	0.54 (0.36-0.80)	
Recessive	G/G-A/G	403 (99.3)	478 (98.3)	1	0.39	638 (97.4)	193 (98.0)	1	0.46
	A/A	3 (0.7)	8 (1.6)	1.77 (0.46-6.87)		17 (2.6)	4 (2.0)	0.65 (0.20-2.11)	
Over-dominant	G/G-A/A	315 (77.6)	402 (82.7)	1	0.012	453 (69.2)	155 (78.7)	1	0.0028
	A/G	91 (22.4)	84 (17.3)	0.65 (0.46-0.91)		202 (30.8)	42 (21.3)	0.55 (0.37-0.82)	
Log-additive	-	-	-	0.75 (0.55-1.02)	0.067	-	-	0.59 (0.41-0.84)	0.0024

Adjusted for sex and age. *P* value of < 0.05 was considered significant. OR: Odd ratio; HTN: Hypertension; T2DM: Type 2 diabetes mellitus; CI: Confidence interval.

CONCLUSION

The current study investigated the impact of the polymorphism rs1137101 on HTN in Mongolian subjects. Mongolian and Han subjects with HTN who had rs1137101 were protected against the development of T2DM, and rs1137101 decreased the risk of HTN and HTN + T2DM for the Han Chinese population of Inner Mongolia. In contrast with its protective role in the Han population, rs1137101 increased the risk of HTN for the Mongolian population.

ARTICLE HIGHLIGHTS

Research background

Hypertension (HTN) and type 2 diabetes mellitus (T2DM) are each considered a risk factor for the other. Both occur frequently in the Inner Mongolia region of China. rs1137101 is a potential risk factor for the occurrence of HTN and T2DM but the association between rs1137101 and HTN + T2DM in the Mongolian and Han population in Inner Mongolia remains unknown.

Research motivation

The association between rs1137101 and occurrence of HTN + T2DM has not been fully elucidated for Mongolian and Han populations in the Inner Mongolia region.

Research objectives

To investigate the relationship between rs1137101 and the occurrence of HTN with T2DM in Mongolian and Han populations in Inner Mongolia. To illuminate the association between the rs1137101 polymorphism and HTN with T2DM by analyzing differences between Han and Mongolian Chinese.

Research methods

Data relating to blood samples, blood pressure, weight, height and other body indices among Chinese populations in Inner Mongolia. The rs1137101 polymorphism was measured. Data was analyzed by SPSS 22.0 and SNPstats software (https://www.snpstats.net/start.htm) to correlate rs1137101 with HTN + T2DM in Mongolian and Han populations in Inner Mongolia.

Research results

The association between the rs1137101 polymorphism and HTN with T2DM was analyzed, and differences between Han and Mongolian individuals were assessed. There was a significant correlation between rs1137101 with both HTN after adjustment for sex and age in individuals of Mongolian origin. rs1137101 was significantly associated with HTN and HTN + T2DM in the Han Chinese population.

Research conclusions

There was significant correlation between rs1137101 and control and HTN/HTN + T2DM in Han and Mongolian subjects. Mongolian and Han subjects with HTN who had rs1137101 were protected against the development of T2DM. rs1137101 decreased the risk of HTN and HTN + T2DM for the Han Chinese population of Inner Mongolia. By contrast, rs1137101 increased the risk of HTN for the Mongolian population.

Research perspectives

The current study analyzed the association between rs1137101 and HTN/HTN + T2DM by comparing control, HTN and HTN + T2DM groups and found rs1137101 to be associated with HTN and HTN + T2DM in Mongolian and Han populations in Inner Mongolia. Further investigations involving larger study populations with further data relating to environmental and lifestyle factors are required to substantiate interactions between genetics and the environment.

FOOTNOTES

Author contributions: Zhao KY, Yuan ML, Wu YN, Cui HW, Han WY, Wang J and Su XL designed the research study; Zhao KY, Wang J, Yuan ML and Su XL performed the research; Su XL and Zhao KY contributed new reagents and analytic tools; Zhao KY, Yuan ML and Su XL analyzed the data and wrote the manuscript; All authors have read and approved the final manuscript.

Supported by National Natural Science Foundation of China, No. 81260058.

Institutional review board statement: The study was reviewed and approved by the Affiliated Hospital of Inner Mongolia Medical University Institutional Review Board, No. 2015003.

Informed consent statement: All study participants or their legal guardian provided informed written consent about personal and medical data collection prior to study enrolment.

Conflict-of-interest statement: All the authors report no relevant conflicts of interest for this article.

Data sharing statement: No additional data are available.

STROBE statement: The authors have read the STROBE Statement—checklist of items, and the manuscript was prepared and revised according to the STROBE Statement—checklist of items.

Country/Territory of origin: China

ORCID number: Xiu-Lan Su 0000-0001-6527-7412.

S-Editor: Fan JR
L-Editor: Filipodia
P-Editor: Fan JR

REFERENCES

1 **Saju MD**, Allagh KP, Scaria L, Joseph S, Thiyagarajan JA. Prevalence, Awareness, Treatment, and Control of Hypertension and Its Associated Risk Factors: Results from Baseline Survey of SWADES Family Cohort Study. *Int J Hypertens* 2020; **2020**: 4964835 [PMID: 32351729 DOI: 10.1155/2020/4964835]

2 **Ojha U**, Ruddaraju S, Sabapathy N, Ravindran V, Worapongsatitaya P, Haq J, Mohammed R, Patel V. Current and Emerging Classes of Pharmacological Agents for the Management of Hypertension. *Am J Cardiovasc Drugs* 2022; **22**: 271-

