Vitamin D Alleviates Type 2 Diabetes Mellitus by Mitigating Oxidative Stress-Induced Pancreatic β-Cell Impairment

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ABSTRACT

Objective Type 2 diabetes mellitus (T2DM) is a common metabolic disorder with rising incidence worldwide. This study explored the anti-T2DM role of vitamin D, thereby providing novel therapeutic strategies.

Methods C57BL/6 J mice and MIN6 cells were used to induce *in vivo* T2DM and damaged β-cell models, respectively. Body weights, fasting blood glucose, and fasting insulin were measured in mice. Oral glucose tolerance test (OGTT) and insulin tolerance test (ITT) were conducted on mice. Lipid indices (TG, TC, LDL-C, and HDL-C) were detected in mouse serum. Hematoxylin-eosin staining was used to evaluate pancreatic tissue injury. ELISA was used to assess insulin and oxidative stress (OS) markers (MDA, GSH, and SOD) in mice and MIN6 cells. Production of ROS was detected in islet β-cells and MIN6 cells. Cell viability and apoptosis were evaluated using CCK-8 and flow cytometry, respectively. QRT-PCR and western blotting were used to detect pro-inflammatory factors (TNF-α and IL-6) and endoplasmic reticulum stress (ERS) markers (CHOP and GRP78), respectively.

Results Vitamin D reduced body weights, fasting blood glucose, and insulin and ameliorated glucose tolerance and insulin sensitivity in T2DM mice. Besides, vitamin D decreased serum TG, TC, LDL-C, and increased HDL-C in T2DM mice. Vitamin D inhibited pancreatic histopathological injury, cell apoptosis, OS, and β-cell decline in T2DM mice. Moreover, vitamin D alleviated cell death, insufficient insulin secretion, inflammation, OS, and ERS in damaged MIN6 cells. Notably, N-acetyl-L-cysteine (an OS inhibitor) enhanced these effects of vitamin D.

Conclusions Vitamin D relieved T2DM symptoms by alleviating OS-induced β-cell impairment.

Introduction

Diabetes mellitus is a prevalent chronic condition induced by endocrine and metabolic disorders [1]. The International Diabetes Federation Global Diabetes Map (the eighth edition) indicated that diabetes has affected nearly 425 million people globally, and the number is estimated to rise to approximately 700 million by 2045 [2, 3]. Type 2 diabetes mellitus (T2DM) accounts for over 90 % of the cases with diabetes mellitus, which brings a great economic burden to the global health system [4, 5]. The main features of T2DM include insulin resistance, hyperglycemia, and the ultimate

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deficiency in insulin secreted by pancreatic β-cells [6]. Besides atherosclerotic cardiovascular disorder, people with T2DM are confronted with a rising risk of diabetic kidney disease and heart failure [7]. Although an increasing number of drugs, including glucose-lowering (e. g., sodium-glucose cotransporter-2 inhibitors and metformin) and anti-obesity (e. g., orlistat and lorcaserin) medications have been used for the treatment of T2DM, the long-term outcomes remain unsatisfactory [8]. Therefore, it is essential to further explore the mechanisms underlying the onset and development of T2DM and provide novel therapeutic strategies.

According to T2DM pathogenesis, dysfunction in pancreatic β-cells is the crucial feature of the early stage, while a total decline in β-cell mass is well recognized as a main feature in subsequent stages [6]. As specific endocrine cells, β-cells tightly regulate blood glucose levels by synthesizing, storing, and secreting insulin through various integrated signals [9]. Under the condition of glucolipotoxicity, β-cell dysfunction can be triggered by multiple mechanisms, such as endoplasmic reticulum stress (ERS), oxidative stress (OS), and inflammation [10]. These factors, in turn, can contribute to glucose intolerance and insulin resistance, resulting in T2DM progression [11]. Notably, β-cells are more susceptible to damage from OS due to their weaker ability to eliminate oxidants compared to other types of cells [10]. Recently, an *in vivo* study has found elevated OS in obese T2DM mice compared with healthy mice [12]. Furthermore, N-acetyl-L-cysteine (NAC; a powerful antioxidant) treatment can rescue impairments in glucose metabolism and β-cells by inhibiting OS [12], suggesting targeting OS can be a potential therapeutic approach against T2DM.

Vitamin D, a steroid hormone, primarily acquired from sun exposure, diet, and dietary supplements, has significant implications for the skeletal system [13]. As the common active form of vitamin D, $1,25(OH)_2D_3$ impacts multiple biological processes, such as cell differentiation and immune and inflammatory responses [14]. Besides its crucial anti-inflammatory function, vitamin D has beneficial effects on suppressing reactive oxygen species (ROS) and nitric oxide, which may restrain oxidative damage [15]. Vitamin D could regulate insulin, restore pancreatic β-cell function, and suppress cell apoptosis and oxidative stress in T2DM rat models [14]. Besides, several clinical studies show the potential of vitamin D to relieve or prevent T2DM [11, 16, 17]. Nevertheless, the anti-T2DM effect of vitamin D and related mechanisms remain to be further investigated.

In this study, we aimed to determine the protective effect of vitamin D on pancreatic β-cells and uncover its molecular mechanisms, thus providing a basis for developing vitamin D treatment as a drug therapy for T2DM.

Materials and Methods

Animal experiments

Thirty specific pathogen-free male C57BL/6 J mice (4 weeks old, 18–22 g) were selected for this study, and were obtained from GemPharmatech Co. Ltd., Nanjing, China. All mice were housed in cages (3 mice/cage) at a temperature of 21–23 °C and humidity of 44–55 % under a 12-h light/dark cycle, with access to food and water *ad libitum*. After a week of adaptation, the mice were randomly categorized into a control group (n = 6) and a T2DM group (n = 24). Mice in the T2DM group were given a high-fat diet (HFD; containing 32 % lard, 28 % casein, 12 % sucrose, 21 % corn starch, 6 % cholesterol, 1 % vitamin mix, and 0.2 % cholic acid; Research Diets, New Brunswick, NJ, USA), while control mice were provided a normal diet (10 % kcal fat; Research Diets). The preparation of HFD accorded with the previous research [18]. After 6 weeks of feeding, mice in the T2DM group were fasted for 12 h and intraperitoneally injected with streptozotocin (50 mg/kg; St. Louis, Sigma Aldrich, MO, United States) dissolved in citrate buffer (1 M) for successive 4 days. The dose of STZ applied to establish a diabetic *in vivo* model conformed to the previous studies [19–22]. Meanwhile, control mice were treated with the same amount of normal saline. The fasting blood glucose level was assessed from the tail vein of mice weekly using a glucometer (LifeScan Inc., Milpitas, CA, USA). Mice with high body weight and fasting blood glucose level ≥ 11.1 mmol/L (72 h after the last streptozotocin injection) were deemed diabetic [23].

