## World Journal of Diabetes

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Type 2 diabetes and bone fragility in children and adults

Maria Felicia Faienza, Paola Pontrelli, Giacomina Brunetti

Abstract

Type 2 diabetes (T2D) is a global epidemic disease. The prevalence of T2D in adolescents and young adults is increasing alarmingly. The mechanisms leading to T2D in young people are similar to those in older patients. However, the severity of onset, reduced insulin sensitivity and defective insulin secretion can be different in subjects who develop the disease at a younger age. T2D is associated with different complications, including bone fragility with consequent susceptibility to fractures. The purpose of this systematic review was to describe T2D bone fragility together with all the possible involved pathways. Numerous studies have reported that patients with T2D show preserved, or even increased, bone mineral density compared with controls. This apparent paradox can be explained by the altered bone quality with increased cortical bone porosity and compromised mechanical properties. Furthermore, reduced bone turnover has been described in T2D with reduced markers of bone formation and resorption. These findings prompted different researchers to highlight the mechanisms leading to bone fragility, and numerous critical altered pathways have been identified and studied. In detail, we focused our attention on the role of microvascular disease, advanced glycation end products, the senescence pathway, the Wnt/β-catenin pathway, the osteoprotegerin/receptor-activator of nuclear factor kappa B ligand, osteonectin and fibroblast growth factor 23. The understanding of type 2 myeloid bone fragility is an important issue as it could suggest possible interventions for the prevention of poor bone quality in T2D and/or how to target these pathways when bone disease is clearly evident.

Key Words: Type 2 diabetes; Bone remodeling; Cytokines; Bone fragility; Bone mineral density; Chronic kidney disease
Core Tip: Type 2 diabetes (T2D) patients show increased susceptibility to bone fractures, despite their bone mineral density being normal or increased, leading to difficult identification for clinicians. The prevalence of T2D in adolescents and young adults is increasing alarmingly. Different researchers highlighted the mechanisms leading to bone fragility, and different critical altered pathways have been identified and studied. In this review, we described the different metabolic pathways responsible for bone fragility in patients with T2D. They can be useful for its management, although further studies are needed to deepen our understanding of the mechanisms underlying bone fragility in T2D.

INTRODUCTION

The prevalence of type 2 diabetes (T2D) mellitus in adolescents and young adults is increasing alarmingly. Data from the SEARCH study showed an annual increase of approximately 7% in the incidence of T2D among people aged 10-years-old to 19-years-old in the United States, with increases in all ethnic groups[1]. Increases in children, adolescents and young adults with T2D have been described across most regions of the world[2]. The highest T2D incidence rates in youth have been registered in Canadian First Nations, American Indian and Navajo nation, Australian Aboriginal and Torres Strait Islander and African American populations (31-94/100000 each year), while youths from non-Hispanic Caucasian populations (i.e., United States and Europe) display the lowest incidence rates (0.1-0.8/100000 each year). Studies show the highest prevalence in youth from Mexico and Brazil, indigenous populations in Canada and the United States, together with Black populations in the Americas (160-3300/100000). Conversely, the lowest prevalence was registered in European populations (0.6-2.7/100000)[2].

A recent literature review examined country-specific prevalence and incidence data of youth-onset T2D published between 2008 and 2019[3]. The highest prevalence rates of youth-onset T2D were observed in China (520 cases/100000 people) and the United States (212 cases/100000) and the lowest in Denmark (0.6 cases/100000) and Ireland (1.2 cases/100000). However, the highest incidence rates were reported in Taiwan (63 cases/100000) and the United Kingdom (33.2 cases/100000), with the lowest in Fiji (0.43 cases/100000) and Austria (0.6 cases/100000). These differences in epidemiology data may be partially explained by variations in the diagnostic criteria used within studies, screening recommendations within national guidelines and race/ethnicity within countries.

The main predisposing risk factors for the development of T2D in pediatric age are represented by obesity, family history and sedentary lifestyle[4]. The mechanisms leading to T2D in young people are similar to those in older patients. However, the severity of onset, reduced insulin sensitivity and defective insulin secretion can be different in subjects who develop the disease at a younger age[5]. In particular, the phase of nutrient-induced insulin secretion might be impaired earlier in children and adolescents than in older subjects[6].

The comorbidities associated with T2D in young people include hypertension, cardiovascular disease, kidney impairment and retinopathy. Furthermore, psychosocial problems are often observed[7]. Altered bone quality has been reported in patients with T2D, possible mechanisms for the effect of T2D on bone mineral density (BMD) include the toxic effects of hyperglycemia, which may impair differentiation and proliferation of osteoblasts[8]. In addition, hyperglycemia can increase urine calcium excretion, which inhibits bone formation[8]. Thus, the objective of this review was to describe T2D bone fragility together with all the possible involved pathways.

T2D AND BONE IMPAIRMENT

Most studies have found that patients with T2D have preserved, or even increased, BMD compared with controls but display bone fragility with consequent increased susceptibility to fractures[9-11]. This apparent paradox is due to the altered bone quality in these patients. In detail, the spine trabecular bone score is decreased in patients with T2D, and it is a predictor of fracture risk independently of the BMD [12].
Furthermore, different studies have evaluated T2D effects on bone microarchitecture of the peripheral skeleton (radius and tibia) through high-resolution peripheral quantitative computed tomography. These studies have generally shown preserved, or even improved, trabecular bone microarchitecture in patients with T2D compared with controls[12-17]. Furthermore, some[14,15,17-19], but not all[16,20,21], studies report augmented cortical porosity in patients with T2D, and interestingly this parameter independently predicts fracture risk.

Another aspect of bone quality that might be impaired in patients with T2D is the mechanical characteristics that can be assessed through the measurement of the bone material strength index. However, discordant results have been reported on this issue. In detail, some authors found reduced bone material strength index in T2D in comparison with controls[20,22,23], whereas others reported no significant variation[16]. These different results could be associated to the different comorbidities characterizing T2D.

Several studies have noted reduced bone turnover in patients with T2D[20,24,25]. It has been reported that patients with T2D have reduced markers of bone formation [serum levels of procollagen type I amino-terminal propeptide and osteocalcin (OC)] as well as resorption [carboxy-terminal telopeptide of type 1 collagen][20,24,26,27]. Moreover, Starup-Linde et al[28] demonstrated an inverse relationship between glycemic control (hemoglobin A1c) and OC levels and a similar trend for carboxy-terminal telopeptide of type I collagen and procollagen type I amino-terminal propeptide. N-terminal telopeptide of type 1 collagen and bone-specific alkaline phosphatase levels are not significantly different between patients with T2D and controls[29]. Very recently, it has been reported that thyroid homeostasis could affect bone turnover markers[30] and that follicle-stimulating hormone levels may contribute to the suppression of the same markers[31].

MECHANISMS AND MOLECULAR PATHWAYS INVOLVED IN T2D BONE FRAGILITY

An indication of mutual regulatory control of both bone and glycemic homeostasis recognizes the close interplay between these two systems. The common regulatory mechanisms involve microvascular disease, advanced glycation end products (AGEs), osteoprotegerin (OPG)/receptor-activator of nuclear factor kappa B ligand (RANKL), the Wnt/β-catenin pathway, osteonectin and fibroblast growth factor 23 (FGF23) (Table 1).

Microvascular disease

Microvascular disease is a common complication (retinopathy, nephropathy or neuropathy) of diabetes[32]. Angiopathy has been demonstrated in the iliac crest of diabetic patients[33]. Recently, decreased microvascular blood flow has been demonstrated to be linked with cortical porosity in patients with T2D, suggesting that microvascular disease negatively affects bone microarchitecture in T2D[16]. Consistently, cortical porosity of the distal radius and tibia is most pronounced in patients with T2D with microvascular disease[19]. In contrast, in 2022 it was found that the poorest femoral trabecular microarchitecture was associated with vascular complications in patients with T2D[34]. Patients with T2D with microvascular disease display a significantly lower trabecular bone score, after adjusting for confounders. Moreover, multivariable analysis demonstrated a significant correlation between low 25(OH) vitamin D levels and microvascular disease[35].

Several mechanisms have been proposed to explain how microvascular disease is associated with bone fragility in T2D. It is important to remember that skeletal blood flow provides growth factors, hormones, oxygen and nutrients affecting bone remodeling, suggesting that alteration in microvasculature leads to bone impairment. In the same manner as perivascular cells show stem-cell like properties and may differentiate in osteoblastic cells, blood vessels also release factors affecting the differentiation and activity of osteoblasts and osteoclasts[36]. Blood flow promotes angiogenesis and thus osteogenesis. Bone blood flow is reduced in T2D rats[37], and hypoxia increases the canal network in rat cortical bone[38], suggesting that insufficient oxygen and blood flow associated with microvascular disease alters bone microarchitecture. The recruitment of osteoprogenitors from blood vessels is fundamental for bone formation following osteoclast resorption[39]. Thus, microvascular disease could uncouple resorption and formation in cortical bone by impairing osteoprogenitor recruitment. However, further studies are needed to deepen our understanding of the mechanisms and in particular whether bone fragility is a comorbidity of T2D or a complication (this item is a matter of debate)[40].

AGEs

Hyperglycemia disturbs both bone cells and the extracellular matrix. The presence of glucose determines the production of intermediate products, which eventually generate the irreversible accumulation of AGE[41]. AGE accumulation leads to the synthesis of defective collagens as well as of reactive oxygen species, with consequent structural changes in the bone[42]. In detail, considering the organic bone matrix, these products lead to diminished bone strength[43,44]. Elevated AGE levels are associated with increased fracture risk[45].
Table 1 Mechanisms of bone fragility in type 2 diabetes

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AGEs: Advanced glycation end products; CKD-MBD: Chronic kidney disease-mineral and bone disorder; DKK1: Dickkopf-related protein 1; FGF23: Fibroblast growth factor 23; OPG/RANKL: Osteoprotegerin/receptor-activator of nuclear factor kappa B ligand; T2D: Type 2 diabetes.

The AGES-receptor for AGE (RAGE) binding generates reactive oxygen species production, macrophage and platelet activation, vascular inflammation and inflammatory cell migration[46]. All these events are involved in the onset and progression of typical macro- and microangiopathy associated with diabetes, thus leading to brittle bones with diminished strength and less capability to deform before fracturing[47].

RAGE is also expressed by immune cells and incites activation of the nuclear factor kappa-light-chain-enhancer of activated B cells, a central transcription factor of the immune and inflammatory response[46]. The AGE-RAGE interaction in immune cells leads to the increased expression of chemokines and adhesion molecules, secreting further RAGE ligands, supporting the inflammatory tissue response, regulating the activated macrophage reaction to enhance the destructive signals in the tissues and inhibiting the repair and remodeling responses[46]. AGES may determine osteoclastogenesis and osteoblast alterations in the bone microenvironment due to the increase in inflammatory cytokines, leading to osteoporosis[48].

In detail, pentosidine, the most studied AGE, accumulates in the trabecular and cortical bone in patients with T2D and negatively affects their bone strength as well as probably leading to functional changes in osteoblasts and the bone mineralization process[49,50]. Consequently, trabecular and cortical bones show impaired biomechanical properties and decreased strength, together with altered osteoblast activity as well as adhesion to the collagen matrix and thus negatively affect bone homeostasis[45,50-52].

AGE bone content correlates with worse bone microarchitecture, including lower volumetric BMD, bone volume/total volume and increased trabecular separation/spacing[53]. High concentrations of AGES blunt insulin-like growth factor I-mediated osteoblast stimulation and determines the resistance of osteoblasts to insulin-like growth factor I effects[54]. Consistently, insulin-like growth factor I serum levels have been found to be inversely correlated with the occurrence of vertebral fractures in T2D postmenopausal women[55].

The role of cellular senescence in mediating skeletal fragility in T2D

Different forms of stress can lead a cell to enter an irreversible permanent growth arrest known as senescence[56]. This is triggered by cyclin-dependent kinase inhibitors, remarkably p16Ink4a and p21Cip1, that antagonize the activity of cyclin-dependent kinases to stop cell proliferation[57,58].

Senescent cells display a transformed gene expression profile with an increase in senescent cell anti-apoptotic pathways as well as a senescence-associated secretory phenotype[59], typically consisting of proinflammatory cytokines, chemokines and matrix remodeling proteins[60,61]. A premature increase in senescent cells is evident in T2D, especially pancreatic β-cells and bone[62,63]. In particular, osteocyte senescence has been demonstrated using an inducible obese mouse model of T2D. These mice display bone quality alterations quite similar to bones from humans with T2D, such as reduced biomechanical strength, defective cortical bone microarchitecture and low bone formation rates[63]. Furthermore, in this model, senescent osteocytes were identified for the high levels of p16Ink4a and p21Cip1, senescence-associated distension of satellites, increased telomere-associated foci (another cell marker of senescence) as well as typical increased expression of proinflammatory senescence-associated secretory phenotype and nuclear factor kappa-light-chain-enhancer of activated B cells[63].
Additionally, cellular senescence in T2D has been linked to the incidence of fracture in murine models and patients[64,65]. In detail, using a murine model of T2D reflecting both hyperinsulinemia caused by insulin resistance induced by a high-fat diet and insulinopenia induced by low dose streptozotocin, increased density of senescent cells has been demonstrated in the callus area in fracture healing [64]. Additionally, the same authors reported that cells of the osteoblastic lineage cultured with sera from patients with T2D displayed increased expression of the p53 responsive genes that are typical of a senescent microenvironment[64]. The decreased levels of serum senescent miR-31-5p in older diabetic women is linked to incidents of fragility fracture and can significantly predict fracture risk if combined with femoral neck and BMD measurements[65].

**The Wnt/β-catenin pathway**

The Wnt/β-catenin pathway activation promotes osteoblastogenesis and bone formation but inhibits osteoclastogenesis. Dickkopf-related protein 1 and sclerostin (encoded by Sost) antagonize the Wnt/β-catenin pathway by binding to low-density lipoprotein receptor-related protein 5 or 6, thus inhibiting osteoblastogenesis and promoting osteoclastogenesis[66].

Bone expression of sclerostin and Dickkopf-related protein 1 has been demonstrated to be high in T2D rat models[67,68]. Circulating sclerostin levels have also been found to be increased in patients with T2D[69] and correlated to the decrease in bone formation markers[70]. In contrast, in T2D postmenopausal women the high circulating levels of sclerostin are related to vertebral fractures[71]. Interestingly, T2D postmenopausal women with previous fractures display thinner cortical bone, together with a tendency towards larger volumetric bone density and elevated circulating levels of sclerostin compared with diabetic women without fractures and nondiabetic controls with fractures[72]. More recently, Piccoli et al[53] reported that Sost expression in RNA extracts from the femoral head of patients with T2D is significantly increased compared with the controls, although circulating sclerostin levels were found to be higher in T2D subjects but not statistically significant.

**OPG/RANKL**

OPG is a soluble tumor necrosis factor receptor superfamily member originally discovered in bone[73,74]. It is an anti-resorptive cytokine that works by binding and neutralizing the receptor activator for RANKL. RANKL is a molecule that induces osteoclast differentiation and activity[73,74]. The OPG/RANKL axis is also linked to the regulation of glucose homeostasis[75,76]. In detail, hyperglycemia downregulates RANKL expression, which inhibits the differentiation and activity of osteoclasts[73,76].

The duration of diabetes seems to negatively affect bone metabolism, but poor glycemic control (hemoglobin A1c ≥ 7.5%) has also been shown to be associated with an increased risk of fracture[77]. Decreased levels of RANKL have been reported in diabetic patients compared to healthy subjects[78]. This seems to be due to the increased number of immature osteoblasts and osteoclasts[79]. Other authors have reported that serum RANKL levels are reduced and OPG increased in diabetic patients with respect to nondiabetics and prediabetic subjects[80,81].

Furthermore, it has also been reported that high RANKL levels are related to a significantly increased risk of T2D development[82]. However, other authors did not measure significant differences in RANKL levels between patients with T2D and controls[29]. Human osteoblast cultures from cancellous bone biopsies of diabetic patients displayed a decreased RANKL/OPG ratio compared to the controls, suggesting that the bone turnover process is suppressed[83].

**Osteonectin**

Osteonectin is produced by osteoblasts and high osteonectin serum levels represent a marker of bone formation[84]. Osteonectin induces osteoblast differentiation, commitment and survival. In vivo, osteonectin-knock out and haploinsufficient mice show osteopenia with low bone turnover, a decreased number of osteoblasts as well as a reduced bone formation rate[85,86]. Additionally, Dole et al[87] reported that a single nucleotide polymorphism in the 3’ untranslated region of osteonectin determined variability in bone mass by modulating its expression. Patients with albuminuria had significantly higher levels of osteonectin compared with normoalbuminuric patients[88].

**T2D and Bone-Kidney Cross-Talk: The Role of Bone-Derived Hormones**

Chronic kidney disease (CKD) represents a serious complication of T2D and impacts 25%-40% of the diabetic population[89], thus leading to end stage renal disease with the need for dialysis or kidney transplantation[90]. Although kidney replacement therapy improves long-term survival and quality of life in CKD patients, this survival highlights bone fragility as an emerging complication[91]. In a large cohort of patients with CKD followed between 1990 and 1999, Bal et al[92] demonstrated that the
Fracture risk was higher with a prolonged period of dialysis before transplantation, and both epidermal growth factor receptor decrease and albuminuria increase were considered important risk factors for fracture[93]. Bone fragility in CKD patients is dependent on several risk factors, and literature data demonstrate the impact of age, race (Caucasian) and sex, low body mass index < 23 kg/m², glucocorticoid duration and immunosuppressive agents[94]. However, in addition to the described factors and dialysis vintage, diabetes and pancreas replacement therapy are also important risk factors for bone fragility[95,96].

In 2009, the Clinical Practice Guidelines for the Diagnosis, Evaluation, Prevention, and Treatment of CKD-Mineral and Bone Disorder (CKD-MBD) were originally published by Kidney Disease: Improving Global Outcomes[97]. This clinical syndrome defines a systemic disorder in CKD patients responsible for abnormalities in mineral metabolism, bone remodeling and vascular calcification. Despite the completion of several key clinical trials since the 2009 publication of the CKD-MBD guidelines, large gaps in the knowledge still remain[98]. Prospective studies are needed to determine the value of BMD and bone biomarkers as predictors of fractures[99] as well as the impact of different therapeutic approaches on bone fragility, especially in patients with both diabetes and kidney disease. Recent studies have demonstrated that CKD patients with T2D are at increased risk of bone diseases[100], which could involve FGF23.

**FGF23**

Ribeiro et al[101] described how the FGF23/klotho axis is a predictive factor for fractures in patients with T2D with early CKD and demonstrated that α-klotho and FGF23 independently influenced the occurrence of bone fractures. FGF23 is a bone-derived hormone secreted by osteocytes that regulates phosphate and vitamin D metabolism. It acts in the kidney through FGF receptors and klotho, thus preventing renal tubular reabsorption of phosphorus. FGF23 plays an important role in the development of bone and mineral disorders, and many studies over recent years, including patients with CKD and diabetes, have demonstrated that FGF23 levels increase in CKD patients and have an impact on bone disease, cardiovascular disease and all causes of mortality[102]. FGF23 can also induce secondary hyperparathyroidism by increasing the 24-hydroxylation of vitamin D, and these changes are associated with an increased risk of fracture in dialysis[103]. FGF23 levels are also further raised in CKD patients with diabetes who had had a previous fracture[104], thus underlying the association of a history of prior fracture with increased risk of hip fracture, as observed in all dialysis patients[105]. Moreover, FGF23 may also promote insulin secretion and insulin resistance[106], thus influencing the risk of adverse outcomes, especially under CKD conditions[107]. Thus FGF23 could represent a potential biomarker for CKD progression in diabetes[107] and be associated with multiple risk factors[108], including bone fragility.

FGF23 signaling on target tissues is mediated by FGF receptors and klotho, which functions as a coreceptor to increase the binding affinity of FGF23 for FGF receptors. Klotho can also circulate as a secreted protein and a physiologically active hormone. It has been demonstrated that insulin can stimulate the release of klotho by inducing the cleavage of the extracellular domain of klotho by ADAM10 and ADAM17 in the kidney[109]. Cleaved klotho can thus regulate both the phosphorus and calcium metabolism in the kidney and mineral homeostasis in the body through 1-alpha hydroxylase activity as well as parathyroid hormone and FGF23 secretion[110]. Klotho expression is significantly reduced by several kidney injuries such as glomerulonephritis, acute kidney injury, ischemia/reperfusion injury and delayed graft function[111,112], chronic allograft dysfunction[113,114] and renal cell carcinoma[115]. Low klotho levels are also associated with accelerated aging that can promote dysregulated mineral metabolism and osteoporosis. Thus, reduced klotho levels are considered early factors in the development of CKD-MBD[116,117]. Klotho levels are also compromised in patients with early CKD and diabetes[101], while lower levels of klotho seem to be an independent predictive factor for bone fracture[101].

**Sclerostin and OC**

The presence of diabetes may also increase sclerostin, an osteocyte-specific protein that inhibits bone formation, and higher serum sclerostin levels are associated with increased fracture rates[118]. Thus, sclerostin has been described as an important factor contributing to CKD-MBD[119]. In diabetic patients with CKD, sclerostin levels start to increase in the CKD-G3 stage, while patients in the CKD-G4/5 stages have dramatically increased levels of circulating sclerostin[120].

OC is another bone-derived hormone whose levels reflect the ability of osteoblasts to form bones [121]. OC is directly associated with glucose metabolism and experimental models show that OC can increase insulin production by pancreatic β cells and insulin sensitivity in peripheral tissues[122]. Moreover, insulin receptor signaling increases the production of OC in osteoblasts[123]. OC levels have been recently associated with the risk of incident diabetes and kidney complications, while increased levels have been described in CKD patients[124,125]. In early CKD patients with diabetes, OC levels independently influence the occurrence of bone fracture[101]. However, further studies are needed to confirm the specific role of OC in the context of diabetes and CKD.
Further research is also needed to assess the diagnostic and prognostic value of these bone turnover biomarkers in the field of CKD-MBD in the context of diabetes. However, the described hormones represent important factors for the development of bone diseases in the context of CKD and may be considered as targets for future clinical trials.

CONCLUSION

The studies reported in the present review describe altered bone quality and the possible mechanisms underlying its pathophysiology. Patients with T2D frequently display bone fragility, which is often an underdiagnosed condition in these subjects. The understanding of its pathophysiology is an important issue as it could suggest possible interventions for the prevention of poor bone quality in T2D. Additionally, the discovery of its pathophysiology could help to target these pathways when bone disease is clearly evident. Thus, the simultaneous use of anti-diabetic drugs and bone treating agents could help to ameliorate the quality of life of patients with T2D. This issue is of particular interest considering the life extension observed. Nevertheless, the possible interventions to improve bone quality in T2D require further investigation, which could determine different treatment approaches through personalized medicine.

FOOTNOTES

Author contributions: Faienza MF wrote the clinical implications; Pontrelli P explored the bone-kidney axis; Brunetti G performed the majority of the writing and coordinated the writing of the paper.

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Orthotic approach to prevention and management of diabetic foot: A narrative review

Min Cheol Chang, Yoo Jin Choo, In Sik Park, Myung Woo Park, Du Hwan Kim

Abstract

Diabetic foot is a common complication affecting more than one-fifth of patients with diabetes. If not treated in time, it may lead to diabetic foot ulcers or Charcot arthropathy. For the management of diabetic foot, shoe modifications and orthoses can be used to reduce pressure on the affected foot or provide foot orthotic devices to relieve patient discomfort during walking. Appropriate shoe modifications include changing the insole material, modifying the heel height, adding a steel shank or rocker sole, and using in-depth shoes. Alternatively, a walking brace or ankle-foot orthosis can be used to reduce the pressure on the affected foot. The purpose of this narrative review was to provide a reference guide to support clinicians in prescribing shoe modifications and foot orthoses to treat diabetic foot ulcers and Charcot arthropathy.

Key Words: Diabetic foot; Foot ulcers; Charcot arthropathy; Shoes; Foot orthosis

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Core Tip: Footwear modifications and orthosis in diabetic foot management are aimed to prevent ulcers, protect the foot from external stimuli, and regulate the pressure on the foot. Types of shoe modifications include using an in-depth shoe, combination of insole materials, lifting the heel, applying a rocker sole, and applying an extended steel shank or flare or stabilizer. Orthosis includes prefabricated removable walking brace (such as control ankle motion walker, pneumatic walker, and conformer walking boot), Arizona ankle-foot orthosis, patellar-tendon-bearing orthosis, and Charcot restraint orthotic walker.

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INTRODUCTION
Diabetic foot is an infection, ulceration, or destruction of the tissues of the foot. It is associated with neuropathy and/or peripheral artery disease in the lower extremity of a person with diabetes mellitus [1]. The global annual incidence of diabetic foot ulcers in patients with diabetes was reported to be approximately 2%-5%, with a lifetime risk ranging from 15%-20%[2]. According to a report by the American Diabetes Association, 20% of patients with diabetes are hospitalized because of foot problems [3]. The main underlying causes of diabetic foot ulcer are peripheral neuropathy and ischemia due to peripheral vascular disease[4,5]. Once a foot ulcer develops, it may progress to foot infection, which if incurable, could eventually require limb amputation[4]. Most diabetic foot ulcers are caused by repetitive trauma due to weight-bearing or poorly fitting footwear, and ulcers often recur if the weight-bearing is not suppressed and the biomechanical abnormality of the foot is not controlled[5,6]. Prevention is important for effective management of the diabetic foot, with methods including continuous education and proper footwear for the patients with diabetes[6]. Such methods have been reported to prevent approximately 80% of all limb amputations due to diabetes[7]. Unfortunately, however, many patients are unaware of the diabetic foot, have never received proper education on its prevention, for example by wearing adequate shoes, or often receive treatment only after the problems, such as foot ulcers and neuropathic arthropathy, occur[8]. Therefore, it is essential for clinicians dealing with diabetic foot to be aware of the possible feet abnormalities due to diabetic complications and the importance of educating the patients with diabetes, including wearing the right footwear.

Another disease that can occur frequently as a complication of diabetes is Charcot arthropathy. The pathogenesis of Charcot arthropathy is not clearly known yet. It is believed to be a phenomenon in which the bone is weakened due to severe bone deficiency caused by autonomic nervous system abnormality or a local increase in blood flow to the bone[9]. Weakened bones are prone to fractures even under a very small weight, and the ligaments may also weaken, resulting in dislocation or subluxation [10]. Patients with Charcot arthropathy fail to sense pain in their limbs and continue to apply their weight, leading to bone deformity that further changes the normal force transmission path; this increases the chance of a fracture or dislocation and, consequently, deforms the weight-bearing part [11]. Over time, the process stops, and the deformity becomes permanent. Owing to the dislocation, the load cannot be distributed effectively and stably during walking, resulting in movement limitations, and the persisting bone protrusion eventually causes foot ulcers[12]. Furthermore, Charcot foot is characterized by a collapsed arch[9,10]. The non-surgical treatment for Charcot arthropathy is based on off-loading and edema control principles. The goal is to prevent the foot ulcers by minimizing mechanical stress, reducing edema, and creating structural stability, all of which help in restricting the weight-bearing and stabilizing the joint until the Charcot arthropathy is sufficiently controlled[12].

Orthoses play a very important role in treating diabetic feet that have already developed foot ulcers and Charcot arthropathy. Orthotic devices not only provide stability, limit the joint movement, and control foot deformity, but also relieve the load and evenly distribute the pressure on the foot. As a result, orthoses can effectively heal foot ulcers and control the symptoms of Charcot arthropathy. This review describes the management of diabetic feet using properly fitting shoes and orthoses.

FOOT ULCER CLASSIFICATION
Wagner classified foot ulcers from Category 0-3 based on the loss of protective sensation, foot deformity, and history of ulcer or ischemia[13]. Category 0 applies to cases where none of the following applies: Loss of protective sensation, deformity, callus, weakness, or history of ulceration or ischemia. Such cases are dealt with educating the patients on basic foot care and recommending conventional footwear. Category 1 solely involves the loss of protective sensation, and the use of in-depth shoes or
sneakers, non-molded soft inlays, and total contact orthoses is recommended. Category 2 involves foot deformity along with the loss of protective sensation and requires the use of in-depth shoes or sneakers, custom-molded foot orthoses, and external shoe modifications, if necessary. Category 3 involves all three factors, namely loss of protective sensation, foot deformity, and history of ulcer or ischemia, and requires custom-fabricated, pressure-dissipating accommodative foot orthoses, with additional recommendations for inlay-depth, soft-leather, adjustable-lacing shoes, and external shoe modifications, if necessary[13]. As illustrated above, the number of requirements for orthoses or properly fitting footwear and the complexity of prescriptions increase with the rising risk of foot ulcers.

ADAPTED FOOTWEAR

Ill-fitting footwear is a common cause of foot ulcers, whereas therapeutic footwear plays an important role in reducing the likelihood of foot ulcers[14]. Foot ulcers recur in about half the patients who wear shoes without modifications, compared to a recurrence rate of approximately 20% when an appropriate protective footwear is worn[5]. While prescribing adapted footwear for patients with diabetes, the goals are to protect the feet from the external environment, relieve excessive pressure, reduce impact and shear force, control foot deformity, and stabilize the movement[14]. The primary role of adapted footwear is to protect the feet from further harm by reducing the pressure on the affected area rather than treating the foot ulcers themselves and preventing their occurrence or recurrence[14,15]. The goal should be to reduce the pressure by at least 30% or to less than 200 kPa, where the pressure on the sole is the highest[16].

GENERAL PRINCIPLES FOR PRESCRIBING SHOES FOR PATIENTS WITH DIABETES

The shoe should conform to the shape of the foot, and there should be enough space inside, similar to an in-depth shoe. To determine the appropriate shoe size, the overall foot length, arch length, and foot width should be measured[17]. The shoes must be manufactured to accommodate the first and fifth metatarsophalangeal joints, which represent the widest part of the foot, while the shoe length should be such that there is a space of 1.3-1.6 cm between the longest toe and the tip of the shoe[17]. Additionally, the ball of the shoe should match in width with the ball of the foot, and the counter should not press the starting point of the Achilles tendon[8,17]. The insole should be removable and of triple-depth; leather insole is not recommended, as the primary aim is to minimize shear and friction[8]. The heel of the shoe is manufactured to be typically 2.5-5 cm high. If the height of the heel is over 5 cm, the pressure on the forefoot increases excessively[8,18]. Regarding the plantar surface, especially when the forefoot has an ulcer, a rigid rocker or a rigid rocker sole can reduce the pressure and help in healing of the ulcer[5]. It is recommended to purchase the shoes in the afternoon when the feet are swollen. In addition to checking the shoe size, the shoes must be tried on to check if it can correctly support the shape and the size of the feet when weight load is applied[8,17].