285 [PMID: 34878631 DOI: 10.1007/s40256-021-00510-9]
3 **Ott C**, Schmieder RE. Diagnosis and treatment of arterial hypertension 2021. *Kidney Int* 2022; **101**: 36-46 [PMID: 34757122 DOI: 10.1016/j.kint.2021.09.026]
4 **Gelaw S**, Yenit MK, Nigatu SG. Self-Care Practice and Associated Factors among Hypertensive Patients in Debre Tabor Referral Hospital, Northwest Ethiopia, 2020. *Int J Hypertens* 2021; **2021**: 3570050 [PMID: 34422407 DOI: 10.1155/2021/3570050]
5 **Jiang B**, Liu Y, Fang F, Wang X, Li B. Association of four insulin resistance genes with type 2 diabetes mellitus and hypertension in the Chinese Han population. *Mol Biol Rep* 2014; **41**: 925-933 [PMID: 24414038 DOI: 10.1007/s11033-013-2937-0]
6 **Xi Y**, Xu PF. Diabetes and gut microbiota. *World J Diabetes* 2021; **12**: 1693-1703 [PMID: 34754371 DOI: 10.4239/wjd.v12.i10.1693]
7 **Alsaadon H**, Afroz A, Karim A, Habib SH, Alramadan MJ, Billah B, Shetty AN. Hypertension and its related factors among patients with type 2 diabetes mellitus - a multi-hospital study in Bangladesh. *BMC Public Health* 2022; **22**: 198 [PMID: 35093064 DOI: 10.1186/s12889-022-12509-1]
8 **Bazalar-Palacios J**, Jaime Miranda J, Carrillo-Larco RM, Gilman RH, Smeeth L, Bernabe-Ortiz A. Aggregation and combination of cardiovascular risk factors and their association with 10-year all-cause mortality: the PERU MIGRANT Study. *BMC Cardiovasc Disord* 2021; **21**: 582 [PMID: 34876013 DOI: 10.1186/s12872-021-02405-8]
9 **Abaturov A**, Nikulina A. Obesity in Children with Leptin Receptor Gene Polymorphisms. *Acta Medica (Hradec Kralove)* 2021; **64**: 158-164 [PMID: 34779381 DOI: 10.14712/18059694.2021.27]
10 **Stefan N**, Vozarova B, Del Parigi A, Ossowski V, Thompson DB, Hanson RL, Ravussin E, Tataranni PA. The Gln223Arg polymorphism of the leptin receptor in Pima Indians: influence on energy expenditure, physical activity and lipid metabolism. *Int J Obes Relat Metab Disord* 2002; **26**: 1629-1632 [PMID: 12461680 DOI: 10.1038/sj.ijo.0802161]
11 **Abella V**, Scotece M, Conde J, Pino J, Gonzalez-Gay MA, Gómez-Reino JJ, Mera A, Lago F, Gómez R, Gualillo O. Leptin in the interplay of inflammation, metabolism and immune system disorders. *Nat Rev Rheumatol* 2017; **13**: 100-109 [PMID: 28053336 DOI: 10.1038/nrrheum.2016.209]
12 **Seoane-Collazo P**, Martínez-Sánchez N, Milbank E, Contreras C. Incendiary Leptin. *Nutrients* 2020; **12** [PMID: 32069871 DOI: 10.3390/nu12020472]
13 **Bełtowski J**. Role of leptin in blood pressure regulation and arterial hypertension. *J Hypertens* 2006; **24**: 789-801 [PMID: 16612235 DOI: 10.1097/01.hjh.0000222743.06584.66]
14 **Kim LJ**, Shin MK, Pho H, Otvos L Jr, Tufik S, Andersen ML, Pham LV, Polotsky VY. Leptin Receptor Blockade Attenuates Hypertension, but Does Not Affect Ventilatory Response to Hypoxia in a Model of Polygenic Obesity. *Front Physiol* 2021; **12**: 688375 [PMID: 34276408 DOI: 10.3389/fphys.2021.688375]
15 **Wauters M**, Mertens I, Rankinen T, Chagnon M, Bouchard C, Van Gaal L. Leptin receptor gene polymorphisms are associated with insulin in obese women with impaired glucose tolerance. *J Clin Endocrinol Metab* 2001; **86**: 3227-3232 [PMID: 11443193 DOI: 10.1210/jcem.86.7.7682]
16 **Cao X**, Huo P, Li W, Li P, He L, Meng H. Interactions among moderate/severe periodontitis, ADIPOQ-rs1501299, and LEPR-rs1137100 polymorphisms on the risk of type 2 diabetes in a Chinese population. *Arch Oral Biol* 2019; **103**: 26-32 [PMID: 31128439 DOI: 10.1016/j.archoralbio.2019.05.014]
17 **Nowzari Z**, Masoumi M, Nazari-Robati M, Akbari H, Shahrokhi N, Asadikaram G. Association of polymorphisms of leptin, leptin receptor and apelin receptor genes with susceptibility to coronary artery disease and hypertension. *Life Sci* 2018; **207**: 166-171 [PMID: 29883719 DOI: 10.1016/j.lfs.2018.06.007]
18 **Salopuro T**, Pulkkinen L, Lindström J, Eriksson JG, Valle TT, Hämäläinen H, Ilanne-Parikka P, Keinänen-Kiukaanniemi S, Tuomilehto J, Laakso M, Uusitupa M; Finnish Diabetes Prevention Study Group. Genetic variation in leptin receptor gene is associated with type 2 diabetes and body weight: The Finnish Diabetes Prevention Study. *Int J Obes (Lond)* 2005; **29**: 1245-1251 [PMID: 15997246 DOI: 10.1038/sj.ijo.0803024]
19 **Illangasekera YA**, Kumarasiri PVR, Fernando DJ, Dalton CF. Association of the leptin receptor Q223R (rs1137101) polymorphism with obesity measures in Sri Lankans. *BMC Res Notes* 2020; **13**: 34 [PMID: 31948470 DOI: 10.1186/s13104-020-4898-4]
20 **Rong G**, Tang W, Wang Y, Qiu H, Chen S. Investigation of *leptin receptor* rs1137101 G>A polymorphism with cancer risk: evidence from 35936 subjects. *Biosci Rep* 2019; **39** [PMID: 31196966 DOI: 10.1042/bsr20182240]
21 **Gu P**, Jiang W, Chen M, Lu B, Shao J, Du H, Jiang S. Association of leptin receptor gene polymorphisms and essential hypertension in a Chinese population. *J Endocrinol Invest* 2012; **35**: 859-865 [PMID: 22293279 DOI: 10.3275/8238]
22 **Zhang L**, Qin Y, Liang D, Li L, Liang Y, Chen L, Tong L, Zhou J, Li H, Zhang H. Association of polymorphisms in LEPR with type 2 diabetes and related metabolic traits in a Chinese population. *Lipids Health Dis* 2018; **17**: 2 [PMID: 29301582 DOI: 10.1186/s12944-017-0644-x]
23 **Al-Makki A**, DiPette D, Whelton PK, Murad MH, Mustafa RA, Acharya S, Beheiry HM, Champagne B, Connell K, Cooney MT, Ezeigwe N, Gaziano TA, Gidio A, Lopez-Jaramillo P, Khan UI, Kumarapeli V, Moran AE, Silwimba MM, Rayner B, Sukonthasan A, Yu J, Saraffzadegan N, Reddy KS, Khan T. Hypertension Pharmacological Treatment in Adults: A World Health Organization Guideline Executive Summary. *Hypertension* 2022; **79**: 293-301 [PMID: 34775787 DOI: 10.1161/HYPERTENSIONAHA.121.18192]
24 **Serbis A**, Giapros V, Kotanidou EP, Galli-Tsinopoulou A, Siomou E. Diagnosis, treatment and prevention of type 2 diabetes mellitus in children and adolescents. *World J Diabetes* 2021; **12**: 344-365 [PMID: 33889284 DOI: 10.4239/wjd.v12.i4.344]
25 **Solé X**, Guinó E, Valls J, Iniesta R, Moreno V. SNPStats: a web tool for the analysis of association studies. *Bioinformatics* 2006; **22**: 1928-1929 [PMID: 16720584 DOI: 10.1093/bioinformatics/btl268]
26 **Weng Z**, Liu Q, Yan Q, Liang J, Zhang X, Xu J, Li W, Xu C, Gu A. Associations of genetic risk factors and air pollution with incident hypertension among participants in the UK Biobank study. *Chemosphere* 2022; **299**: 134398 [PMID: 35339527 DOI: 10.1016/j.chemosphere.2022.134398]
27 **Teo KK**, Rafiq T. Cardiovascular Risk Factors and Prevention: A Perspective From Developing Countries. *Can J Cardiol*

2021; **37**: 733-743 [PMID: 33610690 DOI: 10.1016/j.cjca.2021.02.009]

28 **Yuan M**, Bi L, Su X. [Association of single nucleotide polymorphisms of LEPR gene with essential hypertension among ethnic Mongolian and Han Chinese from Inner Mongolia region]. *Zhonghua Yi Xue Yi Chuan Xue Za Zhi* 2018; **35**: 561-566 [PMID: 30098257 DOI: 10.3760/cma.j.issn.1003-9406.2018.04.023]

29 **Vashi N**, Stryjecki C, Peralta-Romero J, Suarez F, Gomez-Zamudio J, Burguete-Garcia AI, Cruz M, Meyre D. Genetic markers of inflammation may not contribute to metabolic traits in Mexican children. *PeerJ* 2016; **4**: e2090 [PMID: 27366637 DOI: 10.7717/peerj.2090]

30 **Yang Y**, Niu T. A meta-analysis of associations of LEPR Q223R and K109R polymorphisms with Type 2 diabetes risk. *PLoS One* 2018; **13**: e0189366 [PMID: 29293570 DOI: 10.1371/journal.pone.0189366]

31 **Queiroz EM**, Cândido AP, Castro IM, Bastos AQ, Machado-Coelho GL, Freitas RN. IGF2, LEPR, POMC, PPARG, and PPARGC1 gene variants are associated with obesity-related risk phenotypes in Brazilian children and adolescents. *Braz J Med Biol Res* 2015; **48**: 595-602 [PMID: 25923461 DOI: 10.1590/1414-431X20154155]

32 **Bains V**, Kaur H, Badaruddoza B. Association analysis of polymorphisms in LEP (rs7799039 and rs2167270) and LEPR (rs1137101) gene towards the development of type 2 diabetes in North Indian Punjabi population. *Gene* 2020; **754**: 144846 [PMID: 32512158 DOI: 10.1016/j.gene.2020.144846]

33 **Yu P**, Ning Y, Gao Y, Zhao Y, Tie L, Wu L, Zhang L, Zhang R, Cui M, Pang H, Wu Q, Wang Z, Chen L, Zhao L. Hypertension among Mongolian adults in China: A cross-sectional study of prevalence, awareness, treatment, control, and related factors: Hypertension among Mongolian adults in China. *J Clin Hypertens (Greenwich)* 2021; **23**: 1786-1801 [PMID: 34399030 DOI: 10.1111/jch.14348]

34 **Sun Z**, Zheng L, Xu C, Li J, Zhang X, Liu S, Hu D, Sun Y. Prevalence of prehypertension, hypertension and, associated risk factors in Mongolian and Han Chinese populations in Northeast China. *Int J Cardiol* 2008; **128**: 250-254 [PMID: 18160149 DOI: 10.1016/j.ijcard.2007.08.127]

35 **Kolb H**, Martin S. Environmental/lifestyle factors in the pathogenesis and prevention of type 2 diabetes. *BMC Med* 2017; **15**: 131 [PMID: 28720102 DOI: 10.1186/s12916-017-0901-x]

36 **Zhou B**, Perel P, Mensah GA, Ezzati M. Global epidemiology, health burden and effective interventions for elevated blood pressure and hypertension. *Nat Rev Cardiol* 2021; **18**: 785-802 [PMID: 34050340 DOI: 10.1038/s41569-021-00559-8]