Furthermore, the 24 diabetic mice were randomly classified into T2DM, T2DM + 150 ng/kg $1,25(OH)_2D_3$, T2DM + 300 ng/kg $1,25(OH)_2D_3$, and T2DM + 600 ng/kg $1,25(OH)_2D_3$ groups (n = 6/group) and fed with HFD. Mice in the three drug-treated groups were correspondingly treated with 150, 300, and 600 ng/kg $1,25(OH)_2D_3$ (vitamin D; Sigma Aldrich) dissolved in olive oil. The doses of $1,25(OH)_2D_3$ used to treat T2DM were based on previous research [24]. Simultaneously, the diabetic control mice (T2DM group) and healthy control mice (control group) were treated with the same amount of olive oil. Mice were subjected to $1,25(OH)_2D_3$ or olive oil treatment daily for 12 weeks consecutively via oral gavage. The body weights and fasting blood glucose were evaluated daily.

After 12 weeks of treatment, mice were fasted for 12 h and euthanatized using ether as previously described [25–27]. Blood was collected through the venous plexus behind the eyeball, and pancreas tissues were collected and stored at −80 °C until analysis. All the animal experiments conformed to the Guide for the Care and Use of Laboratory Animals and were approved by Xiamen University (XMULAC20220034–13).

Cell culture and treatments

MIN6 cells (a mouse pancreatic β-cell line) were obtained from the American Type Culture Collection (Manassas, VA, USA). MIN6 cells were cultured in Dulbecco's Modified Eagle's medium (Gibco, Grand Island, NY, USA) supplemented with 10 % fetal bovine serum (#16140089, Gibco) at 37 °C with 5 % CO_2. Cells were pretreated with different concentrations of $1,25(OH)_2D_3$ (1, 2, and 4 nM). To induce an *in vitro* β-cell damage model, MIN6 cells were treated with palmitic acid (PA; 0.5 mM) for 24 h. The mechanisms underlying $1,25(OH)_2D_3$ against T2DM were further determined by treating cells with 1 mmol/L NAC for 20 min before PA induction. Cells treated with 10 % w/v bovine serum albumin (BSA) were used as controls.

Oral glucose tolerance test (OGTT), insulin tolerance test (ITT), and lipid profile detection

For OGTT, mice were fasted for 12 h with unlimited access to water and subjected to glucose (2 g/kg) treatment via oral gavage. For ITT, mice fasted for 6 h with *ad libitum* water were treated with insulin (1 U/kg) via intraperitoneal injection. Blood glucose levels of mice in each group were assessed at different time points (0, 30, 60, 90, and 120 min) using the glucometer. Lipid indices, including total cholesterol (TC), triglyceride (TG), low-density lipoprotein cholesterol (LDL-C), and high-density lipoprotein cholesterol (HDL-C), were assessed in the serum of mice using an Indiko Plus Clinical Chemistry Analyzer (#98640000, Thermo Fisher Scientific, Waltham, MA, USA).

Hematoxylin-eosin (HE) staining

Pancreas tissues of mice were fixed in 4 % paraformaldehyde for 24 h, followed by dehydration with gradient concentrations of ethanol (50 %, 70 %, 85 %, 95 %, and 100 %). Then, the tissues were paraffin-embedded, sliced into 5 μm thick sections, and dewaxed using xylene. After hydration with ethanol, tissue sections were stained with hematoxylin for 5 min and eosin for 2 min. Stained sections were observed under a microscope (BX53, Olympus, Tokyo, Japan).

Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) analysis

A One Step TUNEL Apoptosis Assay Kit (#C1086, Beyotime, Shanghai, China) was used following instructions of the manufacturer, to detect cell apoptosis in the pancreas tissues of mice. In brief, after being dewaxed and hydrated, tissue sections were stained with prepared TUNEL detection solution (50 μL) at 37 °C for 1 h and in and 4, 6-diamidino-2- phenylindole (DAPI; #C1005, Beyotime) for 10 min at 25 °C the dark. Images of stained sections were photographed by a fluorescence microscope (CKX53, Olympus) and cell apoptosis was quantified using ImageJ 1.8.0 software (National Institutes of Health, Bethesda, MD, USA).

Enzyme-linked immunosorbent assay (ELISA)

ELISA kits were used following the instructions of the manufacturer, to assess the level of insulin in the plasma of mice and MIN6 cells, as well as malonaldehyde (MDA), glutathione (GSH), and superoxide dismutase (SOD) in the serum of mice and MIN6 cells. To detect glucose-stimulated insulin secretion (GSIS) in MIN6 cells, MIN6 cells were respectively incubated in KRBH buffer (pH 7.4; 115 mM NaCl + 5 mM KCl + 24 mM $NaHCO_3$ + 2.5 mM $CaCl_{2+}$ 1 mM $MgCl_{2+}$ 10 mM HEPES + 2 % w/v BSA) containing 2.5 or 16.7 mM glucose for 1 h. Optical density (OD) values were measured at 450 nm for assessment of insulin levels, 532 nm and 600 nm for MDA, 420 nm for GSH, and 550 nm for SOD using a microplate reader (DR-3518G, Hiwell Diatek). ELISA kits used were as follows: insulin (#CSB-E05071m, CUSABIO, Wuhan, China), MDA (#BC0025, Solarbio, Beijing, China), and GSH (#A006–1–1) and SOD (#A001-1-1) from Nanjing Jiancheng Bioengineering Institute.

Pancreatic β-cell detection

Pancreatic sections were dewaxed and hydrated, followed by three rounds of washing using phosphate-buffered saline (PBS). Then, the sections were made transparent using 5 % TritonX-100 (#T8200, Solarbio) for 20 min and blocked by 5 % normal goat serum (#SL038, Solarbio) for 1 h. The sections were incubated with anti-insulin (1:200; #ab181547, Abcam, Cambridge, UK) and anti-glucagon (1:500; #G2654, Sigma-Aldrich, St. Louis, MO, USA), primary antibodies at 4 °C overnight. Subsequently, the sections were incubated with goat anti-mouse IgG H&L (Alexa Fluor 488) (1:500; #ab150113, Abcam) and goat anti-rabbit IgG H&L (Alexa Fluor Cy3) (1:1,000; #ab6939, Abcam) secondary antibodies at 37 °C for 15 min. The sections were stained with DAPI and washed thrice with PBS. Images of stained sections were captured by a confocal imaging system (UltraVIEW VoX; Perkin Elmer, MA, USA). The fluorescence intensity of insulin was quantified by ImageJ 1.8.0 software, which indicated the β-cell level.

