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Efficacy and Mechanism of Alprostadil in Diabetes Mellitus Combined with Peripheral Atherosclerosis

ORIGINAL INVESTIGATION

ABSTRACT

Background: The aim is to investigate the clinical efficacy of Alprostadil in diabetes mellitus (DM) combined with peripheral atherosclerosis and to investigate the molecular mechanisms.

Methods: Patients included 154 cases with DM combined with peripheral atherosclerosis and were divided into the conventional group (77 cases) and the Alprostadil group (77 cases). Both groups of patients were given conventional treatment, and the Alprostadil group was given Alprostadil treatment on the basis of the conventional group. The therapeutic efficacy and clinical symptom improvement were compared, and the adverse reactions were observed. An in vitro cell model was constructed using high glucose (HG) (50 mM) and oxidized low-density lipoprotein (50 μg/mL) treatment.

Results: The total effective rate of treatment in the Alprostadil group was higher than that in the conventional group. The biochemical indices of whole blood viscosity, plasma viscosity, erythrocyte pressure volume, and fibrinogen, as well as the level of inflammatory factors in the Alprostadil group were lower than those in the conventional group. The incidence rate of adverse reactions of Alprostadil administration was lower than that in the conventional group ($P = .030$). Alprostadil inhibited platelet aggregation and promoted platelet spreading. Alprostadil had an ameliorative effect on HG- and oxidized low-density lipoprotein cholesterol (ox-LDL)-induced human umbilical vascular endothelial cells (HUVECs), and promoted apoptosis and inflammatory response of HUVECs.

Conclusion: Clinically, the use of Alprostadil as an adjunct to conventional therapy for the treatment of DM combined with peripheral atherosclerosis has high clinical efficacy. In addition, Alprostadil has a significant ameliorative effect on high glucose- and ox-LDL-induced HUVECs.

Keywords: Diabetes mellitus, peripheral atherosclerosis, Alprostadil, clinical efficacy

INTRODUCTION

Diabetes mellitus (DM), marked by unusually high blood glucose levels, stands as one of the most rapidly escalating issues in the 21st century. The complexities of DM exert significant health and financial strain on people and the community.[1] Diabetes mellitus is recognized for its role in fostering atherosclerosis, a condition that results in cardiovascular disease.[2] Atherosclerosis, stemming from hyperlipidemia, is a vascular disorder responsible for over half of the elderly's fatalities. Cardiovascular issues significantly contribute to morbidity and mortality associated with DM.[3] Most clinical and experimental data on the pathogenesis of diabetic complications are related to atherosclerosis, suggesting that hyperglycemia may lead to impaired vascular homeostasis, mainly due to endothelial dysfunction. Clinical management of DM combined with peripheral atherosclerosis includes glycemic control, antihypertensive drugs, lipid-lowering therapy, and antiplatelet agents. However, the combined clinical control rate is only 8.1%.[4] How to comprehensively alleviate DM combined with peripheral atherosclerosis is a challenging clinical problem.

Secretion of chemokines and adhesion molecules and deposition of platelet-derived chemokines aggregate mononuclear cells into the vascular endothelium,

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leading to sprouting, formation, and rupture of atherosclerotic plaques.[5,6] Elevated levels of oxidized low-density lipoprotein cholesterol (ox-LDL-C) increases tumor necrosis factor-alpha (TNF-α), predominantly in vascular tissues. Elevated concentrations of TNF-α enhance the permeability of blood macromolecules, facilitating the infiltration of inflammatory cells into damaged vascular tissues, thereby fostering the development of atherosclerotic plaques.[7]

Alprostadil is a typical natural prostaglandin drug, the main component of which is prostaglandin E1. Alprostadil is widely used in the clinic for its vasodilatory effect of dilating blood vessels and its antiplatelet effect of inhibiting platelet aggregation.[8] Alprostadil has been shown to have antioxidant and cell membrane stabilizing activities.[9] The application of Alprostadil combined with Doxium in patients with diabetic nephropathy can effectively reduce microvascular wall permeability and inhibit platelet aggregation.[10] Alprostadil has also been reported to play a role in improving and protecting the kidney by inhibiting the immune response, reducing renal inflammation, and decreasing renal apoptosis.[11-13] However, the therapeutic effects and mechanisms of Alprostadil in DM combined with peripheral atherosclerosis need to be further investigated.

The aim of this study is to investigate the clinical adjuvant efficacy of Alprostadil in DM combined with peripheral atherosclerosis and its mechanism of action based on conventional drug therapy, so as to provide a reference basis for the clinical treatment of this disease.

