Journal of Dental Sciences 19 (2024) 310–320

Available online at www.sciencedirect.com

ScienceDirect

journal homepage: www.e-jds.com

Original Article

Impaired pulp healing associated with underlying disorders in the dental pulp of rats with type 2 diabetes

Shuo Tao [a,b], Ting Yang [a,b], Jia-Ni Zhou [a,b], Qi Zhang [a,b*]

[a] *Department of Endodontics, Stomatological Hospital and Dental School of Tongji University, Shanghai, China*
[b] *Shanghai Engineering Research Centre of Tooth Restoration and Regeneration, Shanghai, China*

Received 20 March 2023; Final revision received 29 March 2023
Available online 10 April 2023

KEYWORDS
Diabetes mellitus;
Dental pulp;
Wound healing;
Pathology;
Metabolism;
Advanced glycation end products

Abstract *Background/purpose:* Type 2 diabetes mellitus (T2DM), characterized by hyperglycemia, is a systematic disease affecting structure and healing ability in various tissues. This study aimed to investigate whether T2DM could impair the dental pulp healing and cause underlying pathological changes in the dental pulp before an injury occurred.
Materials and methods: Goto-Kakizaki rats were used as T2DM model animals and performed with direct pulp capping procedures on the first maxillary molars. The molars at 1, 2, 4 weeks after operation and non-injured molars were examined using hematoxylin and eosin staining, immunohistochemical staining, immunofluorescence staining, and Masson's trichrome staining. The fresh dental pulp of maxillary incisors was collected for transmission electron microscopy and glucose content evaluation.
Results: The T2DM rats showed deficient reparative dentin formation compared with the healthy rats. Before the occurrence of an injury, underlying pathological changes of major components in the pulp tissue were observed in T2DM rats, including vasculopathy; collagen abnormalities; decreased proliferation, decreased odontogenetic differentiation and damaged ultrastructure of dental pulp cells. High glucose content and advanced glycation end products accumulation were further found in the pulp tissue in T2DM rats.
Conclusion: T2DM can impede pulp healing process in rats, which is associated with underlying pathological changes in the non-injured pulp caused by the advanced glycation end products accumulation under high-glucose conditions.

* Corresponding author. Department of Endodontics, Stomatological Hospital and Dental School of Tongji University, Shanghai Engineering Research Centre of Tooth Restoration and Regeneration, 399 Middle Yanchang Road, Shanghai, 200072, China.
E-mail address: qizhang@tongji.edu.cn (Q. Zhang).

https://doi.org/10.1016/j.jds.2023.03.021

Introduction

Diabetes mellitus (DM) is a group of metabolic disorders characterized by hyperglycemia. It is considered one of the most common systemic diseases worldwide and its incidence has been increasing progressively each year.[1] Accumulating clinical data have demonstrated that chronic hyperglycemia can cause structural changes and impair self-repairing capability of various tissues in diabetic patients, thus leading to diabetes-related complications, such as diabetic foot ulcer,[2] diabetic retinopathy,[3] and diabetic periodontitis.[4]

With the widespread prevalence of DM, researches on the relationship between DM and dental pulp healing has recently garnered attention. In 2009, Garber et al.[5] firstly reported deficient reparative dentin formation after direct pulp capping procedures in streptozotocin-induced type 1 diabetic rats. Later a few in vitro studies reported that dental pulp cells (DPCs) exhibited aberrant proliferation and odontogenic differentiation in high-glucose (HG) environment.[6,7] The biologic basis of vital pulp therapy (VPT) is the self-renewal and -repair ability of dental pulp, which rely on the proliferation and odontogenic differentiation of DPCs to generate reparative dentin.[8] These researches suggested that DM may impair the self-repair ability and cause the poor wound healing in the dental pulp, thereby resulting in an increased failure risk when administering VPT in the clinic. However, this statement has not been confirmed yet owing to the absence of sufficient in vivo evidence. Hitherto, the effect of type 2 DM (T2DM, ~90% DM) on the healing process of the dental pulp remains unclear. Furthermore, the possible mechanism that underlies the impairment of diabetic pulp healing requires elucidation.

A wide variety of factors is believed to contribute to impaired diabetic wound healing, involving vascular diseases, collagen abnormalities and aberrant cell biological behaviors.[9–11] These lesions, caused by metabolic alterations, could exist in diabetic skin before an injury occurs, thus affecting all phases of wound healing and resulting in a chronic wound.[12] Studies have demonstrated that persistent hyperglycemia can trigger the activation of abnormal metabolic pathways. Nonenzymatic glycation is one of the major metabolic remodeling activities and its biochemical outcome involves the accumulation of advanced glycation end products (AGE).[13] Hyperglycemia and AGE accumulation are identified as characteristic features of metabolic alterations that occur during diabetes, which may disturb the homeostasis in the microenvironment and are responsible for the formation of the abovementioned lesions in non-injured diabetic skin.[13] Similar to the dermis, the dental pulp is a type of connective tissue and it predominantly contains blood vessels (BVs), collagen and fibroblasts (i.e., DPCs), all of which are known to play vital roles in pulp healing.[14,15] However, whether T2DM causes alterations in these major components that are associated with HG contents and AGE accumulation in the non-injured dental pulp remains unclear. It is worth mentioning that the dental pulp has a specific structure feature, which contains a microcirculation system without collateral BVs. It makes the pulp prone to irreversible inflammation, ultimately leading to infection and necrosis.[16] If vasculopathy occurs in the diabetic pulp, the inflammatory response after injury may be more significant, which more likely leads to impaired wound healing.[16]

Therefore, the current study aims to investigate whether T2DM could impair pulp healing and cause underlying pathological changes of major components in the dental pulp before an injury occurs. Addressing the issue may help provide a theoretical basis for the establishment of VPT strategies in diabetic patients.

Materials and methods

Animal procedures

All experimental animal studies were approved by the local ethics committee ([2021]-DW-21). Male Goto-Kakizaki (GK) and Wistar (age, 8 weeks; n = 25) rats were used in the experiments. All rats were obtained from Shanghai SLAC Laboratory Animal Co., Ltd (SLAC, Shanghai, China). All animals in both groups were anesthetized via an intraperitoneal injection of 30 mg/kg pentobarbital sodium (Sigma–Aldrich, St. Louis, MO, USA). Then, 7 rats in each group were sacrificed via intracardiac perfusion with 4% paraformaldehyde (PFA) (Sigma–Aldrich). The dental pulp of bilateral maxillary first molars was collected for follow-up experiments. The remaining animals in each group were used to perform direct pulp capping procedures. The dental pulp of bilateral maxillary first molars in each group was exposed on the mesial half of the occlusal surface with No. 1/4 surgical round burs (Shofu, San Marcos, CA, USA). All cavities were immediately restored with ProRoot MTA (Dentsply, Tulsa, OK, USA) and composite resin (Kerr Corp, Orange, CA, USA). After 1, 2 and 4 weeks postoperatively, the animals were sacrificed via intracardiac perfusion with 4% PFA and maxillary first molars were collected for follow-up experiments.