Detection of reactive oxygen species

For detection of ROS production in islet β-cells in mice, islets were separated from pancreas tissues using collagenase V (3.3 mg/mL, 30 min) and digested using neutral protease (0.3 mg/mL, 12 min). Islet β-cells were isolated and cultured at 37 °C with 5 % CO_2 for one week. ROS Assay Kit (DCFH-DA; #S0033–1, Beyotime) was used following the instructions of the manufacturer, to measure ROS production in β-cells and MIN6 cells. Briefly, cells were suspended in diluted DCFH-DA and incubated at 37ºC for 20 min. Then, cells were washed thrice with serum-free culture solution and suspended with PBS. ROS production was measured under a CytoFLEX S flow cytometer (Beckman, FL, USA) and analyzed using FlowJo 7.6 software (BD Biosciences, Franklin Lake, NJ, USA).

Cell counting kit-8 (CCK-8) assay

MIN6 cells were seeded in 96-well plates (2×10^3 cells/well), treated with specific reagents, and cultured at 37 °C with 5 % CO_2 for 24 h. Subsequently, a CCK-8 kit (10 μL; #C0037, Beyotime) was added to each well, and cells were incubated for another 2 h. OD values were measured at 450 nm using a DR-3518G microplate reader (Hiwell Diatek, Wuxi, China) and cell viability was quantified by ImageJ 1.8.0 software.

Flow cytometry

Flow cytometry was applied to detect in apoptosis MIN6 cells using Annexin V-FITC Cell Apoptosis Detection Kit (#C1062S, Beyotime). After washing twice with PBS, cells were suspended with a binding buffer (300 μL). Next, cells were stained with Annexin V-FITC (5 μL) for 15 min and propidium iodide (10 μL) for 10 min at 25 °C in the dark. Cell apoptosis was observed under the CytoFLEX S flow cytometer and quantified using Cell Quest software (BD Biosciences).

Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was extracted from MIN6 cells using TRIzol (#15596018, Invitrogen) based on the manufacturer's instructions. The reverse transcription for cDNA synthesis was carried out using FastKing-RT SuperMix (#KR118–02, Tiangen, Beijing, China). QRT-PCR was conducted on a CFX Connect Real-Time PCR Detection System (Bio-Rad, CA, USA) using SYBR Green PCR Master Mix (#4364344; Thermo Fisher Scientific). The reaction procedures were set to "95 °C, 3 min; 95 °C, 12 s; 62 °C, 40 s; 40 cycles". The $2^{-\Delta\Delta Ct}$ method was applied for quantification of gene expression using glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as the internal reference. The primers used are shown in ▶ **Table 1**.

Western blotting

Total protein from MIN6 cells was extracted using radioimmunoprecipitation assay (RIPA) lysis buffer (#P0013B, Beyotime), followed by protein quantification using a bicinchoninic acid (BCA) kit (#P0010S, Beyotime). Proteins were separated by 10 % sodium do-

▶ **Table 1** Primers used in this study.

Gene	Primer sequences (5′ to 3′)
TNF-α	Forward: AGGCACTCCCCCAAAAGATG
	Reverse: CCACTTGGTGGTTTGTGAGTG
IL-6	Forward: GGGACTGATGCTGGTGACAA
	Reverse: AGCATTGGAAATTGGGGTAGGA
GAPDH	Forward: GGAGAGTGTTTCCTCGTCCC
	Reverse: ACTGTGCCGTTGAATTTGCC

decyl sulfate-polyacrylamide gel electrophoresis (#P0015A, Beyotime) and transferred to polyvinylidene difluoride membranes (#FFP24, Beyotime). Then, membranes were sealed with 5 % nonfat milk (Beyotime) for 1 h. Membranes were incubated with anti-CHOP (1:500; #AF6277, Affinity Biosciences, Cincinnati, OH, USA), anti-GRP78 (1:500, #AF5366, Affinity Biosciences), and anti-GAPDH (1:10,000; #ab8245, Abcam) primary antibodies at 4 °C overnight, and then with goat anti-rabbit IgG H&L conjugated with horseradish peroxidase (1:2,000, #ab6721, Abcam) secondary antibody for 1 h. Enhanced chemiluminescence kits (#P1000, Applygen Technologies Inc., Beijing, China) were used to visualize protein bands. Protein expression levels were analyzed using ImageJ 1.8.0 software and normalized to GAPDH expression.

Statistical analysis

All data are presented as mean ± standard deviation. Statistical analyses were carried out using GraphPad 7.0 software (La Jolla, CA, USA). Differences between groups were determined by one-way analysis of variance with Tukey's test. The criterion of statistical significance was set to $p < 0.05$.

Results

Vitamin D alleviates glucose and lipid metabolism disorders and pathological injury in T2DM mice model