IN-DEPTH SHOE

In-depth shoes are usually blucher-type Oxford shoes that are 0.6-1.3 cm deeper than the conventional shoes. This extra space helps when using insoles and foot orthoses, which are necessary in cases of foot deformity due to Charcot arthropathy[8,17]. The in-depth shoes should have a light weight, good shock absorption capacity, and strong heel counter[8]. In the past, the upper material was mostly made of soft leather, but nowadays, breathable synthetic material is commonly used[8]. In addition, the relatively softer insoles are layered, and the lower the density of the insole, the more is the cushioning at the interface between the foot and the insole[19]. The insole is generally thermoformed to contour the patient’s foot, and the outer sole is also modified to further reduce the pressure[19]. In addition to the Oxford shoes, sneakers may also be used. Sneakers have several advantages in terms of the depth, removable insoles, variety of ball widths offered by manufacturers, and diversity of design compared to that of the traditional Oxford shoes, allowing the patient to choose a model according to their personal preference[20]. When purchasing a ready-made footwear, the shoes should be modified if the foot deformity is severe. If the shoes cannot be modified, they must be custom-made[8].

SHOE INSOLE

Well-made custom insoles are necessary to properly distribute the pressure around the foot deformity. One of the ways is to increase the ambient pressure in order to relieve the pressure on a certain part of
the foot; however, this approach tends to be inaccurate and can result in damage to the foot because of an increase in the partial pressure[8]. The insole serves as the backbone of the shoe and secures the upper part to the sole[17]. The insole is manufactured in an accommodative form and divided into a soft, semi-rigid, or rigid material. Soft materials include cross-linked polyethylene foam, open-cell polyurethane foam, sponge rubber, and closed-cell expanded rubber. Although these soft materials are excellent for pressure distribution, they wear out quickly and have poor durability[21]. Semi-rigid materials include firm cross-linked polyethylene foam, ethylene vinyl acetate, and cork composite, which have a longer lifespan than the soft materials. They also function as a support in addition to providing shock absorption and cushioning. Semi-rigid materials are fabricated as custom insoles with three or more layers and typically used together with soft materials to provide a combination of support and compliance[22]. Soft and moldable polyethylene foam is used in the area beneath the plantar surface. Urethane polymer is used in the middle layer to prevent wear and absorb shock. Rigid ethylene vinyl acetate or cork is used as the bottommost layer for support and control of movement[8]. Insoles with rigid materials are made of thermoplastics, acrylics, and carbon fiber composites. Although they are highly durable and offer ample support and control, the rigid materials are difficult to modify and have much lesser shock absorption capacity and offer reduced cushioning and protection. In general, the use of rigid materials is contraindicated for patients with diabetes and neuropathy or a history of foot ulcers[23]. Compression paper or leather is commonly used to make insoles for patients with diabetes. The high-strength compression paper insoles are light in weight and inexpensive. Leather insoles are highly durable and adapt well to the plantar surface, absorb moisture, and provide excellent ventilation. However, the leather insoles are not always used owing to their relatively higher price and heavier weight compared to those of compression paper[17].

**EXTERNAL SHOE MODIFICATIONS**

**Rocker sole**

Rocker soles are an effective modification method for changing the plantar pressure and improving gait[17,24]. Rocker soles help to transition smoothly from heel-strike to toe-off without bending the shoe or foot. From the biomechanical perspective, this improves the overall gait by restoring the movement of the foot or ankle joint that was lost due to foot pain or deformation. This also relieves the pressure in a specific area of the plantar surface[8]. The apex of the rocker sole should be located proximal to the area where the pressure should be relieved, and the front end of the rocker sole should be arched from the proximal part of the metatarsal head to the distal end of the outsole. If there is an angle in the rocker sole instead of an arch, the gait will not be smooth[17]. Several types of rocker soles are available, all of which require appropriate modifications to meet the needs of the user. For example, the double rocker sole is a soft rocker sole without an outsole in the midfoot region. The forefoot rocker sole has a rocker angle only in the toe area. The heel-to-toe rocker sole has a rocker angle on both the heels and toes[16,17].

**Solid ankle cushion heel**

The shoe heel provides stability to the foot heel and distributes the force applied on the foot to the entire sole[17]. The typical height of the shoe heel is 1.5-2 cm for men and 2.5-3 cm for women, but this could be modified according to the user’s needs[25]. Most shoe heels are made up of rigid materials, but, if needed, a flexible heel may also be used to allow some plantar flexion[18]. A typical example is the solid ankle cushion heel (SACH), which has a wedge-shaped shock-absorbing material inserted into the heel. The SACH acts as a buffer during heel contact and mechanically increases the heel traction during the gait cycle, creating a smooth transition from heel-strike to toe-off[26]. Typically, the angle of the SACH is within 30°[17]. The SACH is indicated for patients with ankle or hindfoot stiffness due to metatarsal ulcers or Charcot deformity. It is also used in cases of degenerative arthritis or ankle fixation[17,26].

**Extended steel shank**

The extended steel shank, made of spring steel or carbon graphite, is located between the layers of the sole from the heel to the toes and serves to reinforce the midfoot region of the shoe[27]. The extended steel shank is typically used in combination with rocker soles to help improve their performance. In addition, the shank prevents the bending of the shoe, restricts the toe and midfoot movement, and further reinforces the driving force after a toe-off during gait[8,17]. However, since the extended steel shank can be easily bent owing to the properties of the material, thermoforming may also be combined for increased rigidity[17].

**Flare and stabilizer**

The flare and stabilizer modify the inside or outside of the shoe to stabilize the foot and serve as a support for the shoe[28,29]. A flare, typically made of ethylene vinyl acetate, is a structure added to the heel and sole of a shoe to widen the support surface of the shoe[17]. The flare, when used in combination with the rocker sole, also serves to increase the stability while walking[8]. A stabilizer is an
extension made of hardened resin or crepe that is added to the side of the sole to provide greater stability than flares do. A stabilizer is used for patients with severe instability on the medial or lateral side of the hindfoot or midfoot[30].

**ORTHOSES**

The International Working Group on Diabetic Foot (IWGDF) has published the off-loading guidelines for the appropriate treatment of diabetic foot ulcers[31]. According to this guideline, for people with diabetic ulcers, a removable knee-high off-loading device with an adequate foot-device interface should be selected as the first-choice treatment. Furthermore, a total contact cast (TCC) or a nonremovable knee-high walker is recommended depending on the patient’s preference or the level of foot deformity. If nonremovable knee-high off-loading devices are contraindicated, a removable knee-high or ankle-high off-loading device is recommended[31].

Previous studies have reported that using a knee-high off-loading device is a faster treatment approach for foot ulcers than using other off-loading devices[32,33]. However, in actual clinical practice, knee-high off-loading devices are not commonly used, because they are contraindicated in at least half of the patients with diabetic foot ulcers with ischemia or infection[34]. Furthermore, many clinicians consider knee-high off-loading devices to be less effective than other types of devices[34]. However, according to the IWGDF guideline, since knee-high off-loading devices are contraindicated only in severe ischemia or infection, they can be used for mild or moderate ischemia or infection[31]. Additionally, TCC has traditionally been considered a gold standard off-loading treatment option, and knee-high off-loading devices are the first suggested option for patients with diabetic foot. Nevertheless, TCC and knee-high off-loading devices are under-used due to the perception that they are time-consuming to make and not cost-effective. However, since instant TCC and removable cast walkers are commonly used nowadays, it is necessary to improve the clinicians’ expertise and awareness of orthoses that can be used in diabetic foot treatment[34-36]. Lastly, some barriers may be related to the patients themselves. The patients often perform weight-bearing activities at home that can strain their feet, as they misunderstand that off-loading treatment should be followed outside the home only[34,37]. Moreover, patients who fail to perceive the seriousness of diabetic foot ulcers are less motivated to use the off-loading devices[37,38]. These challenges can be addressed by educating the patients on their condition and the importance of the off-loading devices[35]. The poor mobility and stability that occur when patients use knee-high off-loading devices can also be problematic. The use of the device may result in a difference in length between the legs making it difficult to walk. For such cases, the shoe height, instead of the device, can be modified or mobility aids, such as a frame, can be used simultaneously[34]. The following paragraphs describe the off-loading devices available for diabetic foot ulcers and Charcot arthropathy.

**NONREMOVABLE OFF-LOADING DEVICE**

A typical example of a nonremovable walker is the TCC. The TCC is considered the standard treatment for managing neuropathic plantar ulcers and is also used to protect the foot in the early stages of vulnerability to Charcot fracture-dislocation[31,39]. The TCC relieves the pressure and load on the foot by distributing the weight across the entire sole and can prevent the risk of injury to bony prominences, such as the malleolus and tibia[40]. However, its limitations include the requirement of a skilled cast technician to apply the TCC appropriately, high manufacturing costs, and time-consuming process[40]. Moreover, the TCC is contraindicated in the very elderly and patients with infection or severe ischemia, visual or balance problems, varicose veins, or contralateral foot ulcers[40].

**PREFABRICATED REMOVABLE WALKING BRACE**

A prefabricated removable walking brace is used to treat diabetic feet with ulcers and Charcot arthropathy. The types of walking braces include the boot-type control ankle motion walker that controls ankle movement and uses an arch filler[41,42] (Figure 1A), the pneumatic walker that applies pneumatic pressure to reduce edema and prevent callus formation[43] (Figure 1B), and the conformer walking boot that consists of a molded inner liner that wraps around the foot and leg[44]. All walking braces consist of rigid rocker soles and a protective insole made up of materials such as Plastazote® foams, propylene terephthalate, and Spenco®. The walking braces are designed to immobilize all joints of the ankle and foot as would a TCC[8]. The general advantage of walking braces is that they are easy to wear, making it possible to manage the affected area at a relatively lower cost. However, if the foot deformity is severe, applying them can be difficult, and their large volume may reduce patient compliance[9].
ARIZONA ANKLE-FOOT ORTHOSIS

The Arizona ankle-foot orthosis (AFO) is mainly used for non-surgical management of posterior tibial tendon dysfunction or spastic deformity\cite{45} (Figure 1C). It is also used in cases of ankle instability, arthritis, Charcot arthropathy, and diabetic peripheral neuropathy\cite{8}. The Arizona AFO, typically custom-made from leather and polypropylene, extends proximal to the mid-axis of the tibia and distal to the metatarsal heads. The Arizona AFO plays a role in minimizing valgus alignment of the hindfoot, lateral calcaneal displacement, and dislocation of the medial ankle or restoring inadequate kinematics by supporting the calf and midfoot\cite{46,47}.

PATELLAR-TENDON-BEARING ORTHOSIS

The patellar-tendon-bearing (PTB) orthosis is used for partial weight-bearing of the lower extremities while restricting ankle movement\cite{48} (Figure 1D). It supports the patellar tendon so that 60%-70% of the weight is supported by the knee joint and approximately 30% by the ankle joint\cite{18}. A PTB orthosis can be applied to diabetic feet with fractures of the tibia or fibula, foot ulcers, and Charcot arthropathy\cite{17}. The PTB orthosis is custom-made using a plastic-type brim based on the same principle as that used for making a PTB socket for below-knee prosthesis. The load is distributed to the ankle joint of the orthosis through the medial and lateral uprights. In general, the PTB orthosis is designed to restrict the movement of the ankle joint and provide internal and external stability\cite{18}. Although this orthosis has the advantage of easily responding to changes in foot circumference caused by severe fluctuations in lower-extremity edemas, its heavy weight may limit patient compliance\cite{8}.

CHARCOT RESTRAINT ORTHOTIC WALKER

The Charcot restraint orthotic walker (CROW) is an orthosis manufactured to completely cover the feet and legs and can be applied to diabetic feet with Charcot arthropathy\cite{8}. In Charcot arthropathy, it is used to prevent the development of ulcers by evenly distributing pressure to the entire leg and foot\cite{49}. For a severe rocker sole deformity in the midfoot, it may be necessary to limit or eliminate the movement of the midfoot and hindfoot to restrict the entire foot and ankle joints. In this case, the CROW eliminates the movement of the foot and ankle joints, reduces the load and shear force applied to the foot, and protects the deformed foot from further damage\cite{50}. The CROW orthosis is similar to a bivalved TCC and consists of a rigid polypropylene anterior and posterior shell with a dorsiflexion stop, heel lift, and rocker sole to counteract the nutcracker effect\cite{8}. Since the CROW orthosis is fastened with a Velcro® strap, it is easy to detach, making it convenient for wound management, including wound cleaning\cite{49}. However, as the CROW orthosis is bulky, it potentially limits patient compliance and sometimes leads to knee and lumbar pain due to immobility of the lower extremities\cite{51}.
CONCLUSION

In patients with diabetes, a foot ulcer or Charcot arthropathy is often the first step to amputation of the lower extremity, which becomes a major obstacle in the patient’s life. To prevent foot ulcers, thorough and repeated patient education on diabetic feet is necessary in addition to preventive skin care and, above all, prescription of appropriate footwear. Once foot ulcers and Charcot arthropathy occur, it is extremely important to prescribe appropriate footwear and orthoses for the diabetic feet to effectively treat the foot lesion and prevent further deterioration. We believe that this review can serve as a reference guide for medical staff when prescribing appropriate footwear and orthoses for patients with diabetes.

FOOTNOTES

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Effects of Chios mastic gum on cardiometabolic risk factors

Athanasia K Papazafiropoulou

Abstract

Chios mastic gum (CMG), the resin produced by the trunk of *Pistachia lentiscus* var Chia, has been used for centuries as a strong phytotherapeutic therapy, primarily for the management of gastrointestinal diseases. Recently, there are studies demonstrating that CMG has hypolipidemic, cardioprotective and antidiabetic properties. Therefore, the aim of the present review is to summarize the existing literature data regarding the potential beneficial effects of CMG on cardiometabolic risk factors.

Key Words: Chios mastic gum; Glucose; Cardioprotection; Low-density lipoprotein-cholesterol; Triglycerides

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INTRODUCTION

The aromatic resin known as Chios mastic gum (CMG) is made by the evergreen plant *Pistacia lentiscus* var Chia (Anacardiaceae). Mastic is traditionally produced by making shallow slits in the bark and trunk of the shrub using specific implements called cedritia[1]. Despite the fact that *Pistacia* species are widely distributed throughout the Mediterranean basin and in the circum-Mediterranean regions, CMG is a distinctive resin of the mastic trees grown exclusively in the southern part of the island of Chios, which is situated in the central Aegean Sea close to the coastline of Minor Asia. The fact that mastic is only produced in one location of the island and nowhere else in the greater Mediterranean region may be explained by thousands of years of selective cultivation and the particular microenvironment. The mastic tree’s cultivation and resin harvesting are part of the area’s cultural heritage, and the total production comes from 24 settlements (Mastichochoria in Greek)[1].

CMG has been used for centuries as a spice, a cosmetic, but its most important usage has been as a strong phytotherapeutic therapy, primarily for the management of gastrointestinal diseases. Galen and Dioscorides, two ancient Greek physicians, highlighted its benefits and suggested using it. Furthermore, the need for CMG has always held a special place in folk medicine throughout Europe and Asia during the Byzantine and Medieval eras, and afterwards in formal Pharmacopeias[2]. The first research revealing the resin’s positive characteristics on gastrointestinal inflammations, and particularly those caused by *Helicobacter pylori*, were published in the 1980s, reigniting the scientific community’s interest in CMG[3].

The most prevalent and traditional therapeutic application of mastic in the treatment of gastrointestinal diseases has been extensively studied in recent decades by several scientific investigations that have focused on CMG. Its antibacterial, anti-inflammatory, antioxidant, hypolipidemic, antidiabetic, and anticancer activities have since been the subject of several investigations[2]. Therefore, the aim of the present review is to summarize the existing literature data regarding the potential beneficial effects of CMG on cardiometabolic risk factors.

CHEMICAL COMPOSITION OF CMG

Numerous chemicals have been isolated and identified after a detailed analysis of the chemical makeup of CMG[8-12]. However, ongoing study continues to uncover novel substances, as seen in the case of mastichinoic acid A, a new tetracyclic triterpenoid that was recently discovered from CMG[13]. About 25% of the total CMG is made up of poly-b-myrcene, a sticky and insoluble polymer. From CMG, a number of triterpenoids have been identified. More specifically, acidic and neutral fractions can be obtained from complete mastic gum extract (without the polymer). All significant triterpenic acids, including masticadienonic, isomasticadienonic, oleanonic acid, moronic acid, masticadienolic acid, and oleanolic acid, are included in the acidic fraction. Triterpenic neutral substances such as oleanolic aldehyde, 28-norolean-17-en-3-one, tirucallol, b-amyrone, isomasticadienolic aldehyde, and dammaradienone are included in the neutral fraction.

Other substances with smaller amounts include verbenone, a-terpinolene, and linalool, which support the antibacterial properties of mastic oil, and camphene, which has hypolipidemic properties[14]. Gallic acid traces have also been found. It is amazing that research describing the antibacterial, hypolipidemic, and anti-inflammatory properties of mastic gum or mastic oil have shown the presence of synergy phenomenon, where the combination of many substances is more potent than any one ingredient alone. With herbal products that include numerous different active ingredients, this synergy phenomenon occurs frequently.

EFFECTS OF CMG ON LIPIDS METABOLISM

Human low-density lipoprotein cholesterol (LDL-C) has been shown to be resistant to copper-induced oxidation *in vitro* through the powerful antioxidant effects of CMG[15]. Peripheral blood mononuclear cells are cytotoxic when exposed to oxidized LDL-C without the presence of CMG, an d whole polar extract of CMG prevents this from happening. While mastic complete polar extract increases glutathione (GSH) levels and lowers CD36 expression, oxidized LDL-C decreases GSH levels and increases CD36 expression[16]. Rats susceptible to detergent-induced hyperlipidemia and naïve rats have both been
used to study the hypolipidemic effects of mastic gum essential oil (MGO). The levels of blood total cholesterol, LDL-C, and triglycerides were decreased in a dose-dependent manner after MGO treatment to untrained rats. MGO injection resulted in a significant decrease in the levels of total cholesterol, LDL-C, and triglycerides in hyperlipidemic rats[16].

In a different study, complete CMG was given as a powder and blended with food for 8 wk in low and high doses to examine the hypolipidemic effects of CMG on diabetic mice. The serum levels of triglycerides, total cholesterol, and LDL-C were all significantly lower in the low-dose group whereas high-density lipoprotein cholesterol (HDL-C) levels were significantly higher. Triglyceride levels were considerably lower in the high-dose group[17].

When administered to hypercholesterolemic rabbits, complete mastic extract without polymer and neutral mastic fraction (NMF) decreased total cholesterol levels by 47% and 88%, respectively, exhibiting strong hypolipidemic actions[18]. Healthy adults over the age of 50 years have received total mastic extract. Subjects were divided into two groups at random and given either a mastic solution (low dose) for 12 mo or a daily dose of 5 g of mastic powder (high dose) for 18 mo. The high-dose group showed a decrease in blood total cholesterol, LDL-C, total cholesterol/HDL-C ratio, apolipoprotein A-1, and apolipoprotein B, but no change in the apoB/apoA-1 ratio[19].

In a prospective, randomized, placebo-controlled, pilot study, healthy volunteers’ total cholesterol and blood sugar levels were considerably reduced over the course of 8 wk by taking three capsules per day containing 330 mg CMG. It is important to note that, despite the absence of side effects, overweight and obese people in particular shown excellent tolerance. CMG activity decreases when polymer is absent. Measurements of cholesterol levels in healthy individuals did not reveal any appreciable reduction after taking mastic gum capsules free of polymers[20].

A major limitation of the above human studies was that the rest of the diet of the participants (apart from the addition of CMG) was not controlled and, therefore, any effect of possible diet changes on the results of the study could not be excluded. It should be mentioned that the effects of CMG on lipids in humans are rather minor and should not be overstated as unique, as there are other natural substances, such as sterols and stanols, that have been shown to cause significant reductions in LDL-C.

**EFFECTS OF CMG ON CARDIOPROTECTIVE ACTIVITY**

Cardiovascular disease risk appears to be decreased by CMG. Perhaps one of the underlying causes of this function is the potent antioxidant activity of CMG and its ability to inhibit the buildup of the oxidized LDL in cells, which can cause atherosclerosis[16]. Two essential adhesion molecules can be decreased by the neutral fraction of CMG (25-200 g/mL) and, more specifically, the chemical tirucallol (0.1–100 mmol/L), according to research in human aortic endothelial cells [vascular cell adhesion molecule (VCAM)-1 and intercellular adhesion molecule (ICAM)-1]. Due to the buildup of monocytes in the artery innermost layer, VCAM-1 and ICAM-1 are linked to the early development of atherosclerosis[21]. In another study, male 12-wk-old diabetic mice were divided into groups receiving low and high doses of CMG. The high-dose CMG group (n = 12) received 500 mg/kg body weight for the same duration as the low dose CMG group (n = 12) for a total of 8 wk. CMG lowered serum lipid and glucose levels in both groups[22]. In 2018, the authors showed that administering CMG to renovascular hypertensive cats at a dose of 40 mg/kg/d for 2 wk after the onset of hypertension lowered their blood pressure. The results showed a relationship between reduced levels of renin, C-reactive protein, and interleukin-6, as well as increased vascular and cardiac remodeling[23].

In a different *in vivo* experiment, for 6 wk, rabbits were fed a specific diet supplemented with the NMF and the total mastic extract without polymer (TMEWP) at the same dose. In rabbits that were given a normal diet while under anesthesia, both extracts appeared to diminish the size of the infarct, and in hypercholesterolemic rabbits, they both had antiatherogenic and hypolipidemic effects. For TMEWP and NMF, the reduction in total cholesterol levels was 47% and 88%, respectively[24].

The beneficial effects of CMG on peripheral and aortic blood pressure hemodynamics in hypertensive patients were established in a randomized double-blind case-controlled crossover design, hinting potential downregulation of the proteasome system and the NADPH oxidase 2 pro-oxidant pathway. The subjects consumed 2800 mg of CMG orally (four tablets of 700 mg or a placebo), and they had evaluations during two subsequent visits spaced by 1 wk[25]. Another pilot investigation also suggested that CMG powder may play a role in human in *vivo* hepato- or cardioprotection. In the group consuming daily 5 g of mastic powder for 18 mo, a reduction in blood total cholesterol, LDL-C, total cholesterol/HDL ratio, lipoprotein (a), apolipoprotein A-1, apolipoprotein B, serum glutamyl oxaloacetic transaminase, serum glutamic pyruvic transaminase, and -glutamyl transferase levels was seen[19]. Since apolipoprotein A-1 is a major component of the HDL-C complex (protective fat removal particles), and thus acts as a cardioprotective molecule, the above observed reduction in the study by Triantafyllou et al[19] has to be translated carefully to daily clinical practice especially in patients with increased cardiovascular risk.
EFFECTS OF CMG ON GLUCOSE METABOLISM

The antidiabetic benefit of CMG is a recent discovery, and there is not a lot of evidence to back it up yet. Triantafyllou et al.[19] presented the first concrete proof of glucose-lowering activity, showing that in the low-dose group, male patients’ glucose levels were markedly reduced. According to Georgiadis et al [17], CGM had an unexpectedly strong antidiabetic effect, significantly decreasing blood sugar levels in both the low- and high-dose groups of mice. It is noteworthy to note that, in line with Triantafyllou et al [19], they found that the low-dose group performed better than the high-dose group. According to a recent study, CMG consumption had positive benefits on blood lipid indicators and insulin resistance in healthy Japanese men. More particularly, 30 min of additional activity three times per week enhanced the effect of the mastic powder intake on insulin, which was lowered by 5 g/d for 6 mol.[26].

CONCLUSION

CMG has a wide spectrum of antimicrobial, antioxidant, hypolipidemic, anti-inflammatory, and antidiabetic activities. Several studies have shown that CMG exerts beneficial effects on lipid and glucose metabolism. However, further studies are required to clarify the formula and the active compounds of CMG that have potential cardioprotective effects as well as their use in clinical practice.

FOOTNOTES

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REFERENCES


Advances in neovascularization after diabetic ischemia

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Abstract

With the high incidence of diabetes around the world, ischemic complications cause a serious influence on people’s production and living. Neovascularization plays a significant role in its development. Therefore, neovascularization after diabetic ischemia has aroused attention and has become a hot spot in recent years. Neovascularization is divided into angiogenesis represented by atherosclerosis and arteriogenesis characterized by coronary collateral circulation. When mononuclear macrophages successively migrate to the ischemia anoxic zone after ischemia or hypoxia, they induce the secretion of cytokines, such as vascular endothelial growth factor and hypoxia-inducible factor, activate signaling pathways such as classic Wnt and phosphatidylinositol 3 kinase (PI3K)/protein kinase B (Akt) pathways, trigger oxidative stress response, activate endothelial progenitor cells or enter the glycolysis or lactic acid process and promote the formation of new blood vessels, remodeling them into mature blood vessels and restoring blood supply. However, the hypoglycemic condition has different impacts on neovascularization. Consequently, this review aimed to introduce the mechanisms of neovascularization after diabetic ischemia, increase our understanding of diabetic ischemic complications and their therapies and provide more treatment options for clinical practice and effectively relieve patients’ pain. It is believed that in the near future, neovascularization will bring more benefits and hope to patients with diabetes.

Key Words: Diabetes mellitus; Angiogenesis; Arteriogenesis; Ischemia; Hypoxia

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Core Tip: This review aimed to give an overview of neovascularization in patients with diabetes. First, we introduced the basic concepts and influencing factors of neovascularization, including angiogenesis and arteriogenesis. Second, the mechanisms regarding cytokines, classical and novel signaling pathways, glycolysis and lactic acid process and so on described in detail. Then, the neovascularization after diabetic ischemia was further described in combination with the complications of diabetes, such as diabetic atherosclerosis, diabetic retinopathy, diabetic nephropathy and diabetic foot ulcer. Last but not least, the treatment plans listed, with advantages and disadvantages, that may offer more treatment options.

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INTRODUCTION
Diabetes mellitus is a complex, heterogeneous, whole-body chronic metabolic disease; it is predicted that by 2045, the number of patients with diabetes, aged 20-79 years, will increase to 783 million[1]. Diabetes-related complications include microvascular plaque formation, ischemia and hypoxia caused by atherosclerosis. They are characterized by severe arterial ischemia, increased risk of amputation of peripheral artery disease and so on. Promoting neovascularization to restore the blood flow of the ischemic area is conducive to disease outcomes while avoiding risk. Hence, it is an urgent issue for scientific researchers.

NEOVASCULARIZATION AFTER ISCHEMIA
Neovascularization is caused by angiogenesis and arteriogenesis. Angiogenesis refers to the budding of new blood vessels in the vascular bed in an original way to form new vasculature, mainly in the capillaries[2]. Arteriogenesis is the formation of new arteries by expanding lumen diameter and remodeling tube walls to restore blood flow in the ischemic area. The new route gradually disappears when the previously blocked artery is recanalized[3].

Angiogenesis
Physiological angiogenesis mainly occurs in embryo development, endometrial thickening and wound healing; the process depends on the ratio of proangiogenic factors to antiangiogenic factors. Pathological angiogenesis is often triggered in disease states, such as atherosclerosis, tumors, systemic lupus erythematosus, etc[4], to form abnormal blood vessels with thinner walls and higher permeability. Angiogenesis includes the following steps: (1) Endothelial cells sprout under the action of angiogenic factors[5]; (2) Pericytes aggregate if their absence leads to increased lumen permeability[6] and vascular instability; and (3) The basement membrane is reconstructed to develop mature and stable blood vessels.

Atherosclerosis can lead to severe complications such as myocardial infarction (MI), resulting in heart failure. Endothelial cells, which sprout under the action of angiogenic factors, significantly improve the patient’s recovery, which is the focus of current research.

After MI, blood congestion, thromboembolism and compression of the surrounding tissue, proinflammatory factors are released, leading to impaired endothelial integrity, loss of myocardial cells, endothelial cell damage, increased capillary permeability and secretion of proinflammatory cytokines to activate white blood cells and endothelial cells. This results in the release of a large number of inflammatory factors into the infarcted myocardium, thus promoting myocardial inflammation[7]. The formation of neovascularization in the ischemic infarct area to provide nutrients and oxygen is the key to post-MI repair. Neovascularization in the surrounding area of infarction increases vascular density and extends to the core area of infarction[8], and hypoxia plays an essential role in this process.

The most studied factor is hypoxia-inducible factor-1α (HIF-1α), whose reduced protein expression under high glucose conditions leads to increased MI[9]. In anoxic environments, the HIF-1α/vascular endothelial growth factor (VEGF) pathway acts by releasing angiogenic factors. After MI, reactive oxygen species (ROS) in cardiac fibroblasts increase by about 50%, resulting in mutations in the HIF phenotype marked by scar contraction and dysfunction[10]. HIF activation induces VEGF release, which activates endothelial cells (ECs) through the paracrine mechanism, and can be expressed in ECs to participate in angiogenesis. In addition, angiopoietin-like protein 4 stabilizes VEGF receptor 2/Ca²⁺-dependent cell adhesion molecule 5 complex to maintain endothelial structural integrity and promote...
macrophage transformation into a repair phenotype to enhance boundary region angiogenesis.

Arteriogenesis
Arteriogenesis refers to the growth of new arteries or the derived collateral vessels, mainly involved in the active proliferation of ECs and smooth muscle cells, resulting in lumen enlargement and wall remodeling. It comprises primarily two stages[3]. In the early stage, the diameter of lateral branches and tube walls increases under the influence of fluid shear stress. Subsequently, monocytes promote lumen remodeling by secreting metalloproteinases and cytokines. In addition, M1-type macrophages promote the progression of myocardial inflammation[11]. The release of inflammatory factors under ischemia and hypoxia promotes the progression of inflammation and the occurrence of glycolysis. Under this action, fibroblasts are transformed into ECs, triggering epigenetic modification. ECs and fibroblasts contribute to the formation of arteries[12-14].

Human coronary circulation has an extensive anastomotic network. Even one-third of ordinary people have collateral circulation to cope with MI caused by transient vascular occlusion[15]. Further, 20%-25% of patients with coronary artery disease can prevent MI, improve survival and reduce mortality by promoting normal blood flow through collateral circulation during coronary artery occlusion. Approximately 1 in 5 patients cannot tolerate percutaneous coronary intervention or coronary artery bypass grafting. Therefore, collateral growth promotion is a promising therapeutic strategy targeting arteriogenesis. In the absence of coronary artery disease, these arteries are only 100-200 μm in diameter, and the lumen is impassable. When coronary artery disease causes a major artery occlusion, the collateral arteries are remodeled and the lumen is expanded to 100-800 μm in diameter to serve as part of the major artery[16], which is in line with normal routes and has one to two layers of smooth muscle cells. At the same time, the expansion of the diameter of the tube is accompanied by a decrease in the number of collateral arteries. The myocardial protection of large blood flow is more significant in the collateral arteries than in the new capillaries surrounding the infarct area[17].