Histology, immunohistochemical and immunofluorescence staining

After fixing the specimens in 4% PFA for 48 h, they were decalcified for approximately 2 months, embedded in paraffin and cut into 5-μm-thick sections. Hematoxylin and eosin (H&E) staining was performed on these sections (Sangon Biotech, Shanghai, China).

For immunohistochemical staining, the sections underwent antigen retrieval by incubating with hyaluronidase, treatment with 3% hydrogen peroxide, blocking with goat serum blocking agent and incubation with primary antibodies. The following primary antibodies were used: VEGF (1:200; Affinity, Cincinnati, OH, USA), Col1A1 (1:200; Boster, Wuhan, China), Col3A1 (1:200; Boster), DMP1 (1:200; gifts from Dr. Chunlin Qin, College of Dentistry, Texas A&M University, College Station, TX, USA) and AGE (1:200; Abcam, Cambridge, UK). For staining, biotin-labelled secondary antibody, streptavidin peroxidase, and DAB Detection Kit (Maxim, Fuzhou, China) were used according to manufacturer's instructions. The nuclei were counterstained with the hematoxylin solution. In each section, positive signals were counted by a blinded observer in six randomly selected areas from both the dental crown and root at ×200 magnification. Three different sections from

one specimen were examined, with seven specimens in each group. Image-Pro Plus 6.0 (Media Cybernetics, Silver Spring, MD, USA) was used to perform immunohistochemical quantification and analyses.

For immunofluorescence staining, the sections underwent antigen retrieval through incubation with hyaluronidase, treatment with 3% hydrogen peroxide, blocking with goat serum blocking agent and incubation with anti-PCNA primary antibody (1:200; Boster). Then sections were incubated with Alexa Fluor 488 IgG labelled secondary antibody (1:1000; Invitrogen, Carlsbad, CA, USA) and counterstained with DAPI. The ImageJ (National Institute of Health, Bethesda, ML, USA) software was used to measure the percentage of positive nuclei.

Masson's trichrome staining

Collagen fibers were evaluated with Masson's trichrome staining according to manufacturer's instructions (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). The morphometric analysis, corresponding to the blue color, was performed using the ImageJ software (National Institute of Health).

Transmission electron microscopy (TEM)

The fresh dental pulp of maxillary first incisors was collected for TEM analysis in rats aged 8 weeks. The tissue was fixed with 2.5% glutaraldehyde, post-fixed with 0.5% OsO_4, dehydrated with standard ethanol series, embedded in epoxy resin and then cut into 70 nm-thick slides. After heavy metal staining with 2% uranium acetate and 2.6% lead citrate, the slides were observed via TEM (Hitachi, Tokyo, Japan) according to the previously reported protocol.[17] In each slide, the BV diameter, basement membrane (BM) thickness and pericyte coverage were counted at one randomly selected BV. Collagen fiber density was measured at one randomly selected field at $\times$20,000 magnification. Furthermore, the collagen fiber diameter was measured at an interval of 50 pixels in a single randomly selected collagen fiber at $\times$40,000 magnification. For every ten slides, the first slide was observed and a total of three different slides were calculated for each specimen, with seven specimens in each group.

Glucose content assay in pulp tissue and blood glucose test

The fresh dental pulp of maxillary first incisors was collected for evaluation of glucose content in pulp tissue via glucose assay reagent (Beyotime, Shanghai, China) in rats aged 8 weeks. At the meanwhile, the random blood glucose in tail vein of each rat was tested with Accu-Chek Active Blood Glucose Meter (Roche Diagnostics GmbH, Mannheim, Germany).

Statistical analysis

Experiment data were statistically tested using SPSS 21.0 (SPSS Inc, Chicago, IL, USA) and GraphPad Prism 9.0 (GraphPad Prism Software, San Diego, Calif, USA). Data were presented as mean $\pm$ SD or median with the first and third quartile. Comparisons between the two groups were performed using the student's t-test. Fisher exact test was performed to analyze H&E results for the formation of reparative dentin between two groups. Difference was considered statistically significant at the $P < 0.05$ level.

Results

Impaired reparative dentin formation in T2DM rats

To evaluate the reparative dentin formation, histological analysis was performed as shown in Fig. 1A. In the majority of the cases, in the postoperative week 1, extensive diffused reparative dentin was observed in healthy rats, whereas no reparative dentin was observed in T2DM rats accompanied by tissue necrosis, the area of which was confined to the exposed cavity. In the postoperative week 2, a thin reparative dentin bridge was observed in healthy rats; however, no reparative dentin was observed in T2DM rats accompanied by tissue necrosis, which covered an area of approximately 34.4% of the pulp chamber. In the postoperative week 4, a thick dentin bridge was observed in healthy rats, and necrosis covered an area of approximately 85.8% of the pulp chamber in T2DM rats. Regarding statistical analyses, at 4 weeks after operation, 83.3% of cases showed the formation of complete reparative dentin bridge in healthy rats; 8.3% of cases showed no dentin bridge but reparative dentin formation; and 8.3% of samples showed no reparative dentin formation. However, in T2DM rats, only an estimated 7.1% of cases showed complete dentin bridge formation; 35.7% of cases showed no bridge but reparative dentin formation; and 57.2% of cases showed no reparative dentin formation (Table 1).

Morphological changes in the non-injured pulp in T2DM rats

To investigate whether impaired diabetic pulp healing was associated with underlying pathological changes in dental pulp prior to the onset of an injury, we performed the histological analysis in non-injured pulp. The results revealed that the shape of pulp cavity in T2DM rats was irregular and constricted. Furthermore, the arrangement of pulp cells was considerably disordered, and thickened predentin was noted in some areas (Fig. 1B). Considering the morphological changes observed, the histological changes of major components in pulp tissue were further evaluated.

Vasculopathy in the non-injured pulp in T2DM rats

Compared with healthy rats, the vascular density in the pulp tissue in T2DM rats appeared to be significantly increased ($P < 0.05$), which was accompanied by an increase in the expression of VEGF protein in BV wall ($P < 0.01$; Fig. 2A and B). Obvious lumen deformation was observed in terms of structural changes (Fig. 2C); however, the BV diameter showed no significant changes in the T2DM rats when compared with healthy rats (1.26 [0.83—1.99] mm vs. 1.73 [0.88—2.30] mm, $P > 0.05$; Fig. 2D). Vascular BM was significantly thickened in the T2DM rats, 1.67 times