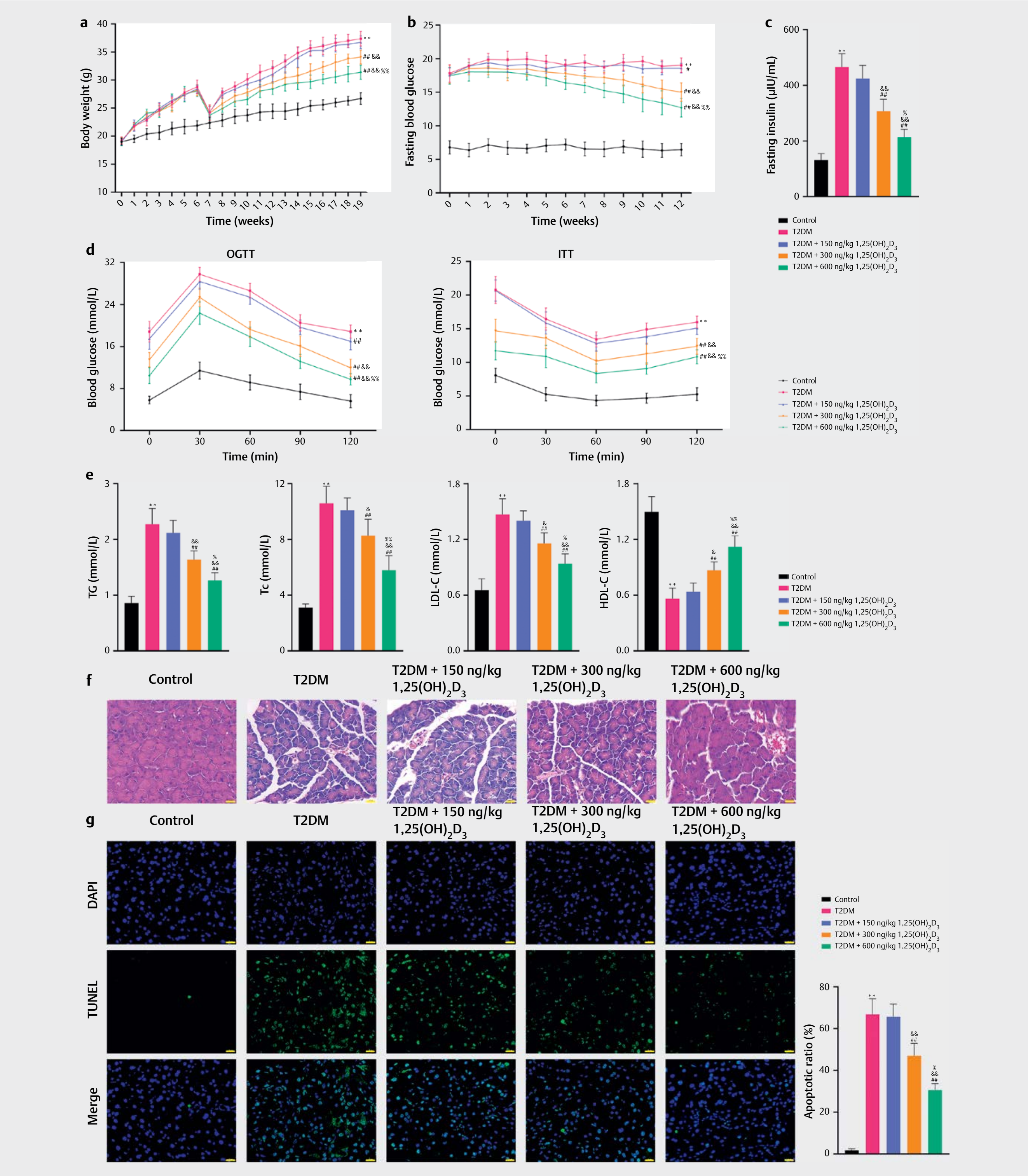
To determine whether vitamin D [$1,25(OH)_2D_3$] can ameliorate the symptoms of T2DM, an *in vivo* model of T2DM was induced by feeding mice with HFD and treating them with streptozotocin. The body weights of T2DM mice were increased markedly compared with those of control mice, however, which were restored by $1,25(OH)_2D_3$ treatments ($p < 0.05$; ▶ **Fig. 1a**). In addition, the fasting blood glucose and insulin levels in T2DM mice were significantly higher than those in control mice, and $1,25(OH)_2D_3$ treatments (300 and 600 ng/kg) decreased these levels in T2DM mice ($p < 0.01$; ▶ **Fig. 1b-c**). The OGTT and ITT results showed significantly higher blood glucose in T2DM mice than in control mice ($p < 0.01$; ▶ **Fig. 1d**). After $1,25(OH)_2D_3$ treatments (300 and 600 ng/kg), glucose tolerance and insulin sensitivity of T2DM mice were noticeably improved, as blood glucose was reduced ($p < 0.01$; ▶ **Fig. 1d**). In terms of lipid metabolism, we found increased levels of serum TG, TC, and LDL-C and decreased HDL-C levels in T2DM mice; however, the levels of these lipid indices were reversed after 1,25(OH)$_2$D3 treatments (300 and 600 ng/kg) ($p < 0.01$; ▶ **Fig. 1e**). Furthermore, $1,25(OH)_2D_3$ treatments also ameliorated the histopathological injury in T2DM mice (▶ **Fig. 1f**). Compared with control mice, T2DM mice exhibited elevated pancreatic cell apoptosis ($p < 0.01$; ▶ **Fig. 1g**), which was reduced after $1,25(OH)_2D_3$ treatments (300 and 600 ng/kg) ($p < 0.01$; ▶ **Fig. 1g**).

Vitamin D ameliorates pancreatic β-cell loss and OS in T2DM mice

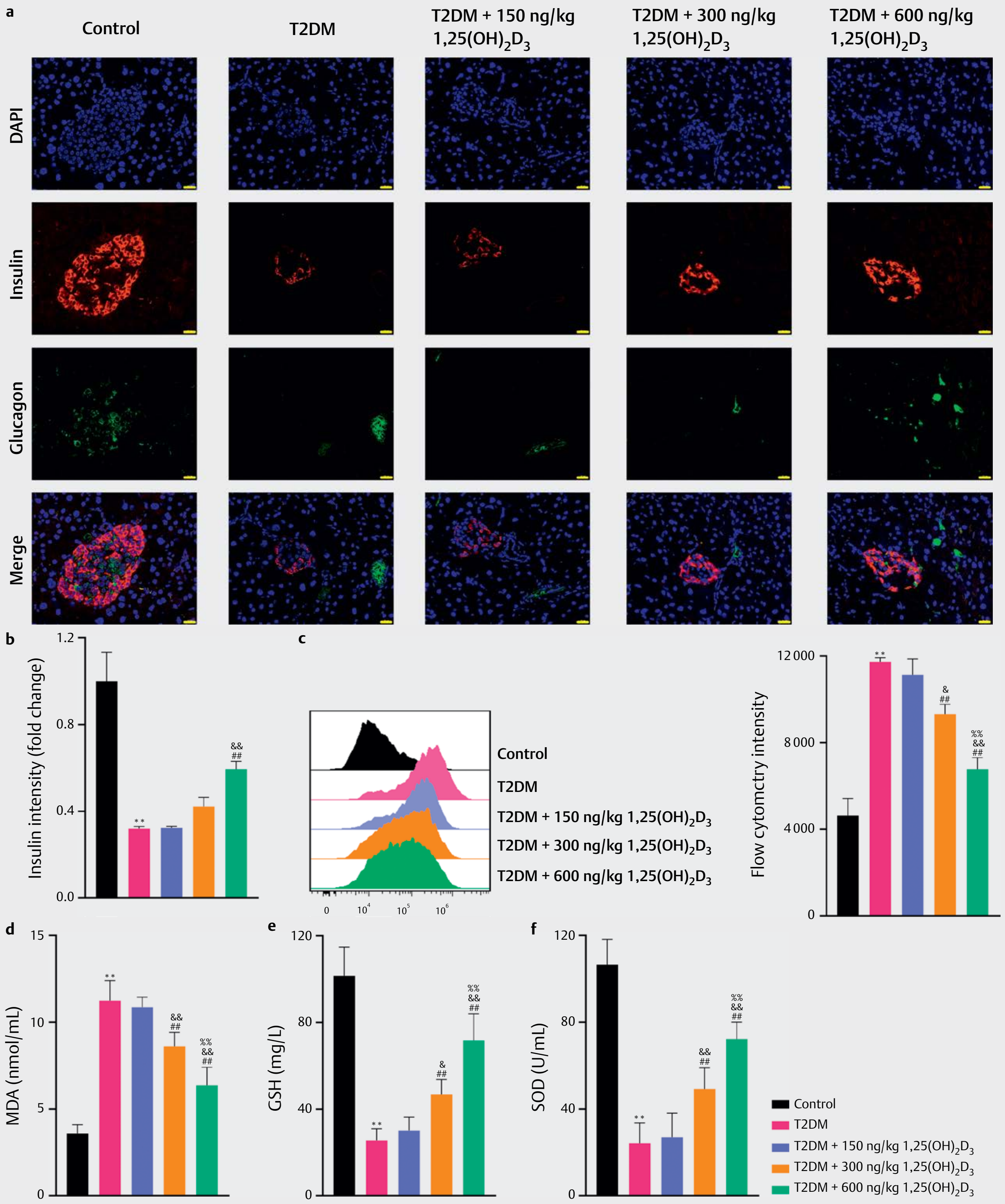
Impairments in pancreatic β-cell function and quality play a key part in the pathogenesis of T2DM [28]. In this study, a notable decline in insulin intensity was observed in T2DM mice compared with control mice, indicating the loss of β-cell function in T2DM mice ($p < 0.01$; ▶ **Fig. 2a, b**). Obviously, 600 ng/kg $1,25(OH)_2D_3$ treatment rescued the β cell loss in T2DM mice ($p < 0.01$; ▶ **Fig. 2a, b**). Evidence demonstrates that pancreatic β-cell dysfunction and loss in T2DM can be attributed to OS [12]. Thus, to investigate the effect of vitamin D on OS, we evaluated OS-related molecules (ROS, MDA, GSH, and SOD) in pancreatic β-cells in mice. Results showed that the levels of ROS and lipid oxidation product MDA were markedly elevated in T2DM mice in comparison with those in control mice ($p < 0.01$; ▶ **Fig. 2c, d**). Meanwhile, T2DM mice showed lower levels of antioxidant enzymes GSH and SOD than control mice ($p < 0.01$; ▶ **Fig. 2e, f**). After $1,25(OH)_2D_3$ treatments (300 and 600 ng/kg), the levels of ROS and MDA were reduced, while GSH and SOD levels were elevated in T2DM mice ($p < 0.01$; ▶ **Fig. 2c-f**).