Although collateral maturation is essential for preserving cardiac function, the related markers are still lacking[18]. Although the influence of coronary artery collateral formation and prognosis has been controversial at present[19], some studies showed[20] that patients with MI having coronary collateral circulation have more severe stenosis and worse cardiac function. Therefore, from a macro point of view, it is believed that collateral circulation benefits at least one-fifth of patients who cannot undergo percutaneous coronary intervention and coronary artery bypass grafting. Physiological or pathological vascular reconstruction and blood flow redistribution can prevent excessive MI and reduce injury to the body. This therapeutic strategy has broad research prospects and is worth further exploration to benefit patients.

Impact factors
As mentioned earlier, neovascularization is affected by various proangiogenic/antiangiogenic factors. The mechanisms and roles of proangiogenic factors, such as HIF, macrophages, VEGF family, noncoding RNA and hepatocyte growth factor, and antiangiogenic factors, such as thrombospondin-1 and interleukin 12 (IL-12), summarized in this study (Tables 1 and 2)[7,21-44].

MECHANISMS OF NEOVASCULARIZATION AFTER DIABETIC ISCHEMIA

Glycolysis
Studies have shown that 80% of ATP in ECs is produced by glycolysis[45], mainly due to the limited number of mitochondria. The energy generated needs to be supplied to the distal tissues. In addition, the ATP generation by glycolysis is faster than that by oxidative phosphorylation. Therefore, glycolysis is the primary energy supply for ECs in normal and hypoxic states. Hypoxia or lack of nutrition can promote the production of VEGF, fibroblast growth factor and other angiogenic factors[46], the concentration and the activity of hexokinase and membrane expression of glucose transporter protein 1. The ECs differentiate into specific cells under high VEGF levels, forming platelet pseudopodia and filiform pseudopodia, promoting migration and stem cell proliferation. Stable glycolysis produces lactic acid[47], promoting VEGF expression and inducing angiogenesis under multiple effects.

6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3 (PFKFB3) transcription through HIF-1α is induced by hypoxia, which is an essential regulator of glycolysis and can be phosphorylated by activating kinases such as mitogen-activated protein kinase[48]. PFKFB3 can promote the synthesis of fructose-2,6-diphosphate, activate phosphofructokinase 1 and promote glycolysis. The Notch-Delta-like ligand 4 signaling pathway can promote stem cell extension, tip cell growth and blood vessel germination[49].

Some intermediate products of glycolysis enter the pentose phosphate pathway. The NADPH produced during this process is necessary for nitric oxide (NO) biosynthesis, promoting angiogenesis. When macrophage metabolism shifts to glycolysis, M1 proinflammatory macrophages facilitate glycolysis, interrupting the tricarboxylic acid cycle and producing lactic acid rather than metabolizing pyruvate to acetyl-CoA. Anti-inflammatory M2 macrophages inhibit the pentose phosphate pathway.
IL-12 stimulates the expression of proinflammatory genes in monocytes. When hypoxia occurs, HIF-α dimerizes with HIF-β, binding to hypoxia response elements in the nucleus, transcribing thousands of genes and promoting angiogenesis.

Macrophages are divided into M1 type, which is proinflammatory and phagocytic, and M2 type, which is anti-inflammatory and promotes angiogenesis.

Monocytes: Angiogenesis is directly dependent on the number of circulating monocytes. HIF-1α, a master regulator of hypoxic genes, dimerizes with HIF-1β, binding to hypoxia response elements in the nucleus, transcribing thousands of genes and promoting angiogenesis.

VEGF activates a variety of downstream signaling pathways and promotes the proliferation, migration and vascular remodeling of ECs; activate ERK1/2 and promote angiogenesis. VEGF-A regulates angiogenesis, vascular permeability and inflammation. VEGF-B regulates angiogenesis and apoptosis. VEGF-C and VEGF-D regulate lymphangiogenesis, apoptosis and fiber formation.

Angiotensin II stimulates angiogenesis by inducing endothelial cell proliferation, migration and tubular blood vessel formation. Angiogenesis is induced by activation of angiotensin 1 receptor and nicotinamide adenine dinucleotide phosphate oxidase. HGF stimulates angiogenesis by inducing endothelial cell proliferation, migration and tubular blood vessel formation. HGF significantly increases the expression of VEGF, decreases the activation of NF-κB and vascular leakage, promotes angiogenesis, is anti-inflammatory, is anti-oxidative and reduces vascular permeability.

Platelet reactive protein-1 (TSP-1) is an antiangiogenic factor. TSP-1 inhibits angiogenesis by stimulating endothelial cell apoptosis and inhibiting endothelial cell migration and proliferation as well as inhibiting VEGR and eNOS. IL-12 stimulates the expression of proinflammatory and antiangiogenic genes in monocytes. Neutralization of IL-12 can enhance angiogenesis in ischemic areas and reduce body dysfunction.

Noncoding RNAs have the dual role of promoting and inhibiting angiogenesis. Uregulation of MiR-15a and MiR-16 reduce Tie-2 protein levels and inhibit angiogenesis.

AS: Atherosclerosis; asTF: Alternatively spliced tissue factor; EC: Endothelial cell; EPO: Erythropoietin; HGF: Hepatocyte growth factor; HIF: Hypoxia inducible factor; MiR: MicroRNA; NF-κB: Nuclear factor kappa-B; VEGF: Vascular endothelial growth factor.

<table>
<thead>
<tr>
<th>Trigger factors</th>
<th>Mechanism</th>
<th>Function</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>HIF</td>
<td>When hypoxia occurs, HIF-α dimerizes with HIF-β, binding to hypoxia response elements in the nucleus, transcribing thousands of genes and promoting angiogenesis.</td>
<td>Promote angiogenesis and increase vascular density; stimulate collateral vessel compensatory formation; regulate EPO and other downstream factors; mobilize endothelial progenitor cells.</td>
<td>[21-24]</td>
</tr>
<tr>
<td>Macrophages</td>
<td>Macrophages are divided into M1 type, which is proinflammatory and phagocytic, and M2 type, which is anti-inflammatory and promotes angiogenesis.</td>
<td>Transform into perivascular cells to control vascular permeability; remodel extracellular matrix to provide conduit for apical cells and promote blood vessel germination; endothelial cells and trim abnormal blood vessels.</td>
<td>[25-30]</td>
</tr>
<tr>
<td>Monocytes</td>
<td>Angiogenesis is directly dependent on the number of circulating monocytes.</td>
<td>Induce HIF-mediated release of chemokines and growth factors to stimulate angiogenesis; express angiogenin receptor Tie-2 and exacerbate inflammation.</td>
<td>[7, 31]</td>
</tr>
<tr>
<td>VEGF family</td>
<td>VEGF-A regulates angiogenesis, vascular permeability and inflammation. VEGF-B regulates angiogenesis and apoptosis. VEGF-C and VEGF-D regulate lymphangiogenesis, apoptosis and fiber formation.</td>
<td>VEGF activates a variety of downstream signaling pathways and promotes the proliferation, migration and vascular remodeling of ECs; activate ERK1/2 and promote angiogenesis.</td>
<td>[32-34]</td>
</tr>
<tr>
<td>Noncoding RNAs</td>
<td>Many noncoding RNAs regulate complex processes of angiogenesis.</td>
<td>MiR-25-3p enhances endothelial permeability and angiogenesis. MiR-990-5p subtype NF90 has angiogenic effects.</td>
<td>[35, 36]</td>
</tr>
<tr>
<td>HGF</td>
<td>Stimulates angiogenesis by inducing endothelial cell proliferation, migration and tubular blood vessel formation.</td>
<td>HGF significantly increases the expression of VEGF, decreases the activation of NF-κB and vascular leakage, promotes angiogenesis, is anti-inflammatory, is anti-oxidative and reduces vascular permeability.</td>
<td>[37]</td>
</tr>
<tr>
<td>Angiogenesins</td>
<td>Angiogenesis is induced by activation of angiotensin I receptor and nicotinamide adenine dinucleotide phosphate oxidase.</td>
<td>Induction of angiotensin II synthesis can lead to a proangiogenic state.</td>
<td>[38]</td>
</tr>
<tr>
<td>asTF</td>
<td>asTF is widely expressed in macrophages and neovascularization in AS plaques.</td>
<td>asTF affects all key stages of angiogenesis, including proliferation, migration and differentiation and induces increased levels of HIF and VEGF to promote angiogenesis.</td>
<td>[39]</td>
</tr>
<tr>
<td>Classical Wnt pathway</td>
<td>Under the influence of Wnt factor, β-catenin isolates and enters the nucleus, binding to TCF/LEF and initiating transcription of downstream genes.</td>
<td>The Wnt pathway promotes angiogenesis by regulating endothelial cell proliferation.</td>
<td>[40, 41]</td>
</tr>
</tbody>
</table>

Lactylation

Lactic acid has been long considered as a metabolic waste. Recent studies have shown that lactic acid can be expressed as a signaling molecule in wound healing and angiogenesis[50]. Lactic acid can modify histones to regulate macrophage polarity and expression of tissue repair genes such as arginase-1[51]. In inflammatory diseases such as atherosclerosis, macrophages secrete proinflammatory cytokines such as tumor necrosis factor γ and interleukin 12 to cause extensive damage to surrounding tissue, and anaerobic glycolysis causes lactic acid accumulation[51]. The macrophage polarity transition is a hallmark of the disease, and lactic acid converts macrophages from a proinflammatory phenotype into a repairing phenotype, removes cell debris and promotes wound healing. Inflammatory macrophages undergo modifications, which promote the repair characteristics of macrophages in response to inflammatory damage[52]. In addition, lactic acid can covalently couple with various histone lysine residues during histone acetylation to promote the transcription of homeostasis-related genes. In the late stage of...
lactic acid and histone lactate modification and accumulation, the cells switch to a steady-state phenotype, in which inflammatory genes are difficult to induce.

Studies have shown that lactic acid can promote the secretion of VEGF, activate the nuclear factor kappa-B/C-X-C motif chemokine 8 pathway and stabilize HIF-1α, playing a role in promoting angiogenesis signaling molecules. The overall function of lactic acid is to transform the inflammatory phenotype of macrophages into a repair phenotype. We hypothesized that lactic acid was associated with angiogenesis. However, the integration mechanism of lactic acid and hypoxia, such as HIF, chromatin remodeling and other processes extending to angiogenesis, is still unclear and needs further research (Figure 1).

**Oxidative stress**

When the body is subjected to various diabetic stimuli, the mitochondria is stimulated to produce superoxide, leading to the formation of the powerful oxidant nitrite, which damages DNA and depletes intracellular NAD (+)[33], resulting in a pathological state. Two common mechanisms[34] contribute to increased oxidative stress[55] in diabetes: One is an increase in free radical production and the other is a decrease in the levels of protective endogenous antioxidants. Also, natural antioxidants include dandelion[56], saffron[57,58], hawthorn[59], vitamin C and vitamin E[60]. However, rhizoma polygonate in traditional Chinese medicine can dephosphorylate DNA to damage DNA[61]. In addition, hyperglycemia activates nuclear factor kappa-B, which can lead to changes in the inflammatory response, upregulation of COX-2, inducible NO synthase (NOS)[62], tumor necrosis factor α and interleukin 1, promotion of cell proliferation and inhibition of cell death. The increased expression of inducible NOS catalyzes the production of large amounts of NO[63]. The inhibition of TLR2/4 signaling can avoid nuclear factor kappa-B translocation, ultimately reducing cell apoptosis[64]. The hyperglycemic environment can stimulate the mitochondria respiratory chain to produce a large number of oxygen free radicals, activate protein kinases C[65] and promote the NADPH-related processes of oxidative stress, leading to endothelial cell apoptosis. A small number of ROS can maintain normal physiological function[66]; however, an excess of ROS causes oxidative stress[67], which can activate multiple stress kinases and related proteases and affect their activities[68], aggravate cytotoxicity and attack cells, leading to endothelial progenitor cell senescence, apoptosis and inhibition of migration and proliferation. Superoxide anions and H₂O₂ in the ROS family play a major role in this process. In addition, the activity of endodermal NOS is reduced, the metabolism of tetrahydrobiopurine (BH₄) is abnormal, and dihydrobiopiperin (BH₂) cannot be recovered in diabetes, resulting in a lower level of BH₄[69]. NO induces the formation of many superoxide anions instead of NO, aggravating oxidative stress. Advanced glycation end products lead to an imbalance in ROS production and clearance and increased endothelial permeability[70]. Oxidative stress impairs angiogenesis through multiple mechanisms.

**Endothelial progenitor cells**

Endothelial progenitor cells (EPCs) are fusiform cells with limited proliferative capacity in the early stage and cells with high proliferative capacity in the late stage[71]. During tissue ischemia, EPCs can be mobilized from bone marrow to damaged blood vessels for vascular repair or angiogenesis, or the number of circulating EPCs increases via various factors, including VEGF, stromal cell-derived factor-1 or stem cell factor. EPCs can proliferate, migrate, adhere and differentiate into ECs, repair damaged ECs or stem cell factor. EPCs can avoid nuclear factor kappa-B translocation, ultimately reducing cell apoptosis[72]. In diabetes, endothelial dysfunction and delayed angiogenesis promote the occurrence and development of diabetic vascular complications. In the high glucose environment, the number of EPCs is reduced and their functions are impaired[73]. In addition, EPCs are less responsive to ischemia, VEGF, stromal cell-derived factor-1 or other stimuli, and the mobilization mechanism is damaged. EPCs may also secrete angiogenic factors. The high glucose environment leads to the excessive production of ROS, excessive activation of NADPH oxidase and a significant decrease in the levels of manganese-containing superoxide dismutase and other antioxidant enzymes, resulting in EPC dysfunction[74]. The excessive production of ROS significantly increases the levels of oxLDL, inhibits Akt phosphorylation and endothelial NOS (eNOS) expression, decreases NO activity, inhibits the PI3K/Akt/eNOS signaling pathway and induces the apoptosis of EPCs, migration of EPCs and formation of functional defects in the lumen[75]. In addition, the severe inflammatory environment of diabetes causes impaired adhesion and proliferation of EPCs as well as neovascularization.

However, when patients with diabetes suffer from vascular complications, the number and function of EPCs in different parts (microvessels and large vessels) are different. For example, when such patients suffer from peripheral artery disease, the number of EPCs decreases. However, the proliferative capacity of EPCs is increased in patients with proliferative retinopathy.

**Slit2/Roundabout 1/PI3K/Akt/VEGF signaling pathways**

Endothelial cell-derived Slit2 plays a proangiogenic role in EC migration and lumen formation through its Roundabout 1 (Robo1) receptor. High glucose levels directly induce Slit2 production or Slit2/Robo1 binding. Robo1 inhibits the activation of the PI3K/Akt pathways and HIF-1α/VEGF signaling pathways and inhibits angiogenesis. PI3K inhibitors also inhibit the HIF-1α/VEGF signaling pathways.
When glucose enters the cell through channels that initiate glycolysis in an aerobic environment, when the anaerobic glycolysis, lactic acid can cause macrophages to convert to a repair phenotype. These macrophages will not produce inflammatory factors such as tumor necrosis factor γ and interleukin 12, which promotes angiogenesis. Lactic acid can activate the nuclear factor kappa-B-C-X-C motif chemokine 8 pathway, promote vascular endothelial growth factor (VEGF) secretion, stabilize hypoxia-inducible factor-1 α (HIF-1α), and promote angiogenesis. When hypoxia occurs in the environment, it promotes hexokinase, which promotes glycolysis, and AMPK, which promotes hypoxia-inducible factor-1. It then acts on 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3. AMPK also acts directly on 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3, and they jointly promote fructose-2,6-biphosphatase, which acts on phosphofructokinase 1 and promotes glycolysis. Hypoxia also promotes VEGF secretion, and a high concentration of VEGF activates the Notch-DLL4 pathway, directly or indirectly promoting the proliferation of tip cells. VEGF also promotes the growth of stalk cells, and both promote angiogenesis.

1,3BPG: 1,3 diphosphoglycerate; 2-PG: 2-phosphoglycerate; 3-PG: 3-phosphoglycerate; F1,6BP: Fructose 1,6-diphosphate; F2,6BP: Fructose 2,6-diphosphate; F6P: Fructose 6-phosphate; G3P: Glyceraldehyde-3-phosphate; G6P: Glucose 6-phosphate; PEP: Phosphoenolpyruvate; Pyr: Pyruvic acid.

**Figure 1** Novel mechanisms of neovascularization. When glucose enters the cell through channels that initiate glycolysis in an aerobic environment, when the anaerobic glycolysis, lactic acid can cause macrophages to convert to a repair phenotype. These macrophages will not produce inflammatory factors such as tumor necrosis factor γ and interleukin 12, which promotes angiogenesis. Lactic acid can activate the nuclear factor kappa-B-C-X-C motif chemokine 8 pathway, promote vascular endothelial growth factor (VEGF) secretion, stabilize hypoxia-inducible factor-1 α (HIF-1α), and promote angiogenesis. When hypoxia occurs in the environment, it promotes hexokinase, which promotes glycolysis, and AMPK, which promotes hypoxia-inducible factor-1. It then acts on 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3. AMPK also acts directly on 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3, and they jointly promote fructose-2,6-biphosphatase, which acts on phosphofructokinase 1 and promotes glycolysis. Hypoxia also promotes VEGF secretion, and a high concentration of VEGF activates the Notch-DLL4 pathway, directly or indirectly promoting the proliferation of tip cells. VEGF also promotes the growth of stalk cells, and both promote angiogenesis.

NEOVASCULARIZATION AFTER DIABETIC ISCHEMIA

**Diabetes atherosclerosis**

Atherosclerotic plaques lead to local hypoxia and trigger angiogenesis of the outer membrane of the vessel. The newly formed vessels are immature and leaky due to the lack of a tight connection between the pericellular cells and ECs. Lipids, inflammatory factors and red blood cells invade the plaques, leading to vessel rupture within the plaques. Proteolytic enzymes released by neovascularization promote inflammatory cell infiltration, and inflammatory factors trigger angiogenesis[77]. At the same time, intraplaque bleeding leads to a rapid increase in plaque volume, the fibrous cap changes to a contraction phenotype, and the fibrous cap becomes thinner[78], increasing the risk of plaque rupture [79]. However, Brezinski et al[80,81] put forward an opposite view that plaque rupture is caused by insufficient angiogenesis. The axial extension of neovascularization allows the healing of most plaques, while insufficient angiogenesis cannot sustain the growth of ECs in long necrotic plaques, resulting in plaque rupture and acute coronary syndrome.

Under high glucose conditions, the senescence and death of EPCs are accelerated, the PI3K/Akt/eNOS signaling pathway is inhibited, advanced glycation end products are increased, and fibrin formation is accelerated[82,83]. This triggers endoplasmic reticulum stress and oxidative stress, resulting in insulin resistance and accelerated atheromatous plaque formation. The endothelial cell differentiation is blocked, and angiogenesis is damaged (Figure 3).
Figure 2 Classical mechanisms of neovascularization. A: Classical Wnt pathway. When there is no Wnt signal, LRP, Frizzled, DV1 and Axin are closely combined with β-catenin. In the presence of Wnt signaling, the complex disintegrates, and β-catenin enters the nucleus, binds to TCF/LEF, transcribes multiple downstream signals such as c-Myc and ultimately promotes neovascularization; B: Under normoxic conditions, hypoxia-inducible factor (HIF)-α inhibits the binding of cAMP-response element binding protein (CREB) and P300, which causes hydroxylation of HIF, then leads to proteasome degradation and inactivation after VHL ubiquitination. When hypoxia occurs, prolyl hydroxylase domains become inactive, allowing HIF-α to migrate to the nucleus, where it dimerizes with HIF-β. The dimer binds to the hypoxia response elements of specific genes in DNA, transcribes thousands of genes such as vascular endothelial growth factor (VEGF) and promotes neovascularization; C: High glucose induces Slit2/Roundabout 1 (Robo1) binding, and Robo1 inhibits the activation of phosphatidylinositol 3kinase (PI3K) /protein kinase B (Akt) and HIF-1α/VEGF signaling pathway and inhibits angiogenesis. PI3K inhibitors also inhibit the HIF-1α/VEGF signaling pathway. After activation of the PI3K/Akt signaling pathway, a variety of cytokines including VEGF will be transcribed to promote angiogenesis.

Diabetic retinopathy
Diabetic retinopathy (DR) can be divided into early nonproliferative DR and late proliferative DR in terms of progression, leading to pathological retinal angiogenesis[84]. Neovascularization extends along the surface of the retina into the vitreous cavity. Still, such vessels are fragile and easily broken, easily leading to vitreous hemorrhage, retinal detachment or macular nonperfusion and related photoreceptor dysfunction[85]. The earliest change is the thickening of the vascular basement membrane. In a high glucose environment, the basement membrane hardens and changes the elasticity of blood vessels, affecting the retinal blood flow and the dynamic balance between the inside and outside of blood vessels[86].

After the blood glucose level increases, the blood-retinal barrier degrades briefly in a few days or weeks, and then Muller cells are activated. Pericytes are lost in about 2 mo, followed by ECs, leading to vascular degeneration 6 mo after diabetes[85]. Vascular degeneration is caused by the loss of pericytes that control vascular patency, resulting in decreased vascular patency, vascular blockage, endothelial cell fusion/degeneration and finally basal membrane dissolution, vascular degeneration and formation of capillaries without ECs. However, under physiological conditions, ECs of retinal capillaries express a high level of tight connections, limiting the circulation of nutrients, soluble factors and cells into tissues [87], and vascular degeneration destroys this structural function.

Consistent with numerous pathological processes, in a hypoxic and ischemic environment, angiogenesis-related factors are promoted, retinal neovascularization occurs, and newly formed blood capillaries migrate to other capillaries and merge to form new blood capillaries[87]. However, the high glucose environment can promote the development of diabetic retinal neovascularization. Proliferative retinopathy is accompanied by tractive retinal detachment and vision loss. Therefore, the treatment aims to inhibit neovascularization. The standard treatment methods include laser therapy and anti-VEGF therapy. The intravitreal injection of VEGF inhibitors has excellent short-term safety. However, whether long-term anti-VEGF may have long-term adverse effects on the function of retinal neurons is unclear.
Diabetic nephropathy

In the early stage of diabetic nephropathy, hypoxia induces HIF generation, and endothelial growth factor or angiopoietin maintains renal vascular density, resulting in increased abnormal angiogenesis, vascular immaturity, plasma protein leakage, increased proteinuria, and significantly increased glomerular filtration rate. In the late stage, glomerular capillaries are sparse, and the production of nephrogenic erythropoietin is increased, which aggravates renal hypoxia, and glomerular cells lose vitality[88]. In this process, eNOS activity declines, the utilization rate of NO decreases, oxidative stress abates HIF activation, and VEGF expression is significantly lowered. Also, the levels of antiangiogenic factors such as platelet response protein 1 and endothelial inhibition significantly increase, the levels of inflammatory cytokines increase, and VEGF expression is inhibited. Further, EPC function is impaired, the inflammatory response is enhanced, and the expression of adherence factors is upregulated, which is accompanied by impaired capillary ECs, endothelial barrier dysfunction, and reduced angiogenesis, resulting in abnormal angiogenesis and vascular leakage. Podocytes are an important source of growth factors that regulate endothelial cell proliferation and angiogenesis. Their number increases in the early stage of diabetes and decreases in the late stage. The number of mesangial cells increases, the capillary basement membrane thickens, and the capillary number and area increase, directly or indirectly resulting in glomerular hyperplasia and mesangial expansion.

At present, no precise treatment is available for diabetic nephropathy to inhibit angiogenesis. VEGFA inhibitors can be used, but their levels need to be maintained at an appropriate level in vivo[89]. A deviation from moderate levels can cause damage.

Diabetic foot ulcer

Diabetic foot ulcer (DFU) is characterized by neuropathy caused by hyperglycemic levels, arterial stenosis caused by lipid deposition, and ischemic lesions of lower extremities. The DFU healing process can be divided into three overlapping phases: early steady-state and inflammation, arteriogenesis and matrix deposition; mid-late reshaping; and epithelial cell remodeling[90]. Neutrophil granulocyte and macrophages produce cytokines and promote cell proliferation; fibroblasts are rich in collagen fibers and induce angiogenesis and vascularization[91]. Neurological and ischemic lesions lead to impaired healing.

DFU arteriogenesis is reduced in diabetes. Hypoxia and ROS decrease transcriptional activities of HIF, VEGF, angiopoietin 2, and fibroblast growth factor and inhibit collateral development and arteriogenesis after limb ischemia[92]. Hyperglycemic levels result in impaired growth factor production and macrophage function, collagen accumulation inhibition and poor migration and proliferation of
keratinocytes and fibroblasts, leading to impaired angiogenesis\cite{91}. At the same time, wound infection reduces the active matrix metalloproteinase 8 (MMP-8) level, increases the active MMP-9 level, inhibits laminin, enhances keratinocyte migration, reduces arteriogenesis and slows wound healing\cite{93}.

For the treatment of DFU, the current strategy is to increase arteriogenesis, eliminate oxidative stress and ulcer infection\cite{94}. The best strategy for DFU treatment is to inhibit MMP-9 without affecting MMP-8, which can reduce inflammation and increase arteriogenesis. Applying prolyl hydroxylase domain inhibitors with clinical potential can stabilize HIF and increase its activity to promote arteriogenesis. Mesenchymal stem cells produce growth hormones that drive arteriogenesis and re-epithelialization.

**TREATMENT**

For treating diabetic ischemic neovascularization, the most crucial way is to control blood glucose levels. On this basis, various therapeutic methods, including gene therapy and vaccine research, have been proposed, which have broad prospects. For example, Ad.trx1 gene therapy can stabilize the microenvironment in the myocardium, reduce oxidative stress and cell death and induce neovascularization and maturation. This is a novel treatment that may improve disease progression and patient recovery\cite{95}. Injection of VEGF and hepatocyte growth factor can promote neovascularization and exert anti-inflammatory and anti-fibrotic effects, but gene therapy is still controversial due to its insignificant effect and needs further study.

Angiogenesis antibodies can be generated by the intramuscular injection of the VEGF DNA vaccine; however, the technology is still immature, and the efficacy and long-term impact are not apparent. Hence, further clinical research is needed. Also, angiotensin-converting enzyme inhibitors can antagonize inflammation, increase the number of EPCs and improve the mobilization ability in patients with diabetes. However, this treatment causes an irritating cough, bilateral renal artery stenosis and other adverse side effects\cite{96}. In addition, EPC mobilization promotes re-endothelialization, repairs damaged ECs, promotes angiogenesis and restores blood flow\cite{97,98}. It is believed that with further research, more treatments can be developed to benefit mankind (Figure 4).
CONCLUSION

The advantages and disadvantages of neovascularization to the body are based on different environments. For MI, peripheral arterial disease, coronary collateral circulation and DFU, neovascularization needs to be promoted to restore the perfusion of the ischemic area and reduce body damage. Neovascularization should be inhibited for DR and tumors to reduce the risk of retinal stripping or tumor metastasis. Although the involvement of neovascularization in disease pathogenesis is still not specific, it has huge prospects for treating ischemia in diabetes.

FOOTNOTES

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Nutritional supplementation on wound healing in diabetic foot: What is known and what is new?

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**Abstract**

Non-healing diabetic foot ulcers (DFU) are the most notable and striking complications of diabetes mellitus. More than 25% of nonhealing DFU can ultimately lead to amputation of the lower extremity within 6-18 mo after the first manifestation of the wound. Although wound healing is complex, nutritional status is crucial in soft tissue repair. Malnutrition is highly prevalent and overlooked in patients with diabetes and chronic wounds. Moreover, to date, we do not have clear recommendations or evidence about the use of nutritional supplements for improving wound healing in patients with DFU. In this article the authors briefly analyzed the current evidence on the use of nutritional supplements of proteins or amino acids, fatty acids, probiotics, vitamins, and trace elements in the wound healing process in patients with DFU.

**Key Words:** Malnutrition; Supplements; Diabetic foot; Diabetes; Wound healing; Nutritional therapy

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**Core Tip:** Malnutrition is common in patients with diabetes and chronic wounds. To date we do not have clear recommendations or evidence about the use of nutritional supplements for improving wound healing in patients with diabetic foot ulcers (DFU). This paper aimed to evaluate current evidence regarding the use of Nutritional supplementation on wound healing in patients with DFU.
INTRODUCTION

The Global Burden of Disease study[1], estimated that 131.0 million (1.77%) people worldwide had diabetes-related lower-extremity complications (DRLECs) in 2016, equal to 34% of the diabetes population. Among these patients almost 105.6 million had neuropathy; 18.6 million had foot ulcers, and 6.8 million undergone to amputation. Moreover, it is known than 84% of lower extremity amputations are preceded by a foot ulcer[2], however ulcer prevention is still an overlooked opportunity[3].

In patients with diabetes known risk factors for developing ulcers are peripheral arterial disease, peripheral neuropathy, repeated trauma, history of previous ulcers, and/or amputation. Male subjects, patients with a longer duration of the disease and a low socioeconomic level have the highest risk[4-7].

Non-healing diabetic foot ulcers (DFU) are the one of the most relevant and dangerous complications of diabetes mellitus[2]. A diabetic foot ulcer is defined as nonhealing in the case of a wound area reduction of less than 50% after 4 weeks of standard care[8]. More than a quarter of non-healing DFU may in the end leads to lower extremity amputation within 6-18 mo after the wound’s outset[9].

Although wound healing is regulated by complex mechanisms, the outcome of the process is the complete repair of the damaged tissue. It is known that infection, wound depth, size, and duration negatively impact healing process and are therefore associated with poor outcomes and possible amputation[10].

Nutritional status is crucial in wound healing. The presence of a wound has a negative impact on nutritional status due to the metabolic cost of repairing damaged tissue, in addition to nutrient loss through wound inflammatory exudate. Malnutrition is highly prevalent in patients with chronic diabetic foot wounds[11], and specific micronutrient deficiencies are common and associated with impaired wound healing and increase the risk of amputation in people with DFU[12,13]. Malnutrition is known to impair the inflammatory phase of the healing process, reducing fibroblast proliferation and collagen synthesis, increasing the risk of developing infections, decreasing T lymphocyte function, phagocytic activity, complement, and antibody levels[14]. Moreover, nutritional defect of polyunsaturated fatty acids (PUFAs) and consequent reduced synthesis of the essential fatty acid-derived resolvins, (crucial regulators of inflammatory phase of wound healing) are particularly deleterious in people with DFU where diabetes itself is associated with an overall decreased production of pro-resolving lipid mediators[15].

Therefore, nutritional interventions can improve DFU healing. Current Australian guidelines recommend the evaluation of nutritional status when progress towards DFU closure is not made[16] and the use of oral nutritional supplements (ONS) when an oral diet is not sufficient to meet nutritional requirements. However, to date, we do not have clear recommendations or evidence on the use of nutritional supplements to improve wound healing in patients with DFU.