World Journal of Diabetes

Submit a Manuscript: https://www.f6publishing.com

World J Diabetes 2022 August 15; 13(8): 654-664

DOI: 10.4239/wjd.v13.i8.654

ISSN 1948-9358 (online)

SYSTEMATIC REVIEWS

Metformin toxicity: A meta-summary of case reports

Deven Juneja, Prashant Nasa, Ravi Jain

Specialty type: Endocrinology and metabolism

Provenance and peer review: Invited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification

Grade A (Excellent): 0
Grade B (Very good): B, B
Grade C (Good): C, C, C
Grade D (Fair): 0
Grade E (Poor): 0

P-Reviewer: Gheshlaghi F, Iran; Neagu TP, Romania; Shen F, China; Swai J, Canada

Received: March 22, 2022
Peer-review started: March 22, 2022
First decision: May 11, 2022
Revised: May 17, 2022
Accepted: July 25, 2022
Article in press: July 25, 2022
Published online: August 15, 2022

Deven Juneja, Institute of Critical Care Medicine, Max Super Speciality Hospital, Saket, New Delhi 110017, India

Prashant Nasa, Department of Critical Care Medicine, NMC Specialty Hospital, Dubai 7832, United Arab Emirates

Ravi Jain, Department of Critical Care Medicine, Mahatma Gandhi Medical College and Hospital, Jaipur 302022, India

Corresponding author: Deven Juneja, DNB, FCCP, MBBS, Director, Institute of Critical Care Medicine, Max Super Speciality Hospital, Saket, 1 Press Enclave Road, New Delhi 110017, India. devenjuneja@gmail.com

Abstract

BACKGROUND

Metformin is arguably the most commonly prescribed oral hypoglycemic agent for the management of diabetes. Due to the lack of randomized control trials, most of the data pertaining to the clinical course, therapeutic interventions and outcomes of patients with metformin induced toxicity has come from case reports or series.

AIM

To analyse the symptomology, clinical interventions and outcomes of patients presenting with severe metformin toxicity by reviewing the published case reports and series.

METHODS

We performed a systematic search from PubMed, Science Direct, *Reference Citation Analysis* (https://www.referencecitationanalysis.com/) and Google Scholar databases using the terms "metformin" AND "toxicity" OR "overdose" OR "lactic acidosis" OR "hyperlactatemia". The inclusion criteria were: (1) Case reports or case series with individual patient details; and (2) Reported toxicity or overdose of metformin in adults, published in the English language. Data regarding baseline demographics, clinical presentation, therapeutic interventions, intensive care unit course and overall outcome were collected.

RESULTS

Two hundred forty-two individual cases were analysed, from 158 case reports and 26 case series, with a cumulative mortality of 19.8%. 214 (88.4%) patients were diabetics on metformin. 57 (23.6%) had acute ingestion, but a great majority (76.4%) were on metformin in therapeutic doses when they developed toxicity.

Metformin associated lactic acidosis (MALA) was the most commonly reported adverse effect present in 224 (92.6%) patients. Most of the patients presented with gastrointestinal and neurological symptoms and a significant number of patients had severe metabolic acidosis and hyperlactatemia. The organ support used was renal replacement therapy (RRT) (68.6%), vasopressors (58.7%) and invasive mechanical ventilation (52.9%). A majority of patients (68.6%) received RRT for toxin removal, renal dysfunction and correction of MALA. Patients with lowest pH and highest serum lactate and metformin levels also had favourable outcomes with use of RRT.

CONCLUSION

Most of the reported cases were on therapeutic doses of metformin but developed toxicity after an acute deterioration in renal functions. These patients may develop severe lactic acidosis, leading to significant morbidity and need for organ support. Despite severe MALA and the need for multiple organ support, they may have good outcomes, especially when RRT is used. The dose of metformin, serum pH, lactate and metformin levels may indicate the severity of toxicity and the need for aggressive therapeutic measures but may not necessarily indicate poor outcomes.

Key Words: Extracorporeal toxin removal; Haemodialysis; Metformin associated lactic acidosis; Metformin overdose; Renal replacement therapy

Core Tip: Metformin may be associated with significant toxicity, even when used in therapeutic doses, of which metformin associated lactic acidosis is the most commonly reported toxicity. These patients may have favourable outcomes in spite of consumption of high doses, severe acidosis, and high serum lactate and metformin concentrations. Early aggressive supportive care, use of renal replacement therapy for toxin removal and organ support may help in improving outcomes.

Citation: Juneja D, Nasa P, Jain R. Metformin toxicity: A meta-summary of case reports. *World J Diabetes* 2022; 13(8): 654-664
URL: https://www.wjgnet.com/1948-9358/full/v13/i8/654.htm
DOI: https://dx.doi.org/10.4239/wjd.v13.i8.654

INTRODUCTION

Metformin is arguably the most commonly prescribed oral hypoglycemic agent (OHA) for the management of diabetes mellitus (DM). In addition to its hypoglycemic properties, it has the potential to reduce micro and macro vascular complications associated with DM and have a clinically beneficial role in reducing serum lipids levels and body weight[1,2]. Use of metformin in the management of type II DM has been shown to reduce all-cause mortality and risk of cardiovascular complications[3].

The primary mode of action of metformin is to reduce hepatic glucose production. In addition, it also exerts hypoglycemic effect through the neuroendocrine axis, enhancing cellular uptake of glucose and reducing insulin resistance[4]. It is considered a very safe drug and is generally not associated with hypoglycemia. Rarely patients may develop toxicity related to its use. Metformin associated lactic acidosis (MALA) has been defined as serum lactate levels above 5 mmol/L and arterial pH below 7.35 in association with metformin exposure[5]. It is a rare complication with a reported incidence of 1-30 cases *per* 100000 patient years but is associated with a high mortality rate of 25%-50%[6,7].

Despite severe acidosis, patients with MALA may have good clinical outcomes if it is recognised early and aggressive resuscitative measures are initiated[8]. In addition, certain therapeutic interventions like extracorporeal toxin removal (ECTR), if instituted timely, may improve survival in select patient subgroups and hence, it is currently recommended in patients with severe metformin toxicity[9]. Even though MALA remains the most dreaded complication associated with metformin use, other complications have also been reported that may require hospitalisation. Due to the lack of randomised control trials, most of the data pertaining to the clinical course, therapeutic interventions and outcomes of these patients have come from case reports and case series. Hence, we conducted this scoping review of case reports and series to analyse the symptomology, clinical interventions and outcomes of patients presenting with severe metformin toxicity requiring hospitalisation and acute intervention.

MATERIALS AND METHODS

We performed a systematic search for this review from PubMed, Science Direct, *Reference Citation Analysis* (https://www.referencecitationanalysis.com/) and Google Scholar databases from January 1, 1975 till December 31, 2021. The search terms used were "metformin" AND "toxicity" OR "overdose" OR "lactic acidosis" OR "hyperlactatemia". The inclusion criteria were: (1) Case reports or case series with individual patient details; and (2) Reported toxicity or overdose of metformin. Further, it was filtered for the literature published in the English language and on adult (> 18 years) humans. We excluded: (1) Conference abstracts; and (2) Case reports or series which did not have individual biochemical data. The authors screened all the search results to include only the relevant literature for metformin toxicity. Duplicate articles from different search databases were excluded.

All the case reports and case series were evaluated, and the data were extracted for patient demographics, clinical symptomatology, clinical interventions including extracorporeal therapies (ECT), intensive care unit (ICU) course, need for organ support and outcomes. Concomitant use of nephrotoxic drugs including nonsteroidal anti-inflammatory drugs (NSAIDs), angiotensin-converting-enzyme inhibitors (ACE-Is), angiotensin II receptor blockers (ARBs), aminoglycoside antibiotics and diuretics was also made. A datasheet for evaluation was further prepared.

Statistical analysis

The prepared datasheet was evaluated by Excel, Microsoft office 2019. Categorical variables were presented as frequency and percentage. Median (interquartile range) or mean ± SD was used for continuous variables. Qualitative correlation statistics were analysed by Chi-square test and Fisher's exact test. A P value of < 0.05 was deemed significant. Unless otherwise indicated, all the statistical analyses were done using SPSS (version 25.0, IBM SPSS Inc., Chicago, IL, United States). Tabulation and final documentation were done using MS Office software (MS office 2019, Microsoft Corp, WA, United States).

RESULTS

This review was performed using PRISMA 2009 checklist (Figure 1). Finally, 184 studies with 242 unique patients that met all the inclusion criteria were included (Supplementary material). Most of the included patients were from the United States of America (66, 27.3%) and the United Kingdom (30, 12.4%) (Figure 2). 185 (76.4%) patients developed toxicity while on chronic therapeutic doses of metformin while 57 (23.6%) patients developed toxicity after an acute overdose of the drug.