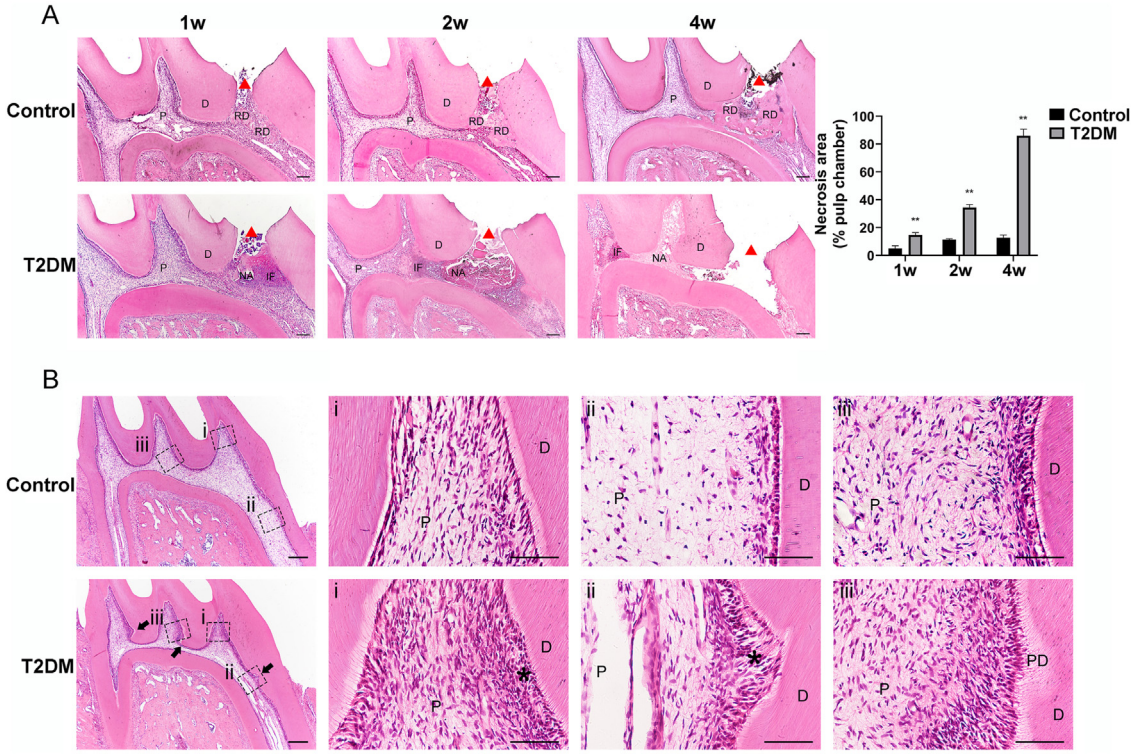

Figure 1 Impaired reparative dentin formation after direct pulp capping procedures and morphological changes in the non-injured dental pulp in T2DM rats. (A) Representative H&E staining images of reparative dentin formation at 1, 2, 4 weeks after direct pulp capping procedures. N = 6 per time point. The red triangles represent the exposed foramen. Scale bar: 400 μm. (B) Representative H&E staining images of non-injured dental pulp. N = 7. The arrows show the irregular shape of pulp cavity. The asterisks show the disordered arrangement of dental pulp cells. Scale bar: 400 μm (left row), 200 μm (right rows). D: dentin; P: pulp; RD: reparative dentin; IF: inflammation infiltration area; NA: necrosis area; PD: predentin. $^{**}P < 0.01$ versus the control group.

Table 1 Proportion of teeth in the type 2 diabetic and healthy rats with dentin bridge formation.

Group	Sample size	Dentin bridge	No bridge but form reparative dentin	No reparative dentin	*P* value for dentin bridge
Control	12	10	1	1	
T2DM	14	1	5	8	<0.01

thicker than that observed in healthy rats (80.15 ± 11.41 nm vs. 47.98 ± 5.00 nm, $P < 0.01$; Fig. 2E). Vascular endothelial cells were damaged in T2DM rats, manefested by mitochondrial swelling, cristae disturbance and disappearance, and the decreased electron density in the mitochondrial matrix (Fig. 2C). Compared with the healthy rats, peripheral cell coverage in T2DM rats appeared to be significantly decreased (15.54 ± 3.52% vs. 41.15 ± 5.72%, $P < 0.01$; Fig. 2F).

Collagen abnormalities in the non-injured pulp in T2DM rats

Compared with the healthy rats, collagen fiber staining was more obvious in the pulp tissues in T2DM rats when observed via optical microscopy ($P < 0.01$; Fig. 3A), whereas the content of type I collagen was decreased ($P < 0.05$), and the content of type III collagen showed no significant changes ($P > 0.05$; Fig. 3B and C). The ratio of type I/III collagen in the pulp tissue in T2DM rats appeared to be significantly decreased (4.15 ± 0.33 vs. 5.45 ± 0.93, $P < 0.05$; Fig. 3D). Notably, no significant change was observed in terms of collagen fiber density in the pulp tissue in T2DM rats via TEM (19.80 ± 3.834 vs. 23.77 ± 5.09, $P > 0.05$; Fig. 3E and F). However, the diameter of collagen fibre showed an irregular increase (39.49 [36.51–44.28] nm vs. 35.62 [33.97–38.75] nm, $P < 0.01$; Fig. 3E, G).

Decreased proliferation, decreased odontogenic differentiation, and damaged ultrastructure of DPCs in the non-injured dental pulp in T2DM rats

Functionally, compared with the healthy rats, the level of PCNA and DMP1 protein in the pulp tissue in T2DM rats were significantly decreased ($P < 0.01$; Fig. 4A and B). This indicated a decreased of cell proliferation and odontogenic differentiation. Structurally, DPCs in the healthy rats were predominantly spindle-shaped, whereas those in rats with T2DM were mostly irregular and showed mitochondrial swelling, cristae disorder and disappearance; decreased electron density in the mitochondrial matrix; and bilayer structure disappeared in some areas. The dilatation and