This article aimed to evaluate current evidence regarding the use of Nutritional (Proteins, Aminoacids, Omega 3, probiotics, vitamins, and Trace Elements) supplementation on wound healing in patients with DFU (Table 1).

PROTEIN AND AMINO ACIDS

Proteins and amino acids are the most frequent nutritional supplementation used to improve the healing of diabetic foot wounds, starting from the evidence of the positive effects of these compounds in wound healing. We identified four randomised controlled trial (RCT) studies that supplemented with protein or amino acid mixture in patients with diabetic foot. Eneroth 2004[17] supplemented 27 malnourished patients with diabetic foot ulcers with 20 g protein per 200 mL bottle and compared them with a placebo (26 patients). It is unknown whether there is a difference in the proportion of ulcers healed at the end of the study period, or an absolute change in individual parameters of ulcer dimensions, or ulcer area, over time, for those treated, or not treated with an ONS. Secondary outcomes too, amputation and adverse events (death), didn’t demonstrate a difference. Armstrong et al[18], an RCT with 270 patients, experimented with supplementation with arginine, glutamine, and b-hydroxy-b-methyl butyrate on foot ulcer healing in people with diabetes. No differences were found between groups in wound closure or time to wound healing after 16 wk. Subgroup analysis shows that the addition of arginine, glutamine, and b-hydroxy-b-methyl butyrate in addition to the standard of care can improve the healing of diabetic foot ulcers in patients with the risk of limb hypoperfusion and/or
Table 1 Strengths and Limitations of the main randomised controlled trial that evaluated the effects of specific nutritional supplement on wound healing in patients with diabetic foot ulcers

<table>
<thead>
<tr>
<th>Ref.</th>
<th>Study design and Number of participants</th>
<th>Nutrient(s) or Supplement Studied</th>
<th>Aim of study</th>
<th>Main results</th>
<th>Limitations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eneroth et al [17], 2004</td>
<td>Double-blind RCT, 53 patients</td>
<td>20 g protein per 200 mL bottle, 1 kcal/mL</td>
<td>To determine the effects of supplementation on wound healing at 6 mo in subjects with DFU</td>
<td>10/27 (37%) participants in the oral nutritional supplement group healed at 6 mo compared with 12/26 (46%) in placebo group</td>
<td>The sample size was small; certainty of the evidence is very low</td>
</tr>
<tr>
<td>Armstrong [18], 2014</td>
<td>Double-blind RCT, 270 patients</td>
<td>Arginine, glutamine and β-hydroxy-β-methylbutyrate</td>
<td>To determine the effects of supplementation on proportion of ulcers healed at 16 wk</td>
<td>65/129 (50%) participants in the arginine, glutamine and β-hydroxy-β-methylbutyrate supplement group healed, compared with 65/141 (46%) in the placebo group</td>
<td>Certainty of the evidence is very low; no difference in quality of life, new ulcers, amputation</td>
</tr>
<tr>
<td>Wong et al [19], 2014</td>
<td>RCT, 27 patients</td>
<td>Amino acid mixture containing (beta)-hydroxy (beta)-methylbutyrate (HMB), arginine and glutamine</td>
<td>Compare pressure ulcer healing rates</td>
<td>Wound area did not decrease significantly in the short term for both groups. The proportion of viable tissues increased within 2 wk on HMB, arginine and glutamine supplementation; pressure Ulcer Scale for Healing scores showed significant improvement within 1 wk of supplementation for the experimental group</td>
<td>The sample size was small. Observation time was short. Amino acid does not appear to reduce wound size but to improve healing process</td>
</tr>
<tr>
<td>Basiri et al [20], 2022</td>
<td>RCT, 29 patients</td>
<td>Nutritional supplementation (total energy of 500 calories, 28 g of protein, and essential vitamins and minerals) and education</td>
<td>Effects of nutrition supplementation and education on inflammatory biomarkers</td>
<td>The mean plasma concentration of IL-6 significantly decreased in the treatment group</td>
<td>The sample size was small</td>
</tr>
<tr>
<td>Basiri et al [20], 2022</td>
<td>RCT, 29 patients</td>
<td>Nutritional supplementation (total energy of 500 calories, 28 g of protein, and essential vitamins and minerals) and education</td>
<td>Nutrient dense formula combined with nutrition education on wound healing</td>
<td>Treatment group experienced a faster wound healing rate (6.43 mm²/wk more reduction in the wound area) than the control group. The mean reduction in the wound area during the first four weeks of the study was almost 13-fold greater in the treatment group compared to the control group</td>
<td>The sample size was small</td>
</tr>
<tr>
<td>Sipahi et al [22], 2013</td>
<td>Retrospective analysis, 11 patients</td>
<td>Beta-hydroxy-beta-methylbutyrate, arginine and glutamine</td>
<td>Wound healing in diabetic dialysis patients</td>
<td>Healing was observed on the wound depth score of 7 (63.6%) patients and on wound appearance score of 8 (72.7%) patients out of 11</td>
<td>Not RCT; the sample size was small</td>
</tr>
<tr>
<td>Soleimani et al [31], 2017</td>
<td>Double-blind RCT, 60 patients</td>
<td>Omega-3 PUFA</td>
<td>To determine the effects of flaxseed oil omega-3 fatty acids supplementation on wound healing and metabolic profiles in subjects with DFU</td>
<td>Significant decrease in ulcer length, width, and depth</td>
<td>The sample size was small. The authors did not report data on ulcer healing (percentage of ulcers healed), cost of surgery, quality of life, adverse events, development of any new foot ulcers, amputation rate, incidence osteomyelitis from surgery</td>
</tr>
<tr>
<td>Mohseni et al [37], 2017</td>
<td>Double-blind RCT, 60 patients</td>
<td>Probiotics (Lactobacillus acidophilus, Lactobacillus casei, Lactobacillus fermentum, and Bifidobacterium bifidum)</td>
<td>To determine the effects of oral probiotic therapy on wound healing and metabolic status in adult patients with diabetes</td>
<td>Significant improvement in length, width, and depth of ulcers in treatment vs placebo groups</td>
<td>The sample size was small. The authors did not report data on ulcer healing (percentage of ulcers healed), quality of life, adverse events, development of any new foot ulcers, amputation rate</td>
</tr>
<tr>
<td>Gunton et al [59], 2021</td>
<td>Double-blind RCT, 19 patients</td>
<td>Vitamin C 500 mg daily in a slow-release capsule</td>
<td>To determine the effects of oral vitamin C supplement on wound healing</td>
<td>Healing at 8 wk was significantly better in the vitamin C group (median 100 v. -14 %, P = 0.041); healing without amputation occurred in all patients in the vitamin C group</td>
<td>The sample size was extremely small</td>
</tr>
<tr>
<td>Yarahmadi et al [40], 2021</td>
<td>Double-blind RCT, 25 patients</td>
<td>Oral vitamin E and C or placebo</td>
<td>To determine the effects of oral vitamin C and E supplement on wound</td>
<td>Significantly higher wound size reduction in intervention (P = 0.019); significant decrease in</td>
<td>The sample size was small. All patients were treated even with platelet-rich</td>
</tr>
</tbody>
</table>

*(DFU = diabetic foot ulcers)*
To determine the effects of oral vitamin D supplement on wound healing and markers of inflammation and insulin resistance. After 12 wk vitamin D treatment resulted in treatment Greater Reduction in ulcer length, width, depth, and erythema rate vs placebo. The sample size was small.

**Zinc oxide + vitamin E**

Razzaghi et al. [46], 2018

Double-blind RCT, 60 patients

50000 IU vitamin D supplements every 2 wks

To determine the effects of oral vitamin D supplement on wound healing and hs-CRP in the intervention group (P < 0.05) plasma-fibrin glue dressing and vitamin E. The sample size was small.

**Magnesium oxide + vitamin E**

Afzali et al. [47], 2019

Double-blind RCT, 70 patients

250 mg magnesium oxide + vitamin E supplement on wound healing Beneficial effects on ulcer size after 12 wk of treatment. The sample size was small.

**Zinc oxide + vitamin E**

Razzaghi et al. [46], 2018

Double-blind RCT, 57 patients

250 mg magnesium oxide plus 400 IU vitamin E supplement on wound healing Magnesium plus vitamin E supplements for 12 wk reduced ulcer length and depth vs placebo. The sample size was small.

**Zinc oxide + vitamin E**

Momenn—Heravi et al. [48], 2017

Double-blind RCT, 60 patients

220 mg zinc sulfate supplements on wound healing Zinc supplementation for 12 wk was associated with significant reductions in ulcer length and width. The sample size was small.

RCT: Randomised controlled trial; DFU: Diabetic foot ulcers; PUFA: Polyunsaturated fatty acids.

hypoalbuminemia. Wong et al [19], use a mixture of amino acid containing (beta)-hydroxy (beta)-methyl butyrate (HMB), arginine, and glutamine in diabetic patients with pressure ulcers without obtaining wound area reduction, but demonstrates a better proportion of viable tissues on HMB, arginine, and glutamine supplementation group. Recently Basiri et al [20], evaluated the effects on wound healing of a treatment regime based on nutritional education in combination with a nutritional supplement (total of 500 kilocalories, 28 g of protein, and essential vitamins and minerals) in a group of patients with DFU getting a faster wound healing rate in treated group. The mean reduction in wound area during the first four weeks was almost 13 times greater in patients treated with nutritional supplement compared to the control group. Moreover, the same authors demonstrated in another publication that nutritional intervention could have favorable effects on controlling inflammation in patients with DFU [21]. Finally, we included in this analysis a retrospective study, from Sipahi et al. [22] for interesting results in diabetic dialysis patients supplemented with aminoacid (beta-hydroxy-beta-methyl butyrate, arginine, and glutamine) obtaining healing in most of the patients (63.6%). Dialysis always represents a challenge in the healing of the diabetic foot.

In summary, the results of the current studies did not give a clear effect of proteins and amino acids in the healing of diabetic foot wounds, but there is some evidence of efficacy with improvement in the healing process. The nutritional status of the participants was not an inclusion/exclusion criterion in the studies included in the review. As such, the supplement was generally given randomly irrespective of the presence or absence of malnutrition at inclusion), thus, the baseline nutritional status of the participants is independently correlated with the severity of infection, increased risk of poor outcome, poor prognosis after vascular interventions and it even predicts amputation. Furthermore, it is unclear what the minimum duration is required to achieve a measurable impact on the wound once the serum level of a particular nutrient has improved beyond the deficient range.

## POLYUNSATURATED OMEGA-3 FATTY ACIDS

Long-chain Omega-3 PUFA and their bioactive metabolites (resolvins, marine, protectin) have long been considered as powerful negative modulators of acute inflammation and/or inducers of its resolution [23], while the role they play in the wound healing process is still largely unknown. Interest is growing in the understanding of the specific effects of PUFA derived metabolites on the cellular and molecular mechanisms involved in the modulation of inflammatory process [24]. The most bioactive PUFAs are eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) that can be obtained directly from many dietary sources as fish and algae, or indirectly through the endogenous metabolic conversion of their precursor, as linolenic acid contained in high concentrations in vegetables and nuts.

Data on the effects of omega-3 fatty acids from flaxseed oil on wound healing in human studies are scarce. Additionally, the studies [25–27] that have evaluated the effects of fish oil supplements (rich in omega-3) on wound healing in patients without diabetes have produced mixed results.

Regarding the consequence of omega-3 fatty acids on inflammatory factors, a meta-analysis showed that supplementation with marine-based omega-3 induce a significative reduction of C-reactive protein,
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IL-6, and tumor necrosis factor α[26]. McDaniel et al[26,27,29] showed that supplementing omega-3 for 4 wk in a healthy subjects resulted in increased production of pro-inflammatory cytokines, including IL-1β at wound sites representing a potential therapeutic option for people with chronic wounds[26-29].

As recommended in the Expert consensus and guidance of the American limb preservation society on nutrition interventions in adults with diabetic foot ulcers, mono and PUFAs, including EPA, DHA, and arachidonic acid should be considered because they contribute to membrane fluidity, membrane and intracellular signals, and modulation of apoptotic pathways[30] (ALPS Nutrition Interventions in Adults with Diabetic Foot Ulcers Expert Consensus and Guidance).

In a double-blind RCT, Soleimani et al[31] evaluated the effects of daily omega-3 fatty acids from flax seeds for 12 wk in 60 subjects with grade 3 DFU according to “Wagner-Meggitt’s criteria”. Participants were assigned to receive 1 g twice daily of omega-3 PUFA or a placebo for 12 wk. A significant reduction in ulcer width, length, and depth and an increase in insulin sensitivity and total antioxidant capacity was observed in the intervention compared to the control group. As reported by Moore et al[32] the study of Soleimani et al[31] had several limitations. The sample size was small. The authors did not report percentage of ulcers healed, cost of surgery, quality of life, amputation rate, and incidence of osteomyelitis from surgery.

PROBIOTICS

An emerging treatment line for wound healing is the use of probiotics, defined by the International Scientific Association of Probiotics and Prebiotics as “live microorganisms which when administered in adequate amounts confer a health benefit on the host”[33].

The most common probiotics used as supplements include members of the Lactobacillus spp, Bifidobacterium genera[34], strains from other bacterial species (Propionibacterium acidilactici, Lactococcus lactis, Leuconostoc mesenteroides, Bacillus subtilis, Streptococcus thermophilus) and yeasts (Saccharomycesboulardii)[35].

Perioperative supplementation of probiotics demonstrated accelerated skin healing in diabetic rats, probably due to a reduction of the inflammatory response, increased neovascularization, and increased type I collagen deposition. Supplementation with probiotics also prevents weight loss and improves glycemic control in animal models[36].

In a double-blind RCT, the effects of daily probiotics supplementation for 12 wk were examined in 60 patients with DFU with cellulitis on wound healing[37]. Participants were randomly assigned to receive a probiotic capsule or placebo daily for 12 wk.

The study founds improvement in length, width, and depth of the ulcers in patients treated with probiotic supplement compared with placebo. Additionally, compared to placebo, a significant decrease in HbA1c (-0.6% ± 0.5% vs -0.2% ± 0.4%), total cholesterol and CRP levels was observed in treated patients. This study had several limitations: the sample size was small; the authors did not report data on ulcer healing, quality of life, adverse events, and amputation rate.

Studies on probiotic use are limited, for this reason more robust evidence are needed to examine the effects of probiotics on wound healing in patients with DFU.

VITAMINS AND TRACE ELEMENTS

Vitamin C

Vitamin C, also known as ascorbic acid, is a water-soluble vitamin that plays a pivotal role in many biological processes involved in wound healing, including collagen formation, regulation of the immune system, and maintenance of cartilage and bones.

The recommended dietary allowance for adults and older is 90 mg daily for men and 75 mg for women. Deficiency of Ascorbic Acid has been associated with increased severity of DFU in a prospective study of 131 patients with diabetic foot[12]. Intriguing data derive from case series of people with diabetes and poorly healing lower limb ulcers in whom there was prompt ulcer healing with vitamin C replacement[38].

Recent evidence from some small randomized controlled trials has raised the idea of a possible benefit of vitamin C supplements on wound healing in DFU. An Australian placebo-controlled randomized trial founds that active treatment with Vitamin C 500 mg daily in a slow-release capsule for 8 wk results in a better percentage reduction in ulcer volume in 7 patients with DFU[39].

With similar findings Yarahmadi et al[40] demonstrate that supplements of vitamin E (200IU/2 d) and C (250IU/2 d) for eight weeks significantly increase wound healing in patients with non-healing DFU by enhancing the wound healing process and reducing oxidative stress in 13 patients treated within platelet-rich plasma-fibrin glue dressing plus[40].
Therefore, although seminal evidence is promising and of interest for future research, more convincing and robust evidence on the role of ascorbic acid in wound healing is required.

**Vitamin D**

Vitamin D is a fat-soluble vitamin that has long been known to help the body absorb certain calcium and phosphorus; both are essential for the building of bone. Circulating levels of 25 (OHD) are low in patients with DFU[12] or patients with severe peripheral artery disease[41]. Furthermore, vitamin D insufficiency has been associated with impaired inflammatory responses, oxidative stress, and wound healing. A meta-analysis of observational studies reports that severe vitamin D deficiency is associated with foot ulceration[42] in people with diabetes. However, evidence of the benefits of vitamin D supplementation in DFU is still lacking. To date, we have only a few RCTs (most of them for the same study group) that evaluated the effects of Vitamin D supplementation on wound healing in DFU. A double-blind, placebo-controlled, randomized clinical trial, conducted among 60 patients with grade 3 DFU according to “Wagner-Meggitt” criteria, showed that after 12 wk of intervention, compared to placebo, vitamin D supplementation resulted in a significant reduction in ulcer length, width, depth, and erythema rate[43]. A very similar study with a comparable number of participants confirmed that a supplement of 60000 IU/weekly of cholecalciferol had a beneficial effect on wound healing when compared with a placebo[44].

More convincing and robust evidence is required on the role of vitamin D supplementation in wound healing.

**Trace elements**

The nutritional relevance of trace elements has grown rapidly due to a better understanding of their biological functions. They are involved with many enzymatic processes such as catalysis, oxidation-reduction, and cellular transport. Therefore, trace elements have a pivotal role in the synthesis and structural stabilization of both proteins and nucleic acid needed for wound healing. In wound healing, the most important trace elements are zinc, copper, chromium, selenium, and magnesium, which are believed to be essential for the regeneration of physiological tissue in humans[45]. However, only a few randomized controlled trials have tested the effect of trace element supplements on wound healing in DFU.

Effects of magnesium supplements on wound healing in people with diabetes were evaluated in 2 small randomized, double-blind, placebo-controlled trial. The first[46] was carried out among 70 subjects with grade 3 DFU according to the “Wagner-Meggitt” criteria and subjects treated with 250 mg of magnesium oxide had beneficial effects on ulcer size after 12 wk of treatment. In a similar study 57 patients with grade 3, DFU were randomized to take either 250 mg magnesium oxide plus 400 IU vitamin E (n = 29) or a placebo per day (n = 28) for 12 wk. Compared to placebo, taking magnesium plus vitamin E supplements reduced ulcer length and depth[47].

Zinc was tough to improve wound healing due to its effects on insulin resistance, inflammation, and oxidative stress. The effect of zinc supplements on wound healing in DFU was evaluated in a randomized, double-blind, placebo-controlled trial conducted among 60 patients with grade 3 diabetic foot ulcers[48]. Participants were randomly assigned to take either 220 mg zinc sulfate supplements containing 50 mg elemental zinc or a placebo daily for 12 wk. Compared with the placebo, zinc supplementation was associated with significant reductions in ulcer length and width.

**CONCLUSION**

Current evidence supports that poor nutritional status is common and associated with the impaired healing process in patients with DFU. However, the number of good quality RCTs evaluating nutritional supplementation in DFU patients is limited with varying effects[30,49,50]. Patients with diabetic foot ulcers require good multidisciplinary care to optimize wound healing, but often an important and overlooked aspect of this is their nutritional status. However, evidence for the impact of nutritional interventions on foot ulcer healing in people with diabetes remains uncertain, with studies showing no clear benefit or harm. For this reason, the IWGDF Guidelines on the use of interventions to improve healing of diabetic foot ulcers (2019) recommend not using interventions aimed at correcting the nutritional status of people with diabetic foot ulcers, to improve healing, preferring it as the best standard of care[51]. Instead, according to the new Australian guidelines on wound healing interventions to improve wound healing, the nutritional status of the foot ulcer should be reviewed, and adequate daily nutritional needs should be met as part of the best standard of care "to ensure that this recommendation has not been misinterpreted[15]. In conclusion more and well-designed studies are needed to better clarify the potential impact of nutritional supplementation on the healing of foot ulcers in people with diabetes.
PERSPECTIVES

Author’s suggestion for future research and design of proper RCT study: (1) Complete evaluation of nutritional status at the beginning, evidence, and agreement on malnutrition, and evidence if the population examined have a specific nutritional defect; (2) guarantee to all patient's real optimal standard therapy; (3) choose uniformity of wound conditions, don’t mix heterogeneous kinds of wounds. Consider that some conditions are time-limited, for example, choosing “infected wounds” we don’t define a homogeneous group, with this definition we could include neuropathic, ischemic, traumatic, or mixed lesions with very different characteristics; (4) adequate duration of supplementation and study too, since mean wound healing of diabetic foot is approximately 3 mo, it is not possible to evaluate healing in a shorter period; (5) evaluate evidence of supplementation with blood samples that demonstrate direct change in parameters; and (6) choose multiple parameters, other than healing, to evaluate the effects of supplementation: Rate of wound reduction, percentage of granulation tissue.

FOOTNOTES

Author contributions: Da Porto A and Miranda C came up with ideas and constructs; Da Porto A, Miranda C, and Da Ros R wrote the manuscript; Michelli A, Brosolo G, and Zanette G approved the main conceptual ideas and made corrections; all authors provided final edits and approved the manuscript.

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MINIREVIEWS

Combination therapy of hydrogel and stem cells for diabetic wound healing

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Abstract

Diabetic wounds (DWs) are a common complication of diabetes mellitus; DWs have a low cure rate and likely recurrence, thus affecting the quality of patients’ lives. As traditional therapy cannot effectively improve DW closure, DW has become a severe clinical medical problem worldwide. Unlike routine wound healing, DW is difficult to heal because of its chronically arrested inflammatory phase. Although mesenchymal stem cells and their secreted cytokines can alleviate oxidative stress and stimulate angiogenesis in wounds, thereby promoting wound healing, the biological activity of mesenchymal stem cells is compromised by direct injection, which hinders their therapeutic effect. Hydrogels form a three-dimensional network that mimics the extracellular matrix, which can provide shelter for stem cells in the inflammatory microenvironment with reactive oxygen species in DW, and maintains the survival and viability of stem cells. This review summarizes the mechanisms and applications of stem cells and hydrogels in treating DW; additionally, it focuses on the different applications of therapy combining hydrogel and stem cells for DW treatment.

Key Words: Combination therapy; Mesenchymal stem cells; Hydrogel; Diabetic wound; Cells delivery; Wound healing

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FUNCTIONAL HYDROGELS FOR DW TREATMENT

Hydrogels are three-dimensional (3D) networks with a high water content and have been intensively studied because they can be functionalized and have good biocompatibility. Several studies have shown that hydrogels provide a moist environment, contribute to cell migration and tissue regeneration, and promote wound healing. Therefore, hydrogels are considered ideal dressings for DWs. Furthermore, hydrogels provide antioxidant, antibacterial, proangiogenic, and proliferative functions owing to the sustained release of bioactive agents encapsulated in hydrogels. Stem cells are bioactive agents that promote wound healing and are effective in skin regeneration.

Stem cells possess self-renewal and differentiation abilities and are essential for post-injury skin repair. Thus, stem cell therapy has become a promising new approach for treating DWs. Local injection of the cell suspension or stent implantation stimulates neovascularization, accelerates wound closure, prevents wound contracture and scar formation, and ultimately improves wound healing. However, the outcome of stem cell therapy is hindered by the poor bioactivity of stem cells and thus the low amounts of secreted cytokines in the hyperglycemic inflammatory microenvironment of DWs. Effective stem cell delivery remains a challenge.

To achieve better healing outcomes combining hydrogel and stem cell treatment is one of the most promising therapies for DWs. Although various reviews on stem cell therapy or hydrogel therapy for DWs have been reported, reviews on combined therapy are limited. Herein, we review the mechanisms of DW therapy combining hydrogel and stem cells and focus on preclinical studies of therapy combining hydrogel and stem cells for DWs.

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INTRODUCTION

Diabetes mellitus (DM) is a significant global public health burden because of its high incidence and mortality rates. In 2019, 1.5 million people died of DM. Diabetic wounds (DWs) are one of the most concerning complications of DM and affect up to 25% of diabetic patients. In addition to causing patient suffering, DW has a low cure rate and high amputation rate, and thus it places a long-term burden on society.

DW is difficult to heal because its healing process is unlike that of normal wounds. Normal wound healing typically includes three phases: Inflammation, proliferation, and remodeling. Various cells, growth factors, and cytokines play important roles in each phase to ensure a smooth wound healing progress. Owing to the elevated levels of reactive oxygen species (ROS), impaired immune function, and cellular dysfunction in the DW microenvironment, the healing stage stagnates in the inflammatory phase. In addition, the peripheral arterial disease leads to a lack of blood perfusion and hypoxia within wounds, thereby increasing ROS release. ROS also induces the expression of extracellular matrix (ECM) degradation enzymes that degrade ECM, thus precluding the normal matrix-cell interaction required for wound healing and prolonging the inflammation phase of DW healing.

DW healing remains a clinical challenge because of several complications in the DW microenvironment, including oxidative stress, chronic inflammation, and angiogenic dysfunction. Current clinical treatments (standard care) involve glycemic control, offloading, debridement, and infection management, which are painful and insufficient for curing DWs. Therefore, new approaches for improving DW healing must be developed. The application of functional hydrogel dressings or scaffolds is a promising advanced therapy.

Hydrogels are three-dimensional (3D) networks with a high water content and have been intensively studied because they can be functionalized and have good biocompatibility. Several studies have shown that hydrogels provide a moist environment, contribute to cell migration and tissue regeneration, and promote wound healing. Therefore, hydrogels are considered ideal dressings for DWs. Furthermore, hydrogels provide antioxidant, antibacterial, proangiogenic, and proliferative functions owing to the sustained release of bioactive agents encapsulated in hydrogels. Stem cells are bioactive agents that promote wound healing and are effective in skin regeneration.

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To achieve better healing outcomes combining hydrogel and stem cell treatment is one of the most promising therapies for DWs. Although various reviews on stem cell therapy or hydrogel therapy for DWs have been reported, reviews on combined therapy are limited. Herein, we review the mechanisms of DW therapy combining hydrogel and stem cells and focus on preclinical studies of therapy combining hydrogel and stem cells for DWs.
the wound, reducing pain by absorbing exudates, preventing infection, and maintaining a moist environment for cell migration. In addition, hydrogels have been used as delivery systems to minimize drug toxicity and improve drug delivery efficiency\[2\]. Functional hydrogels, such as antioxidant, immune regulation, and vascularization hydrogels, have been designed according to the wound microenvironment of DWs.

DWs are often accompanied by oxidative and antioxidant imbalance in vivo. Hydrogels are designed to alleviate excessive oxidative reactions. Self-antioxidant materials, such as 2-hydroxyethyl methacrylate\[18\] and polyvinyl alcohol, can directly act on wounds; additionally, gel-loaded antioxidant drugs, such as curcumin\[19\], or bioactive substances can be used to achieve antioxidant effects. These materials act as reducing agents.

Because the inflammatory phase has an active defense response to external stimuli, the inflammatory response aids in cleaning the wound during the healing process\[20\]. However, in chronic wounds, such as DWs, owing to repeated tissue damage, cytokines continue to recruit immune cells to the wound, thereby resulting in an excessive inflammatory response and blocked healing\[21\]. Therefore, the inhibition of excessive immune responses is also considered. Hydrogels, such as sodium alginate and zwitterionic hydrogels, can provide a protective microenvironment for wounds and regulate the transformation of macrophages between proinflammatory and anti-inflammatory\[20\]. Meanwhile, anti-inflammatory drug-loaded hydrogel dressings have a local sustained-release effect\[22\]. Responsive hydrogels that can change their properties according to environmental clues to achieve sustained release of entrapped drugs are also desirable.

Angiogenesis is essential for tissue regeneration, whereas the formation of healthy blood vessels is hindered by various microenvironment conditions in DWs\[23\]. Therefore, promoting blood vessel formation is conducive to DW healing. Studies have shown that some hydrogel materials, such as chitosan and hyaluronic acid, regulate the activity and distribution of cytokines or growth factors\[24\]. These materials simulate the microenvironment of the ECM, thereby promoting tissue formation. Bioactive components, including epidermal growth factor and vascular endothelial growth factor, can also be encapsulated by hydrogels, which can promote the regeneration of blood vessels\[25\].

In general, the mechanism of hydrogels in DWs is relatively clear and positively affects DW healing.

CURRENT STUDIES OF MESENCHYMAL STEM CELLS FOR DW HEALING

In addition to selecting different hydrogel materials, drugs, and biological factors, using stem cells to treat DWs is desirable. Stem cells can asymmetrically replicate and differentiate into different cell types\[26\]. With the unlimited replication capacity, they can provide numerous “sister” stem cells\[15\]. Furthermore, because stem cells secrete pro-regenerative cytokines, stem cell therapy, which treats diseases or injuries by administering stem cells into damaged tissues, has been used as an intervention for DWs\[27\]. Stem cells used for wound healing and tissue regeneration include embryonic stem cells, induced pluripotent stem cells, and mesenchymal stem cells (MSCs)\[15\].

Allogeneic, xenogeneic, and autologous MSCs have been widely used in skin regeneration and wound healing owing to their significant proliferation, migration ability, and long-term self-renewal potential\[28\]. Considering the impaired function of MSCs derived from patients with diabetes and the risk of tissue rejection, allogeneic MSCs are more widely used\[29\].

MSCs that are locally injected into wounds are involved in various stages of wound healing. They reduce inflammatory responses through immunomodulation and growth factor production\[15\], accelerate neovascularization and epithelialization, and stimulate collagen synthesis\[30\], thereby accelerating wound healing\[30\]. Additionally, clinical studies have demonstrated the efficacy of MSCs in treating diabetic ulcers\[30\]. For example, injecting allogeneic MSCs into the dermis-epidermal junction\[31\] or subcutaneous and intramuscular tissue around wounds\[32\] facilitated DW healing in patients.

The potential benefits of MSC therapy have been demonstrated in several studies. Although simple transplantation methods, such as intravenous, subcutaneous, intramuscular, and local injection of MSCs, have achieved some preclinical and clinical success\[5\], MSC performance still has numerous limitations. Premature senescence and apoptosis of MSCs transplanted in DWs are some of the biggest limitations\[33,34\]. Owing to hyperglycemia caused by DM, DWs generate a chronic inflammatory microenvironment and accumulate advanced glycation end products, which is not conducive to the survival of stem cells\[35\] and increases the degradation of growth factors secreted by the effector cells, thus compromising efficacy\[36\]. Hence, the delivery strategy must be optimized to ensure cell viability, paracrine function, and differentiation function, which in turn ensures MSC therapy outcomes.

Abundant evidence has shown that using hydrogels to deliver MSCs improves DW healing. Hydrogels are ideal carrier systems for stem cells because they produce a relatively uniform distribution of transplanted cells and retain high water content, close to that of the native tissue, thus improving the retention and survival of stem cells at transplantation sites. Transplanted stem cells can exert their functions through paracrine signals and differentiate into the various cell types required in healthy tissues (Figure 1).
Figure 1 Therapy combining hydrogels and mesenchymal stem cells promotes diabetic wound healing. Mesenchymal stem cells (MSCs) in hydrogels are long-lasting in the wound and regulate wound healing. These cells release exosomes, growth factors, and cytokines, reduce the levels of interleukin-1, tumor necrosis factor-α, and other pro-inflammatory cytokines to modulate the inflammatory response, enhance angiogenesis via increasing vascular endothelial growth factor and hepatocyte growth factor, and promote fibroblast and keratinocyte migration. MSCs can also be transdifferentiated into other cell types to increase wound closure. MSCs: Mesenchymal stem cells; IL-1: Interleukin-1; TNF-α: Tumor necrosis factor-α; VEGF: Vascular endothelial growth factor; HGF: Hepatocyte growth factor.