The commonly reported symptoms were gastrointestinal (vomiting 52.5%, abdominal pain 40%) and neurological (altered mental status 36%, loss of consciousness 11.6%). Two hundred fourteen (88.4%) patients had underlying diabetes and were on metformin (Table 1).

MALA was the most commonly reported adverse effect in 224 (92.6%) patients. Other reported isolated adverse effects were encephalopathy in 6 (2.5%) patients, hepatitis or acute liver failure in 5 (2.1%) patients, hypoglycemia in 4 (1.6%) patients, and psychosis, vitamin B12 deficiency and acute pancreatitis in 1 (0.4%) patient each.

Overall, 57 (23.6%) patients had acute ingestion, out of which 52 were suicidal, three were reported as accidental and in two cases the cause was not reported or was unclear. The reported median dose consumed by these patients was 42.5 gms (interquartile range 24.8–61.5 gms). Out of these 57 cases, there were 11 deaths with a cumulative mortality rate of 19.3%. Sixteen patients with acute intoxications had a history of co-ingestion of other drugs.

The mean duration of presentation after acute intoxication was 10.9 (± 13.8) hours. Activated charcoal (6.6%) and gastric lavage (5.8%) was rarely employed to reduce the absorption of metformin in patients with acute intoxication. Intravenous soda-bicarbonate was most commonly used among the other therapies employed in 65.3% of patients.

Arterial blood gas at presentation was available in 214 (88.4%) patients and serum metformin concentration was measured only in 58 (24%) cases (Table 2).

Overall, 185 (76.4%) patients were on long-term therapeutic doses of metformin when they developed metformin toxicity. These patients were on metformin doses ranging from 250–3000 mg/day (median 1625 mg/day). The cumulative mortality was 37/185 (20%) in this group of patients. Out of these 185 patients, 38 patients had underlying chronic kidney disease (CKD) and 73 patients had documented reasons, which may have caused acute renal dysfunction precipitating metformin toxicity. These reasons included acute gastroenteritis leading to dehydration (36 patients), ACE-I inhibitors (22 patients), NSAIDs (18 patients), diuretics (16 patients), ARBs (8 patients), IV contrast (7 patients), post-operative (5 patients), acute urinary tract infection (5 patients), anti-retroviral drugs like tenofovir (4 patients), aminoglycosides (2 patients), and obstructive uropathy (1 patient). Several patients had multiple risk factors for acute kidney injury.

Renal dysfunction was the most common organ dysfunction (74%), followed by cardiac (59.5%) and pulmonary (47.1%). One hundred sixty-six (68.6%) patients underwent renal replacement therapy (RRT)

Table 1 The commonly reported symptoms (mean ± SD)

Parameter	Number of patients (*n* = 242)
Age	59.3 (16) yr
Gender, *n* (%)	Females, 126 (52.1)
	Males, 115 (47.5)
	Not mentioned, 1 (0.4)
Clinical presentation, *n* (%)	Vomiting, 127 (52.5)
	Abdominal pain, 96 (40)
	Altered mental status, 87 (36)
	Shock, 43 (17.8)
	Breathlessness, 41 (16.9)
	Loss of consciousness, 28 (11.6)
	Anuria, 22 (8.3)
	Cardiac arrest, 5 (2)
	Others, 15 (6.2)
Comorbidities, *n* (%)	Diabetes, 214 (88.4)
	Hypertension, 94 (38.8)
	Coronary artery disease, 34 (14.1)
	Chronic kidney disease, 41 (16.9)
	Chronic liver disease, 6 (2.5)
	Others, 24 (9.9)
	None, 22 (9.9)
	Not mentioned, 2 (0.8)
History of psychiatric illness, *n* (%)	30 (12.4)
History of metformin use, *n* (%)	214 (88.4)
Type of ingestion, *n* (%)	Chronic use, 185 (76.4)
	Suicidal, 52 (21.5)
	Accidental, 3 (1.2)
	Unclear, 1 (0.4)
	Not mentioned, 1 (0.4)
Urine toxicology screen, *n* (%)	15 (6.2)
Time to presentation after acute intoxication (h)	10.9 ± 13.8
Hypoglycemia, *n* (%)	59 (24.4)
Therapies to reduce absorption, *n* (%)	Activated charcoal, 16 (6.6)
	Gastric lavage, 14 (5.8)
	Whole bowel irrigation, 1 (0.4)
Need for organ support, *n* (%)	RRT, 166 (68.6)
	Vasopressors, 142 (58.7)
	Invasive mechanical ventilation, 128 (52.9)
	Extracorporeal membrane oxygenation, 2 (0.8)
Type of RRT, *n* (%)	Haemodialysis, 83 (34.3)
	Continuous RRT, 60 (24.8)
	Slow low-efficiency dialysis, 13 (5.4)

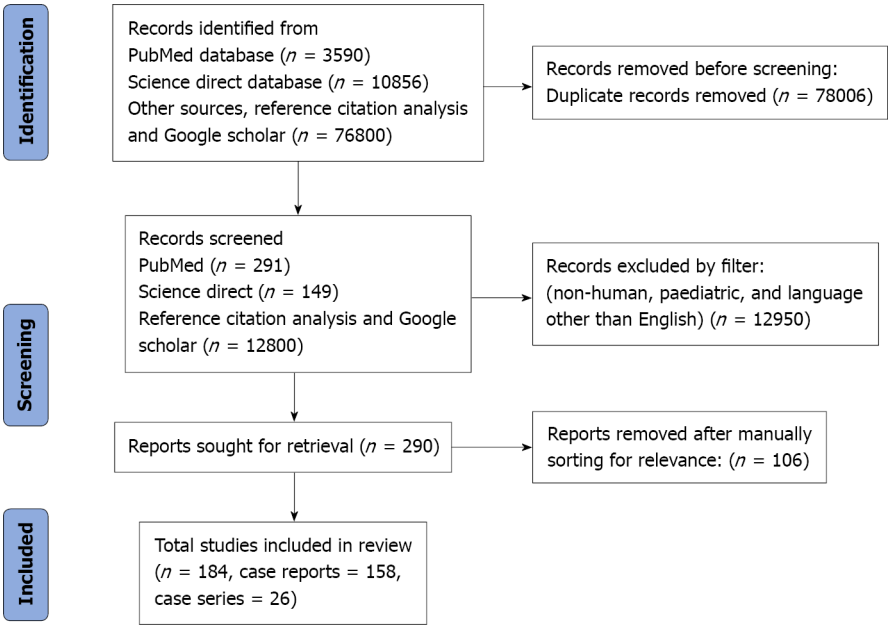
	Peritoneal dialysis, 6 (2.5)
	Haem-adsorption columns, 3 (1.2)
	Plasmapheresis, 1 (0.4)
Other treatments given, *n* (%)	Sodium bicarbonate, 158 (65.3)
	Glucose/insulin, 15 (6.2)
	Methylene blue, 2 (0.8)
	ECMO, 2 (0.8)
	L-carnitine, 1 (0.4)
	High dose vitamin C, 1 (0.4)
Development of organ failure, *n* (%)	Renal, 179 (74)
	Cardiac, 144 (59.5)
	Pulmonary, 114 (47.1)
	Neurological, 88 (36.4)
	Liver, 18 (17.4)
	Haematological, 2 (0.8)
Days on RRT	3.1 ± 6.7
Days on IMV	2.2 ± 5.1
Number of sessions of RRT	2 ± 2.6
Time of initiation of RRT after presentation (h)	6.3 ± 12.7
Days in hospital	7.3 ± 11.4
Days in ICU	4.1 ± 6.6
Outcome, *n* (%)	Alive, 192 (79.3)
	Death, 48 (19.8)
	Not mentioned, 2 (0.8)

RRT: Renal replacement therapy; IMV: Invasive mechanical ventilation; ICU: Intensive care unit.