Vitamin D mitigates pancreatic β-cell impairment *in vitro*

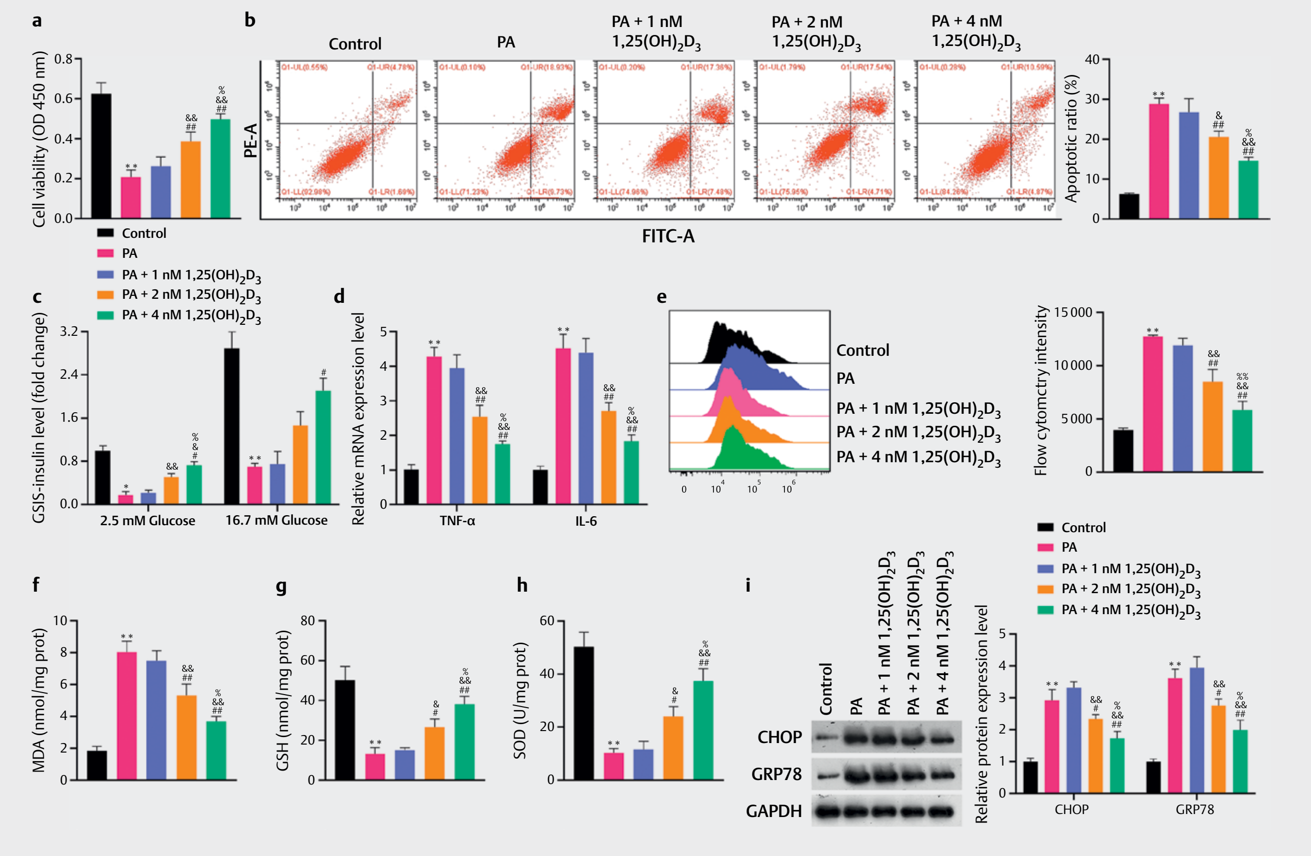
To further confirm the alleviating effect of vitamin D on pancreatic β-cell impairment, the pancreatic β-cell line (MIN6 cells) was pretreated with 1, 2, and 4 nM $1,25(OH)_2D_3$, followed by PA treatment to induce an *in vitro* model of β-cell damage. We observed that cell viability was markedly suppressed in PA-treated MIN6 cells in comparison with that in control cells, which was dose-dependently reversed by $1,25(OH)_2D_3$ pretreatments (2 and 4 nM) ($p < 0.05$; ▶ **Fig. 3a**). Moreover, $1,25(OH)_2D_3$ pretreatments (2 and 4 nM) notably inhibited PA-induced MIN6 cell apoptosis in a dose-dependent way ($p < 0.05$; ▶ **Fig. 3b**). We also evaluated the GSIS-insulin level in MIN6 cells to further ascertain whether vitamin D can ameliorate β-cell dysfunction. The insulin secretion level was markedly decreased in PA-induced MIN6 cells under the conditions of both 2.5 mM (basal concentration) and 16.7 mM (stimulating concentration) glucose compared with that in control cells ($p < 0.05$; ▶ **Fig. 3c**). Notably, 4 nM $1,25(OH)_2D_3$ pretreatment increased the GSIS-insulin level in PA-induced MIN6 cells ($p < 0.05$; ▶ **Fig. 3c**), suggesting the rescue of β-cell dysfunction. Inflammation has been demonstrated to be closely linked with β cell dysfunction, and PA can induce the release of pro-inflammatory mediators, such as tumor necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6) [29]. As expected, PA-induced MIN6 cells had higher expression of TNF-α and IL-6 mRNAs than control cells, which were markedly reversed by $1,25(OH)_2D_3$ pretreatments (2 and 4 nM) in a dose-dependent way ($p < 0.05$; ▶ **Fig. 3d**). Furthermore, OS was significantly enhanced in MIN6 cells after PA treatment, as evidenced by elevated levels of ROS and MDA, but reduced levels of GSH and SOD ($p < 0.01$; ▶ **Fig. 3e-h**). Also, $1,25(OH)_2D_3$ pretreatments (2 and 4 nM) dose-dependently inhibited OS in PA-induced MIN6 cells ($p < 0.05$; ▶ **Fig. 3e-h**). ERS has been indicated to accompany OS and inflammation in T2DM and is crucial for β-cell dysfunction [30]. Accordingly, we assessed the levels