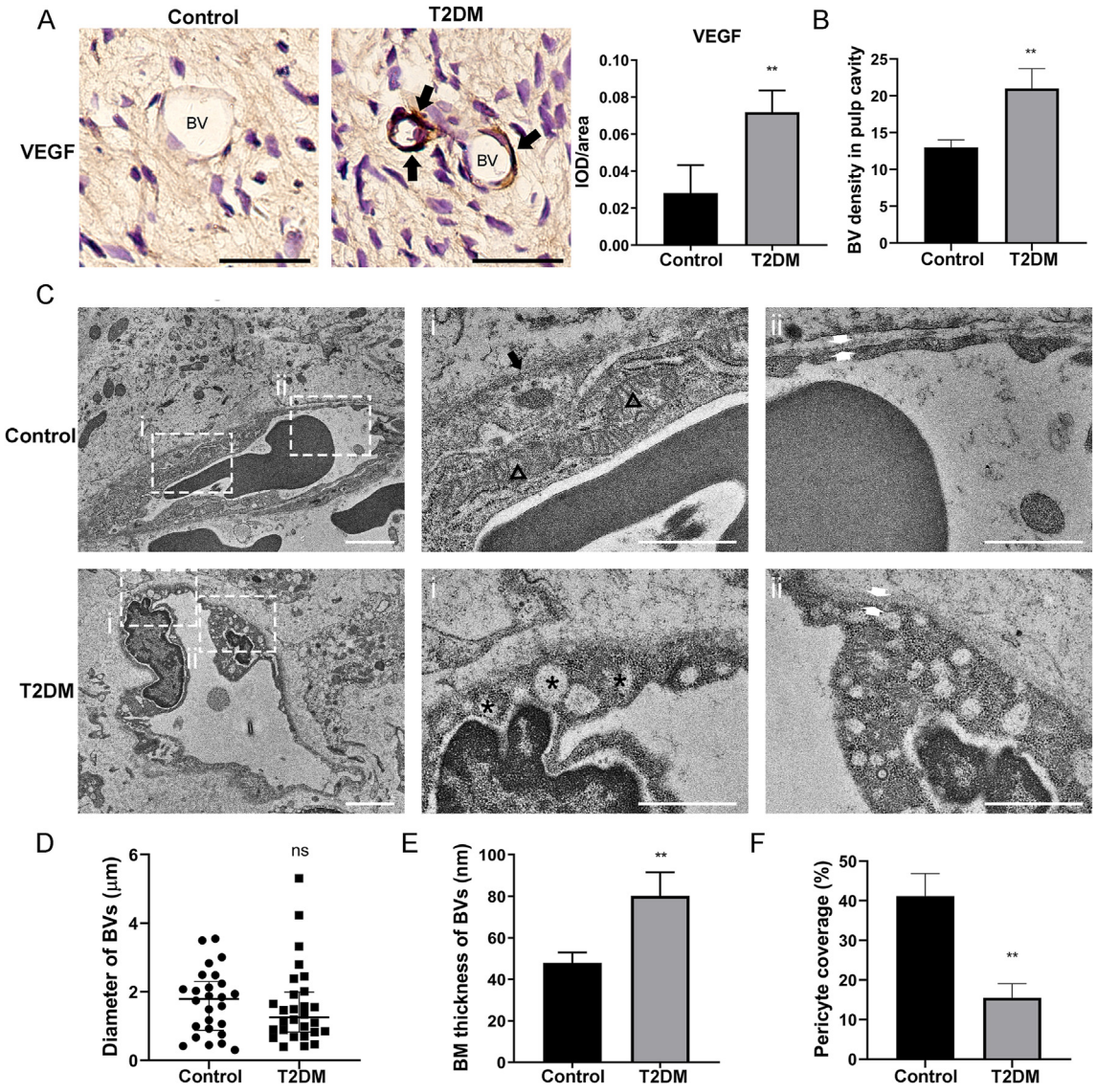

Figure 2 Vasculopathy in the non-injured dental pulp in T2DM rats. (A) Immunohistochemistry staining of VEGF in the pulp tissue. The black arrows indicate the strong positive signals. Scale bar: 100 μm. Right panel shows the quantitative measurement of IOD/area of VEGF. BV: blood vessel. (B) Blood density was calculated on VEGF staining images. (C) TEM images of blood capillary in the pulp tissue. The triangles show the healthy mitochondria. The asterisks show the damaged mitochondria. The black arrow shows the pericyte. The couple white arrows show the basement membrane (BM). Scale bar: 2 μm (left row) and 1 μm (right rows). The diameter of blood capillary diameter (D), BM thickness in capillary (E), and pericyte coverage (F) were calculated on TEM images. Data are presented as the mean ± SD or median with the first and third quartile of seven independent experiments. $^{**}P < 0.01$ versus the control group. NS, non-significantly difference between two different groups.

vesiculation of the rough endoplasmic reticulum (ER) were also observed (Fig. 4C).

High glucose content and AGE accumulation in the non-injured pulp in T2DM rats

T2DM rats showed significantly higher blood glucose levels than healthy rats (18.90 ± 3.05 mmol/L vs. 6.67 ± 0.81 mmol/L, $P < 0.01$). Glucose content in the pulp tissue in T2DM rats also showed a significant increase (12.61 ± 2.72 mg/g tissue vs. 3.25 ± 0.56 mg/g tissue, $P < 0.01$). Immunohistochemical results revealed that the AGE levels in both the BV wall and extracellular matrix (ECM) in the pulp tissue in T2DM rats were significantly higher than those in healthy rats ($P < 0.01$; Fig. 5A–C). In addition, periodic acid-Schiff (PAS)-positive substances were excessively deposited in both the BV wall and ECM in the pulp tissue in T2DM rats when compared with the healthy rats ($P < 0.01$; Fig. S1).

Discussion

In this study, the dental pulp in T2DM rats showed impaired wound healing and underlying disorders before an injury