Table 2 Arterial blood gas parameters

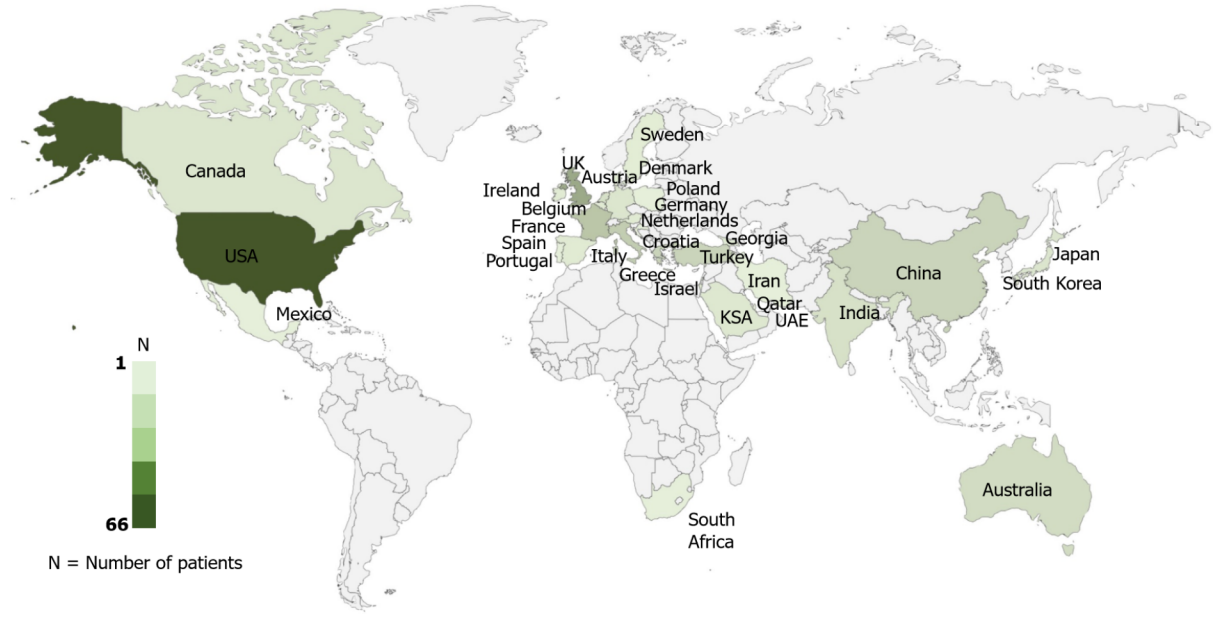
Parameter	Mean	Standard deviation	Range
pH, at presentation	7.00	0.11	6.38-7.5
Lactates, at presentation (mmol/L)	15.7	7.6	2.1-40.2
Bicarbonate, at presentation (mmol/L)	7.7	6	1–23.7
Anion Gap, at presentation	32	10.8	10-61
Lowest pH reported	6.97	0.22	6.28–7.5
Highest lactates reported (mmol/L)	18	8.6	2.4-48
Serum metformin concentration (mcg/mL)	108.7	280	0.9-2020

for underlying renal dysfunction, metabolic acidosis correction, or metformin removal. Intermittent haemodialysis (IHD, 34.3%) was the most commonly employed method of RRT followed by continuous RRT (CRRT, 24.8%), which included both continuous veno-venous hemofiltration and continuous veno-venous hemodiafiltration. Overall, 41 (16.9%) patients had underlying CKD and were already on dialysis support. Only 17 survivors, who did not have pre-existing CKD, required RRT after hospital discharge, rest all showed complete recovery of renal function.



DOI: 10.4239/wjd.v13.i8.654 **Copyright** ©The Author(s) 2022.

Figure 1 PRISMA flow diagram of the selected literature for this Meta summary. The inclusion criteria were (1) Case reports or case series with individual patient details; and (2) Reported toxicity or overdose of metformin.



DOI: 10.4239/wjd.v13.i8.654 **Copyright** ©The Author(s) 2022.

Figure 2 Geographical distribution of the patients reported with metformin toxicity.

DISCUSSION

This review evaluated data of 242 individual patients from 158 case reports and 26 case series. Most of the patients had gastrointestinal or neurological symptoms at presentation. A great majority of patients (76.4%) developed metformin toxicity on chronic therapeutic doses. The most commonly reported side effect was MALA (92.6%). These patients had severe metabolic acidosis with hyperlactatemia and required multi organ support. RRT was employed in 68.6% of patients, and the cumulative mortality rate was 19.8%.

Several other isolated serious complications, in the absence of MALA, were also reported in many case reports. These included encephalopathy[10-14], psychosis[15], vitamin B12 deficiency[16], acute pancreatitis[17] and acute liver failure[18-22]. In our analysis, many patients (24.4%) developed hypoglycemia, attributed to sulphonylureas or other OHA that the patients were co-prescribed.

However, in a few case reports no other cause could be ascertained and hypoglycemia was attributed to metformin toxicity[23-26]. The reported incidence for development of moderate to severe hypoglycemia is 60 *per* 100000 for patients on metformin therapy, with an odds ratio of 1.42[27].

As metformin is primarily excreted by the kidneys, it is generally recommended not to use metformin in patients with underlying renal dysfunction[28]. Nonetheless, in our review we observed that 16.9% patients who developed metformin related side effects had underlying CKD. However, emerging literature supports the use of metformin in patients with mild to moderate renal dysfunction, when used in reduced dosage and regular monitoring[29].

Even though renal dysfunction is a major contributing factor for metformin toxicity, other factors like hypotension, dehydration, sepsis, ischemia, and liver impairment, which lead to increased production or impaired clearance of lactates, may also contribute to lactic acidosis. In our analysis, most of the patients on therapeutic doses of metformin who developed toxicity had some insult causing acute renal dysfunction which could have precipitated metformin toxicity. This acute insult included concomitant use of nephrotoxic drugs, acute infection, post-operative state or dehydration secondary to severe diarrhoea. Hence, it may be suggested that patients on long-term metformin should be closely monitored for the development of any side-effects in case of any acute renal insult, and concomitant use of nephrotoxic drugs should be avoided in these patients.

Patients with MALA had severe metabolic acidosis and hyperlactatemia, with the reported nadir of pH being 6.28[30]. The mainstay of therapy for MALA remains early aggressive resuscitation and organ support, as there is no specific antidote. In our analysis, intravenous soda-bicarbonate was used in a large proportion of patients (65.3%). Even though it may help in the correction of acidosis, it may lead to electrolyte imbalance and fluid overload. In addition, it does not help in the correction of the underlying cause. However, it may be reasonable to use intravenous bicarbonate in patients with severe acidemia and patients with an arterial pH less than 7.20 in the presence of underlying cardiovascular disease or hemodynamic compromise[31].

Low serum pH levels, and high lactate and metformin concentration have been associated with severe toxicity and higher mortality[32]. However, in our review, the patients with the lowest pH and highest serum lactate and metformin levels survived. All three patients with acute ingestion of massive doses of metformin (more than 100 g) survived after the institution of ECTR with complete recovery of renal functions[33-35]. In addition, patients with the highest serum metformin levels (2020 and 678 mcg/mL)[36,37] and patients with the highest presenting lactate levels (40.2 and 35.3 mmol/L)[38,39] also had favourable outcomes. The lowest pH reported was 6.28, in a post-operative diabetic CKD patient on prolonged metformin therapy, who survived after aggressive intensive care[30]. Similar findings have also been reported by other authors who found that blood pH, lactate and metformin levels were poor predictors of mortality in patients with MALA[40].

Even though metformin has a relatively large volume of distribution (1-5 L/kg), but its small molecular size (165 Da) and lack of protein binding makes it amenable for removal through ECT and use of ECTR is recommended in the management of patients with severe toxicity[9,41]. The currently recommended indications of ECTR include lactate levels above 20 mmol/L, pH less than 7.0, presence of shock, reduced consciousness or in patients with failure of standard supportive measures. The current recommendations suggest discontinuing ECTR when serum lactate levels fall below 3 mmol/L and the pH becomes more than 7.35[9].

Intermittent HD has been recommended as the RRT modality of choice for ECTR in patients with metformin toxicity as it provides rapid and superior correction of acidemia and removal of metformin and lactates[9]. Lactate clearance may also be enhanced with use of higher effluent rates and high-flux/high-efficiency dialyzers[33,42]. In addition, IHD has wider availability, lesser costs and a better safety profile. Hence, it was the most commonly used mode of ECTR as observed in our review.

CRRT may be used as the second line therapy in patients with haemodynamic instability who cannot tolerate IHD[9]. As many patients in our analysis had haemodynamic instability requiring vasopressor support, CRRT was the second most common mode of RRT, employed in 24.8% patients.

Slow low efficiency dialysis (SLED) is increasingly becoming a popular RRT option, especially in ICU patients as it can achieve rapid and efficient solute clearance while offering good haemodynamic tolerability. This fact was evidenced in our review, where SLED was used in a few patients (5.4%) for initial RRT. There are a few reports of effective use of resin or charcoal based haem-adsorbent filters in managing patients with severe metformin toxicity[43,44]. However, lack of widespread availability, higher cost, limited data regarding their efficacy and risk of complications especially haemolysis, precludes haemoperfusion (HP) using haemadsorption filters from becoming the modality of choice for ECTR. Additionally, as metformin is not protein-bound, HP and plasmapheresis do not offer any advantage over IHD. Peritoneal dialysis is rarely used for ECTR because of inefficient and slow correction of hyperlactatemia and acidosis[9].