▶ **Fig. 1** Vitamin D mitigates glucose and lipid metabolism disorders and pathological injury in T2DM mice. (**a-c**) Detection of mouse body weights, fasting blood glucose, and fasting insulin were measured in each group. (**d**) Performance of OGTT and ITT on mice in each group. (**e**) Detection of lipid indices (TG, TC, LDL-C, and HDL-C) in the serum of mice in each group. (**f**) Detection of the histopathological injury in the pancreatic tissues of mice in each group using HE staining (Scale bar = 20 μm). (**g**) Detection of cell apoptosis in the pancreatic tissues of mice in each group using TUNEL assay (Scale bar = 20 μm). Data were presented as mean ± standard deviation (n = 6 mice/group). $^{**}p<0.01$ *vs* Control group; $^{\#}p<0.05$ and $^{\#\#}p<0.01$ *vs* T2DM group; $^{\&\&}p<0.01$ *vs* T2DM + 150 ng/kg 1,25(OH)$_2$D$_3$ group; $^{\%}p<0.05$ and $^{\%\%}p<0.01$ *vs* T2DM + $_3$00 ng/kg 1,25(OH)$_2$D$_3$ group. T2DM· type 2 diabetes mellitus; OGTT, oral glucose tolerance test; ITT, insulin tolerance test; TG, triglyceride; TC, total cholesterol; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; HE, hematoxylin-eosin; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling.



▶ **Fig. 2** Vitamin D rescues pancreatic β-cell loss and inhibits OS in T2DM mice. (**a**-**b**) Detection of β-cell levels in the pancreatic tissues of mice in each group (Scale bar = 20 μm). (**c**) Detection of ROS production in the islet β-cells of mice in each group. (**d**-**f**) Detection of MDA, GSH, and SOD in the serum of mice in each group using ELISA. Data were presented as mean ± standard deviation (n = 6 mice/group). $^{**}p<0.01$ *vs* Control group; $^{\#\#}p<0.01$ *vs* T2DM group; $^{\&\&}p<0.01$ *vs* T2DM + 150 ng/kg 1,25(OH)$_2$D$_3$ group; $^{\%\%}p<0.01$ *vs* T2DM + 300 ng/kg 1,25(OH)$_2$D$_3$ group. OS, oxidative stress; T2DM, type $_2$ diabetes mellitus; ROS, reactive oxygen species; MDA, malonaldehyde; GSH, glutathione; SOD, superoxide dismutase; ELISA, enzyme-linked immunosorbent assay.



▸ **Fig. 3** Vitamin D alleviates pancreatic β-cell impairment *in vitro*. (**a**) Detection of MIN6 cell viability by CCK-8 assay. (**b**) Detection of MIN6 cell apoptosis using flow cytometry. (**c**) Detection of the GSIS-insulin level in MIN6 cells using ELISA. (**d**) Detection of the mRNA expression levels of TNF-α and IL-6 in MIN6 cells by qRT-PCR. (**e**) Detection of ROS production in MIN6 cells. (**f-h**) Detection of MDA, GSH, and SOD in MIN6 cells by ELISA. (**i**) Detection of the protein expression levels of CHOP and GRP78 in MIN6 cells by western blotting. Data were presented as mean ± standard deviation. $^{*}p<0.05$ and $^{**}p<0.01$ *vs* Control group; $^{\#}p<0.05$ and $^{\#\#}p<0{\cdot}01$ *vs* PA group; $^{\&}p<0.05$ and $^{\&\&}p<0.01$ *vs* PA + 1 nM $1{,}25(OH)_2D_3$ group; $^{\%}p<0.05$ and $^{\%\%}p<0.01$ *vs* PA + 2 nM $1{,}25(OH)_2D_3$ group. CCK-8, cell counting kit-8; GSIS, glucose-stimulated insulin secretion; ELISA, enzyme-linked immunosorbent assay; qRT-PCR, quantitative real-time polymerase chain reaction; ROS, reactive oxygen species; MDA, malonaldehyde; GSH, glutathione; SOD, superoxide dismutase; PA, palmitic acid.