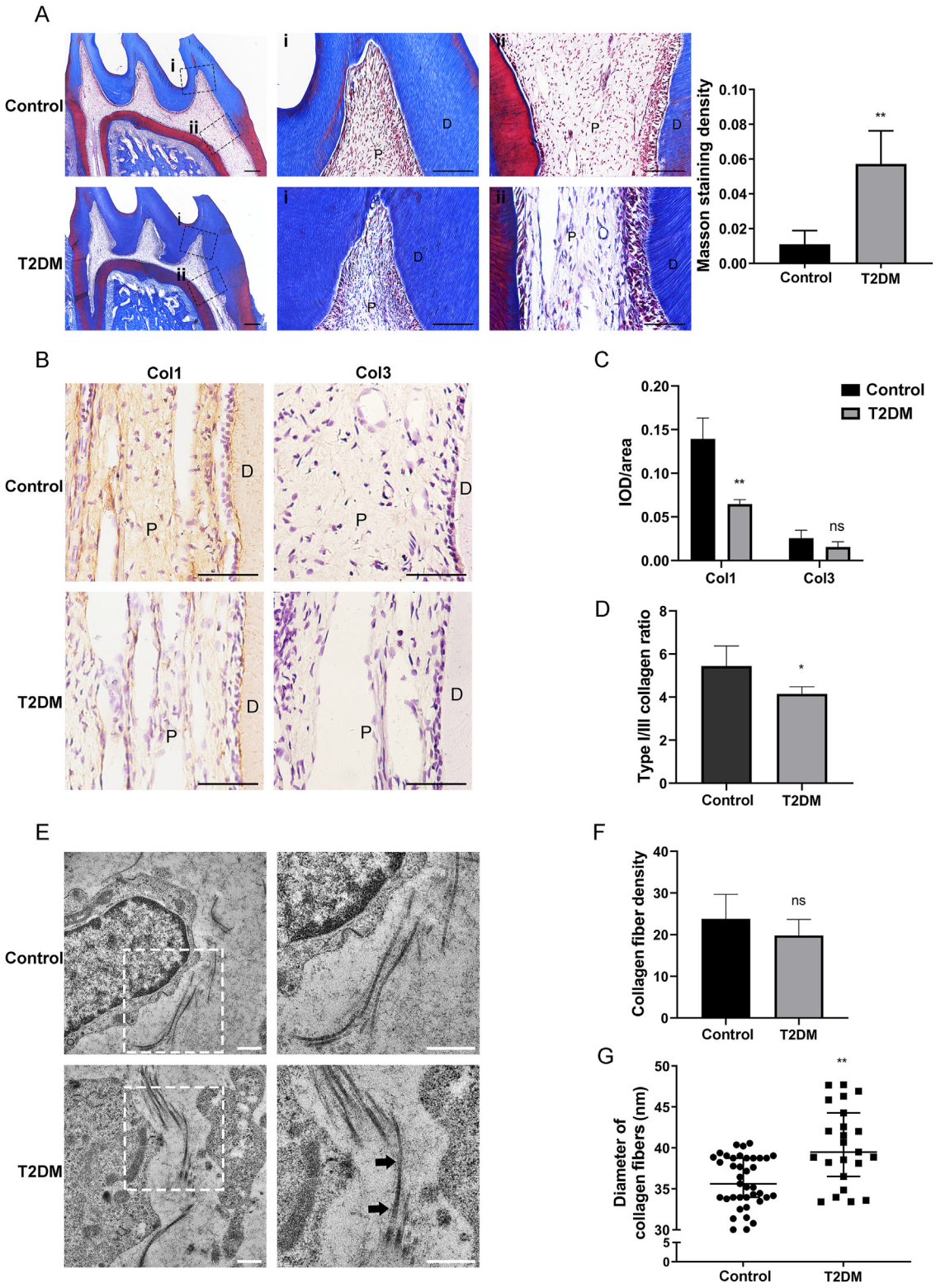

Figure 3 Collagen abnormalities in the non-injured dental pulp in T2DM rats. (A) Masson staining of collagen fibres in the pulp tissue. Scale bar: 200 μm (left row), 400 μm (right rows). Right panel shows the quantitative measurement of Masson staining density, corresponding to the blue colour. (B) Immunohistochemistry staining of Col1 and Col3 in the pulp tissue. Scale bar: 200 μm. (C) The quantitative measurement of IOD/area of type I and III collagen staining, respectively. (D) The ratio of type I/III collagen content was calculated. (E) TEM images of collagen fibres in the pulp tissue. The black arrows show the irregular thickened collagen fibre. Scale bar: 500 nm. The collagen fibre density (F) and diameter of collagen fibres (G) were calculated on TEM images. D: dentin; P: pulp. Data are presented as the mean ± SD or median with the first and third quartile of seven independent experiments. *$P < 0.05$ and **$P < 0.01$ versus the control group. NS, non-significantly difference between two different groups.

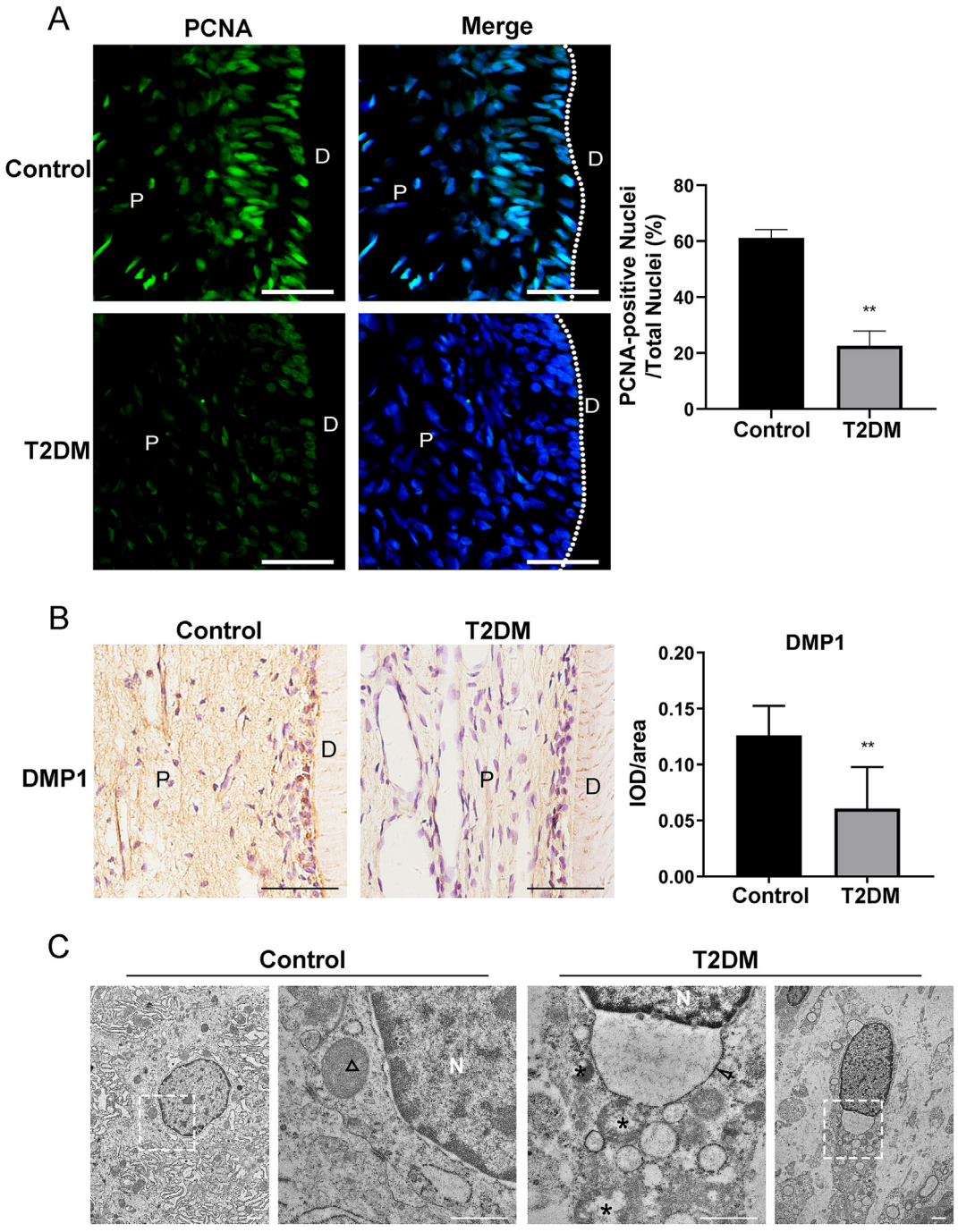

Figure 4 Decreased proliferation, decreased odontogenic differentiation, and damaged ultrastructure of DPCs in the non-injured dental pulp in T2DM rats. (A) Immunofluorescence staining of PCNA in the pulp tissue. Scale bar: 100 μm. Right panel shows the quantitative measurement of the percentage of PCNA-positive nuclei/total nuclei. (B) Immunohistochemistry staining of DMP1 in the pulp tissue. Scale bar: 200 μm. Right panel shows the quantitative measurement of IOD/area of DMP1. (C) TEM images of DPC in the pulp tissue. The triangle shows the healthy mitochondria. The asterisks show the damaged mitochondria. The arrow shows the dilation of endoplasmic reticulum. Scale bar: 1 μm. D: dentin; P: pulp; N: nuclear. Data are presented as the mean ± SD of seven independent experiments. $^{**}P < 0.01$ versus the control group.

occurred. In the clinic, expanding the indications for root canal therapy or proposing a specific VPT strategy for T2DM patients may be necessary.