APPLICATIONS OF COMBINATION THERAPY OF HYDROGEL AND STEM CELLS FOR DW HEALING

As previously discussed, although stem cell therapy has promising potentials for DW healing, the lack of an optimal delivery strategy is one of the biggest obstacles to its therapeutic efficacy. Traditional injection of MSCs always results in low cell viability and transient engraftment, whereas using advanced biomaterial scaffolds (such as films, nanofibers, and hydrogels) to maintain cellular viability, proliferation, and differentiation has received considerable attention. Hydrogels have physical and biological characteristics similar to those of natural tissues; this renders them as ideal candidates for stem cell delivery. Inspired by the encouraging outcomes of hydrogels on DW healing and their function as a carrier system for drugs, the efficacy of MSCs has been improved with hydrogels. For a successful clinical application of the therapy, the optimal hydrogel composition for cell delivery must be considered, and appropriate application methods to ensure stem cell viability and promote DW healing must be designed. Currently, the most common application methods of hydrogels and stem cell combination therapy for DW healing are divided into hydrogel sheets, in situ forming hydrogels, and hydrogel microspheres (MS) (Figure 2).

HYDROGEL SHEETS

Applying hydrogel sheets on wounds is a convenient stem cell delivery method, wherein hydrogels are typically preformed in molds, with stem cells seeded onto or inside hydrogels. Rustad et al. seeded MSCs onto collagen-pullulan hydrogels and significantly accelerated wound healing and skin appendage recovery in mice within 11 d. The amount of microangiogenesis was approximately doubled in wounds treated with MSC-seeded hydrogel sheets compared with those treated with MSC injection. Given that the biomimetic hydrogel provides a functional niche to augment the regenerative potential of MSCs, the implanted MSCs differentiated into dermal fibroblasts, pericytes, and endothelial cells, which contribute to wound healing. In another study, Guo et al. demonstrated the improved retention and survival rate of MSCs in hydrogel sheets when transplanted into mouse hearts compared to cell suspension alone. Cells were observed inside the hydrogel sheets for over 9 d in ICR mice.

In vitro culturing of stem cells within hydrogels was found to promote cell adhesion and enhance stem cell functions by supporting normal phenotype maintenance and empowering the transdifferenti-
Huang JN et al. Combination therapy for diabetic wound healing

Figure 2 Three application methods of hydrogels and mesenchymal stem cells combination therapy for diabetic wound healing. A: Hydrogel sheets preformed before application; B: In situ forming hydrogels injected at the wound for sol-gel transition; C: Hydrogel microspheres applied onto the diabetic wound. MSCs: Mesenchymal stem cells.

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tiation capacity into specific skin lineages compared with the immediate transplantation of stem cell-seeded hydrogels[40]. Da Silva et al[41] pre-cultured adipose-derived stem cells (ADSCs) in hyaluronic acid-based sponge hydrogel in neurogenic/standard media for 14 d before transplantation onto the DWs of mice. Wounds treated with pre-cultured ADSCs-loaded spongy hydrogels improved wound closure rates compared to the untreated control and acellular spongy hydrogel groups after healing for 4 wk. The hydrogel sheet promoted the polarization of M1-type macrophages to the M2 type (anti-inflammatory) and improved successful neoinnervation.

Because of the high concentration of inflammatory cytokines in the DW microenvironment, which impairs the activity of MSCs and degrades growth factors secreted by stem cells, single functional hydrogel sheets may not be sufficient for DW healing[42]. To be more suitable for DW treatment, hydrogel sheets that inhibit inflammatory responses or protease activity are more effective[43]. Ahmed et al[44] studied the wound healing efficacy of bone marrow-derived mesenchymal stem cells (BMSCs) delivered by nitric oxide (NO)-releasing hydrogels on diabetic rabbits. As an endogenous molecule, NO increased angiogenesis and improved immune responses during acute infections. NO-releasing hydrogels increased the viability and proliferation of BMSCs under oxidative stress. In addition to improving collagen deposition and promoting re-epithelialization and angiogenic activity, the NO-releasing hydrogel with BMSC treatment upregulated the expression of growth and cytoactive factors for DW healing within 16 d[44].

In addition to traditional manufacturing technology, 3D bioprinting builds special structures layer-by-layer according to a predetermined computer model that better fits the skin’s architecture and geometry, providing hydrogel sheets with more complex structures[45]. Xia et al[46] developed curcumin-incorporated 3D bioprinting gelatin methacryloyl (GelMA) to seed ADSCs and promote DW healing within 21 d. Curcumin encapsulation in 10% GelMA hydrogel exhibited inhibitory effects on ROS generation and ADSC apoptosis, and living cells were detected after scaffolds embedded with ADSCs were implanted into the backs of nude mice for 21 d. Further, the scaffold increased the amount of collagen deposition and induced angiogenesis in DWs[46]. In addition to 3D bioprinting, multifunctional hydrogel sheets with complex 3D structures can be produced by folding or weaving microfiber-shaped hydrogels[47]. Hydrogel sheets can also be easily functionalized, such as the thermally responsive release of stem cells or drugs[48] for oxidative stress resistance, antibacterial activity, and other functions.
Because stem cells can be cultured separately and the hydrogel sheet is easy to handle, combination therapy with hydrogel sheets and MSCs is easily translated into a clinical setting. A clinical report, Ravari et al. applied BMSCs along with platelets, fibrin glue, and bone marrow-impregnated collagen matrix onto wounds, which resulted in the complete wound closure in 3 of 8 patients with aggressive, refractory DWs within 4 wk of treatment. Additionally, topical administration of placenta-derived mesenchymal stem cells in a sodium alginate hydrogel completely healed diabetic foot ulcers. However, this clinical case report must be evaluated further because of the limited sample size of the report. Although functionalizing or changing shapes is very convenient, hydrogel sheets must be pre-formed before application. Because hydrogel sheets are not conducive to long-term storage and the bonding between the sheets and wound surface is limited, in situ forming hydrogels have attracted attention.

**IN SITU FORMING HYDROGELS**

**In situ** forming hydrogels are another mainstream application of combination therapy, with stem cells suspended in the precursor solution before application. After the mixed precursor solution is injected into the wound site, the hydrogel containing stem cells is formed in situ on wound beds via chemical bonds. Compared with hydrogel sheets, injectable hydrogels are more flexible in their application; this flexibility allows them to adapt to complex-shaped wounds and fit closely. Eke et al. designed a precursor solution composed of GelMA and methacrylated hyaluronic acid containing ADSCs, which can be crosslinked within 40 s of ultraviolet irradiation to form hydrogels in situ. Reportedly, the hydrogel promoted cell proliferation, and in vivo studies revealed a three-fold increase in vascularization for the ADSC-loaded hydrogel group compared to the hydrogels without cells.

However, because ultraviolet irradiation may induce chromosomal and genetic instability, ultraviolet-crosslinked hydrogels on exposed wounds negatively affect cell viability and differentiation, which is detrimental to wound healing. Owing to its high biocompatibility and specificity, enzymatic crosslinking has received considerable attention. Yao et al. developed a gelatin-hydroxyphenyl hydrogel with the dual enzyme crosslinking of horseradish peroxidase and galactose oxidase, and the hydrogel encapsulated with BMSCs achieved gelation within 5 min at the wound site. The gelatin-hydroxyphenyl hydrogel provides a friendly 3D microenvironment for BMSCs, thereby improving the transplanted cells’ survival and accelerating wound closure.

Given that frequently studied natural hydrogels, such as gelatin, collagen, or hyaluronic acid, contain a single component of ECM, their potential to provide the optimum microenvironment for stem cell proliferation and differentiation is limited. ECM maintains the original components of the native tissue and is considered an ideal scaffold for tissue regeneration. Chen et al. developed an ECM-derived hydrogel from human decellularized adipose tissue matrix to deliver ADSCs to DWs. The hydrogel was prepared via pepsin digestion and pH neutralization. The paracrine activity of ADSCs encapsulated in the hydrogel was enhanced, whereas the secretion of hepatocyte growth factor increased, thus promoting neovascularization during wound healing. Compared with the untreated control, local ADSC injection, and acellular hydrogel groups, treatment with ADSC-hydrogel composites accelerated wound closure in diabetic mice and restored cutaneous appendages within 14 d.

For better DW healing outcomes, specific materials are co-entrapped inside the hydrogel for hemostasis and anti-inflammatory properties, and the stem cell viability in the hydrogel can reach an ideal state by optimizing its mechanical strength. Xu et al. encapsulated MSCs in an injectable hydrogel system of GelMA and chitosan-catechol cross-linked with dithiothreitol to repair full-thickness DWs. Chitosan-catechol has a good hemostatic effect, and zinc ions were introduced into the hydrogel to enhance angiogenesis. The cell adhesion, proliferation, and differentiation potency of umbilical cord-derived mesenchymal stem cells in vitro were well maintained in GelMA with optimal stiffness. At the same time, the hydrogel-umbilical cord-derived mesenchymal stem cells combined treatment promoted DW healing by inhibiting the inflammatory factors TNF-α and IL-1β in vivo, with a wound closure rate of 92.2% within 14 d. Compared with the untreated control, local umbilical cord-derived mesenchymal stem cell injection, and acellular hydrogel groups, collagen deposition was significantly abundant on day 7, whereas the most vascular regeneration with the earliest hair follicle formation was found on day 14.

Dispersive MSCs are usually loaded inside hydrogels. Recently, 3D MSC spheroids were found to possess better differentiation potential than dispersive MSCs, which exhibited enhanced vascularization and anti-inflammatory effects, thereby promoting wound closure. Yang et al. combined injectable thermosensitive chitosan/collagen/β-glycerophosphate hydrogels with 3D MSC spheroids, rapidly converted to a gel by physical cross-linking at body temperature, and then completely covered the wound surface and fitted to any shape of the wound bed. Compared with the local 2D monolayer MSC injection and 2D monolayer MSC-encapsulated hydrogel groups, angiogenic factors were much higher for wounds treated with 3D MSC spheroid-encapsulated hydrogel (almost 3-fold), and neovascularization was enhanced, thereby achieving complete re-epithelialization within 3
Although in situ forming hydrogels adapt to complex-shaped wounds and fit tightly, thus enabling flexible use at the wound bed, the bulk hydrogel formed at the wound site produces poor tissue infiltration and thus low stem cell survival. Compared with in situ forming hydrogels, hydrogel MSs have a larger specific surface area and more specific functions, thus playing an essential role in the medical field.

HYDROGEL MS

Hydrogel MSs exhibit good dispersion and stability in physiological environments with a high drug-loading capacity[68]. Their drug-carrying[69] and bioactive factors[70] are highly effective in wound healing. We previously demonstrated that antibiotic and growth factor separately loaded alginate/CaCO3 MSs prepared using microfluidic technology sustainably released drugs and exhibited pH sensitivity. These MSs were embedded in the regenerated tissue and functioned as scaffold materials. They improved wound healing with thicker granulation tissue and stimulated angiogenesis, ideally meeting the requirements of different stages of wound healing[71]. Lei et al[70] developed biohybrid agarose MSs conjugated with basic fibroblast growth factor, which achieved local growth factor delivery, stimulated angiogenesis, and enhanced wound healing in diabetic mice.

The special geometry of hydrogel MSs is conducive to the diffusion of nutrients and wastesc[72]. MSs that deliver stem cells can release stem cells, thereby promoting proliferation and differentiation of surrounding cells and enhancing the formation of integrated functional tissues[73]. Stem cell-loaded MSs have been applied in various tissue systems, including cartilage[74], bone[75], bone marrow[72], and brain[76]. Intracerebral implantation of stem cells using MSs in the rat brain improved stroke treatment[76]. Mao et al[72] demonstrated that microgel encapsulation sustained MSC survival after intravenous injection in mice and enhanced the immunoregulatory capacity of MSCs in a bone marrow transplantation model.

Considering that our previous study demonstrated that hydrogel MSs act as scaffolds and gradually integrate into regenerated skin tissue, we designed gelatin MSs encapsulated with ADSCs from rats (rADSC/MS) with an ideal mechanical strength and degradation rate that matched tissue regeneration to improve DW healing[77]. Gelatin MSs promoted the adhesion and proliferation of fibroblast cells and maintained the viability of encapsulated rADSCs. Slowly released exosomes from rADSCs were eventually internalized by HUVECs, which suggested a potential exosome mechanism for improving wound healing. The implanted rADSC/MS gradually integrated into the regenerated skin tissue, thus facilitating the arrangement of neat collagen fibers. Compared with the untreated group and the MS group, rADSCs embedded in rADSC/MS promoted M2 macrophage polarization and recovery of peripheral nerves, formed larger blood vessels, and eventually generated a dermis close to normal tissue within 14 d[77].

Previous studies have demonstrated that hydrogels provide a functional niche for MSCs, which enhances MSC regeneration potential and promotes wound healing. Preclinical studies on the combined treatment of DWs with hydrogels and stem cells are summarized in Table 1.

CONCLUSION

This review discussed the benefits associated with therapy combining hydrogels and MSCs for DW healing. Researchers have explored different application methods for stem cell delivery with hydrogels, including hydrogel sheets, in situ forming hydrogels, and hydrogel MSs. In addition to providing a friendly microenvironment for stem cells, this strategy enhances the adhesion between the dressing and wound and facilitates the function of stem cells, ultimately benefiting vascular and neural regeneration in DWs. Among these application methods, hydrogel MSs have the advantages of a larger specific surface area, more uniform dispersibility, and more specific functions; additionally, they can effectively deliver various types and functions of cells into the wound. Therefore, hydrogel MSs loaded with stem cells are expected to play an important role in clinical practice.

Therapy combining hydrogels and MSCs has shown great potential for DW healing. However, the plasticity of MSCs has led to their double-sidedness for clinical applications. Although the multi-differentiation ability provides them with good application prospects, it increases the risk of tumorigenicity[78]. As a solution, cell-free treatments, such as exosomes and artificial cell products derived from the MSCs secretome have attracted recent interest. Exosomes and secretomes retain the paracrine factors of stem cells[7]. Although extensive studies have explored the combination therapies of hydrogels and MSCs for DW healing, additional work is required to optimize parameters, such as the storage and transport stability of cells, and avoid their tumorigenic and immunogenic risks. Further improvement and testing of this technology in vivo will also contribute to the clinical transformation of combination therapy.
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<th>Full re-epithelialization efficiency</th>
<th>Outcome</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>UMSCs from human, xenogeneic, 1 × 10⁶</td>
<td>Self-assembled nanopeptide hydrogels based on RADA16-I, RGD, and KLT peptide solutions</td>
<td>Self-assembled nanopeptide hydrogels with easy biomimetic functionalization</td>
<td>Cells were encapsulated into the in situ forming hydrogels</td>
<td>NOD/SCID mice</td>
<td>8 mm, dorsal</td>
<td>10 d</td>
<td>Accelerated skin wound healing by inhibiting inflammation and promoting angiogenesis</td>
<td>[14]</td>
</tr>
<tr>
<td>BMSCs from rats, allogeneic, 2 × 10⁶</td>
<td>N-chitosan/HA-ALD hydrogel</td>
<td>Hemostasis and antimicrobial hydrogels</td>
<td>Cells were encapsulated into the in situ forming hydrogels</td>
<td>STZ-induced diabetic rats</td>
<td>5 mm, foot</td>
<td>12 d</td>
<td>Promoted wound healing; stimulated the secretion of growth factors from rBMSCs, and modulated the inflammatory environment by inhibiting the expression of M1 macrophages and promoting the expression of M2 macrophages, resulting in granulation tissue formation, collagen deposition, nucleated cell proliferation, neovascularization</td>
<td>[37]</td>
</tr>
<tr>
<td>ADSCs from human, xenogeneic, 3 × 10⁶</td>
<td>GG-HA spongy hydrogel</td>
<td>Vascularization hydrogels</td>
<td>Cells were seeded onto the top of spongy-like hydrogel sheets</td>
<td>STZ-induced diabetic mice</td>
<td>9 mm, dorsal</td>
<td>4 wk</td>
<td>Accelerated excisional skin wound healing; induced the healing phase switch from the inflammatory to the proliferative phase; presented a thicker epidermis with a high number of proliferative keratinocytes in the basal layer; increased the number of intraepidermal nerve fibers in the regenerated epidermis</td>
<td>[41]</td>
</tr>
<tr>
<td>BMSCs from rabbits, allogenic, 1 × 10⁶</td>
<td>SNAP-loaded chitosan-PVA hydrogel</td>
<td>Vascularization hydrogels</td>
<td>Cells were intradermally injected and topically covered with hydrogel sheets</td>
<td>Alloxan monohydrate induced diabetic rabbits</td>
<td>20 mm, dorsal</td>
<td>14 d</td>
<td>Augmented the wound closure, decreased inflammation, and upregulated expression of CD31, VEGF and TGF-β1; promoted angiogenesis by forming new capillaries and improving the microvascular and vessel maturation; showed an abundant expression of collagen type I on day 14</td>
<td>[44]</td>
</tr>
<tr>
<td>ADSCs from human, xenogeneic, 5 × 10⁵</td>
<td>Curcumin-incorporated 3D bioprinting GelMA hydrogel</td>
<td>Antioxidant hydrogels</td>
<td>Cells were encapsulated into hydrogel sheets</td>
<td>STZ-induced diabetic nude mice</td>
<td>15 mm, dorsal</td>
<td>21 d</td>
<td>Promoted wound healing; improved hADSCs apoptosis and increased the amount of collagen</td>
<td>[46]</td>
</tr>
<tr>
<td>ADSCs from human, xenogeneic, 2.5 × 10⁵</td>
<td>hDAM hydrogel</td>
<td>Intact ECM-derived hydrogels from living tissues</td>
<td>Cells were suspended in the in situ forming</td>
<td>KK/Upi-Ay-J mice (diabetic mice)</td>
<td>8 mm, dorsal</td>
<td>14 d</td>
<td>Accelerated wound closure and improved skin architecture regeneration,</td>
<td>[62]</td>
</tr>
</tbody>
</table>
### Combination therapy for diabetic wound healing

**UMSCs from human, xenogeneic, $5 \times 10^6$**

| GelMA/Chi-C hydrogel | Vascularization hydrogels | Cells were mixed with the *in situ* forming hydrogels | Diabetic mice (db/db) | 8 mm, dorsal | 14 d | Promoted the wound healing process by inhibiting protein expression of TNF-α and IL-1β to decrease inflammation. Accelerated angiogenesis and re-epithelialization, promoted collagen deposition, and induced regeneration of skin appendages such as hair follicles. |

**PDSs from human, xenogeneic, $1 \times 10^3$**

| Chitosan/collagen/β-GP hydrogel | Thermostensitive and pH-responsive hydrogels | 3D-spheroids were encapsulated in the *in situ* forming hydrogels | Diabetic mice (db/db) | 7 mm, dorsal | 3 wk | Accelerated wound closure by enhancing angiogenesis and paracrine effects. The hydrogel provided an environment favorable for the attachment and proliferation of encapsulated hPDSCs, accelerating cell proliferation and paracrine factor secretion. |

**ADSCs from rats, allogenic, $5 \times 10^5$**

| Gelatin hydrogel | Adaptive hydrogel microspheres with degradation rates well-matched to tissue regeneration | Hydrogel microspheres | STZ-induced diabetic rats | 8 mm, dorsal | 14 d | Significantly accelerated wound healing by promoting M2 macrophage polarization, collagen deposition, angiogenesis associated with peripheral nerve recovery, and hair follicle formation. The microspheres well embedded in the tissue, exhibited good biocompatibility and adaptive biodegradation rates. |

**BMSCs from human, xenogeneic, $5 \times 10^3$**

| PEGDA hydrogel | Vascularization hydrogels | Cells were encapsulated into hydrogel sheets | Genetically diabetic mice (BKS.Cg-m +/− Leprdb/J) | 1 cm × 1 cm, dorsal | 14 d | Accelerated wound healing; the co-encapsulation of hBMSCs and insulin secreting cells resulted in healing wounds without scab or scar. |

**ADSCs from human, xenogeneic, $3 \times 10^5$**

| PEG-gelatin hydrogel | Vascularization hydrogels | Cells were mixed with the *in situ* forming hydrogels | Diabetic mice (db/db) | 6 mm, dorsal | 15 d | Significantly accelerated wound closure; the encapsulated cells attached and diffused well inside the hydrogel, improving cell retention *in vivo*, reduced inflammatory cell infiltration and enhanced neovascularization. |

---

1 Wound size (side length × side length).

3D: Three dimensional; ADSCs: Adipose-derived stem cells; β-GP: β-glycerophosphate; BMSCs: Bone marrow-derived mesenchymal stem cells; Chi-C:...
Huang JN et al. Combination therapy for diabetic wound healing

Chitosan-catechol; ECM: Extracellular matrix; GelMA: Gelatin methacryloyl; GG-HA: Gellan gum-hyaluronic acid; HA-ALD: Hyaluronic acid-aldehyde; hADSCs: Human adipose-derived stem cells; hBMSCs: Human bone marrow-derived mesenchymal stem cells; hDAM: Human decellularized adipose tissue matrix; hPDSCs: Human placenta-derived mesenchymal stem cells; N-chitosan: N-carboxyethyl chitosan; PEG: Poly(ethylene glycol); PEGDA: Polyethylene glycol diacrylate; PVA: Polyvinyl alcohol; rBMSCs: Rat bone marrow-derived mesenchymal stem cells; SNAP: S-nitroso-N-acetyl-penicillamine; STZ: Streptozotocin; UMSCs: Umbilical cord-derived mesenchymal stem cells; VEGF: Vascular endothelial growth factor.

FOOTNOTES

Author contributions: Li Y designed the research study; Huang JN and Cao H searched the literature and drafted the initial manuscript; Liang KY and Cui LP provided further revision; all authors have read and approved the final version of the manuscript.

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Role of defensins in diabetic wound healing

Zhi-Xiang Tan, Rui Tao, Si-Cheng Li, Bing-Zheng Shen, Lan-Xia Meng, Zhan-Yong Zhu

Abstract

The adverse consequences resulting from diabetes are often presented as severe complications. Diabetic wounds are one of the most commonly occurring complications in diabetes, and the control and treatment of this is costly. Due to a series of pathophysiological mechanisms, diabetic wounds remain in the inflammatory phase for a prolonged period of time, and face difficulty in entering the proliferative phase, thus leading to chronic non-healing wounds. The current consensus on the treatment of diabetic wounds is through multidisciplinary comprehensive management, however, standard wound treatment methods are still limited and therefore, more effective methods are required. In recent years, defensins have been found to play diverse roles in a variety of diseases; however, the molecular mechanisms underlying these activities are still largely unknown. Defensins can be constitutively or inductively produced in the skin, therefore, their local distribution is affected by the microenvironment of these diabetic wounds. Current evidence suggests that defensins are involved in the diabetic wound pathogenesis, and can potentially promote the early completion of each stage, thus making research on defensins a promising area for developing novel treatments for diabetic wounds. In this review, we describe the complex function of human defensins in the development of diabetic wounds, and suggest potential therapeutic benefits.

Key Words: Defensin; Diabetic wound; Wound healing; Inflammation; Re-epithelialization; Tissue regeneration

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Core Tip: Although previous studies have suggested that defensins have a function in the promotion of wound healing, their mechanism is still unclear. In this review, we discuss the potential role of various defensins in refractory diabetic wounds and their properties, including immunoregulation, promotion of re-epithelialization, collagen deposition, vascular regeneration, and neurological recovery, as well as antimicrobial activity.

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INTRODUCTION

Diabetes was estimated to affect over 536.6 million people worldwide in 2021, and an increase in prevalence occurs at a faster rate among middle-income countries[1]. Diabetes mellitus gradually causes a series of complications, such as neuropathy, retinopathy, nephropathy, cardiovascular diseases, and diabetic wounds, as it develops. Due to lesions in the small blood vessels and peripheral nerves that are particularly prominent in the feet, diabetic wounds are usually presented as diabetic foot ulcers (DFUs), and are characterized by a delayed tissue growth and increased susceptibility to infection. Patients with DFUs have an increased risk of lower limb amputation, which has poor short-term prognosis associated with high mortality[2]. Statistically, the five-year mortality and direct costs of care for patients with DFUs have been comparable to that of cancer[3]. The large patient population and medical expenditure urgently requires an effective treatment method.

The mechanisms for diabetic wound development involve multifactorial etiologies, including a hyperglycemic microenvironment, abnormal host immune resistance, and neuropathy (Figure 1)[4]. These mechanisms influence each other, instead of occurring independently, causing irreversible diabetic complications. The local damage to vessels and nerves, reduced growth factors expression, and lower collagen accumulation contribute to repeated outbreaks and the protracted course of diabetic wounds, leading to further infections. Although current treatments, including glycemic control, antibiotic infection, and advanced dressing application, promote wound healing by regulating the local microenvironment, they also have disadvantages, such as protracted treatment periods, high costs, and occasionally inefficient results[5]. Hence, studying methods that promote diabetic wound healing is ongoing.

With concern about antibiotic resistance growing more prominent, antimicrobial peptides (AMPs) have garnered attention as a new method of antibacterial therapy, including development of different formulation strategies for effective delivery to wounds, including AMPs loaded in nanoparticles, hydrogels, creams, gels, etc. As a representative AMP, the defensins properties are gradually being researched (Table 1)[6-23]. Human defensins are divided into α-defensins and β-defensins[22]. Human α-defensins mainly occur in neutrophils (human neutrophil peptide1-4, HNP1-4) or small intestinal Paneth cells (human defensin5-6, HD5-6). More extensively, 31 human β-defensins (HBDs) have been described, and HBD1-4 is most widely-studied[24]. Reportedly, the direct primary role of defensins is controlling microbial infections by killing bacteria and modulating the immune system. Moreover, defensins play different roles in different environments within the body, such as infected wounds, malignancy, atherosclerosis, pulmonary fibrosis, etc. In this review, we focused on investigating their mechanism of action in wound healing, especially chronic diabetic wounds.

Although little is known about defensins involvement in diabetic wounds, existing studies indicate that they play potential roles in complex pathophysiological changes of diabetic wounds[25,26]. This review summarizes and analyzes known experimental data about the role of defensins in diabetic wound healing, particularly for inflammation, cell proliferation and migration, regeneration of blood vessels and nerves, and antibacterial activities. Research articles on the role of defensins in diabetic wounds, published between inception and September 10, 2022, were collected from various search engines, such as PubMed, Google Scholar, Web of Science, and Science Direct using the following keywords: AMPs, defensins, host defense peptides, diabetic, refractory, and chronic wounds, wound healing, etc. Identified studies and relevant citations within these studies were reviewed.

MULTIFACTORIAL MECHANISM OF DEFENSINS DURING WOUND HEALING

The response to tissue injury involves multiple cellular and extracellular events, including inflammation, re-epithelialization, and angiogenesis, followed by fibroplasia with collagen synthesis, and tissue remodeling. Defensins may be a multifactorial modulator in the management of this process,
which interferes in diabetic wounds (Table 2, Figure 2).

**Defensins triggered by inflammation**

The first phase of wound healing is the inflammatory phase, characterized by platelet aggregation and leukocytes migration, including neutrophils and macrophages that secrete defensins and consequently clear the wound area[27]. Studies suggest defensins promote recruitment and accumulation of leukocytes at inflammatory sites, and simultaneously release a series of chemokines[28,29]. In diabetic wounds, the number of neutrophils increases abnormally and macrophage polarization is suppressed, leading to an excessive inflammatory expression[30,31]. As defensins are released in response to inflammation from neutrophils and macrophages, which act as a signal to instigate recruitment of immune cells, a positive-feedback loop is created. HBDs can reportedly participate in degranulation of mast cells and induce secretion of proinflammatory factors by keratinocytes via the p38 and ERK1/2 MAPK pathways activation[32,33]. Through the same action sites, HNP5s produce vasoactive by-products in endothelial cells via ROS-dependent mechanisms, and stimulate the increased expression of IL-6 and IL-8 by activating p42/44 MAPK pathways[34,35].

In contrast, studies investigating associations between defensins and inflammatory mediators exhibited controversial results. HBDs have demonstrated an immunosuppressive effect by down-regulating the TIR, TRAF-6, and NF-κB of TLR signaling pathways[36]. Moreover, HBDs contribute to their anti-inflammatory ability by inducing M2 macrophage differentiation[37]. Previous experiments established that HBDs can be beneficial in inflammatory diseases, such as periodontitis, considering its anti-inflammatory properties[38]. A study on HNP5s from dying neutrophils exhibited an immunosuppressive effect of the α-defensins that inhibited macrophage stimulation[39]. The HNP1 “bipolar effect” represents the reduction of inflammatory responses with a physiological dose, enhanced expression of inflammatory factors with a high dose, and significant reductions in cell viability and interleukin-10 expression with increased concentration levels[40]. Overall, defensins perform different functions under different conditions, including concentration levels[40]. Defensins are often used as disease-related markers as dysregulation of their levels is caused by immune system disorders and effectors produced themselves or through associated cells[41]. However, the causal relationship and sequence of cascades remain unclear. Several studies emphasized the relationship between delayed wound healing and uncontrolled inflammatory responses, and defensins as efficient adjustors playing a regulatory role in the process.