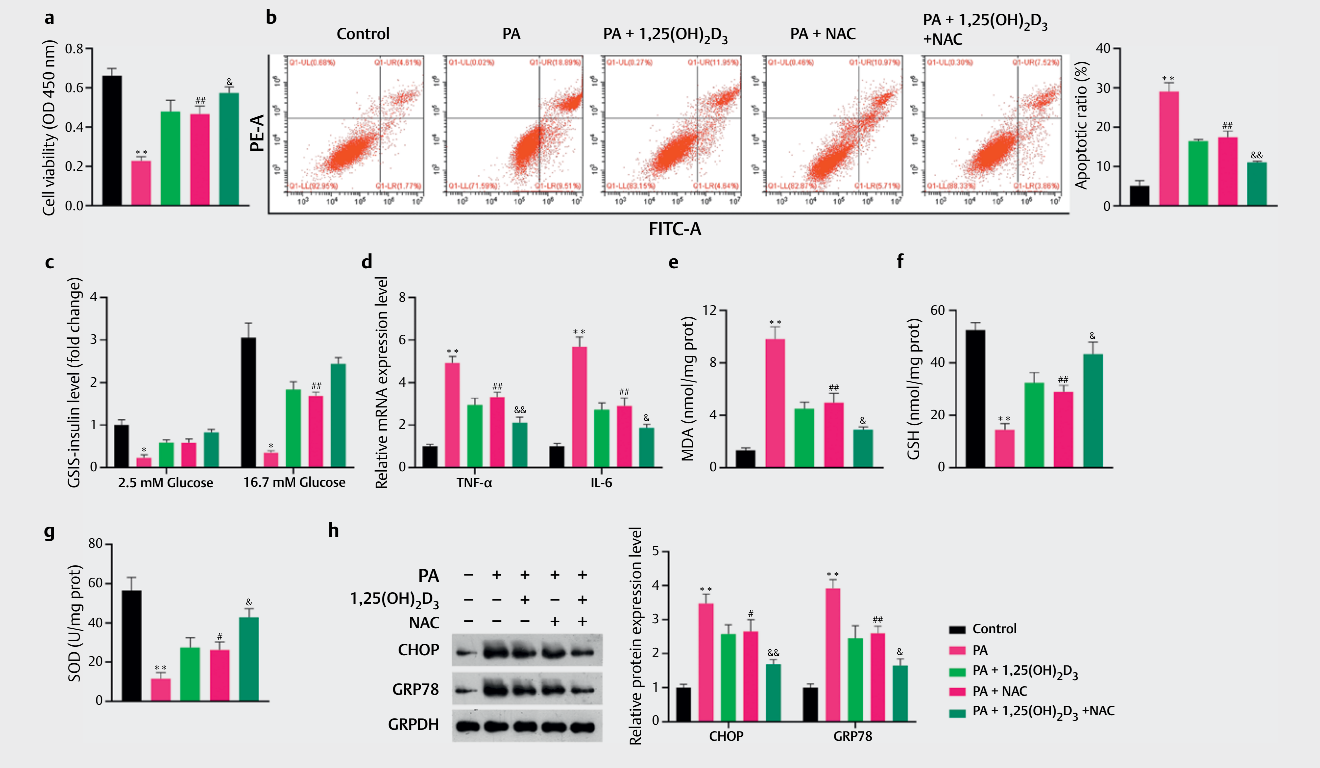
of ERS markers (C/-EBP homologous protein, CHOP; glucose-regulated protein, GRP78) in MIN6 cells to investigate the effects of vitamin D on ERS. Results showed that the protein expression levels of CHOP and GRP78 were significantly higher in PA-induced MIN6 cells than those of control cells, whereas this increasing trend was mitigated by $1{,}25(OH)_2D_3$ pretreatments (2 and 4 nM) ($p<0.05$; ▸ **Fig. 3i**). As shown in ▸ **Fig. 3**, $1{,}25(OH)_2D_3$ (4 nM) exerted the optimal protective effect on β-cells; thus, this dose was chosen for subsequent functional assays.

Vitamin D alleviates pancreatic β-cell impairment *in vitro* by inhibiting oxidative stress

To further verify whether vitamin D mitigates pancreatic β-cell damage by suppressing OS, PA-induced MIN6 cells were treated with $1{,}25(OH)_2D_3$ or/and OS inhibitor NAC. We found that NAC markedly promoted cell viability and inhibited apoptosis in PA-induced MIN6 cells, which strengthened the beneficial effects of $1{,}25(OH)_2D_3$ on viability and apoptosis in these cells ($p<0.05$; ▸ **Fig. 4a, b**). Besides, NAC exerted an up-regulatory effect on the GSIS-insulin level in PA-induced MIN6 cells ($p<0.01$; ▸ **Fig. 4c**). Meanwhile, NAC showed an inhibitory effect on the mRNA expression of TNF-α and IL-6 in PA-induced MIN6 cells, which reinforced the anti-inflammatory function of $1{,}25(OH)_2D_3$ in these cells ($p<0.05$; ▸ **Fig. 4d**). Notably, NAC enhanced the suppressive effect of $1{,}25(OH)_2D_3$ on OS ($p<0.05$; ▸ **Fig. 4e-g**). In terms of ERS, NAC notably reduced the protein expression of CHOP and GRP78 in PA-induced MIN6 cells and it strengthened the inhibitory effect of $125(OH)_2D_3$ on ESR in these cells ($p<0.05$; ▸ **Fig. 4h**).

Discussion

T2DM is a multi-factor metabolic disorder with growing incidence worldwide [31]. Previous clinical and basic studies indicated that vitamin D might exert beneficial effects on the symptoms or the prevention of T2DM [14, 16, 17, 32]. Furthermore, a latest meta-analysis suggested the beneficial effects of vitamin D on the con-



▶ **Fig. 4** Vitamin D alleviates pancreatic β-cell dysfunction *in vitro* by suppressing OS. (**a**) Detection of MIN6 cell viability using CCK-8 assay. (**b**) Detection of MIN6 cell apoptosis using flow cytometry. (**c**) Detection of the GSIS-insulin level in MIN6 cells by ELISA. (**d**) Detection of the mRNA expression levels of TNF-α and IL-6 in MIN6 cells by qRT-PCR. (**e-g**) Detection of MDA, GSH, and SOD in MIN6 cells by ELISA. (**h**) Detection of the protein expression levels of CHOP and GRP78 in MIN6 cells by western blotting. Data were presented as mean ± standard deviation. $^{*}p<0.05$ and $^{**}p<0.01$ *vs* Control group; $^{\#}p<0.05$ and $^{\#\#}p<0.01$ *vs* PA group; $^{\&}p<0.05$ and $^{\&\&}p<0.01$ *vs* PA + 1,25(OH)$_2$D$_3$ group. OS, oxidative stress; CCK-8, cell counting kit-8; GSIS, glucose-stimulated insulin secretion; ELISA, enzyme-linked immunosorbent assay; qRT-PCR, quantitative real-time polymerase chain reaction; MDA, malonaldehyde; GSH, glutathione; SOD, superoxide dismutase.

trol of blood glucose in patients with T2DM and the prevention of diabetic complications [33]. However, the application of vitamin D in the prevention and treatment of T2DM remains controversial and the understanding of the anti-T2DM mechanisms of vitamin D is still lacking. In this study, the active form of vitamin D, 1,25(OH)$_2$D$_3$, was indicated to ameliorate glucose and lipid metabolism disorders, pathological injury, and β-cell dysfunction by inhibiting OS.