A previous report on the effect of diabetes on pulp healing after direct pulp capping procedures has been aforementioned,[5] in which streptozotocin-induced type 1 diabetic animal model was used. In this study, GK rat was selected as a spontaneous T2DM animal model, which is a preferrable animal model for T2DM with typical complications such as microangiopathy and macroangiopathy.[18] Our

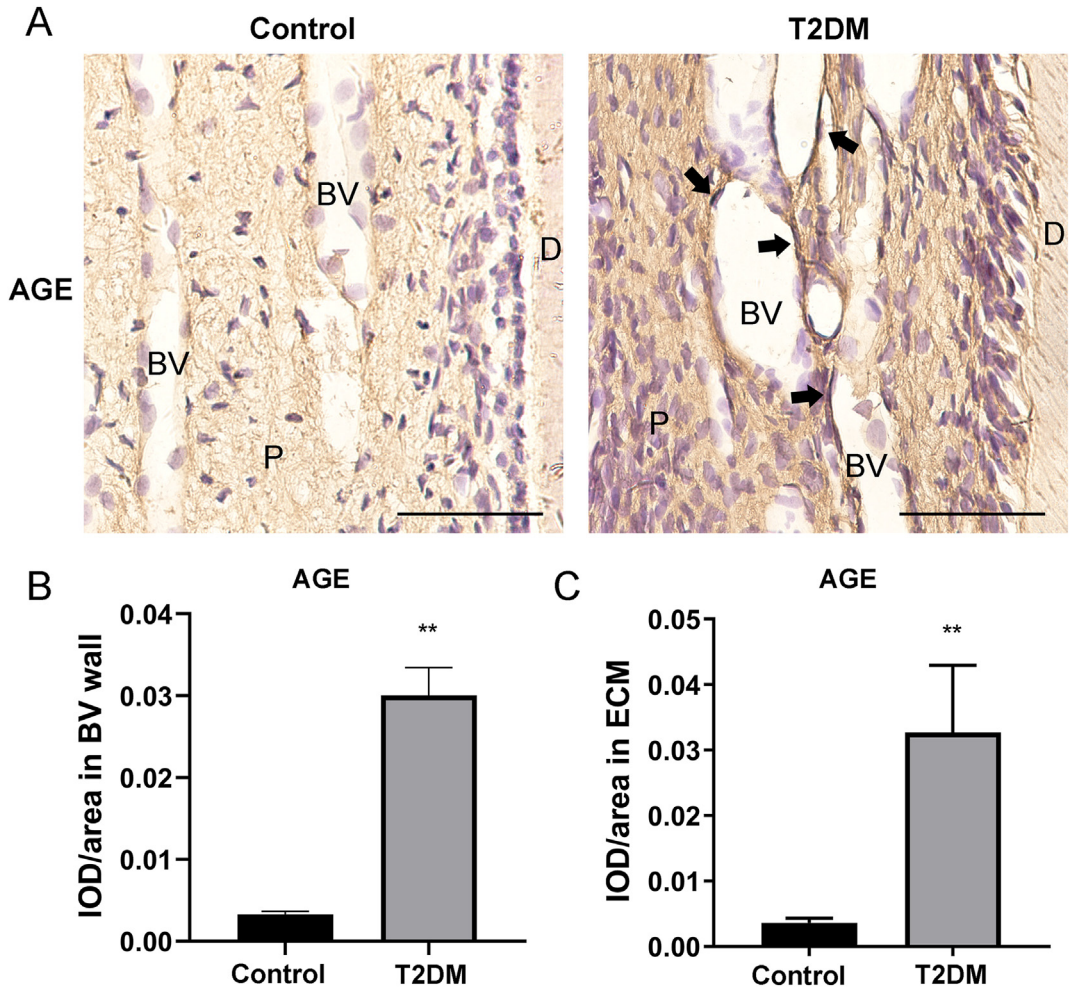

Figure 5 AGE accumulation in the non-injured dental pulp in T2DM rats. (A) Immunohistochemistry staining of AGE in the pulp tissue. Scale bar: 200 μm. The quantitative of IOD/area in BV wall (B) and ECM (C) of AGE levels were measured. The black arrows indicate the strong positive signals. D: dentin; P: pulp; BV: blood vessels. Data are presented as the mean ± SD of seven independent experiments. $^{**}P < 0.01$ versus the control group.

findings revealed that T2DM could also impede the dental pulp healing process in rats, manifested by the deficient formation of reparative dentin after direct pulp capping procedures. Moreover, the success rate of dentin bridge formation at 4 weeks after operation in T2DM rats was lower than that reported by Garber et al., which may be related to the different type or duration periods of DM.[5] In GK rats, hyperglycemia is detectable around 3.5 weeks of age.[19] Thus the duration periods of DM were 4.5 weeks in our study, whereas it was 1 week in the study from Garber et al.[5]

Several factors, involving BVs, collagen and DPCs, are believed to contribute to physical pulp healing.[14,15] Moreover, vasculopathy, collagen abnormalities and fibroblast damage were reportedly known to exist in the diabetic skin before injuries occur, thereby resulting in poor wound healing.[12] Therefore, we further investigated whether these alterations could exist in the diabetic non-injured dental pulp. Vasculopathy in combination with BM thickening, ultrastructural damage of ECs, decreased pericyte coverage, lumen deformation and increased vascular density was observed in the pulp BVs in T2DM rats. BM thickening and damaged ultrastructure of ECs indicated the endothelial dysfunction and were considered as early typical changes in diabetic vasculopathy, which was not conducive to vasodilation and leukocyte migration to the wound site.[20] Additionally, an over-deposition of PAS-positive substances was observed in the pulp BV wall in T2DM rats, which may explain for the BM thickening in the diabetic pulp (Fig. S1). Pericyte, an important component in the BV wall, constitutes the barrier between the BV and interstitial tissue in combination with ECs.[21] Pericyte deficiency may manifest exocentric dilatation and tortuosity in BVs.[22] In the current study, the decreased pericyte coverage may explain the severe lumen deformation in the BVs of the diabetic dental pulp, which may affect local blood flow and interfere with nutrient diffusion. In addition, increased BV density in the diabetic pulp may have occurred due to VEGF overexpression. However, studies previously reported a reduction in the number of BVs in the pulp tissue of patients diagnosed with T2DM for more than one year.[16,23] Considering that the pulp BVs were observed

in the early stages of T2DM (age, 8 weeks) in our study, the duration periods of diabetes may be an important factor affecting angiogenesis. Our data indicated that although the BV density was increased, the endothelial function remained limited and more studies were required to further elucidate this finding. Retrospective analyses of previous studies on the vasculopathy in the diabetic pulp revealed that Russell and Bissada et al.[20] have firstly reported controversial vascular structural changes in the pulp tissue of diabetics in the 1960s and 1970s — the former reported angiopathies and thickened BM and the latter reported no vascular changes in diabetics. However, relevant reports on this issue are lacking and the influence of diabetes on dental pulp microcirculation remains predominantly theoretical. Further research is warranted to acquire additional valuable data on potential structural/molecular alterations in pulp vessels to understand their correlation with poor pulp healing in diabetic patients.