A similar meta-summary included 253 cases and reported a cumulative mortality of 17.2% in patients with MALA[45]. The authors reported that non-survivors had significantly higher levels of lactates and metformin. Additionally, lactate levels above 20 mmol/L were significantly associated with mortality. Even though the cumulative mortality rate in our review was 19.8%, which is close to that reported by Yeh *et al*[45], our review has significant differences. The previous meta-summary had included patients only up to September 2014, so must have missed the recent changes in clinical practices which might

have happened after EXtracorporeal TReatments In Poisoning guidelines, released in 2015, recommending ECTR for metformin toxicity[9]. Yeh *et al*[45], also included conference abstracts from the EMBASE database and included publications in all languages, explaining their relatively higher case numbers[45]. On the other hand, we included only English language papers and excluded conference abstracts. In addition, we also included all patients with metformin toxicity and even those in whom ECTR was not employed.

Strength and limitations

This review compiled 184 global studies involving 242 unique patients who had developed metformin toxicity. In addition, we included only studies with individual patient's details to compare demographics, therapeutic interventions and outcomes.

The included studies were only case reports and case series without a control arm. Hence, the efficacy and cost-benefit analysis of ECTR compared to pharmacological therapy could not be performed. The studies were heterogeneous, with a high risk of bias and missing data, which may impact the generalisability of the results. As we excluded case reports or series which did not have individual biochemical data, we might have missed some relevant case reports or series.

CONCLUSION

Metformin is associated with significant toxicity, of which MALA is most commonly reported. Most of the reported cases were on therapeutic doses of metformin but developed toxicity after an acute deterioration in renal function. These patients may develop severe lactic acidosis, leading to significant morbidity and need for organ support. However, in spite of severe lactic acidosis and need for multiple organ support they may have good outcomes, especially when RRT is used for toxin removal. The dose of metformin, serum pH, lactate and metformin levels may indicate the severity of toxicity and the need for aggressive therapeutic measures but may not necessarily indicate poor outcomes.

ARTICLE HIGHLIGHTS

Research background

Metformin is arguably the most commonly prescribed oral hypoglycemic agent for the management of diabetes. Due to the lack of randomized control trials, most of the data pertaining to the clinical course, therapeutic interventions and outcomes of patients with metformin induced toxicity has come from case reports or series.

Research motivation

Despite severe acidosis, patients with metformin associated lactic acidosis (MALA) may have good clinical outcomes, if it is recognized early and aggressive resuscitation measures are initiated.

Research objectives

This study aimed to analyse the symptomology, clinical interventions and outcomes of patients presenting with severe metformin toxicity by reviewing the published case reports and series.

Research methods

We performed a systematic search from PubMed, Science Direct, *Reference Citation Analysis* (https://www.referencecitationanalysis.com/) and Google Scholar databases using the terms "metformin" AND "toxicity" OR "overdose" OR "lactic acidosis" OR "hyperlactatemia". The inclusion criteria were case reports or case series with individual patient details; and reported toxicity or overdose of metformin in adults, published in the English language. Data regarding baseline demographics, clinical presentation, therapeutic interventions, intensive care unit course and overall outcome were collected.

Research results

Two hundred forty-two individual cases were analyzed, from 158 case reports and 26 case series, with a cumulative mortality of 19.8%. 214 (88.4%) patients were diabetics on metformin. 57 (23.6%) had acute ingestion, but 76.4% were on metformin in therapeutic doses when they developed toxicity. MALA was the most commonly reported adverse effect present in 224 (92.6%) patients. Patients with lowest pH and highest serum lactate and metformin levels also had favorable outcomes with use of renal replacement therapy.

Research conclusions

Most of the reported cases were on therapeutic doses of metformin but developed toxicity after an acute

deterioration in renal function. These patients may develop severe lactic acidosis, leading to significant morbidity and need for organ support. Despite severe MALA and the need for multiple organ support, they may have good outcomes, especially when renal replacement therapy is used. The dose of metformin, serum pH, lactate and metformin levels may indicate the severity of toxicity and the need for aggressive therapeutic measures but may not necessarily indicate poor outcomes.

Research perspectives

Larger trials may be required to identify the risk factors associated with poor outcomes in patients with MALA.

FOOTNOTES

Author contributions: Juneja D contributed to acquisition of data, analysis and interpretation of data, drafting the article, final approval; Nasa P contributed to acquisition of data, analysis and interpretation of data, drafting the article, final approval; Jain R contributed to interpretation of data, revising the article, final approval.

Conflict-of-interest statement: All the authors report no relevant conflicts of interest for this article.

PRISMA 2009 Checklist statement: The authors have read the PRISMA 2009 Checklist, and the manuscript was prepared and revised according to the PRISMA 2009 Checklist.

Country/Territory of origin: India

ORCID number: Deven Juneja 0000-0002-8841-5678; Prashant Nasa 0000-0003-1948-4060; Ravi Jain 0000-0001-9260-479X.

S-Editor: Fan JR
L-Editor: A
P-Editor: Fan JR

REFERENCES

1 **Graham GG**, Punt J, Arora M, Day RO, Doogue MP, Duong JK, Furlong TJ, Greenfield JR, Greenup LC, Kirkpatrick CM, Ray JE, Timmins P, Williams KM. Clinical pharmacokinetics of metformin. *Clin Pharmacokinet* 2011; **50**: 81-98 [PMID: 21241070 DOI: 10.2165/11534750-000000000-00000]
2 **Gong L**, Goswami S, Giacomini KM, Altman RB, Klein TE. Metformin pathways: pharmacokinetics and pharmacodynamics. *Pharmacogenet Genomics* 2012; **22**: 820-827 [PMID: 22722338 DOI: 10.1097/FPC.0b013e3283559b22]
3 Effect of intensive blood-glucose control with metformin on complications in overweight patients with type 2 diabetes (UKPDS 34). UK Prospective Diabetes Study (UKPDS) Group. *Lancet* 1998; **352**: 854-865 [PMID: 9742977]
4 **Cho YM**, Kieffer TJ. New aspects of an old drug: metformin as a glucagon-like peptide 1 (GLP-1) enhancer and sensitiser. *Diabetologia* 2011; **54**: 219-222 [PMID: 21116606 DOI: 10.1007/s00125-010-1986-3]
5 **Luft D**, Deichsel G, Schmülling RM, Stein W, Eggstein M. Definition of clinically relevant lactic acidosis in patients with internal diseases. *Am J Clin Pathol* 1983; **80**: 484-489 [PMID: 6624712 DOI: 10.1093/ajcp/80.4.484]
6 **Finkle SN**. Should dialysis be offered in all cases of metformin-associated lactic acidosis? *Crit Care* 2009; **13**: 110 [PMID: 19216723 DOI: 10.1186/cc7161]
7 **Kajbaf F**, Lalau JD. Mortality rate in so-called "metformin-associated lactic acidosis": a review of the data since the 1960s. *Pharmacoepidemiol Drug Saf* 2014; **23**: 1123-1127 [PMID: 25079826 DOI: 10.1002/pds.3689]
8 **Friesecke S**, Abel P, Roser M, Felix SB, Runge S. Outcome of severe lactic acidosis associated with metformin accumulation. *Crit Care* 2010; **14**: R226 [PMID: 21171991 DOI: 10.1186/cc9376]
9 **Calello DP**, Liu KD, Wiegand TJ, Roberts DM, Lavergne V, Gosselin S, Hoffman RS, Nolin TD, Ghannoum M; Extracorporeal Treatments in Poisoning Workgroup. Extracorporeal Treatment for Metformin Poisoning: Systematic Review and Recommendations From the Extracorporeal Treatments in Poisoning Workgroup. *Crit Care Med* 2015; **43**: 1716-1730 [PMID: 25860205 DOI: 10.1097/CCM.0000000000001002]
10 **Jung EY**, Cho HS, Seo JW, Kim DW, Kim HJ, Chang SH, Park DJ. Metformin-induced encephalopathy without lactic acidosis in a patient with contraindication for metformin. *Hemodial Int* 2009; **13**: 172-175 [PMID: 19432690 DOI: 10.1111/j.1542-4758.2009.00358.x]
11 **Kang YJ**, Bae EJ, Seo JW, Jeon DH, Cho HS, Kim HJ, Chang SH, Park DJ. Two additional cases of metformin-associated encephalopathy in patients with end-stage renal disease undergoing hemodialysis. *Hemodial Int* 2013; **17**: 111-115 [PMID:

22515914 DOI: 10.1111/j.1542-4758.2012.00698.x]
12 **Hanazono A**, Takahashi Y, Sanpei Y, Kamada S, Sugawara M. Focal brain lactate accumulation in metformin-induced encephalopathy without systemic lactic acidosis: A case report suggesting mitochondrial vulnerability in lentiform fork sign. *eNeurologicalSci* 2021; **25**: 100383 [PMID: 34917782 DOI: 10.1016/j.ensci.2021.100383]
13 **Simon SP**, Thomas J. Metformin-associated Encephalopathy in Hemodialysis. *Indian J Nephrol* 2019; **29**: 194-196 [PMID: 31142967 DOI: 10.4103/ijn.IJN_257_17]
14 **Vander T**, Hallevy H, Ifergane G, Herishanu YO. Metformin-induced encephalopathy without lactic acidosis. *Diabet Med* 2004; **21**: 194-195 [PMID: 14984458 DOI: 10.1046/j.1464-5491.2003.01087.x]
15 **Cheng HY**, Ko KT, Tzang RF. Metformin-induced first-episode psychosis in patient with polycystic ovary syndrome using drospirenone. *Psychiatry Clin Neurosci* 2019; **73**: 196 [PMID: 30719814 DOI: 10.1111/pcn.12825]
16 **Tung ML**, Tan LK. Long term use of metformin leading to vitamin B 12 deficiency. *Diabetes Res Clin Pract* 2014; **104**: e75-e76 [PMID: 24674102 DOI: 10.1016/j.diabres.2013.12.054]
17 **Mallick S**. Metformin induced acute pancreatitis precipitated by renal failure. *Postgrad Med J* 2004; **80**: 239-240 [PMID: 15082849 DOI: 10.1136/pgmj.2003.011957]
18 **Hashmi T**. Probable hepatotoxicity associated with the use of metformin in type 2 diabetes. *BMJ Case Rep* 2011; **2011** [PMID: 22679227 DOI: 10.1136/bcr.04.2011.4092]
19 **Nammour FE**, Fayad NF, Peikin SR. Metformin-induced cholestatic hepatitis. *Endocr Pract* 2003; **9**: 307-309 [PMID: 14561576 DOI: 10.4158/EP.9.4.307]
20 **Zheng L**. Metformin as a Rare Cause of Drug-Induced Liver Injury, a Case Report and Literature Review. *Am J Ther* 2016; **23**: e315-e317 [PMID: 24263160 DOI: 10.1097/MJT.0000000000000007]
21 **Cone CJ**, Bachyrycz AM, Murata GH. Hepatotoxicity associated with metformin therapy in treatment of type 2 diabetes mellitus with nonalcoholic fatty liver disease. *Ann Pharmacother* 2010; **44**: 1655-1659 [PMID: 20647417 DOI: 10.1345/aph.1P099]
22 **Babich MM**, Pike I, Shiffman ML. Metformin-induced acute hepatitis. *Am J Med* 1998; **104**: 490-492 [PMID: 9626034 DOI: 10.1016/s0002-9343(98)00088-6]
23 **Aldobeaban S**, Mzahim B, Alshehri AA. Recurrent hypoglycemia secondary to metformin toxicity in the absence of co-ingestions: a case report. *J Med Case Rep* 2018; **12**: 223 [PMID: 30119705 DOI: 10.1186/s13256-018-1758-0]
24 **Al-Abri SA**, Hayashi S, Thoren KL, Olson KR. Metformin overdose-induced hypoglycemia in the absence of other antidiabetic drugs. *Clin Toxicol (Phila)* 2013; **51**: 444-447 [PMID: 23544622 DOI: 10.3109/15563650.2013.784774]
25 **Joseph CMC**. Symptomatic Hypoglycemia During Treatment with a Therapeutic Dose of Metformin. *Am J Case Rep* 2021; **22**: e931311 [PMID: 34075013 DOI: 10.12659/AJCR.931311]
26 **Sharma MP**, Kar SK. Surreptitious metformin abuse in anorexia nervosa presenting as periodic hypoglycaemia. *Aust N Z J Psychiatry* 2015; **49**: 851-852 [PMID: 25916797 DOI: 10.1177/0004867415584642]
27 **Zitzmann S**, Reimann IR, Schmechel H. Severe hypoglycemia in an elderly patient treated with metformin. *Int J Clin Pharmacol Ther* 2002; **40**: 108-110 [PMID: 11911598 DOI: 10.5414/cpp40108]
28 **Glucophage**. Metformin hydrochloride [final printed labelling]. [cited 17 March 2022]. Available from: http://packageinserts.bms.com/pi/pi_glucophage.pdf
29 **Inzucchi SE**, Lipska KJ, Mayo H, Bailey CJ, McGuire DK. Metformin in patients with type 2 diabetes and kidney disease: a systematic review. *JAMA* 2014; **312**: 2668-2675 [PMID: 25536258 DOI: 10.1001/jama.2014.15298]
30 **Gonçalves BM**, Coelho D. Metformin-associated lactic acidosis: A case reporting a serious complication in the perioperative period. *Rev Esp Anestesiol Reanim (Engl Ed)* 2019; **66**: 483-486 [PMID: 31601432 DOI: 10.1016/j.redar.2019.02.009]
31 **Sabatini S**, Kurtzman NA. Bicarbonate therapy in severe metabolic acidosis. *J Am Soc Nephrol* 2009; **20**: 692-695 [PMID: 18322160 DOI: 10.1681/ASN.2007121329]
32 **Dell'Aglio DM**, Perino LJ, Kazzi Z, Abramson J, Schwartz MD, Morgan BW. Acute metformin overdose: examining serum pH, lactate level, and metformin concentrations in survivors versus nonsurvivors: a systematic review of the literature. *Ann Emerg Med* 2009; **54**: 818-823 [PMID: 19556031 DOI: 10.1016/j.annemergmed.2009.04.023]
33 **Akoglu H**, Akan B, Piskinpasa S, Karaca O, Dede F, Erdem D, Albayrak MD, Odabas AR. Metformin-associated lactic acidosis treated with prolonged hemodialysis. *Am J Emerg Med* 2011; **29**: 575.e3-575.e5 [PMID: 20708887 DOI: 10.1016/j.ajem.2010.06.005]
34 **Dell'Aglio DM**, Perino LJ, Todino JD, Algren DA, Morgan BW. Metformin overdose with a resultant serum pH of 6.59: survival without sequalae. *J Emerg Med* 2010; **39**: e77-e80 [PMID: 18343080 DOI: 10.1016/j.jemermed.2007.09.034]
35 **Chiew AL**, Wright DFB, Dobos NM, McArdle K, Mostafa AA, Newth A, Roberts MS, Isbister GK. 'Massive' metformin overdose. *Br J Clin Pharmacol* 2018; **84**: 2923-2927 [PMID: 29534338 DOI: 10.1111/bcp.13582]
36 **Almaleki N**, Ashraf M, Hussein MM, Mohiuddin SA. Metformin-associated lactic acidosis in a peritoneal dialysis patient. *Saudi J Kidney Dis Transpl* 2015; **26**: 325-328 [PMID: 25758883 DOI: 10.4103/1319-2442.152498]
37 **Ives Tallman C**, Zhang Y, Black N, Lynch K, Fayed M, Armenian P. Refractory vasodilatory shock secondary to metformin overdose supported with VA ECMO. *Toxicol Rep* 2022; **9**: 64-67 [PMID: 35004183 DOI: 10.1016/j.toxrep.2021.12.010]
38 **Avcı D**, Çetinkaya A, Karahan S, Oğuzhan N, Karagöz H, Başak M, Erden A. Suicide commitment with metformin: our experience with five cases. *Ren Fail* 2013; **35**: 863-865 [PMID: 23742066 DOI: 10.3109/0886022X.2013.801299]
39 **Friesecke S**, Abel P, Kraft M, Gerner A, Runge S. Combined renal replacement therapy for severe metformin-induced lactic acidosis. *Nephrol Dial Transplant* 2006; **21**: 2038-2039 [PMID: 16449278 DOI: 10.1093/ndt/gfl011]
40 **Kajbaf F**, Lalau JD. The prognostic value of blood pH and lactate and metformin concentrations in severe metformin-associated lactic acidosis. *BMC Pharmacol Toxicol* 2013; **14**: 22 [PMID: 23587368 DOI: 10.1186/2050-6511-14-22]
41 **Juneja D**, Singh O. Extracorporeal Therapies: Specific Poisons. In: Singh O, Juneja D. Principles and Practice of Critical Care Toxicology. New Delhi: Jaypee Brothers Medical Publishers, 2019: 274-287
42 **Liu Y**, Ouyang B, Chen J, Chen M, Ma J, Wu J, Huang S, Li L, Liu Z, Guan X. Effects of different doses in continuous

veno-venous hemofiltration on plasma lactate in critically ill patients. *Chin Med J (Engl)* 2014; **127**: 1827-1832 [PMID: 24824239]

43 **Liu S**, Xu L, Ma J, Huang R, Lin T, Li Z, Liang H, Li S, Li R, Zhang L, Tao Y, Chen Y, Ye Z, Zhang B, Wang W, Xiao H, Liang X, Shi W. High-volume continuous venovenous hemodiafiltration plus resin hemoperfusion improves severe metformin-associated toxicity. *J Diabetes Investig* 2018; **9**: 975-978 [PMID: 28980449 DOI: 10.1111/jdi.12757]