Initially, we found that the body weights of T2DM mice were increased compared with healthy mice, which were effectively reduced by 1,25(OH)$_2$D$_3$ treatment. Obesity is one of the primary hazardous factors for T2DM development, and most of the patients with T2DM are simultaneously overweight or obese [34, 35]. Thus, weight loss interventions play a crucial part in the management of T2DM. Consistent with a previous study [32], our results showed that 1,25(OH)$_2$D$_3$ treatment reduced the fasting blood glucose and insulin levels and ameliorated glucose tolerance and insulin sensitivity. Dyslipidemia is a prevalent comorbidity of T2DM, which is marked by augmented serum TG, TC, and LDL-C levels, and/or lowered HDL-C levels [36]. In T2DM, a decline in lipase activity compared to the insulin level leads to elevated TG and LDL-C levels, along with reduced HDL-C level [37]. Several studies have demonstrated the link between vitamin D and lipid profile in T2DM [38–40]. A recent meta-analysis has suggested that vitamin D does not exert significant effects on the levels of TC, LDL-C, and HDL-C in children and adolescents [41]. Nevertheless, this analysis has also indicated that total vitamin D supplementation (≥ 200,000 IU) could decrease fasting blood glucose and TG levels [41]. Here, we found that 1,25(OH)$_2$D$_3$ treatment markedly downregulated the levels of serum TG, TC, and LDL-C and upregulated the HDL-C level in T2DM mice, which was in line with the results of a previous study [40]. We also observed that the histopathological injury and cell apoptosis in the pancreatic tissues of T2DM mice were mitigated following 1,25(OH)$_2$D$_3$ treatment. Consistent with our findings, a recent study has revealed that 1,25(OH)$_2$D$_3$ intervention effectively ameliorated histological lesions and cell apoptosis in the pancreatic tissues of T2DM rats [14].

Pancreatic β-cell impairment is deemed the leading cause of T2DM progression [42]. In T2DM, both dysfunction and loss of β-cells can contribute to insulin secretion deficiency [42]. In the present study, a notable decline in β-cells was observed in T2DM mice comparative to healthy mice, which however, was rescued after 1,25(OH)$_2$D$_3$ treatment. The positive effect of vitamin D on β-cell function has been reported, which might be ascribed to the activation of vitamin D receptor (VDR) in β-cells [43]. A previous study indicated that activated VDR ameliorated β-cell dysfunction in both mouse and human islets [44]. One study demonstrated that 1,25$(OH)_2D_3$ could regulate the insulin-secreting ability of β-cells via modulating calcium influx during GSIS [45]. Another study revealed that 1,25$(OH)_2D_3$ could rescue β-cell dysfunction induced by high glucose via activating the AMP-activated protein kinase axis [46].

On the other hand, increasing studies reveal that OS is the core mechanism related to pancreatic β-cell impairment in the development of diabetes mellitus [47–49]. In T2DM, elevated metabolic stress induced by hyperglycemia and insulin resistance can cause mitochondrial dysfunction, resulting in ROS production [50]. Increased ROS concentrations can lead to OS and β-cell apoptosis [50]. β-cells are deemed poor in antioxidant defense and vulnerable to OS [14]. Cells utilize the antioxidant defense system to eliminate ROS, thereby lightening the accumulative burden of OS. The first line of defense against OS, consisting of GSH and SOD, can suppress the formation of free radicals and restrain oxidative damage [51]. MDA is the outcome of lipid peroxidation, which is widely used as an indicator of OS [14]. Herein, we observed elevated ROS and MDA levels, as well as reduced GSH and SOD levels, in the β-cells of T2DM mice in comparison with healthy mice, suggesting that OS was induced in T2DM. Serval animal studies have reported the anti-OS function of vitamin D [52–54]. Similar to previous findings [14], the present study demonstrated inhibition of OS after 1,25$(OH)_2D_3$ treatment in the β-cells of T2DM mice.

Given that a decline in pancreatic β-cell function and excess OS were detected in T2DM mice, we hypothesized that vitamin D might exert anti-T2DM effects by ameliorating OS-induced β-cell dysfunction. Subsequently, we conducted *in vitro* experiments to confirm our hypothesis and found that 1,25$(OH)_2D_3$ treatment enhanced cell viability, inhibited apoptosis, and increased the GSIS-insulin level in PA-induced MIN6 cells, indicating the protective role of vitamin D in β-cells. Treatment with 1,25$(OH)_2D_3$ effectively suppressed OS in PA-induced MIN6 cells, as the ROS and MDA levels were downregulated along with upregulated GSH and SOD levels. Apart from OS, inflammation and ERS have been demonstrated to be the significant factors for β-cell decline in T2DM [55]. In line with the previous study [29], upregulated expression of the pro-inflammatory cytokines, TNF-α and IL-6, was observed in PA-induced MIN6 cells compared to normal cells. Significantly, 1,25$(OH)_2D_3$ treatment reduced the expression of TNF-α and IL-6 in PA-induced MIN6 cells. A previous investigation indicated that 1,25$(OH)_2D_3$ could be a crucial immunity regulator in alleviating inflammation by downregulating pro-inflammatory cytokines, including TNF-α and IL-6 [56]. Elevated CHOP and GRP78 usually serve as ERS indicators [57]. In this study, we found increased expression of CHOP and GRP78 in PA-induced MIN6 cells compared with normal cells, which was reversed after 1,25$(OH)_2D_3$ treatment. A recent study has demonstrated that vitamin D combined with resveratrol can inhibit ERS to alleviate the symptoms of T2DM [58]. Notably, we found that the OS inhibitor, NAC, enhanced the alleviating effects of vitamin D on cell death, GSIS-insulin deficiency, inflammation, OS, and ERS in PA-induced MIN6 cells. NAC treatment can exert positive effects on glucose metabolism and β-cell function in T2DM mice [12]. Collectively, these results suggest that vitamin D might rescue β-cell impairment in T2DM partly due to its anti-OS effect.

Nevertheless, this study still has some limitations. The anti-T2DM effect of vitamin D was investigated in animal and cell models; thus, additional studies are required to verify the efficacy of vitamin D in the treatment of T2DM. Besides, we merely detected stimulated inflammation and ERS in damaged β-cells without further analyzing the underlying crosstalk among inflammation, OS, and ERS. These limitations will be ameliorated in our subsequent research.

Conclusion

To conclude, vitamin D ameliorated the disorders in glucose and lipid metabolism, alleviated pathological injury, and inhibited OS in T2DM. Vitamin D also rescued β-cell dysfunction, which might be partly owing to its anti-OS effect. Overall, this study suggests that vitamin D has the potential as a drug for T2DM and provides novel directions for follow-up in animal and clinical research. Future studies would further explore the specific pathways and molecular targets by which vitamin D alleviates T2DM, which is beneficial for the development of more targeted interventions. Besides, it is important to explore the potential synergistic effects of vitamin D with other therapeutic strategies commonly used in T2DM management.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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