METHODS

Clinical Information

A total of 154 patients with Type 2 DM (T2DM) combined with peripheral atherosclerosis from February 2016 to March 2018 were taken as the study objects and divided into the conventional group (77 cases) and the Alprostadil group (77 cases) using the method of a random number table. In the Alprostadil group, there were 47 males and 30 females; the age range was 44-65 years, with a mean age of 54.9 ± 10.2 years. The patients in the observation group had a duration of T2DM of 1-9 years, with a mean of 5.2 ± 4.1 years, and combined atherosclerosis of 2-5 months, with a mean of 3.5 ± 1.5 months. In the conventional group, there were 53 males and 24 females; the age range was 46-64 years, with a mean age of 55.2 ± 8.9 years. Secondly, the patients in the observation group had a duration of T2DM of 2-8 years, with a mean of 5.5 ± 2.9 years, and combined atherosclerosis of 2-5 months, with a mean of 3.6 ± 1.6 months. Detection of FBG ($P = .797$) and 2-h postprandial blood glucose ($P = .160$) to diagnose DM in both groups showed that the blood glucose level in the Alprostadil group was higher than that in the conventional group, but there was no statistical difference. There was no significant difference in the comparison of complications of the 2 groups. The differences between end-diastolic blood flow rate ($P = .003$), peak systolic blood flow rate ($P < .001$), and resistance index ($P = .019$) were also statistically significant between the 2 study groups, as shown in Table 1.

HIGHLIGHTS

- Alprostadil improves clinical indicators in patients with DM combined with peripheral atherosclerosis.
- Alprostadil inhibits platelet aggregation and promotes platelet spreading.
- Alprostadil has ameliorative effects on HG- and ox-LDL-induced HUVECs.
- Alprostadil modulates the transforming growth factor-β1 (TGF-β1)/SMAD pathway.

Inclusion Criteria

Inclusion criteria include (1) meeting the diagnostic criteria for DM combined with peripheral atherosclerosis;[14] (2) the diagnosis of T2DM was made by laboratory tests, and the presence of atherosclerotic lesions in the arteries was detected by ultrasonography; (3) the patients had DM for at least 3 years and atherosclerosis for at least 1 month; (3) the clinical symptoms of DM included nausea, vomiting, dizziness, rash, and weakness; and (4) patients volunteered for treatment were informed about the study and signed an informed consent form.

Exclusion Criteria

Exclusion criteria include (1) pregnant and lactating women; (2) patients with a diagnosis of malignant disease, autoimmune disease other than DM, or immunosuppression; (3) subclinical atherosclerosis diagnosed on the basis of Doppler color ultrasonography; (4) patients who have taken drugs affecting the metabolism of Alprostadil in the last 12 weeks, smoked cigarettes, or consumed alcohol; (5) patients with a history of chronic diseases such as cardiac, hepatic, renal, and cancerous diseases; and (6) patients with a history of allergic reactions to drugs used in the present study.

Treatment Methods

Patients in both groups were given conventional medication prior to treatment. The conventional group continued conventional treatment with oral Alprostadil tablets, atorvastatin calcium tablets, and metoprolol tartrate tablets, with the possibility of increasing the dosage of the current treatment or adding oral hypoglycemic agents at the same time. The Alprostadil group received 250 mL of saline containing 60 mg of Alprostadil intravenously based on the conventional group. The medication was administered once daily for 16 months. Before and after treatment, 4-8 mL of fasting venous blood was collected from the patients, and the blood samples were analyzed biochemically using the ES-480 automatic biochemical analyzer (Bioelab, Nanjing, China). Fasting blood glucose (FBG) and 2-h postprandial glucose (2hPG) were detected, and biochemical indices including whole blood viscosity, plasma viscosity, erythrocyte pressure volume, and fibrinogen were determined. Anticoagulant tubes containing blood samples were inverted several times and centrifuged at 300 × *g* to separate the plasma.

Clinical Efficacy

This study mainly refers to the 2013 China guideline for T2DM to evaluate the clinical efficacy of the 2 groups of patients. Ineffective, in case of atherosclerosis did not improve or

Table 1. Clinical Data

Categorization		Alprostadil Group (n = 77)	Conventional Group (n = 77)	χ^2/t	*P*
Gender	Male	47	53	0.108	.652
	Female	30	24	0.112	.711
Age (years)		54.9 ± 10.2	55.2 ± 8.9	0.471	.52
Duration of diabetes (years)		5.2 ± 4.1	5.5 ± 2.9	0.214	.731
Doppler color ultrasonography					
Combined atherosclerosis (months)		3.5 ± 1.5	3.6 ± 1.6	0.234	.507
Peak systolic flow rate (cm/s)		27.25 ± 3.25	33.67 ± 3.92	11.060	<.001
End-diastolic flow rate (cm/s)		99.69 ± 16.31	107.43 ± 15.32	3.035	.003
Resistance index		0.75 ± 0.21	0.81 ± 0.07	2.378	.019
Fasting blood glucose (mmol/L)		10.3 ± 2.3	10.2 ± 2.5	0.361	.467
Two-hour postprandial blood glucose (mmol/L)		14.2 ± 1.6	13.8 ± 1.9	0.375	.724

even worsened after treatment, and the blood glucose level was still in a high state; effective, in case of atherosclerosis improved, and the blood glucose level tended to stabilize; and significantly effective, in case of atherosclerosis significantly improved, and the blood glucose level was in a stable state. The total effective rate = effective rate + significantly effective rate.