44 **Guo PY**, Storsley LJ, Finkle SN. Severe lactic acidosis treated with prolonged hemodialysis: recovery after massive overdoses of metformin. *Semin Dial* 2006; **19**: 80-83 [PMID: 16423187 DOI: 10.1111/j.1525-139X.2006.00123.x]

45 **Yeh HC**, Ting IW, Tsai CW, Wu JY, Kuo CC. Serum lactate level and mortality in metformin-associated lactic acidosis requiring renal replacement therapy: a systematic review of case reports and case series. *BMC Nephrol* 2017; **18**: 229 [PMID: 28693440 DOI: 10.1186/s12882-017-0640-4]

World Journal of Diabetes

Submit a Manuscript: https://www.f6publishing.com

World J Diabetes 2022 August 15; 13(8): 665-667

DOI: 10.4239/wjd.v13.i8.665

ISSN 1948-9358 (online)

LETTER TO THE EDITOR

Loss of skeletal muscle mass is not specific to type 2 diabetes

Bo Zhou, Ying-Qi Jin, Lian-Ping He

Specialty type: Endocrinology and metabolism

Provenance and peer review: Unsolicited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification

Grade A (Excellent): 0
Grade B (Very good): B
Grade C (Good): C
Grade D (Fair): 0
Grade E (Poor): 0

P-Reviewer: Kobyliak N, Ukraine; Ulasoglu C, Turkey

Received: March 22, 2022
Peer-review started: March 22, 2022
First decision: May 30, 2022
Revised: May 30, 2022
Accepted: July 6, 2022
Article in press: July 6, 2022
Published online: August 15, 2022

Bo Zhou, Ying-Qi Jin, Lian-Ping He, School of Medicine, Taizhou University, Taizhou 318000, Zhejiang Province, China

Corresponding author: Lian-Ping He, PhD, Academic Fellow, Director, Teacher, School of Medicine, Taizhou University, No. 1139 Shifu Avenue, Jiaojiang District, Taizhou 318000, Zhejiang Province, China. lianpinghe@tzc.edu.cn

Abstract

Skeletal muscle is a massive insulin-sensitive tissue in the body. Loss of muscle mass is associated with mitochondrial dysfunction, and is often a result of diabetes. Insulin deficiency or insulin resistance can only be seen as reduced skeletal muscle mass. Diabetes is caused by insulin deficiency or insulin resistance; however, insulin resistance is not unique to diabetics. Insulin resistance also exists in many diseases.

Key Words: Diabetics; Insulin deficiency; Insulin resistance; Skeletal muscle mass

Core Tip: Insulin resistance is present in hypertension, and in this case, loss of skeletal muscle mass occurs. At the same time, insulin resistance also results in obesity, and in this case, there is also a reduction in skeletal muscle mass. Loss of skeletal muscle mass can occur in many diseases.

Citation: Zhou B, Jin YQ, He LP. Loss of skeletal muscle mass is not specific to type 2 diabetes. *World J Diabetes* 2022; 13(8): 665-667
URL: https://www.wjgnet.com/1948-9358/full/v13/i8/665.htm
DOI: https://dx.doi.org/10.4239/wjd.v13.i8.665

TO THE EDITOR

We read with great interest the study by Chen LY *et al*[1] which discovered that there is a relationship between loss of skeletal muscle mass and the presence of diabetic mellitus in males, but not in females. The findings have positive implications for the treatment and prevention of diabetes. Nonetheless, it appears to me that there are still some issues worth rethinking.

In the study, loss of skeletal muscle mass was shown to be associated with diabetes in men; however, the loss of skeletal muscle mass is not unique to diabetes. High insulin resistance occurs in both type 2 diabetes and high blood pressure. Insulin resistance plays a major role in the development of hypertension. Previous animal studies have also found that the spontaneously hypertensive rat manifests insulin resistance[2]. At the same time, there is a loss of skeletal muscle mass in insulin-resistant diseases. Skeletal muscle is the largest insulin-sensitive tissue in the body. Decreased muscle mass is associated with mitochondrial dysfunction and increased fat infiltration. This leads to a decrease in glucose processing capacity. Therefore, loss of skeletal muscle mass is also associated with hypertension.

In addition, insulin resistance also appears in adolescent obesity. Lipid accumulation is evident in skeletal muscles in adolescents with obesity. Intermuscular fat may impair insulin action through reducing blood flow to muscles[3,4]. Obesity is associated with biological dysfunction in skeletal muscles[5]. Sarcopenic obesity is a symptom of obesity with loss of muscle mass and physical dysfunction. Obesity can cause several biological dysfunctions, including insulin resistance, mitochondrial dysfunction, and inflammation. These changes further aggravate skeletal muscle loss and physical dysfunction. There is a study that shows that in the early stages of juvenile obesity development, the microvasculature and prefrontal cortex exhibit impaired insulin signaling[6]. This study suggests that obesity has insulin resistance. At the same time, there is a loss of skeletal muscle mass in insulin-resistant diseases. This further suggests that skeletal muscle mass loss is not unique to diabetes.

In summary, decreased skeletal muscle mass occurs in both hypertension and obesity. Insulin resistance is not just a loss of skeletal muscle mass. Loss of skeletal muscle mass is also present in many diseases and is not a specific feature of diabetes. More research is needed to determine the relationship between reduced skeletal muscle mass and diabetes.

FOOTNOTES

Author contributions: Zhou B and He LP came up with ideas and constructs; Zhou B and Jin YQ wrote the manuscript; He LP approved the main conceptual ideas and made corrections; all authors provided final edits and approved the manuscript.

Conflict-of-interest statement: Every author stated that there is no commercial, professional, or personal conflict of interest relevant to the study and hereby proves that it complies with the principles of publishing ethics.

Country/Territory of origin: China

ORCID number: Bo Zhou 0000-0002-2141-4523; Ying-Qi Jin 0000-0003-4805-449X; Lian-Ping He 0000-0002-9627-5599.

S-Editor: Wang LL
L-Editor: Filipodia
P-Editor: Wang LL

REFERENCES

1 **Chen LY**, Xia MF, Wu L, Li Q, Hu Y, Ma H, Gao X, Lin HD. Skeletal muscle loss is associated with diabetes in middle-aged and older Chinese men without non-alcoholic fatty liver disease. *World J Diabetes* 2021; **12**: 2119-2129 [PMID: 35047125 DOI: 10.4239/wjd.v12.i12.2119]

2 **Coan PM**, Hummel O, Garcia Diaz A, Barrier M, Alfazema N, Norsworthy PJ, Pravenec M, Petretto E, Hübner N, Aitman TJ. Genetic, physiological and comparative genomic studies of hypertension and insulin resistance in the spontaneously hypertensive rat. *Dis Model Mech* 2017; **10**: 297-306 [PMID: 28130354 DOI: 10.1242/dmm.026716]

3 **Miljkovic I**, Kuipers AL, Cauley JA, Prasad T, Lee CG, Ensrud KE, Cawthon PM, Hoffman AR, Dam TT, Gordon CL, Zmuda JM. Greater Skeletal Muscle Fat Infiltration Is Associated With Higher All-Cause and Cardiovascular Mortality in Older Men. *J Gerontol A Biol Sci Med Sci* 2015; 70: 1133-1140 [DOI: 10.1093/gerona/glv027]

4 **Scott D**, de Courten B, Ebeling PR. Sarcopenia: a potential cause and consequence of type 2 diabetes in Australia's ageing population? *Med J Aust* 2016; **205**: 329-333 [PMID: 27681976 DOI: 10.5694/mja16.00446]

5 **Guo A**, Li K, Tian HC, Fan Z, Chen QN, Yang YF, Yu J, Wu YX, Xiao Q. FGF19 protects skeletal muscle against obesity-induced muscle atrophy, metabolic derangement and abnormal irisin levels *via* the AMPK/SIRT-1/PGC-α pathway. *J Cell Mol Med* 2021; **25**: 3585-600 [DOI: 10.1111/jcmm.16448]

6 **Olver TD**, Grunewald ZI, Jurrissen TJ, MacPherson REK, LeBlanc PJ, Schnurbusch TR, Czajkowski AM, Laughlin MH, Rector RS, Bender SB, Walters EM, Emter CA, Padilla J. Microvascular insulin resistance in skeletal muscle and brain occurs early in the development of juvenile obesity in pigs. *Am J Physiol Regul Integr Comp Physiol* 2018; **314**: R252-R264 [PMID: 29141949 DOI: 10.1152/ajpregu.00213.2017]

Published by **Baishideng Publishing Group Inc**
7041 Koll Center Parkway, Suite 160, Pleasanton, CA 94566, USA
Telephone: +1-925-3991568
E-mail: bpgoffice@wjgnet.com
Help Desk: https://www.f6publishing.com/helpdesk
https://www.wjgnet.com