Collagen, the major component of pulp ECM, not only is the structural protein for mineralization but also provides tissue scaffold for DPCs and growth factors during dentin formation.[24,25] Our findings showed that under an optical microscope, collagen fibers were more obvious in the dental pulp in T2DM rats, and these results were consistent with those reported by Moraru et al.[16] Indeed, we found that collagen fiber density did not change in the diabetic pulp when examined via TEM, whereas the diameter of collagen fiber showed irregularly thickening. These data indicated that only qualitative changes occurred in collagen fibers in the early stage of T2DM, and quantitative changes may occur over time. The thickened collagen fibers might transform the pulp from a loose connective tissue into a fibrous tissue in the diabetic pulp, which may impede the formation of the tissue scaffold for cell recruitments, and was not beneficial for mineralization during pulp healing. Furthermore, a decline in the content of type I collagen was observed in the diabetic pulp. A previous report indicated that HG directly down-regulated type I collagen synthesis in pulp tissue in vitro.[26] It raised the possibility that declined content of type I collagen in diabetic pulp may have occurred as a result of the decreased collagen synthesis in DPCs under hyperglycemia condition. Type III collagen, another component of collagen fiber,[24] was found no intergroup changes. Therefore, a relative decline in the ratio of type I/III collagen was noted in the diabetic pulp. Previous studies have reported that this low ratio is attributable to the impairment of the stability of connective tissue as indicated in studies on hernia,[27] which may also facilitate the decrease in the mechanical properties of diabetic skin and impaired diabetic wound healing.[28] This may provide a perspective regarding the pathogenesis of poor diabetic pulp healing. Hitherto, researches on the influence of diabetes on the pulp collagen are scarce. A thorough understanding of diabetic pulp collagen is needed to elucidate its pathological role in diabetic pulp healing.

DPCs are the most abundant cell type in pulp tissue and play pivotal roles in pulp healing.[15] A Few in vitro studies have suggested that HG may inhibit DPC proliferation and odontogenic differentiation.[6,7] The in vivo experiments performed in this study demonstrated that there was a decrease in the proliferation and odontogenic differentiation of DPCs in the pulp tissue in T2DM rats. Furthermore, damaged ultrastructure in DPCs in the diabetic pulp was observed, manifested by mitochondrial swelling, cristae disorder or disappearance and the ER dilatation. An increasing number of studies have reported that mitochondria are critical modulators of various cells, involving osteoblast,[29] keratinocyte,[30] periodontal ligament stem cells[31] under diabtic condition. Recently, studies reported that the ER is a central organelle involved in cellular function and metabolic adaptation. Disturbed ER function, also known as "ER stress," is an essential feature of metabolic disorders, including T2DM.[32] The aberrant ultrastructure of mitochondria and ER observed in our study may provide important clues to the pathogenesis of impaired DPC functions under diabetic conditions.

AGE formation is one of the main characteristics of dysregulated metabolism in HG environment and plays an important role in underlying disorders in diabetic non-injured skin.[13] AGEs are nonenzymatic glycated forms of free amino acids and can cause abnormal crosslinking of extra-cellular and intracellular proteins through the disruption of the normal structure.[33] Components of the connective tissue matrix or BV wall are prime targets, such as collagen, which is abundant in the dental pulp.[34] Nakajima et al.[35] reported a higher AGE content in diabetic pulp tissue, however, the deposition location was not mentioned. In the present study, AGE accumulated extensively in the pulp ECM and BV walls in T2DM rats. Glycosylation modification of collagen can alter its spatial conformation and make it resistant to proteolytic degradation of matrix metalloproteinases, thereby preventing its replacement with new functional fibers. This indicated that the thickening of collagen fibers may be partially a compensatory response to AGE.[36] Studies have reported that AGE can directly cross-link with vascular BM proteins and lead to BM thickening;[33]; AGE in ECs and pericytes can directly impair the conformation and function of intracellular proteins, and result in endothelial dysfunction;[33,37]; AGE can exert a stimulatory role in endothelial cell production of VEGF, which is involved in the development of diabetic retinopathy.[33] These suggested that vasculopathy in the diabetic pulp may be attributable to the AGE accumulation.

Apart from aforementioned histological changes, the morphological changes were the first to be noticed in the dental pulp in T2DM rats, which were constricted pulp cavity and the irregularly thickened predentin. The structural changes in the pulp tissue was previously reported in STZ-induced type 1 diabetic rats, and the possibility was raised that constricted pulp cavity and extend predentin might be due to AGE stimulation, which was further supported by our study.[6,38] However, this causative relationship warrants further elucidation, and the relationship among constricted pulp cavity, thickened predentin and deficient diabetic pulp healing also needs to be investigated. In addition, the pulp matrix in T2DM rats was much more viscous than that in healthy rats; this was observed during sample collection, and it might be associated with the over-deposition of PAS-positive substances (i.e., polysaccharides such as glycogen and emplastic substances such as mucopolysaccharide) in the pulp ECM in T2DM rats (Fig. S1), which further supported the sign of metabolic alterations in the diabetic pulp.

In conclusion, T2DM can inhibit the reparative dentin formation in rats, which may be associated with underlying pathological changes in the dental pulp before an injury occurs, including vasculopathy; collagen abnormalities; decreased proliferation, decreased odontogenic differentiation and damaged ultrastructure of DPCs. Furthermore, these preexisting pathological changes may be attributable to the AGE accumulation under HG condition. In the clinic, it has been a dilemma whether to conduct VPT or extend the indication for root canal treatment in medically compromised diabetic patients. Our study provides a preliminary histological basis for solving this problem; however, this research is relatively incomplete. Additional clinical data need to be collected. Extensive fundamental pathological data is also needed to reveal and describe cellular and molecular mechanisms of recognized alterations in the non-injured pulp and reparative response to injuries during diabetes to provide potential therapeutic targets for VPT in diabetic patients.

Declaration of competing interest

The authors have no conflicts of interest relevant to this article.

Acknowledgments

This work was supported by the National Natural Science Foundation of China [grant numbers 82170945, 81870760]. We appreciate the antibodies provided by Dr. Chunlin Qin (College of Dentistry, Texas A&M University).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jds.2023.03.021.