**Defensins promote skin reconstruction**

Failure to re-epithelialize is one of the most significant indicators for chronic wounds. Re-epithelialization is achieved through keratinocyte migration, proliferation, and differentiation. The HNP1, HBD2, HBD3, and HBD4 can reportedly induce proliferation and migration of keratinocytes, which can consequently secrete HBDs, thereby promoting reconstruction of the cellular barrier to accelerate wound healing[42,43]. Subsequent studies reported that HBD3 enhances phosphorylation of the FGFR1/JAK2/STAT3 pathways to promote keratinocyte proliferation and migration[20]. HBD1 potentially acts as a relevant transcription factor by protecting keratinocytes from apoptosis during epithelial reorganization[44]. In other words, defensins have properties that promote wound epithelial-

---

**Table 1 Defensins play multiple roles in different diseases**

<table>
<thead>
<tr>
<th>Defensin</th>
<th>Main cellular source</th>
<th>Action</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-defensin</td>
<td>HNP1 Neutrophils; monocytes; macrophages; natural killer cells</td>
<td>Increase the healing rate of MRSA-infected wounds[6]; promote hemostasis[7]; t/affect the cardiovascular system[8]; inhibit thrombus formation[9]</td>
</tr>
<tr>
<td>HNP2-3</td>
<td>Anti-tumor activity[10]</td>
<td></td>
</tr>
<tr>
<td>HD5-6</td>
<td>Intestinal Paneth cells</td>
<td>Reverse dyslipidemia and improve glucoregulatory capacity[12]; anti-tumor ability[13]; amyloid inhibitor[14]</td>
</tr>
<tr>
<td>β-defensin</td>
<td>HBD1 Epithelial cells; monocytes; macrophages</td>
<td>Anti-tumor activity[15]; potentiate osteoclastogenesis[16]</td>
</tr>
<tr>
<td>HBD2</td>
<td>Accelerate wound healing[17]; Oncolytic activity[18]; reduce alcoholic liver injury[19]</td>
<td></td>
</tr>
<tr>
<td>HBD3</td>
<td>Accelerate wound healing[20]; induce IL-8 release and apoptosis in airway smooth muscle cells[21]</td>
<td></td>
</tr>
<tr>
<td>HBD4</td>
<td>Epithelial cells</td>
<td>Stimulate/suppress cancer cell proliferation and viability[22]</td>
</tr>
</tbody>
</table>

HNP: Human neutrophil peptide; HBD: Human β-defensins.
Table 2 Defensins play a potential role in wound healing

<table>
<thead>
<tr>
<th>Stage</th>
<th>Defensin</th>
<th>Activation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inflammation</td>
<td>HNP1-2, HBD1-3[28]</td>
<td>Recruitment of leukocytes</td>
</tr>
<tr>
<td></td>
<td>HNP1-4[29]</td>
<td>Secretion of inflammatory cytokines like IL-8</td>
</tr>
<tr>
<td></td>
<td>HBD2-4[32]</td>
<td>Activation of the p38 and ERK1/2 MAPK pathways</td>
</tr>
<tr>
<td></td>
<td>HNPI, HBD2[35]</td>
<td>Activation of the p42/44 MAPK pathways</td>
</tr>
<tr>
<td></td>
<td>HBD2, HBD3[36]</td>
<td>Down-regulate the TIR, TRAF-6, NF-xB of TLR signaling pathways</td>
</tr>
<tr>
<td></td>
<td>HBD3[37]</td>
<td>Induce M2 macrophage differentiation</td>
</tr>
<tr>
<td>Re-epithelialization</td>
<td>HNP1[43], HBD2-3[42]</td>
<td>Induce keratinocyte migration and proliferation</td>
</tr>
<tr>
<td></td>
<td>HBD1[44]</td>
<td>Protect keratinocytes from apoptosis</td>
</tr>
<tr>
<td>Collagen synthesis</td>
<td>HNP1[45]</td>
<td>Enhance extracellular matrix deposition</td>
</tr>
<tr>
<td></td>
<td>HBD3[20,37]</td>
<td>Increase the expression of MMP-2, and down-regulate the expression of MMP-9</td>
</tr>
<tr>
<td>Fibroplasia</td>
<td>HNP1[45], HBD2[46]</td>
<td>Promote the proliferation and activation of fibroblasts</td>
</tr>
<tr>
<td>Angiogenesis</td>
<td>HNP1[51], HBD2[52], HBD3[53]</td>
<td>Induce VEGF</td>
</tr>
<tr>
<td></td>
<td>HBD1-4[54]</td>
<td>Induce angiogenin</td>
</tr>
<tr>
<td></td>
<td>HNPI[55]</td>
<td>Inhibit adhesion and migration of endothelial cell</td>
</tr>
<tr>
<td>Nerve reconstruction</td>
<td>HNP1[40]</td>
<td>Promote the recovery of neurological function</td>
</tr>
<tr>
<td></td>
<td>HBD3[57]</td>
<td>Modulate the expression of nerve elongation factors</td>
</tr>
<tr>
<td>Antimicrobial activity</td>
<td>HNP1-4, HBD1-4[61]</td>
<td>Exhibit a broad range of antimicrobial properties</td>
</tr>
<tr>
<td></td>
<td>HBD2[60]</td>
<td>Reduce biofilm formation</td>
</tr>
<tr>
<td></td>
<td>HNPI-3[62]</td>
<td>Neutralize bacterial toxins</td>
</tr>
<tr>
<td></td>
<td>HNP1, HBD1, HBD3[63]</td>
<td>Show synergy of action with antibiotics</td>
</tr>
</tbody>
</table>

HNP: Human neutrophil peptide; HBD: Human β-defensins; MMP: Matrix metalloproteinase.

ization by affecting keratinocyte activity, and thus facilitating early wound closure.

Furthermore, defensins seemingly play an important role in fibroblasts and collagen matrix accumulation, which is essential for dermal reconstitution. HNPI can reportedly promote proliferation and activation of fibroblasts more effectively than HBD2 at the same concentration, and the increased collagen gene expression can only be observed by its stimulation[45]. A study also proved that HBDs indirectly stimulate fibroblast migration by activating protein kinase C[46]. High levels of pro-inflammatory cytokines and inflammatory chemokines in diabetic wounds lead to an increased production of matrix metalloproteinases (MMPs), especially MMP-2 and MMP-9, thereby inhibiting extracellular matrix formation and dermis reconstruction[47,48]. Studies have suggested that the use of an inhibitor for MMP-2 and MMP-9 accelerates wound healing in diabetic mice by maintaining the balance between systematic inflammation and cytokine biosynthesis[49]. HBD3 may potentially reverse the pathological condition as they have shown an inhibitory effect on MMP-9, which may result from cytotoxicity for dendritic cells in high concentrations[50]. Instead, HBD3 reportedly increases the expression of MMP-2, which is essential for angiogenesis and prolonged matrix remodeling[20]. To explain these contradictory findings, further clarification and a comprehensive analysis on the mechanism of wound healing is necessary, as well as verification through specific experiments.

**Defensins involved in regeneration of blood vessels and nerves**

Angiogenesis is a vital physiological process in wound healing and largely regulated by growth factors, specifically vascular endothelial growth factor (VEGF) and angiogenin. HNP1, HBD2, and HBD3 was proven to bind to cell surface receptor proteins, thus promoting VEGF expression and improvement of vascularization[51-53]. The novel role of HBDs in angiogenesis was also identified, revealing that HBD1-4 increases secretion of angiogenin dose-dependently[54]. However, the opposing actions can be described as a consequence of on-site recruitment of distinct subpopulations from circulation. HNP1s can reportedly inhibit adhesion and migration of endothelial cells, and block VEGF-induced endothelial cell proliferation and capillary formation upon inflammatory stimulation[55]. These studies have shed a light on the mechanistic complexity of HNPs angiogenesis.
Figure 1 Mechanism of refractory diabetic wounds. The mainstream views include: Hyperglycemic microenvironment, abnormal immune system and neuropathy. Hyperglycemic microenvironment results in the complex formation of advanced glycation end products (AGEs) and cytokines, as well as circulating progenitor cell dysfunction. AGEs can significantly inhibit the proliferation of endothelial cells and alter the structure of collagen and elastin in the vascular wall, causing microvascular injury in the wound. The hallmarks of abnormal immune system are polymorphonuclear cell dysfunction, late neutrophil infiltration and suppressed macrophage polarization. As a result, diabetic wound healing is delayed and susceptible to bacterial infections and even biofilm formation. Neuropathy is occasion of subcutaneous hemorrhage, ultimately leads to skin breakdown.

Neuropathy caused by diabetes is the influencing factor for subcutaneous hemorrhage underneath the callus formation, ultimately leading to skin breakdown[56]. Studies have proven that HNP1 administration can promote recovery of neurological function following sciatic nerve injury[40]. Additionally, HBD3 modulates the expression of nerve elongation factors that are involved in epidermal hyperinnervation and hypersensitivity to warm sensations[57]. As a result, application of defensins can help prevent delayed treatment due to peripheral neuropathy and difficulty in mastering wound conditions in patients with diabetic wounds.

**Defensins exhibit antimicrobial activity**

Healing of refractory diabetic wounds is often associated with susceptibility to bacterial infections and formation of biofilms[38]. As a class of small cationic molecule peptides with broad-spectrum antimicrobial activity, defensins are produced to eliminate invading pathogens during the initial stages of wound formation[59]. While the important role of the pore-formation mechanism has been recognized in many studies, other mechanisms, such as disruption of cell wall synthesis, metabolic activity, ATP and nucleic acid synthesis, and amino acid uptake, have also been proposed in recent years[60]. HNPs and HBDs both exhibit a strong tendency to eliminate various pathogens, including *Staphylococcus aureus* and *Escherichia coli*, which often invades chronic wounds[61]. Specifically, HBD2 exhibits biofilm inhibitory activity by inducing structural changes that interfere with the biofilm precursor’s transport into the extracellular space[60]. Additionally, HNPs were proven to protect leukocytes from neutralization by gram-positive pathogenic bacterial toxins[62]. Furthermore, they can potentially avoid the emergence of resistance when implemented with other antimicrobial therapies[63]. Defensins are not only more effective against drug-resistant bacteria, as compared to antibiotics, but can also preserve the resident bacteria, despite the lack of target specificity as an intractable problem preventing their use as a therapeutic drug[64].
CONCLUSION

Although there is ambiguity regarding its exact role, refractory healing of diabetic wounds is speculated to result from interactions between multiple pathophysiological changes in the microenvironment of hyperglycemic and persistent inflammation. This affects immune cell function and composition of defensins at the wound site. In human skin, HBD1 is constitutively expressed in epithelial cells, while inducible HNP1-4 by neutrophils and HBD2-3 by keratinocytes mainly[65]. It was obtained through a biopsy that HBD2-4 were overexpressed in the border area of DFUs[25]. Studies generally agree that inadequate HBD expression is associated with poor wound healing, and many methods that promote diabetic wound healing are seemingly carried out by promoting defensins expression[66,67]. In diabetic wounds, higher HNP1, HNP3, and HNP4 expressions are more common in the central part than in the marginal areas, thus causing a significant increase in IL-8 expression under the influence of advanced glycation end products (AGEs)[29].

Defensins affect the expression and secretion of cytokines, cell proliferation, migration, and apoptosis, and are also involved in all stages of wound healing. Contrary to its proven activity in fighting pathogens and promoting tissue reconstruction, the role of defensins in inflammation and vascularization remains unclear. This discrepancy could be due to pro-inflammatory and anti-inflammatory properties being attributed to HBDs at lower concentrations[28,29], compared to antibacterial effects at higher concentrations exhibited in different experiments[48]. Thus, effects of defensins may vary depending on concentration. Furthermore, HNP1 and HBD3 exhibit increased cytotoxic effects with the increased concentration, which can also be related to a greater hydrophobicity[43,68]. Therefore, changing the local distribution or structure of defensins can have beneficial effects and prevent toxic side effects.

Studying every type of defensins within a single experiment is difficult. Additionally, certain defensins can exhibit different or contradictory effects within the same environment due to differences in experimental complexity and aims of the experiment. These factors create a huge obstacle in horizontal comparison among similar experiments. Cytotoxicity caused by defensins is difficult to assess, which indicates that topical application may be more appropriate than the systemic administration. Considering the unstable biochemical properties of defensins, topical application alone may be insufficient. To overcome this limitation, researchers are studying biological dressings as an alternative; however, formulation of an ideal material has not yet been achieved. However, animal studies on defensins exhibit improved healing outcomes, and display stable effects through application of new materials or genetic engineering methods[17,20,69]. These findings present defensins as a promising therapeutic approach owing to modern techniques, such as development of new materials to efficiently load active factors and novel protein sequences to highlight their beneficial effects.

Defensins regulate chronic inflammation, tissue regeneration, angiogenesis, and nerve recovery, as well as antimicrobial properties; therefore, they are a promising treatment for diabetic wounds. There is an urgent need to find the appropriate dosing regimens and develop new biological dressing alterna-
atives to incorporate active factors. Hence, further preclinical investigations are necessary to understand extensive molecular mechanisms of defensins in the treatment of diabetic wounds, and consequently determine suitable therapeutic strategies.

**FOOTNOTES**

**Author contributions:** Tan ZX and Tao R wrote the manuscript and proposed research subtopics; Shen BZ was responsible for navigating the literature, sharing the relevant studies, and drawing the tables included in this review; Meng LX and Li SC drew the figures in the manuscript, formatted citations and compiled references, verified spelling, punctuation, and grammatical errors; Zhu ZY revised and formatted the body of the manuscript, and coordinated the whole work.

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Dietary Nε-(carboxymethyl) lysine affects cardiac glucose metabolism and myocardial remodeling in mice

Zhong-Qun Wang, Zhen Sun

BACKGROUND
Myocardial remodeling is a key factor in the progression of cardiovascular disease to the end stage. In addition to myocardial infarction or stress overload, dietary factors have recently been considered associated with myocardial remodeling. Nε-(carboxymethyl)lysine (CML) is a representative foodborne toxic product, which can be ingested via daily diet. Therefore, there is a marked need to explore the effects of dietary CML on the myocardium.

AIM
To explore the effects of dietary CML (dCML) on the heart.

METHODS
C57 BL/6 mice were divided into a control group and a dCML group. The control group and the dCML group were respectively fed a normal diet or diet supplemented with CML for 20 wk. Body weight and blood glucose were recorded every 4 wk. 18F-fluorodeoxyglucose (FDG) was used to trace the glucose uptake in mouse myocardium, followed by visualizing with micro-positron emission tomography (PET). Myocardial remodeling and glucose metabolism were also detected. In vitro, H9C2 cardiomyocytes were added to exogenous CML and cultured for 24 h. The effects of exogenous CML on glucose metabolism, collagen I expression, hypertrophy, and apoptosis of cardiomyocytes were analyzed.

RESULTS
Our results suggest that the levels of fasting blood glucose, fasting insulin, and serum CML were significantly increased after 20 wk of dCML. Micro-PET showed that 18F-FDG accumulated more in the myocardium of the dCML group than in the control group. Histological staining revealed that dCML could lead to myocardial fibrosis and hypertrophy. The indexes of myocardial fibrosis, apoptosis, and hypertrophy were also increased in the dCML group, whereas the activities of glucose metabolism-related pathways and citrate synthase (CS) were
Cardiovascular disease is the leading cause of mortality worldwide[1]. Myocardial remodeling can lead to decreased cardiac function, which is an important factor in increasing mortality due to cardiovascular disease[2]. Cardiac remodeling is characterized by cardiomyocyte hypertrophy, apoptosis, fibrosis, and increased fibrocollagen deposition[3]. Short-term compensatory remodeling increases cardiac contractility, whereas long-term sustained pathological remodeling leads to a decline in cardiac function or even heart failure[4]. Cardiac remodeling can be caused by myocardial infarction, stress overload, inflammatory cardiomyopathy, idiopathic dilated cardiomyopathy, or diabetes[5]. Although these causes differ, they share similar mechanisms such as oxidative stress, endoplasmic reticulum stress, and inflammatory response[6]. Attempts have been made to improve myocardial remodeling with drugs or other interventions, albeit with unsatisfactory results[7].

Recent studies have shown that in addition to diseases such as diabetes and coronary heart disease, foodborne factors are also associated with myocardial remodeling[8]. Nakamura et al[9] found that carbohydrate content in the diet could affect myocardial remodeling. Zeng et al[10] reported that a high-fat diet promoted myocardial remodeling. Therefore, it is important to identify the key pathogenic components in the diet for the prevention and treatment of myocardial remodeling.

Advanced glycation end products (AGEs) are a class of heterogeneous irreversible products formed by non-enzymatic reactions[11]. AGEs can accumulate in various tissues resulting in adverse health effects by increasing disease pathogenesis[12,13]. AGEs can accumulate via endogenous and exogenous mechanisms. Food is the main source of exogenous AGEs[14]. N-carboxymethyl)lysine (CML) is considered a representative of food-derived AGEs[15]. CML has been found in a variety of foods such as milk, bakery products, and coffee. Ahmed et al[16] reported that the concentration of CML is 877 ± 47 nM in pasteurized milk. Assar et al[17] reported that bread crust contains 46.1 mg/kg of CML. Ingestion of CML via routine diet is substantially higher than the level of CML in plasma and tissues[18]. Daily exposure to high levels of CML is a health risk for humans[19]. The dietary intake of CML is positively correlated with prevalent vertebral fractures[20]. Studies have also shown that foodborne CML can accelerate the progression of atherosclerosis, Alzheimer’s disease, and other diseases[21]. Our previous studies reported that long-term exposure to CML leads to osteogenic differentiation of vascular smooth muscle cells and calcification in diabetic plaques[12]. Exogenous CML can lead to the continuous evolution of atherosclerotic plaques[22]. However, it is currently unknown whether CML intake affects myocardial remodeling.

**INTRODUCTION**

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Glucose metabolism is an important energy source for heart activities. Physiologically, cardiomyocytes uptake and transport glucose via the glucose transporter (Glut) family and obtain energy via aerobic glucose oxidation[23]. Under pathological conditions such as myocardial infarction, glucose metabolism is altered and glucose uptake is increased to counteract the decline in cardiac function[24]. Impaired glucose metabolism is an independent risk factor for the progression of heart failure[25]. However, whether foodborne factors have an impact on myocardial glucose metabolism needs further study.

In the present study, we hypothesized that dietary CML (dCML) can lead to myocardial glucose metabolism dysfunction and myocardial remodeling. We fed mice a CML-supplemented diet and used $^{18}$F-fluorodeoxyglucose (FDG) to track the glucose uptake by the mouse myocardium in vitro and in vivo. Our study provides new insights into the relationship between dCML and myocardial remodeling.

**MATERIALS AND METHODS**

**Animals**

All animal experiments were approved by the Experimental Animal Use Ethics Committee of Jiangsu University and followed the ARRIVE guidelines. Ten-wk-old male C57 BL/6 mice (Cavens, ChangZhou, China) were stored in a light:dark (12:12) cycle environment at a temperature of 26 °C and humidity of 70%. Mice were randomly divided into two groups: control (Ctrl) group and dCML group ($n = 25$). Mice in the Ctrl group were freely administered a standard pelleted diet (XueTong, Nanjing, China). The dCML group received a daily standard pelleted diet mixed with CML (1 g/kg)[26]. The body weight and blood glucose of mice were measured every 4 wk. Blood glucose was detected with a glucose meter (Roche, Basel, Switzerland). After 20 wk of feeding, the fasting insulin levels of the mice were detected via the enzyme-linked immunoassay (ELISA) (Mercodia, Sweden), and the homeostatic model assessment insulin resistance (HOMA-IR) was calculated [$\text{HOMA-IR} = \frac{\text{glucose (mmol/L) } \times \text{insulin (mU/L)}}{22.5}$]. Then the mice were subjected to the oral glucose tolerance test (OGTT)[27]. Each mouse was fasted for 8 h prior to the tests. During fasting, mice had access to adequate water. The serum CML level in mice was detected via ELISA (Cloud-Clone, Wuhan, China). The operation steps followed the instructions. The citrate synthase (CS) in mouse tissues was detected with the Citrate Synthase Activity Detection Kit (Solarbio, Beijing, China). The mouse tissues were homogenized and centrifuged at 11000 g for 10 min. The protein concentration of supernatants was measured using the bicinchoninic acid assay. The samples were reacted with the detection reagent for 10 s or 2 min, and the absorbance at 412 nm was measured. Then the activity was calculated and expressed as nmol/min/mg protein.

**Histology**

Mice were euthanized with carbon dioxide (CO$_2$). Mouse hearts were isolated and the left ventricles were used for subsequent analysis. Masson’s trichrome staining was performed to assess the degree of myocardial fibrosis in mice using an appropriate kit (Solarbio). Myocardial glycogen accumulation was measured using the Glycogen Periodic Acid Schiff (PAS) Stain Kit (Solarbio)[28]. The cytoplasm and nuclei were stained with hematoxylin and eosin (H&E) dye according to the manufacturer’s instructions (Solarbio), followed by imaging using a microscope (Olympus, Tokyo, Japan). Areas testing positive with Masson’s trichrome and PAS stains were measured using ImageJ software.

**Positron emission tomography imaging**

To assess glucose uptake in the mouse myocardium in vivo, $^{18}$F-FDG was used to trace the glucose metabolism and visualized with micro-position emission tomography (PET) (Inveon; Siemens, Munich, Germany)[29]. Mice were fasted for 12 h before scanning. The mice were weighed and anesthetized with isoflurane/oxygen mixture (15-20 mL/L). $^{18}$F-FDG (7.4 MBq, 150 μL) was injected through the tail vein. Micro-PET scanning was performed 2 h after injection for 10 min. The scanned images were iteratively reconstructed with ordered set expectation maximization three-dimensional software. The average level of radioactive material uptake in the cardiac region was analyzed using ASIProVM software. The mean of standard uptake value ($\text{SUV}_{\text{mean}}$) was calculated according to the formula: $\text{SUV}_{\text{mean}} = \frac{\text{cardiac radioactive material uptake (μCi/g)} \times \text{total injected dose (μCi)}}{\text{body weight (g)}}$. Similarly, the in vitro micro-PET scanning of mouse hearts was also performed[30]. Two hours after the injection of $^{18}$F-FDG, the mice were euthanized with CO$_2$ and the hearts were isolated. The isolated heart was placed in a tube filled with ultrasound gel and scanned via micro-PET. The calculation of $\text{SUV}_{\text{mean}}$ of isolated mouse hearts was the same as described above.

**Cell culture**

H9C2 cells (Procell, Wuhan, China) were cultured in Dulbecco’s Modified Eagle Medium supplemented with 100 mL/L fetal bovine serum[31]. Cells were divided into a Ctrl group and a CML group, followed by seeding of $2 \times 10^5$ cells and addition of 10 mmol/L CML to the CML group, and incubated for 24 h.
Cell viability was detected with Cell Counting Kit-8 (C0037; Beyotime, Shanghai, China). All cells were cultured at 37 °C with 50 mL/L CO₂. Collagen I content in H9C2 cells was assessed via immunocytochemical staining. The SP Rabbit & Mouse HRP Kit (CoWin Century, Beijing, China) was used for immunocytochemical staining. Briefly, after fixating in 40 g/L paraformaldehyde for 30 min, the cell samples were washed with phosphate-buffered saline. The samples were incubated with 100 mL/L goat serum and 2.5 mL/L Triton X-100 at room temperature, followed by incubation with primary and secondary antibodies. The primary antibody used for the staining was: anti-collagen I (1:500, 14695-1-AP; Proteintech, Rosemont, IL, United States). To assess the cardiomyocyte area, cells were labeled with Phalloidin (P5282; Sigma-Aldrich, St. Louis, MO, United States). After fixing in 40 g/L paraformaldehyde, the cells were incubated with 2.5 mL/L Triton X-100 for 15 min, followed by labeling with Phalloidin (5 μmol/L) to analyze cell morphology. The nuclei were subsequently stained with DAPI. All stained images were acquired under a microscope (Olympus) and quantified with a computer-assisted image analysis system. Six high-resolution fields in each independent experiment were randomly selected and the area of at least 100 cells was calculated.

Western blotting

The experimental steps were performed as previously described[32]. Protein samples were prepared using RIPA lysis buffer supplemented with protease and phosphatase inhibitors (Beyotime). Proteins were transferred to PVDF membranes after sodium dodecyl sulfate-polyacrylamide gel electrophoresis. After blocking in 50 g/L milk powder for 1 h, the membranes were incubated with suitable diluted primary antibodies overnight. The membranes were incubated with horseradish peroxidase-labeled secondary antibodies and imaged using a chemiluminescence system (Amersham Imager 600; GE Healthcare, Chicago, IL, United States). The acquired images were analyzed using ImageJ software. Primary antibodies used for western blotting were anti-CML (1:1000, ab125145; Abcam, Cambridge, United Kingdom), anti-collagen I (1:500, 14695-1-AP; Proteintech), anti-Glut-1 (1:2000, 21829-1-AP; Proteintech), anti-Glut-4 (1:2000, 66846-1-lg; Proteintech), anti-Akt (1:5000, 10176-2-AP; Proteintech), anti-B-cell leukemia/lymphoma 2 (Bcl-2) (1:2000, 26593-1-AP; Proteintech), anti-AMP-activated protein kinase (AMPK) (1:5000, 66536-1-lg; Proteintech), anti-phospho-AMPK (phospho-Thr183 and Thr172; 1:5000, ab133448; Abcam) and anti-β-actin (1:2000, E1702-67; HUABIO, Hangzhou, China).

Quantitative PCR

Total RNA from tissues/cells was obtained with the RNA-Quick Purification Kit (ES-RN001; YISHAN BIOTECH, Shanghai, China). The mRNA was reverse-transcribed into cDNA using a reverse transcriptase kit (R222; Vazyme Biotech, Nanjing, China)[33]. The SYBR qPCR Master Mix Kit (Q311, Vazyme Biotech) was used to detect the level of each gene. All experimental operations were carried out according to the manufacturer’s instructions. The primer sequences are shown in Table 1.

Statistical analysis

All data were presented as the mean ± SD. The differences between the two groups were analyzed with the Student’s t-test. Statistical significance was set at P < 0.05. All data were analyzed using SPSS 22.0 software.

RESULTS

Dietary CML affects glucose metabolism and insulin resistance in mice

After feeding the mice a diet supplemented with CML, we assessed their body weight and blood glucose every 4 wk until week 20. Body weight of the Ctrl group increased with feeding time, whereas the weight gain of the dCML group slowed down from the 8th wk of feeding (Figure 1A). There was no significant difference in body weight between the Ctrl group and the dCML group. From the 12th wk of feeding, the fasting blood glucose of mice in the dCML group was significantly higher than in the Ctrl group (Figure 1B). Further, we detected the glucose tolerance of mice. The OGTT test showed that the glucose AUC of the dCML group was significantly increased compared with the Ctrl group, suggesting impaired glucose tolerance of the dCML group (Figure 1C and D). There were significant differences in fasting insulin and HOMA-IR values between the two groups of mice. The fasting insulin and HOMA-IR levels of the dCML group were higher than those of the Ctrl group, suggesting insulin resistance in the dCML group (Figure 1E and F). Similarly, the serum CML level of mice in the dCML group was also significantly increased due to the consumption of CML, which was 2.6-fold higher than that in the Ctrl group (Figure 1G).

Dietary CML promotes glucose uptake in mouse myocardium

18F-FDG is a glucose analog that can be used to track the glucose uptake in tissues and organs. Micro-
Table 1 Sequences of the primers used for quantitative PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward, 5’-3’</th>
<th>Reverse, 5’-3’</th>
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</thead>
<tbody>
<tr>
<td>Bax (mouse)</td>
<td>GAACAGATCATGAAGACAGGG</td>
<td>CAGTTCATCTCAAATTCGCC</td>
</tr>
<tr>
<td>Bcl-2 (mouse)</td>
<td>AGGGGGAACACACAGGAGGA</td>
<td>GGTAGCCAGCAGGAGAAGT</td>
</tr>
<tr>
<td>ANP (mouse)</td>
<td>CTTGTGCATGTTGGGTTCGTC</td>
<td>CTTAGGACACTGAGTCGCT</td>
</tr>
<tr>
<td>Glut-1 (mouse)</td>
<td>AGCCGCCCAAGAGTTGAT</td>
<td>GCTTGAATGTTGCTGGAT</td>
</tr>
<tr>
<td>Glut-4 (mouse)</td>
<td>TTGACCGGCTCAGGAGAAG</td>
<td>ATCTCCTTCTGATTCGCTCA</td>
</tr>
<tr>
<td>β-actin (mouse)</td>
<td>TGCAGAGGATGATTGCTGAC</td>
<td>TGCAGATGTTGCTGGAT</td>
</tr>
<tr>
<td>Bcl-2 (rat)</td>
<td>AGTGGGATGCGGGAGATGTG</td>
<td>GGGGCGTACAGTTCCACAA</td>
</tr>
<tr>
<td>ANP (rat)</td>
<td>CCTGACAGCAGGAGAAGT</td>
<td>CTATGCCTCAGGAG</td>
</tr>
<tr>
<td>Glut-1 (rat)</td>
<td>CCTCCTAATCGTCAGAGG</td>
<td>GTGCTACAGTTCCACAA</td>
</tr>
<tr>
<td>Glut-4 (rat)</td>
<td>GACACCTTCTCAATGAG</td>
<td>TGCAGATGTTGCTGGAT</td>
</tr>
<tr>
<td>Cs (rat)</td>
<td>GCCACGATAGATGAG</td>
<td>ACCACAGTCCATGCAAC</td>
</tr>
<tr>
<td>β-actin (rat)</td>
<td>ACCACAGTCCATGCAAC</td>
<td>TCCACACCTGTTGCTGA</td>
</tr>
</tbody>
</table>

ANP: Atrial natriuretic peptide; BAX: Bcl-2-associated X; Bcl-2: B-cell leukemia/lymphoma 2; Cs: Citrate synthase; Glut: Glucose transporter.

Figure 1 Dietary Nε-(carboxymethyl)lysine increases blood glucose and induces insulin resistance in mice. A: Body weight of mice; B: Fasting blood glucose of mice; C: Oral glucose tolerance test (OGTT) test of mice; D: Area under the curve (AUC) of OGTT test; E: Fasting insulin of mice; F: Mouse homeostatic model assessment insulin resistance; G: Mouse serum Nε-(carboxymethyl)lysine (CML) level. Ctrl: Control; dCML: Dietary CML; n = 6, *P < 0.05, **P < 0.01, compared with the Ctrl group.

PET scanning was performed to detect the uptake of 18F-FDG in the mouse myocardium. Compared with the Ctrl group, the myocardial SUV mean of the dCML group was significantly increased (6.40 ± 0.70 vs 3.67 ± 0.60; P < 0.01) (Figure 2A and B). Then the mouse hearts were isolated and detected via micro-PET scanning in vitro. The SUV mean of hearts in the dCML group was still significantly higher than that in the Ctrl group (0.71 ± 0.10 vs 0.41 ± 0.06; P < 0.01) (Figure 2C and D), suggesting that the glucose uptake of the myocardium was increased after supplementation with the CML diet.
Dietary CML promotes myocardial remodeling in mice

Histopathological changes in the myocardium of mice were detected after 20 wk of dCML. Glycogen PAS staining showed a significant increase in the accumulation of glycogen in the myocardium of the dCML group, which was 3.7-fold higher than that of the Ctrl group, suggesting impaired glucose metabolism (Figure 3A and B). Masson staining indicated that myocardial fibrosis was aggravated in the dCML group (Figure 3A and C). H&E staining showed that the cell area in the myocardium of the dCML group was 1.32-fold higher than that of the Ctrl group (Figure 3A and D). The level of CML in the myocardium of the dCML group also significantly increased (Figure 3E and F). Protein levels of collagen 1 and the apoptosis regulator BAX were significantly upregulated, whereas the anti-apoptotic factor Bcl-2 was significantly inhibited (Figure 3E, G, H and I). The mRNA level of the cardiac hypertrophy indicator atrial natriuretic peptide (ANP) was significantly increased in the dCML group (Figure 3J). Compared with the Ctrl group, Bax mRNA was significantly upregulated, whereas Bcl-2 was significantly downregulated in the dCML group, suggesting increased myocardial apoptosis in the dCML group (Figure 3K and L).

Dietary CML inhibits glucose metabolic signaling pathways in mouse myocardium

Glut-1 and Glut-4 play a key roles in myocardial glucose transport[34]. Glut-1 mRNA and protein levels were significantly increased in the mouse myocardium of the dCML group, whereas Glut-4 was significantly decreased (Figure 4A-E). Akt and AMPK signaling are key regulatory pathways in glucose metabolism[34,35]. Compared with the Ctrl group, the dCML group showed significant inhibition of Akt and AMPK activities of the myocardium (Figure 4C, F and G). These results suggest that the myocardial glucose metabolism of mice is impaired after dCML. CS is the rate-limiting enzyme in the aerobic oxidation of glucose. The activity of CS was significantly inhibited in the dCML group (Figure 4H).

Exogenous CML inhibits glucose metabolism and promotes fibrosis, hypertrophy, and apoptosis in cardiomyocytes

Given that dCML inhibits myocardial glucose metabolism and promotes myocardial remodeling in mice, we further investigated the direct effects of CML in vitro. Exogenous stimulation with 10 mmol/L CML did not affect the viability of H9C2 cells (Figure 5A). Exogenous CML stimulation significantly decreased Glut-4 expression and significantly reduced the levels of Akt and AMPK phosphorylation in H9C2 cardiomyocytes, whereas the mRNA and protein levels of Glut-1 were significantly increased (Figure 5B-H). Immunocytochemical staining indicated that CML increased the content of collagen I in the myocardium of these cells (Figure 5I).
Figure 3 Dietary N-(carboxymethyl)lysine increases myocardial fibrosis, hypertrophy and apoptosis in mice. A: Mouse myocardial glycogen Periodic Acid Schiff (PAS) staining, Mason’s trichrome staining, and hematoxylin and eosin staining; Scale 100 μm; B and C: Percentage of PAS-positive and fibrotic areas in the mouse myocardium; D: Relative area of myocardial cells in the myocardium; E-I: Western blotting and its relative level of N-(carboxymethyl)lysine (CML), collagen I, B-cell leukemia/lymphoma 2 (Bcl-2) and Bcl-2-associated X (BAX) in the mouse myocardium; J-L: Atrial natriuretic peptide (ANP), Bax, and Bcl-2 mRNA
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levels in the mouse myocardium. dCML: Dietary CML. n = 6. *P < 0.05, **P < 0.01, compared with the control (Ctrl) group.

Figure 4 Dietary N\(^{\text{ε}}\)-(carboxymethyl)lysine impairs glucose metabolism in mouse myocardium. A and B: mRNA levels of glucose transporter (Glut) -1 and Glut-4 in the mouse myocardium; C-G: Western blotting and its relative quantification of Glut-1, Glut-4, phospho-Akt, and phospho-AMP-activated protein kinase (AMPK) in the mouse myocardium; H: Citrate synthase (CS) activity in the mouse myocardium. dCML: Dietary N\(^{\text{ε}}\)-(carboxymethyl)lysine; Bcl-2: B-cell leukemia/lymphoma 2; BAX: Bcl-2-associated X; ANP: Atrial natriuretic peptide. *P < 0.05, **P < 0.01, compared with the control (Ctrl) group.

cardiomyocytes (Figure 5I and J). Phalloidin staining showed that the cardiomyocyte area in the CML group was significantly increased, and the expression of cardiac hypertrophy marker ANP was also significantly upregulated (Figure 5I, K and L). Meanwhile, the mRNA level of the apoptosis indicator Bax was increased in the CML group, and the level of the anti-apoptotic marker Bcl-2 was significantly downregulated, suggesting that CML induced cardiomyocyte apoptosis (Figure 5M and N). The Cs mRNA level was also decreased in the CML group (Figure 5O).

DISCUSSION

This study investigated the effects of dCML on myocardial glucose metabolism and myocardial remodeling using experimental mouse models and cells. In the in vivo model, myocardial glucose uptake was tracked using \(^{18}\)F-FDG micro-PET scans. The glucose uptake in the dCML group was significantly increased. Histological staining and detection of related molecular indicators suggested that dCML inhibited glucose metabolism in the myocardium and promoted myocardial fibrosis, cardiac hypertrophy, and apoptosis (Figure 6). In vitro, H9C2 cardiomyocytes were treated with exogenous CML to analyze changes in cardiac remodeling and glucose metabolism. Consistent with the in vivo evidence, CML inhibited glucose metabolism and promoted hypertrophy, collagen I expression, and apoptosis of cardiomyocytes. Our study may reveal new clues for underlying foodborne factors associated with myocardial injury and provide new ideas for the prevention and treatment of myocardial remodeling.

AGEs are a class of non-enzymatic reaction products composed of complex components, and the pathogenic role of AGEs has been previously reported[11,12]. The effects of AGEs may be receptor-
Figure 5 Exogenous N\(^{-}\)(carboxymethyl)lysine inhibits the glucose metabolism and promotes collagen I expression, hypertrophy and apoptosis in H9C2 cells. A: Cell viability after the simulation of N\(^{-}\)(carboxymethyl)lysine (CML); B and C: Quantitative PCR detection of glucose transporter (Glut) -1 and Glut-4 mRNA; D-H: Relative expression of Glut-1, Glut-4, phospho-Akt, and phospho-AMP-activated protein kinase (AMPK) in H9C2 cells; I: Upper: Detection of collagen I content with immunocytochemical staining; bottom: Phalloidin-labeled H9C2 cardiomyocytes; J: Quantification of collagen I-positive areas; K: Quantitative analysis of cardiomyocyte size; L-O: Atrial natriuretic peptide (ANP), Bcl-2-associated X (Bax), B-cell leukemia/lymphoma 2 (Bcl-2), and citrate synthase (CS) mRNA levels in H9C2 cardiomyocytes. dCML: Dietary CML. \(n\) = 8 independent experiments. *\(P\) < 0.05, **\(P\) < 0.01, compared with the control (Ctrl) group.

Dependent or receptor-independent. In the receptor-independent pathway, AGEs cross-link with the extracellular matrix and change the physicochemical properties, which affects cell physiology and tissue function[36]. In the receptor-dependent pathway, AGEs bind to cell surface receptors, change the original signal transmission pathway, and lead to pathological outcomes[37]. In addition to AGEs, the precursors of AGEs, such as methylglyoxal, also accumulate in the body. These precursors can play a direct pathogenic role or continue to form AGEs[38]. Adverse effects of dietary AGEs have been...
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Figure 6 Dietary N\(^{\varepsilon}\)-(carboxymethyl)lysine alters myocardial glucose metabolism and promotes myocardial remodeling.

previously reported. Wang et al\[39\] found that dietary AGEs disrupted gut microbiota and induced insulin resistance. Thornton et al\[40\] suggested that dietary AGEs affected ovarian function. A western diet rich in AGEs can also induce changes in the cardiovascular system\[41\]. Given that AGEs are multi-component, but whether each component has a similar effect is unclear. It is still unknown which component plays the most critical role. CML is one of the most active components of AGEs. Due to its ease of formation, CML is also found in high concentrations in food\[42\]. Therefore, our study explored the role of dCML, and showed that dCML can lead to disorders of myocardial glucose metabolism and myocardial remodeling. This finding may provide more evidence underlying the negative effects of foodborne AGEs.

Some studies have also reported the limited pathogenic role of dietary AGEs. Koyama et al\[43\] found no significant association between dietary AGEs and all-cause mortality in adults with diabetes. The double blind parallel study by Linkens et al\[44\] suggested that a short-term AGE diet did not affect the sensitivity, secretion and clearance of insulin, vascular function, and overall inflammation in individuals with abdominal obesity. The Maastricht Study also revealed that dietary AGEs are not associated with stiffness of the aorta or carotid arteries\[45\]. These studies are population-based, suggesting additional confounding factors than in animal models. Moreover, surveys of dietary structure or dietary interventions in these subjects were conducted for relatively short periods of time, which may not be sufficient to represent the long-term dietary habits of individuals. In our study, we focused on myocardial glucose uptake and its remodeling and found adverse effects of dCML. We also evaluated the systemic effects of dCML on mice. Exposure to 20 wk of dCML resulted in glucose intolerance and insulin resistance, but the fasting glucose did not reach the level of diabetes, suggesting a progressive pathogenic effect of dCML. It may take more than 20 wk of a CML diet to further increase blood glucose and worsen insulin resistance. Also, the weight gain of dCML mice became more obvious starting from the 12\(^{th}\) wk. Prolonged dCML time may induce significant changes in mouse body weight.

Myocardium is one of the most energy-consuming organs, with 70% of the energy supply of adult myocardium derived from ATP produced by fatty acid oxidation, and glucose metabolism plays a secondary but important role\[46\]. Under physiological conditions, glucose is converted to pyruvate. ATP is produced via tricarboxylic acid cycle and respiratory chain. Under specific pathological conditions, glucose is the main energy substrate as a result of the reorganization of enzymes involved in energy metabolism. However, glycolysis is the main energy source rather than aerobic oxidation. The energy provided by glycolysis does not meet the long-term needs of myocardial activity. The overall cardiac metabolic activity is subsequently reduced, eventually leading to cardiomyocyte apoptosis and malignant remodeling of the myocardium\[47\]. However, an increase or decrease in myocardial glucose metabolism is also associated with specific pathological changes. For example, in diabetic cardiomyopathy, the lipotoxicity caused by diabetes increases the fatty acid metabolism in the heart, thus inhibiting glucose metabolism\[48\]. The interaction between fatty acids and glucose metabolism is also known as Randle Cycle\[49\]. However, the relationship between myocardial metabolic reprogramming and myocardial pathological remodeling is unclear. Whether myocardial metabolic disturbance is a cause or a consequence of myocardial remodeling is still inconclusive. In this study, we observed impaired myocardial glucose metabolism but increased glucose uptake after long-term dCML in mice.
The glucose metabolic pathways Akt and AMPK were significantly inhibited. The long-term dCML may alter the metabolic substrates for myocardial energy supply. CS, an enzyme initiating the tricarboxylic acid cycle, was also inhibited after exposure to dCML. Therefore, the myocardium has to absorb more glucose to provide adequate substrates for energy metabolism. The specific mechanism will be further explored in future studies.

To analyze the glucose uptake in the myocardium, we used micro-PET imaging based on the $^{18}$F-FDG probe. Since $^{18}$F-FDG was synthesized in 1969, it has been widely used in the diagnosis, staging and prognostic assessment of clinical diseases. $^{18}$F-FDG is a glucose analog and is therefore involved in glucose processing in vivo. Under pathological conditions, inflammation or hypoxia can lead to impaired glucose metabolism but increased glucose uptake to provide adequate energy. Therefore, $^{18}$F-FDG usually accumulates in the lesions. In the study of cardiovascular disease, $^{18}$F-FDG imaging also plays an important role. $^{18}$F-FDG is the reference standard for molecular imaging of myocardial inflammation. Our study found an increased $^{18}$F-FDG uptake but impaired glucose transport and metabolism in the myocardium of dCML mice, which may be related to the elevated levels of inflammation. Consistent with our study, in the spontaneously hypertensive rat model, myocardial $^{18}$F-FDG imaging SUV was elevated, whereas glucose aerobic oxidation-related transporters and metabolic pathways were significantly inhibited.

We investigated the detrimental effects of dietary CML on myocardial remodeling to draw attention to the CML content in the diet. However, this study has some limitations. The exploration of specific mechanisms needs to be further continued in the future, and clinical evidence is also needed. We speculate that dietary CML may also promote myocardial remodeling through non-receptor and receptor approaches. CML could increase collagen cross-linking in the extracellular matrix and bind to its receptors to activate related signals. In the future, we will further explore the mechanism of cardiac remodeling induced by dietary CML to identify effective targets for intervention. There is an endogenous CML generation system in human body. Compared with reducing endogenous CML, reducing exogenous CML from dietary sources appears to be more controllable. Previous studies have also reported some CML inhibitors, such as antioxidants and aminoguanidine. However, these additives may change the original methods of food production, and their high cost and unclear safety also limit the application. Therefore, in the future, we will also focus on strategies to inhibit CML in diet preparation.

CONCLUSION

Our study focused on the adverse effects of food-derived CML on myocardial glucose metabolism and remodeling. Long-term dCML leads to impaired myocardial glucose metabolism and induces myocardial hypertrophy, fibrosis, and apoptosis. This study offers new clues associated with myocardial remodeling and also provides an experimental basis for dietary planning to prevent cardiovascular disease prevention.

ARTICLE HIGHLIGHTS

Research background
N-ε-(carboxymethyl)lysine (CML), a major component of advanced glycation end products, exists in the daily diet and poses a threat to health after ingestion. It is necessary to evaluate the effect of dietary CML on the heart.

Research motivation
Previous studies have confirmed that the toxic metabolite CML can cause pathological changes in a variety of tissues such as blood vessels and bones. Foodborne CML, as the main source of CML, may lead to cardiac injuries.

Research objectives
To investigate the effects of dietary CML on cardiac remodeling and glucose metabolism.

Research methods
C57 BL/6 mice received a 20-wk CML diet (1 g/kg). The body weight, fasting blood glucose, fasting insulin and serum CML levels of mice were recorded. Exogenous CML was given to establish an in vitro H9C2 cell model. Micro-positron emission tomography was used to evaluate the glucose uptake of the mouse heart. Myocardial remodeling and glucose metabolism were detected by histological/cytological staining, Western blotting, and polymerase chain reaction.
Research results
The 20 wk of CML diet could cause insulin resistance in mice and increase CML levels in serum and heart. Myocardial fibrosis, hypertrophy and apoptosis in mice were significantly aggravated after dietary CML. Moreover, dietary CML increased myocardial glucose uptake but disrupted glucose metabolism. In vitro, exogenous CML inhibited glucose metabolism-related signaling pathways and promoted H9C2 cell hypertrophy, apoptosis and collagen I expression.

Research conclusions
Dietary CML promoted cardiac remodeling and abnormal glucose metabolism.

Research perspectives
This study emphasizes the cardiac hazards of dietary CML and provides new suggestions for the diet preparation in the prevention and treatment cardiovascular diseases.

ACKNOWLEDGEMENTS
We thank the Experimental Animal Center of Jiangsu University for guiding animal experiments.

FOOTNOTES
Author contributions: Sun Z designed the study and wrote the manuscript; Wang ZQ performed the experiments, analyzed the data, and prepared the images; all authors edited and approved the final version of the article.

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Observational Study

Risk factor analysis and clinical decision tree model construction for diabetic retinopathy in Western China

Yuan-Yuan Zhou, Tai-Cheng Zhou, Nan Chen, Guo-Zhong Zhou, Hong-Jian Zhou, Xing-Dong Li, Jin-Rui Wang, Chao-Fang Bai, Rong Long, Yu-Xin Xiong, Ying Yang

Abstract

BACKGROUND
Diabetic retinopathy (DR) is the driving force of blindness in patients with type 2 diabetes mellitus (T2DM). DR has a high prevalence and lacks effective therapeutic strategies, underscoring the need for early prevention and treatment. Yunnan province, located in the southwest plateau of China, has a high prevalence of DR and an underdeveloped economy.

AIM
To build a clinical prediction model that will enable early prevention and treatment of DR.

METHODS
In this cross-sectional study, 1654 Han population with T2DM were divided into groups without (n = 826) and with DR (n = 828) based on fundus photography. The DR group was further subdivided into non-proliferative DR (n = 403) and proliferative DR (n = 425) groups. A univariate analysis and logistic regression analysis were conducted and a clinical decision tree model was constructed.

RESULTS
Diabetes duration ≥ 10 years, female sex, standing- or supine systolic blood
pressure (SBP) ≥ 140 mmHg, and cholesterol ≥ 6.22 mmol/L were risk factors for DR in logistic regression analysis (odds ratio = 2.118, 1.520, 1.417, 1.881, and 1.591, respectively). A greater severity of chronic kidney disease (CKD) or hemoglobin A1c increased the risk of DR in patients with T2DM. In the decision tree model, diabetes duration was the primary risk factor affecting the occurrence of DR in patients with T2DM, followed by CKD stage, supine SBP, standing SBP, and body mass index (BMI). DR classification outcomes were obtained by evaluating standing SBP or BMI according to the CKD stage for diabetes duration < 10 years and by evaluating CKD stage according to the supine SBP for diabetes duration ≥ 10 years.

**CONCLUSION**

Based on the simple and intuitive decision tree model constructed in this study, DR classification outcomes were easily obtained by evaluating diabetes duration, CKD stage, supine or standing SBP, and BMI.

**Key Words:** Diabetic retinopathy; Type 2 diabetes; Western China; Decision tree

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**Core Tip:** Due to the underdeveloped economy and higher prevalence of diabetic retinopathy (DR), Yunnan province is facing a serious task of prevention. Based on a large sample of the Han population with type 2 diabetes mellitus in Yunnan province, this study constructed a cost-effective predictive model that may facilitate the timely and individualized estimation of DR risk.

**INTRODUCTION**

Type 2 diabetes mellitus (T2DM), a common chronic disease, poses a severe threat to human health and quality of life. By 2045, an estimated 552 million people worldwide will suffer from T2DM[1]. Given the socio-economic developments over the past 40 years, China has become increasingly urbanized, with a growing aging population. Moreover, obesity and overweight caused by lifestyle changes contribute to the growing burden of T2DM in China[2]. As a major vascular complication of T2DM, diabetic retinopathy (DR) is currently largely responsible for blindness in the working-class[3,4].

The two major challenges associated with DR include the high disease prevalence and lack of effective treatments. The global prevalence of DR is 34.6%. As of 2011, 126.6 million people suffered from DR, and this number is estimated to reach 191.0 million in 2030 without effective and timely measures[5]. The concerning prevalence and severity of DR worldwide may be modulated by racial/ethnic disparities, socio-economic status, health care systems, lifestyles, research methods, and other factors[6]. Regarding Asian populations, the prevalence of DR varies according to the region. For example, the prevalence of DR is 20.1%[7], 25.7%[8], and 35.0%[9] in Chinese Singaporeans, Chinese Americans, and Taiwanese Chinese, respectively. Further, the prevalence of DR in inland areas of China is 23%. It is also higher in rural areas than in urban areas and in northern areas than in southern and eastern coastal areas[10-12].

Nevertheless, there is currently a paucity of effective treatments for DR. In addition to systematic interventions for controlling blood glucose levels, blood pressure, and blood lipid levels, several modern therapies have been developed, such as laser photocoagulation[13] and intravitreal injections of anti-vascular endothelial growth factor (VEGF) antibodies or glucocorticoids, which can delay the progress of proliferative DR (PDR)[14,15]. However, several side effects associated with these therapies should be noted. For instance, photocoagulation may cause potential retinal damage, anti-VEGF injections are associated with relapse after drug withdrawal, and glucocorticoid use contributes to cataracts and elevated intraocular pressure in a considerable number of patients. Moreover, intraocular injections may cause complications such as endophthalmitis, intraocular hemorrhage, vitreous hemorrhage, and even retinal detachment[14,15]. Therefore, the utilization of these therapeutic options in clinical practice should be based on systematic evaluation and strict indications.
The high prevalence of DR and lack of efficient therapeutic strategies are associated with reduced quality of life of patients and pose a substantial socio-economic burden on individuals, families, and the society[16]. According to an analysis of the pedigree of T2DM in Yunnan province, the prevalence of DR [17] approximates the national average[10]. Therefore, the active search for associated risk factors is a fundamental priority for the prevention of DR. In this regard, a decision tree model established using identified risk factors is a useful tool. Distinct from traditional statistical methods such as logistic regression analysis, decision trees are effective machine-learning algorithms that solve classification problems. This method obtains a set of effective classification rules through systematic learning of multiple attributes of samples with known classification results. When faced with new unknown samples, the choice of classification or characteristic attributes can be quickly obtained based on the set of rules extracted from the established decision tree[18-20]. Thus, a decision tree is a prediction model with a simple and intuitive flowchart structure that is particularly suitable for use in clinical practice. This study examined the risk factors associated with DR in the Han population with T2DM in Yunnan province and constructed a clinical decision tree model.

MATERIALS AND METHODS

Study subjects
Patients from the Han population with T2DM were enrolled from the Department of Endocrinology, Affiliated Hospital of Yunnan University. All patients fulfilled the Chinese Diabetes Association criteria for the diagnosis of T2DM[21]. The criteria for exclusion were as follows: (1) Age < 18 years; (2) positive islet autoantibodies [including islet cell autoantibodies and autoantibodies to glutamic acid decarboxylase-65, insulin, the tyrosine phosphatases islet antigen 2 (IA-2) and IA-2β, and zinc transporter 8]; (3) acute complications of diabetes mellitus (including diabetic ketoacidosis or diabetic hyperosmolar state); (4) severe hepatic damage; (5) malignant tumors; (6) acute or chronic infectious diseases; (7) other eye diseases (including glaucoma, retinal vascular occlusion, and ischemic optic neuropathy); and (8) pregnancy. Finally, 1654 patients with T2DM were enrolled.

Ethical principles
This study was approved by the Ethics Committee of Affiliated Hospital of Yunnan University (No. 2021062), and written informed consent was obtained from all participants according to the principles of the Helsinki Declaration.

This trial registration was registered at ChiCTR (ChiCTR2100041888; registration on January 9, 2021, http://www.chictr.org.cn/index.aspx).

Clinical information collection
Patient sex, age, diabetes duration, height, weight, waist circumference, hip circumference, waist-hip ratio (WHR), body mass index (BMI), and systolic blood pressure (SBP) and diastolic blood pressure (DBP) (both in the standing and supine positions) were recorded.

Laboratory assessments
All patients fasted at 22:00 the day before blood collection. At 8:00 the next day, 6 mL of venous blood was collected. Fasting blood glucose (Glu0), hemoglobin A1c (HbA1c), serum creatinine (Scr), blood urea nitrogen (BUN), uric acid (UA), triglycerides (Trig), cholesterol (Chol), high-density lipoprotein Chol (HDL-C), and low-density lipoprotein Chol (LDL-C) were measured. Based on the formula eGFR (mL/min/1.73 m²) = 175 × Scr (mg/dL) - 1.234 × age - 0.179 × (0.790 for women)[22], the estimated glomerular filtration rate (eGFR) was calculated.

Ophthalmological measurements
All patients underwent non-mydriatic fundus photography and were evaluated according to the international clinical grading standards for DR established by the American Academy of Ophthalmology[23].

Definitions
According to the DR Preferred Practice Pattern[23], DR was clinically classified into two types—non-PDR (NPDR) and PDR; the latter was identified by neovascularization and preretinal or vitreous hemorrhage. To further study the effect of blood pressure-related indicators (SBP and DBP in both the standing and supine positions), obesity-related indicators (BMI and waist circumference), blood glucose-related indicators (Glu0 and HbA1c), blood lipid-related indicators (Chol, Trig, LDL-C, and HDL-C), and renal function related indicators (UA and eGFR) on DR, these indicators were defined according to relevant guidelines or expert consensus. Abnormal blood pressure was defined as SBP ≥ 140 mmHg and/or DBP ≥ 90 mmHg[24]. Overweight was defined as BMI ≥ 24 kg/m², obesity was defined as BMI ≥ 28 kg/m², and abdominal obesity was defined as waist circumference ≥ 90 cm in men.
As shown in Decision tree modeling of DR-related risk factors, patients with CKD stages G3 and G4 was 2.109 (95%CI: 1.362 to 3.266) and 2.290 (95%CI: 1.016 to 5.165) times higher than that in patients with CKD stage G1. In other words, a greater severity of CKD was associated with a higher risk of DR.

The risk of progression to PDR in patients with 7% ≤ HbA1c < 8% or HbA1c ≥ 8% was 1.787 (95%CI: 1.198 to 13.512), 9.693 (95%CI: 3.255 to 28.862), and 20.691 (95%CI: 2.540 to 168.581) times higher than that in patients with CKD stage G2, G3, G4, and G5 was 2.206 (95%CI: 1.678 to 2.899), 7.860 (95%CI: 4.573 to 13.512), 9.693 (95%CI: 3.255 to 28.862), and 20.691 (95%CI: 2.540 to 168.581) times higher than that in patients with normal Chol, those with Chol ≥ 6.22 mmol/L had a 1.591 times higher risk of DR (95%CI: 1.046 to 1.919). Compared to patients with supine SBP < 140 mmHg, those with supine SBP ≥ 140 mmHg had a 1.881-fold higher risk of DR (95%CI: 1.399 to 2.528). Compared to patients with normal Chol, those with Chol ≥ 6.22 mmol/L had a 1.591 times higher risk of DR (95%CI: 1.104 to 2.291). The risk of DR in patients with CKD stages G2, G3, G4, and G5 was 2.206 (95%CI: 1.678 to 2.899), 7.860 (95%CI: 4.573 to 13.512), 9.693 (95%CI: 3.255 to 28.862), and 20.691 (95%CI: 2.540 to 168.581) times higher than that in patients with CKD stage G1.

Women had a 1.520 times higher risk of DR [95% confidence interval (CI): 1.218 to 1.897] compared to men (Figure 1). Patients with diabetes duration ≥ 10 years had a 2.118-fold higher risk of DR than those with diabetes duration < 10 years (95%CI: 1.661 to 2.700). The risk of DR in patients with standing SBP ≥ 140 mmHg was 1.417 times higher than that in patients with standing SBP < 140 mmHg (95%CI: 1.046 to 1.881). Patients with diabetes duration ≥ 10 years had a 2.118-fold higher risk of DR than those with diabetes duration < 10 years (95%CI: 1.661 to 2.700). The risk of DR in patients with standing SBP ≥ 140 mmHg was 1.417 times higher than that in patients with standing SBP < 140 mmHg (95%CI: 1.046 to 1.881).

Logistic regression analysis of PDR-related risk factors
Women had a 1.520 times higher risk of DR [95% confidence interval (CI): 1.218 to 1.897] compared to men (Figure 1). Patients with diabetes duration ≥ 10 years had a 2.118-fold higher risk of DR than those with diabetes duration < 10 years (95%CI: 1.661 to 2.700). The risk of DR in patients with standing SBP ≥ 140 mmHg was 1.417 times higher than that in patients with standing SBP < 140 mmHg (95%CI: 1.046 to 1.881).

Logistic regression analysis of PDR-related risk factors
As shown in Figure 1, the importance of variables in the decision tree model was presented as a root-to-

Statistical analysis
Based on fundus photography, participants were divided into groups without DR (WDR group, n = 826) or with DR (DR group, n = 828). The DR group was further classified into the NPDR (n = 403) and PDR (n = 425) groups according to severity. All statistical analyses were performed using SPSS (SPSS 20.0, IBM, United States). Differences between the WDR and DR groups or the NPDR and PDR groups were assessed (Table 1). For continuous variables, Welch’s t-test was used for normal distributions, while for categorical variables, the chi-square test was performed. Using DR or PDR as the dependent variable, logistic regression analysis was explored to analyze DR or PDR-related risk factors. Logistic regression analysis was performed using forward selection (likelihood ratio), with P < 0.05 as the entry criterion and P > 0.1 as the removal criterion. The decision tree method was performed using the chi-squared automatic interaction detector, with 70% participants as the training dataset, and the remaining 30% as the test dataset. The variable assignments used in both logistic regression analysis and decision tree model are presented in Table 2.

RESULTS

Baseline clinical characteristics
The baseline clinical characteristics of all participants are presented in Table 1.

Univariate analysis of DR-related risk factors
As shown in Table 1, the proportion of women, diabetes duration, supine SBP, standing SBP, supine DBP, Chol, BUN, UA, Scr, Glu0, and HbA1c were higher in the DR group than in the WDR group (all P values < 0.05). In contrast, the DR group had a lower BMI, hip circumference, and eGFR than the WDR group (all P values < 0.05).

Univariate analysis of PDR-related risk factors
The proportion of women, supine SBP, supine DBP, Chol, LDL-C, and BUN were significantly higher in the PDR group than in the NPDR group (all P values < 0.05). However, age, hip circumference, and eGFR were significantly lower in the PDR group than in the NPDR group (all P values < 0.05) (Table 1).

Logistic regression analysis of DR-related risk factors
Women had a 1.520 times higher risk of DR [95% confidence interval (CI): 1.218 to 1.897] compared to men (Figure 1). Patients with diabetes duration ≥ 10 years had a 2.118-fold higher risk of DR than those with diabetes duration < 10 years (95%CI: 1.661 to 2.700). The risk of DR in patients with standing SBP ≥ 140 mmHg was 1.417 times higher than that in patients with standing SBP < 140 mmHg (95%CI: 1.046 to 1.881).