References

1. Chatterjee S, Khunti K, Davies MJ. Type 2 diabetes. *Lancet* 2017;389:2239—51.
2. Armstrong DG, Boulton AJM, Bus SA. Diabetic foot ulcers and their recurrence. *N Engl J Med* 2017;376:2367—75.
3. Vujosevic S, Aldington SJ, Silva P, et al. Screening for diabetic retinopathy: new perspectives and challenges. *Lancet Diabetes Endocrinol* 2020;8:337—47.
4. Nibali L, Gkranias N, Mainas G, Di Pino A. Periodontitis and implant complications in diabetes. *Periodontol* 2000 2022;90:88—105.
5. Garber SE, Shabahang S, Escher AP, Torabinejad M. The effect of hyperglycemia on pulpal healing in rats. *J Endod* 2009;35:60—2.
6. Inagaki Y, Yoshida K, Ohba H, et al. High glucose levels increase osteopontin production and pathologic calcification in rat dental pulp tissues. *J Endod* 2010;36:1014—20.
7. Yeh CK, Harris SE, Mohan S, et al. Hyperglycemia and xerostomia are key determinants of tooth decay in type 1 diabetic mice. *Lab Invest* 2012;92:868—82.
8. Duncan HF. Present status and future directions-vital pulp treatment and pulp preservation strategies. *Int Endod J* 2022;55(Suppl 3):497—511.
9. Chang M, Nguyen TT. Strategy for treatment of infected diabetic foot ulcers. *Acc Chem Res* 2021;54:1080—93.
10. Patel S, Srivastava S, Singh MR, Singh D. Mechanistic insight into diabetic wounds: pathogenesis, molecular targets and treatment strategies to pace wound healing. *Biomed Pharmacother* 2019;112:108615.
11. Hu SC, Lan CE. High-glucose environment disturbs the physiologic functions of keratinocytes: focusing on diabetic wound healing. *J Dermatol Sci* 2016;84:121—7.
12. Quondamatteo F. Skin and diabetes mellitus: what do we know? *Cell Tissue Res* 2014;355:1—21.
13. Hines A, Alavi A, Davis MDP. Cutaneous manifestations of diabetes. *Med Clin* 2021;105:681—97.
14. Rombouts C, Giraud T, Jeanneau C, About I. Pulp vascularization during tooth development, regeneration, and therapy. *J Dent Res* 2017;96:137—44.
15. Alvarez-Vasquez JL, Castaneda-Alvarado CP. Dental pulp fibroblast: a star cell. *J Endod* 2022;48:1005—19.
16. Moraru AI, GheorghiTa LM, Dascalu IT, et al. Histological and immunohistochemical study on the dental pulp of patients with diabetes mellitus. *Roman J Morphol Embryol = Rev rouma morphol embryol* 2017;58:493—9.
17. Hu XF, Wang L, Lu YZ, et al. Adiponectin improves the osteointegration of titanium implant under diabetic conditions by reversing mitochondrial dysfunction via the AMPK pathway in vivo and in vitro. *Acta Biomater* 2017;61:233—48.
18. Chen D, Wang MW. Development and application of rodent models for type 2 diabetes. *Diabetes Obes Metabol* 2005;7:307—17.
19. Suzuki N, Aizawa T, Asanuma N, et al. An early insulin intervention accelerates pancreatic beta-cell dysfunction in young Goto-Kakizaki rats, a model of naturally occurring noninsulin-dependent diabetes. *Endocrinology* 1997;138:1106—10.
20. Bender I, Bender A. Diabetes mellitus and the dental pulp. *J Endod* 2003;29:383—9.
21. Huang H. Pericyte-endothelial interactions in the retinal microvasculature. *Int J Mol Sci* 2020;21:7413.
22. Santos GSP, Magno LAV, Romano-Silva MA, Mintz A, Birbrair A. Pericyte plasticity in the brain. *Neurosci Bull* 2019;35:551—60.
23. Alsamahi S, Milne TM, Hussaini H, Rich AM, Friedlander LT. Type 2 diabetes and the clinically normal pulp: an in vitro study. *Int Endod J* 2022;55:660—71.
24. Goldberg M, Kulkarni AB, Young M, Boskey A. Dentin: structure, composition and mineralization. *Front Biosci* 2011;3:711—35.
25. Ravindran S, George A. Biomimetic extracellular matrix mediated somatic stem cell differentiation: applications in dental pulp tissue regeneration. *Front Physiol* 2015;6:118.
26. Valikangas L, Pekkala E, Larmas M, Risteli J, Salo T, Tjaderhane L. The effects of high levels of glucose and insulin on type I collagen synthesis in mature human odontoblasts and pulp tissue in vitro. *Adv Dent Res* 2001;15:72—5.
27. Henriksen NA, Yadete DH, Sorensen LT, Agren MS, Jorgensen LN. Connective tissue alteration in abdominal wall hernia. *Br J Surg* 2011;98:210—9.
28. Bermudez DM, Herdrich BJ, Xu J, et al. Impaired biomechanical properties of diabetic skin implications in pathogenesis of diabetic wound complications. *Am J Pathol* 2011;178:2215—23.
29. Buccoliero C, Dicarlo M, Pignataro P, et al. The novel role of PGC1a in bone metabolism. *Int J Mol Sci* 2021;22:4670.
30. Rizwan H, Pal S, Sabnam S, Pal A. High glucose augments ROS generation regulates mitochondrial dysfunction and apoptosis via stress signalling cascades in keratinocytes. *Life Sci* 2020;241:117148.
31. Fang H, Yang K, Tang P, et al. Glycosylation end products mediate damage and apoptosis of periodontal ligament stem cells induced by the JNK-mitochondrial pathway. *Aging* 2020;12:12850—68.

32. Lemmer IL, Willemsen N, Hilal N, Bartelt A. A guide to understanding endoplasmic reticulum stress in metabolic disorders. *Mol Metabol* 2021;47:101169.
33. Lee J, Yun JS, Ko SH. Advanced glycation end products and their effect on vascular complications in type 2 diabetes mellitus. *Nutrients* 2022;14:3086.
34. Singh R, Barden A, Mori T, Beilin L. Advanced glycation end-products: a review. *Diabetologia* 2001;44:129—46.
35. Nakajima Y, Inagaki Y, Kido J, Nagata T. Advanced glycation end products increase expression of S100A8 and A9 via RAGE-MAPK in rat dental pulp cells. *Oral Dis* 2015;21:328—34.
36. Baltzis D, Eleftheriadou I, Veves A. Pathogenesis and treatment of impaired wound healing in diabetes mellitus: new insights. *Adv Ther* 2014;31:817—36.
37. Lin WJ, Ma XF, Zhou HR, et al. Autophagy modulates the migration of retinal pericytes induced by advanced glycation end products. *Evid Based Complement Alternat Med* 2022; 2022:2760537.
38. Lee YH, Kim HS, Kim JS, et al. C-myb regulates autophagy for pulp vitality in glucose oxidative stress. *J Dent Res* 2016;95: 430—8.