Logistic regression analysis of PDR-related risk factors
Compared to men, women had a 2.161-fold higher risk of progression to PDR (95%CI: 1.615 to 2.890) (Figure 2). Compared to patients with diabetes duration < 10 years, patients with diabetes duration ≥ 10 years had a 1.483 times higher risk of PDR (95%CI: 1.099 to 2.001). The risk of progression to PDR in patients with CKD stages G3 and G4 was 2.109 (95%CI: 1.362 to 3.266) and 2.290 (95%CI: 1.016 to 5.165) times higher than that in patients with CKD stage G1.
Table 1 Univariate analysis of risk factors for diabetic retinopathy or proliferative diabetic retinopathy

<table>
<thead>
<tr>
<th>Variable</th>
<th>WDR group (n = 826)</th>
<th>DR group (n = 828)</th>
<th>NPDR group (n = 403)</th>
<th>PDR group (n = 425)</th>
<th>P value¹</th>
<th>P value²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (yr)</td>
<td>53.9 ± 10.3</td>
<td>54.9 ± 10.7</td>
<td>56.2 ± 10.9</td>
<td>53.6 ± 10.3</td>
<td>0.056</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Sex (male/female)</td>
<td>487/339</td>
<td>424/404</td>
<td>238/165</td>
<td>186/239</td>
<td>0.002</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Diabetes duration (yr)</td>
<td>6.9 ± 5.2</td>
<td>11.2 ± 6.8</td>
<td>10.9 ± 7.1</td>
<td>11.4 ± 6.4</td>
<td>&lt; 0.001</td>
<td>0.221</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>165.2 ± 8.3</td>
<td>162.5 ± 8.3</td>
<td>164.2 ± 8.5</td>
<td>160.8 ± 7.8</td>
<td>&lt; 0.001</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>68.1 ± 11.7</td>
<td>64.2 ± 11.3</td>
<td>65.7 ± 11.5</td>
<td>62.8 ± 10.9</td>
<td>&lt; 0.001</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>24.9 ± 3.3</td>
<td>24.3 ± 3.4</td>
<td>24.3 ± 3.3</td>
<td>24.2 ± 3.5</td>
<td>&lt; 0.001</td>
<td>0.880</td>
</tr>
<tr>
<td>Waist circumference (cm)</td>
<td>90.8 ± 9.5</td>
<td>89.9 ± 10.0</td>
<td>90.2 ± 9.7</td>
<td>89.6 ± 10.3</td>
<td>0.060</td>
<td>0.399</td>
</tr>
<tr>
<td>Hip circumference (cm)</td>
<td>97.6 ± 7.6</td>
<td>95.9 ± 7.4</td>
<td>96.4 ± 7.2</td>
<td>95.4 ± 7.6</td>
<td>&lt; 0.001</td>
<td>0.046</td>
</tr>
<tr>
<td>WHR</td>
<td>1.0 ± 0.5</td>
<td>0.9 ± 0.1</td>
<td>0.9 ± 0.1</td>
<td>0.9 ± 0.1</td>
<td>0.284</td>
<td>0.092</td>
</tr>
<tr>
<td>Standing SBP (mmHg)</td>
<td>129.0 ± 16.9</td>
<td>136.4 ± 22.9</td>
<td>135.9 ± 22.6</td>
<td>136.9 ± 23.2</td>
<td>&lt; 0.001</td>
<td>0.517</td>
</tr>
<tr>
<td>Standing DBP (mmHg)</td>
<td>83.4 ± 11.6</td>
<td>83.8 ± 13.4</td>
<td>83.5 ± 13.0</td>
<td>84.0 ± 13.7</td>
<td>0.501</td>
<td>0.578</td>
</tr>
<tr>
<td>Supine SBP (mmHg)</td>
<td>130.1 ± 16.3</td>
<td>141.0 ± 21.3</td>
<td>139.4 ± 20.6</td>
<td>142.4 ± 22.0</td>
<td>&lt; 0.001</td>
<td>0.045</td>
</tr>
<tr>
<td>Supine DBP (mmHg)</td>
<td>82.6 ± 11.2</td>
<td>85.0 ± 12.3</td>
<td>83.9 ± 12.0</td>
<td>86.1 ± 12.4</td>
<td>&lt; 0.001</td>
<td>0.012</td>
</tr>
<tr>
<td>Glu0 (mmol/L)</td>
<td>9.4 ± 3.3</td>
<td>9.9 ± 3.7</td>
<td>10.0 ± 3.7</td>
<td>9.8 ± 3.7</td>
<td>0.008</td>
<td>0.647</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>9.1 ± 2.5</td>
<td>9.8 ± 2.4</td>
<td>9.9 ± 2.5</td>
<td>9.7 ± 2.3</td>
<td>&lt; 0.001</td>
<td>0.402</td>
</tr>
<tr>
<td>Chol (mmol/L)</td>
<td>4.4 ± 1.5</td>
<td>5.0 ± 1.5</td>
<td>4.8 ± 1.5</td>
<td>5.1 ± 1.4</td>
<td>&lt; 0.001</td>
<td>0.013</td>
</tr>
<tr>
<td>Trig (mmol/L)</td>
<td>2.8 ± 2.3</td>
<td>2.6 ± 2.4</td>
<td>2.6 ± 2.5</td>
<td>2.6 ± 2.4</td>
<td>0.033</td>
<td>0.856</td>
</tr>
<tr>
<td>HDL-C (mmol/L)</td>
<td>1.1 ± 0.3</td>
<td>1.1 ± 0.3</td>
<td>1.1 ± 0.3</td>
<td>1.1 ± 0.4</td>
<td>0.057</td>
<td>0.095</td>
</tr>
<tr>
<td>LDL-C (mmol/L)</td>
<td>2.9 ± 0.9</td>
<td>3.0 ± 1.0</td>
<td>2.9 ± 1.0</td>
<td>3.1 ± 1.1</td>
<td>0.101</td>
<td>0.004</td>
</tr>
<tr>
<td>BUN (μmol/L)</td>
<td>5.1 ± 1.7</td>
<td>6.6 ± 2.8</td>
<td>6.4 ± 2.8</td>
<td>6.8 ± 2.8</td>
<td>&lt; 0.001</td>
<td>0.021</td>
</tr>
<tr>
<td>UA (μmol/L)</td>
<td>347.6 ± 103.4</td>
<td>359.4 ± 102.0</td>
<td>355.4 ± 106.4</td>
<td>363.2 ± 97.6</td>
<td>0.019</td>
<td>0.270</td>
</tr>
<tr>
<td>Scr (μmol/L)</td>
<td>69.8 ± 28.3</td>
<td>90.0 ± 61.8</td>
<td>86.0 ± 64.1</td>
<td>93.9 ± 59.4</td>
<td>&lt; 0.001</td>
<td>0.068</td>
</tr>
<tr>
<td>eGFR (mL/min)</td>
<td>116.9 ± 37.3</td>
<td>98.3 ± 42.4</td>
<td>103.5 ± 40.9</td>
<td>93.3 ± 43.2</td>
<td>&lt; 0.001</td>
<td>0.001</td>
</tr>
</tbody>
</table>

¹WDR group vs DR group.
²NPDR group vs PDR group.

WDR: Without diabetic retinopathy; DR: Diabetic retinopathy; NPDR: Non-proliferative diabetic retinopathy; PDR: Proliferative diabetic retinopathy; BMI: Body mass index; WHR: Waist-hip ratio; SBP: Systolic blood pressure; DBP: Diastolic blood pressure; Glu0: Fasting blood glucose; HbA1c: Glycated hemoglobin A1c; Chol: Cholesterol; Trig: Triglyceride; HDL-C: High-density lipoprotein Chol; LDL-C: Low-density lipoprotein Chol; BUN: Blood urea nitrogen; UA: Uric acid; Scr: Serum creatinine; eGFR: Estimated glomerular filtration rate.

leaf structure, with diabetes duration being the first variable or root node, followed by CKD stage, supine SBP, standing SBP, and BMI, in order of importance. As presented in Table 3, seven “if-then” rules summarized the path from the root node to each leaf node.

DISCUSSION

In this study, we attempted to reveal the DR-related risk factors in Han population with T2DM in Yunnan province and construct a predictive model for personalized DR risk assessment and early preventive effect.

Studies have reported that various factors modulate the effects of age on DR. Although sporadic cases have been reported, the onset of DR before puberty is extremely rare[28,29]. Researchers have suggested that patients with diabetes during adolescence are prone to develop serious vascular complications, including DR, compared to patients with diabetes after adolescence. This could be partly due to the characteristics of adolescent patients; for example, patients at this stage tend to be accompanied by dramatic hormone level fluctuations, and most patients present with type 1 diabetes tend to have relatively poor blood glucose self-management ability[30]. Therefore, this study focused on Han
Table 2 Variable assignment

<table>
<thead>
<tr>
<th>Variable</th>
<th>Assignment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex</td>
<td>0 = male</td>
</tr>
<tr>
<td></td>
<td>1 = female</td>
</tr>
<tr>
<td>Age</td>
<td>0 = age &lt; 60 years</td>
</tr>
<tr>
<td></td>
<td>1 = age ≥ 60 years</td>
</tr>
<tr>
<td>Diabetes duration</td>
<td>0 = duration &lt; 10 years</td>
</tr>
<tr>
<td></td>
<td>1 = duration ≥ 10 years</td>
</tr>
<tr>
<td>Blood pressure</td>
<td>0 = normal (SBP &lt; 140 mmHg and/or DBP &lt; 90 mmHg)</td>
</tr>
<tr>
<td></td>
<td>1 = abnormal (SBP ≥ 140 mmHg and/or DBP ≥ 90 mmHg)</td>
</tr>
<tr>
<td>BMI</td>
<td>0 = normal (BMI &lt; 24 kg/m²)</td>
</tr>
<tr>
<td></td>
<td>1 = overweight (24 kg/m² ≤ BMI &lt; 28 kg/m²)</td>
</tr>
<tr>
<td></td>
<td>2 = obesity (BMI ≥ 28 kg/m²)</td>
</tr>
<tr>
<td>Waist circumference</td>
<td>0 = normal (male &lt; 90 cm, female &lt; 85 cm)</td>
</tr>
<tr>
<td></td>
<td>1 = increased (male ≥ 90 cm, female ≥ 85 cm)</td>
</tr>
<tr>
<td>Glu0</td>
<td>0 = well controlled (Glu0 ≤ 7.0 mmol/L)</td>
</tr>
<tr>
<td></td>
<td>1 = poorly controlled (Glu0 &gt; 7.0 mmol/L)</td>
</tr>
<tr>
<td>HbA1c</td>
<td>0 = well controlled (HbA1c &lt; 7%)</td>
</tr>
<tr>
<td></td>
<td>1 = generally controlled (7% ≤ HbA1c &lt; 8%)</td>
</tr>
<tr>
<td></td>
<td>2 = poorly controlled (HbA1c ≥ 8%)</td>
</tr>
<tr>
<td>Blood lipids</td>
<td>0 = normal</td>
</tr>
<tr>
<td></td>
<td>1 = dyslipidemia (met any of the following criteria: TG ≥ 2.26 mmol/L; TC ≥ 6.26 mmol/L; LDL ≥ 4.14 mmol/L; HDL &lt; 1.04 mmol/L)</td>
</tr>
<tr>
<td>UA</td>
<td>0 = normal</td>
</tr>
<tr>
<td></td>
<td>1 = hyperuricemia (UA &gt; 420 μmol/L)</td>
</tr>
<tr>
<td>CKD stage</td>
<td>0 = G1 (eGFR ≥ 90 mL/min)</td>
</tr>
<tr>
<td></td>
<td>1 = G2 (eGFR = 60-89 mL/min)</td>
</tr>
<tr>
<td></td>
<td>2 = G3 (eGFR = 30-59 mL/min)</td>
</tr>
<tr>
<td></td>
<td>3 = G4 (eGFR = 15-29 mL/min)</td>
</tr>
<tr>
<td></td>
<td>4 = G5 (eGFR &lt; 15 mL/min)</td>
</tr>
</tbody>
</table>

SBP: Systolic blood pressure; DBP: Diastolic blood pressure; BMI: Body mass index; Glu0: Fasting blood glucose; HbA1c: Glycated hemoglobin A1c; Trig: Triglyceride; Chol: Cholesterol; LDL-C: Low-density lipoprotein Chol; HDL-C: High-density lipoprotein Chol; UA: uric acid; CKD: Chronic kidney disease; G: Grade; eGFR: Estimated glomerular filtration rate.

population with T2DM aged ≥ 18 years in Yunnan province to exclude the potential confounding effects of adolescence and type 1 diabetes on the results.

DR has emerged as the leading cause of blindness among 27-75 year olds worldwide[3,21]. A Chinese meta-analysis reported that the prevalence of DR in patients with T2DM was age related, increasing from 12.55% in adults aged 30-39 years to 20.44% in adults aged 60-69 years and decreasing to 11.22% in those aged ≥ 80 years[12]. Of the 1654 patients enrolled in this study, 33.49% and 16.56% patients with DR were < 60 years of age (n = 554) and ≥ 60 years of age (n = 274), respectively. This suggests that the peak of DR prevalence in Han population with T2DM in Yunnan province is concentrated in the population aged < 60 years, which accounts for the majority of the social labor force. In addition, univariate analysis (Table 1) revealed no significant difference in age between the DR and WDR groups (P = 0.056). However, overall age was dramatically lower in the PDR group than in the NPDR group (P < 0.001). As shown in Figures 1 and 2, age was not retained in the logistic regression equation. In conclusion, the correlation between age and DR is complex; this association depends on age stratification and may be affected by the degree of vision. This relationship warrants further exploration in future studies.
### Table 3 “If-then” rules extracted from decision tree

<table>
<thead>
<tr>
<th>Rule</th>
<th>If</th>
<th>Then</th>
</tr>
</thead>
<tbody>
<tr>
<td>R1</td>
<td>Diabetes duration &lt; 10 years, CKD stage = G1, standing SBP &lt; 140 mmHg</td>
<td>WDR</td>
</tr>
<tr>
<td>R2</td>
<td>Diabetes duration &lt; 10 years, CKD stage = G1, standing SBP ≥ 140 mmHg</td>
<td>DR</td>
</tr>
<tr>
<td>R3</td>
<td>Diabetes duration &lt; 10 years, CKD stage = G2, BMI &lt; 24 kg/m²</td>
<td>DR</td>
</tr>
<tr>
<td>R4</td>
<td>Diabetes duration &lt; 10 years, CKD stage = G2, BMI ≥ 24 kg/m²</td>
<td>WDR</td>
</tr>
<tr>
<td>R5</td>
<td>Diabetes duration &lt; 10 years, CKD stage = G3/G4/G5</td>
<td>DR</td>
</tr>
<tr>
<td>R6</td>
<td>Diabetes duration ≥ 10 years, supine SBP &lt; 140 mmHg, CKD staging = G1/G5</td>
<td>WDR</td>
</tr>
<tr>
<td>R7</td>
<td>Diabetes duration ≥ 10 years, supine SBP &lt; 140 mmHg, CKD staging = G2/G3/G4</td>
<td>DR</td>
</tr>
<tr>
<td>R8</td>
<td>Diabetes duration ≥ 10 years, supine SBP ≥ 140 mmHg, CKD staging = G1/G2</td>
<td>WDR</td>
</tr>
<tr>
<td>R9</td>
<td>Diabetes duration ≥ 10 years, supine SBP ≥ 140 mmHg, CKD staging = G3/G4/G5</td>
<td>DR</td>
</tr>
</tbody>
</table>

R: Rule; CKD: Chronic kidney disease; SBP: Systolic blood pressure; WDR: Without diabetic retinopathy; DR: Diabetic retinopathy.

### Variable OR (95% CI) P value

<table>
<thead>
<tr>
<th>Variable</th>
<th>OR (95% CI)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age ≥ 60 yr</td>
<td>0.709 (0.556, 0.905)</td>
<td>0.006</td>
</tr>
<tr>
<td>Female sex</td>
<td>1.520 (1.218, 1.897)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Diabetes duration ≥ 10 yr</td>
<td>2.118 (1.661, 2.700)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>24 kg/m² ≤ BMI &lt; 28 kg/m²</td>
<td>0.759 (0.598, 0.963)</td>
<td>0.023</td>
</tr>
<tr>
<td>BMI ≥ 28 kg/m²</td>
<td>0.608 (0.435, 0.851)</td>
<td>0.004</td>
</tr>
<tr>
<td>Standing SBP ≥ 140 mmHg</td>
<td>1.417 (1.046, 1.919)</td>
<td>0.024</td>
</tr>
<tr>
<td>Supine SBP ≥ 140 mmHg</td>
<td>1.881 (1.399, 2.528)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Chol ≥ 6.22 mmol/L</td>
<td>1.591 (1.104, 2.291)</td>
<td>0.013</td>
</tr>
<tr>
<td>Trig ≥ 2.26 mmol/L</td>
<td>0.688 (0.546, 0.867)</td>
<td>0.002</td>
</tr>
<tr>
<td>CKD stage = G2</td>
<td>2.206 (1.678, 2.899)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>CKD stage = G3</td>
<td>7.860 (4.573, 13.512)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>CKD stage = G4</td>
<td>9.693 (3.255, 28.862)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>CKD stage = G5</td>
<td>20.691 (2.540, 168.581)</td>
<td>0.005</td>
</tr>
<tr>
<td>7% ≤ HbA1c &lt; 8%</td>
<td>1.787 (1.198, 2.664)</td>
<td>0.004</td>
</tr>
<tr>
<td>HbA1c ≥ 8%</td>
<td>3.073 (2.225, 4.245)</td>
<td>&lt; 0.001</td>
</tr>
</tbody>
</table>

Figure 1 Logistic regression analysis of diabetic retinopathy-related risk factors. Female sex, diabetes duration ≥ 10 years, standing systolic blood pressure (SBP) ≥ 140 mmHg, supine SBP ≥ 140 mmHg, cholesterol ≥ 6.22 mmol/L, greater severity of chronic kidney disease, and worse control of glycated hemoglobin A1c are associated with a higher risk of diabetic retinopathy. Values are shown using a base 10, logarithmic scale. DR: Diabetic retinopathy; OR: Odds ratio; CI: Confidence interval; BMI: Body mass index; SBP: Systolic blood pressure; Chol: Cholesterol; Trig: Triglyceride; CKD: Chronic kidney disease; HbA1c: Glycated hemoglobin A1c.

The relationship between sex and DR is unclear. A study from Germany and Australia based on 120000 samples suggested that women are more likely to develop DR than men[31]. Similarly, studies in the United Kingdom and Japan have reported that women are more prone to visual impairments than men[32,33]. Other studies from the United States and India, however, have reported that the men have a higher risk of DR than women[6,34,35]. In particular, the United Kingdom Prospective Diabetes Study (UKPDS) has proposed that DR progression is associated with the male sex[36]. Therefore, it is necessary to further explore the correlation between sex and DR. Univariate analysis (Table 1) revealed that the proportion of women was significantly higher in the DR group than in the WDR group (P = 0.002). Moreover, the proportion of women was significantly higher in the PDR group than in the NPDR group (P < 0.001). Indeed, further logistic regression analysis (Figures 1 and 2) re-emphasizes the importance of female sex. These findings suggest that female sex not only is a risk factor for the development of DR in patients with T2DM but also contributes to the progression of DR to PDR, at least
in Yunnan province.

It is well-established that the diabetes duration majorly affects the occurrence and progression of DR [23,37]. A case-control study in South Korea demonstrated that of 523 patients with T2DM, 44.9% developed DR, and 13.6% developed PDR. The average diabetes duration to mild NPDR, moderate-severe NPDR, and PDR was 14.8, 16.7, and 17.3 years, respectively[38]. Based on the Chinese population, a meta-analysis indicated that the prevalence of DR in patients newly diagnosed with diabetes and patients with a disease course of ≥ 10 years was 9.00% and 55.52%, respectively[12]. Univariate analysis (Table 1) revealed that the diabetes duration in the DR group was substantially longer than that in the WDR group (P < 0.001). In contrast, the diabetes duration did not differ significantly between the PDR and NPDR groups (P = 0.221). However, logistic regression analysis (Figures 1 and 2) revealed that a diabetes duration ≥ 10 years was an extremely risk for the occurrence and progression of DR. Crucially, the diabetes duration was classified as the root node of the DR decision tree model (Table 3 and Figure 3), emphasizing that the diabetes duration is critical in DR risk assessment.

In addition to the diabetes duration, good glycemic control is considered a key factor for reducing vascular complications of diabetes[39]. This study focused on two blood-glucose-related indicators, Glu0 and HbA1c. Compared with the transient characteristics of Glu0, HbA1c reflects the overall level of blood glucose control of patients in the prior 2 to 3 months. In this study, univariate analysis (Table 1) indicated that both Glu0 and HbA1c were substantially greater in the DR group than in the WDR group (all P < 0.05). As shown in Figure 1, HbA1c but not Glu0 was retained in the logistic regression equation. These findings suggest that poor HbA1c control is associated with a higher risk of DR. In conclusion, compared to Glu0, HbA1c, which reflects long-term glucose control levels, is more relevant for the prevention of DR. However, HbA1c did not negatively affect the progression of DR. Of note, large clinical studies such as the UKPDS[40,41] and the Diabetes Control and Complications Study[42] have demonstrated that early and intensive glucose control can reduce the occurrence and progression of diabetic vascular complications, including DR. However, good glycemic control is not equivalent to excessive control. Indeed, extensive evidence indicates that recurrent hypoglycemic episodes caused by excessive strict glycemic control with insulin are associated with the early deterioration of DR, but the underlying mechanisms are unclear[43-45].

Previous studies have demonstrated that hypertension is linked to the development and severity of DR[46,47]. Since patients with diabetes are prone to have complications of postural blood pressure changes[48,49], data on standing and supine blood pressure were collected simultaneously. Univariate analysis (Table 1) revealed that standing or supine SBP and supine DBP were significantly lower in the WDR group than in the DR group (all P < 0.001), but there was no obvious difference in standing DBP between the groups (P = 0.501). Although supine SBP and supine DBP were lower in the NPDR group than in the PDR group (all P < 0.05), no significant intergroup differences existed in standing SBP and standing DBP (all P > 0.05). Further logistic regression analysis (Figures 1 and 2) indicated that SBP had an effect on DR occurrence but not DR progression. In addition, supine SBP ≥ 140 mmHg was the leaf node of the decision tree model, second only to diabetes duration. Our results emphasize the detrimental effects of elevated SBP (especially supine SBP) on DR, suggesting that good blood pressure control is vital for the prevention of DR. Furthermore, the benefits of certain antihypertensive drugs, particularly angiotensin-converting enzyme inhibitors and angiotensin receptor blockers, are not limited to lowering blood pressure[50,51] and may also benefit DR through neuroprotection[52,53], increasing insulin sensitivity[54], anti-inflammatory effects[55], and inhibiting the blood-eye barrier[56,57].

To date, the complex link between dyslipidemia and DR has remained controversial[10,47,58,59]. In this study, the occurrence and development of DR seemed to be more strongly affected by Chol than by

---

### Table 1

<table>
<thead>
<tr>
<th>Variable</th>
<th>OR (95%CI)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age ≥ 60 yr</td>
<td>0.458 (0.335, 0.626)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Female sex</td>
<td>2.161 (1.615, 2.890)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Duration of diabetes ≥ 10 yr</td>
<td>1.483 (1.099, 2.001)</td>
<td>0.010</td>
</tr>
<tr>
<td>CKD stage = G2</td>
<td>1.368 (0.966, 1.938)</td>
<td>0.078</td>
</tr>
<tr>
<td>CKD stage = G3</td>
<td>2.109 (1.362, 3.266)</td>
<td>0.001</td>
</tr>
<tr>
<td>CKD stage = G4</td>
<td>2.290 (1.016, 5.165)</td>
<td>0.046</td>
</tr>
<tr>
<td>CKD stage = G5</td>
<td>1.541 (0.443, 5.364)</td>
<td>0.497</td>
</tr>
</tbody>
</table>

---

**Figure 2** Logistic regression analysis of proliferative diabetic retinopathy-related risk factors. Female sex, diabetes duration ≥ 10 years, and chronic kidney disease stage G3 or G4 are risk factors for the progression to proliferative diabetic retinopathy. Values are shown using a base 10, logarithmic scale. Abbreviations: PDR: Proliferative diabetic retinopathy; NPDR: Non-proliferative diabetic retinopathy; OR: Odds ratio; CI: Confidence interval; CKD: Chronic kidney disease.
Figure 3 Training dataset of decision tree model for diabetic retinopathy. Based on the decision tree model constructed in this study, the diabetic retinopathy classification outcomes are obtained by evaluating standing systolic blood pressure (SBP) or body mass index according to the chronic kidney disease (CKD) stage for patients with a diabetes duration < 10 years and the evaluation of CKD stage according to the supine SBP for patients with a diabetes duration ≥ 10 years. WDR: Without diabetic retinopathy; DR: Diabetic retinopathy; CKD: Chronic kidney disease; SBP: Systolic blood pressure; BMI: Body mass index.
Trig, HDL-C, and LDL-C Univariate analysis (Table 1) revealed that Chol was significantly higher in the DR group than in the WDR group ($P < 0.001$) and significantly higher in the PDR group than in the NPDR group ($P = 0.013$). Moreover, as shown in Figures 1 and 2, Chol increased the risk of developing DR in patients with T2DM, but had no remarkable impact on DR progression. A recent meta-analysis revealed that lipid-lowering drugs exerted a protective effect on the progression of DR[60] but did not affect the deterioration of visual acuity or aggravation of hard exudate. Therefore, further large-scale clinical trials are urgently needed to substantiate the necessity of early application of lipid-lowering drugs in patients with DR.

In parallel with the tremendous rise in the global prevalence of obesity, the prevalence of obesity-related T2DM has also increased annually[2]. However, the relationship between obesity and DR has not been fully elucidated. Reports from Wisconsin illustrated that obesity (defined by BMI) was not independently implicated in the occurrence or progression of DR in patients with T2DM within 10 years [61]. Similarly, in the Hoorn study, WHR, but not BMI, was related to the occurrence of DR[62]. However, data based on Asian populations have provided the opposite conclusions. For example, based on a sample of 420 Asian patients with T2DM, Man et al[63] reported that BMI was negatively correlated with mild, moderate, and severe DR, but WHR was positively correlated with DR severity. Subsequently, a Korean study demonstrated that higher BMI, increased waist circumference, and higher body fat content (measured by dual-energy X-ray) were notably correlated with a lower risk of DR[64]. In this study, data regarding relevant body mass indicators were collected, including those on BMI, waist circumference, hip circumference, and WHR. Univariate analysis (Table 1) revealed that BMI and hip circumference in the DR group were remarkably lower than those in the WDR group (all $P < 0.001$), but waist circumference was did not differ across the groups ($P > 0.05$). Although the PDR group had a lower hip circumference than the NPDR group ($P = 0.046$), there were no significant intergroup differences in BMI and waist circumference ($P > 0.05$). In addition, an obvious difference in WHR was not noted among groups (all $P > 0.05$). Although the logistic regression model ultimately did not retain BMI, waist circumference, hip circumference, and WHR (Figures 1 and 2), the decision tree model (Table 3 and Figure 3) supported the protective effect of higher BMI for the assessment of DR risk. In general, although the relationship between these obesity-relevant indicators and DR is yet to be confirmed, it can be conjectured that excessively low BMI is not conducive to protection against DR in the Asian population.

Among BUN, Scr, and UA, eGFR calculated using Scr is the gold standard for CKD staging[65]. In addition, CKD staging is an essential approach to evaluating the severity of diabetic nephropathy in clinical practice. Univariate analysis (Table 1) indicated that eGFR was significantly lower in the DR group than in the WDR group ($P < 0.001$) and was significantly lower in the PDR group than in the NPDR group ($P = 0.001$). Furthermore, Figures 1 and 2 highlight the importance of CKD staging in the risk assessment of DR. In this regard, the risk of occurrence and progression of DR increased stepwise with each additional risk level of CKD staging. Notably, in the decision tree model (Table 3 and Figure 3), CKD staging was second only to diabetes duration for DR risk assessment. In particular, CKD staging is a key indicator of diabetic nephropathy, and the current results also suggest that DR is often comorbid with diabetic nephropathy. This highlights the need to simultaneously screen for diabetic nephropathy in patients with DR.

Differing from traditional statistical methods such as logistic regression analysis, decision trees are successfully employed in the field of medicine with its advantages in solving classification problems, that is, qualitatively judging the possibility of each risk factor at a specific level. Decision trees obtain a set of effective classification rules by systematically learning multiple attributes of the samples with known classification results. When faced with new unknown samples, the appropriate classification or characteristic attributes can be quickly obtained based on the set of rules extracted from the established decision tree[18-20]. In other words, three basic elements compose the decision tree: root node, internal node, and leaf node. The root node is the main feature attribute in the model, the internal node is the secondary attribute judgment based on the root node, and the leaf node is the final classification outcome of the model.

This study extended traditional statistical analysis by building a DR decision model using machine learning based on the attributes of T2DM samples. In the decision tree model, diabetes duration was demonstrated to primarily affect the occurrence of DR in patients with T2DM, namely, the root node. The extraction rules were interpreted as follows: for patients with diabetes duration < 10 years, if they met the criteria of: (1) CKD stage = G3/G4/G5; or (2) supine SBP ≥ 140 mmHg and CKD stage = G3/G4/G5, then DR was prone to occur. In contrast, for patients with diabetes duration ≥ 10 years, if they met the criteria of: (1) Supine SBP < 140 mmHg and CKD stage = G3/G4/G5; or (2) supine SBP ≥ 140 mmHg and CKD stage = G3/G4/G5, then DR was prone to occur. This model may assist clinicians in Yunnan province (particularly primary medical staff who lack relevant DR detection approaches such as ophthalmoscope) to make more effective clinical predictions of DR risk in patients with T2DM. Our decision tree model is simple and intuitive, highlighting its potential for application in clinical practice.

However, there are several limitations that should be noted. First of all, this study did not record in detail the medication of patients, especially the use of anti-diabetic, lipid-lowering and antihypertensive drugs. Secondly, Yunnan province is located in the western plateau of China, and its climate, cultural
and economic conditions are very different from those of plain areas. Therefore, these confounding factors should be included in the future to enhance the integrity and reliability of research conclusions.

CONCLUSION

Female sex, diabetes duration ≥ 10 years, standing or supine SBP ≥ 140 mmHg, Chol ≥ 6.22 mmol/L, deterioration of CKD stage, and HbA1c are key DR-related risk factors in the Han population with T2DM in Yunnan province. The concise and intuitive DR prediction model developed through machine learning in this study could help clinicians quickly predict DR outcomes based on patients' potential risk factors and conduct early individualized interventions.

ARTICLE HIGHLIGHTS

Research background
Yunnan province has a high prevalence of diabetic retinopathy (DR). Accordingly, it is of great significance to explore the DR-related factors and to construct an economic and intuitive clinical prediction model.

Research motivation
The research motivation is early intervention using the DR-related risk factors from the perspective of a predictive model to reduce the prevalence of DR in patients with type 2 diabetes mellitus (T2DM).

Research objectives
The research intends to establish a prediction model that allows clinically early prevention and treatment of DR.

Research methods
A total of 1654 Han population with T2DM were recruited in this study and were grouped in the without DR and DR groups. The DR group was further subgrouped according to the severity of DR. Then, univariate analysis, logistic regression analysis, and clinical decision tree models of clinical data were performed.

Research results
Based on the decision tree model constructed in this study, DR classification outcomes were obtained by evaluating diabetes duration followed by stages of chronic kidney disease, supine systolic blood pressure (SBP), standing SBP, and body mass index.

Research conclusions
Personalized interventions for DR-related risk factors based on a decision tree model may potentially reduce the prevalence of DR.

Research perspectives
In this study, patients with T2DM in Western China were taken as samples to analyze the influencing factors of DR and build a clinical prediction model. In the future, it is hoped that the prediction model can produce certain social and economic benefits in clinical practice. In addition, when comparing with other clinical studies on DR, we found some controversies, such as the impact of sex and body mass index on DR, which opened up a new direction for future research.

FOOTNOTES

Author contributions: Zhou YY contributed to the conception and design, acquisition of data or analysis and interpretation of data, and drafting the article or revising it critically for important intellectual content; Yang Y and Zhou TC were responsible for supervision, project administration, and funding acquisition; Chen N and Zhou GZ were responsible for literature and formal analysis; Wang JR, Bai CF, Long R, Xiong YX, Zhou HJ, and Li XD were responsible for patient recruitment and clinical data curation; all authors gave final approval of the version to be published.

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Institutional review board statement: The study was reviewed and approved by the Ethics Committee of Affiliated Hospital of Yunnan University (Approval No. 2021062).

Informed consent statement: Written informed consent was obtained from all participants.

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.

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L-Editor: A
P-Editor: Chen YL

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Zhou YY et al. Clinical prediction model for diabetic retinopathy