Observation Indicators

Clinical tests—end-diastolic blood flow rate, peak systolic blood flow rate, and resistance index, as well as glycemic metabolic indices were compared. Clinical efficacy—at least 3 months of follow-up were carried out to compare the efficacy of the 2 groups of patients. Therapeutic effect—after treatment, 3 mL of fasting venous blood was collected from patients in both groups, and after static precipitation, serum was prepared for examination with a centrifugation rate of 3500 r/min and a centrifugation time of 15 minutes. Changes in FBG and 2hPG, as well as inflammatory factors, were detected. The levels of serum C-reactive protein (CRP), interleukin-6 (IL-6), IL-8, and TNF-α were detected using an enzyme-linked immunosorbent assay.

Platelet Separation and Aggregation Assays

Blood was collected from the posterior elbow vein of the subject under strict sterilization into a centrifuge tube preloaded with the anticoagulant ACD (Sigma-Aldrich; 85 mmol/L sodium citrate, 71.38 mmol/L citrate, 27.78 mmol/L glucose) in a ratio of ACD to whole blood of 1 : 7. Centrifugation was performed in a centrifuge at 300 *g* at room temperature for a time dependent on the volume of blood, with 20 mL of whole blood centrifuged for approximately 10 min. After centrifugation, the whole blood was stratified, and the upper layer of platelet-rich plasma (PRP) was carefully aspirated and centrifuged at 740 *g* at room temperature for a volume-dependent period of time of approximately 10 minutes for 10 mL of PRP. After centrifugation, platelets were precipitated and resuspended by gently blowing with pre-warmed Tyrode's buffer (TB) to obtain the washed platelet (WP) suspension. The platelet concentration was measured, and the WP concentration was adjusted to approximately 3 × 10^8 platelets/mL by adding TB in appropriate amounts.

Platelet aggregation was determined by the turbidimetric method using a dual-channel platelet aggregometer from Chrono-Log. The WP prepared in the above steps was placed in a 37°C oven. The platelet aggregometer was turned on, preheated to 37°C, and connected to a computer. Then, 400 μL of TB was added to the control tube as a reference. The turbidimetric tube was added with 300 μL of WP and inserted into the aggregometer. The drug and agonist were added when the baseline was stable to record the aggregation at a constant temperature and speed. Aggregation was induced by adenosine diphosphate, collagen, or thrombocyte adhesion, respectively.

Platelet Spreading Assay

The operation area was marked on the back of the slide, and 200 μL of a final concentration of 200 μg/mL fibrinogen (pH = 8.3, 0.1 mmol/L $NaHCO_3$) was added dropwise and left at 4°C overnight, followed by 200 μL of 5% BSA (Beyotime) for 60 minutes at room temperature. The concentration of the platelet suspension was adjusted to 1 × 10^8 platelets/mL in both groups and allowed to stand for 30 minutes at 37°C. The platelets were added dropwise to the encapsulated slides at 37°C overnight and covered with 200 μL of Blocking Solution (BD Company) at 4°C for 20 minutes and wash buffer for 10 minutes. The wash buffer was aspirated, and 100 μL of FITC-phalloidin in 3% BSA was added dropwise and incubated at room temperature for 1 hour. The membrane was washed with PBS for 10 minutes 3 times, and the excess liquid was aspirated. Then, 20 μL of antifluorescence quencher was added, and nail polish was covered on the coverslip. The platelets were observed by fluorescence microscope (Leica), and the platelet spreading area was counted.

Cell Culture

Primary human umbilical vascular endothelial cells (HUVECs; CC-2517, Lonza) were routinely cultured (37°C, 5% CO_2) in endothelial cell medium (1001, Sciencell) containing 5% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.) and endothelial cell growth supplement (MilliporeSigma). Human umbilical vascular endothelial cells with fewer than 7 passages and 80-90% confluence were used for the experiments. Human umbilical vascular endothelial cells were

placed in serum starvation for 4 hours and then treated with HG (50 mM, Beyotime) and ox-LDL (50 μg/mL, ThermoFisher) for 24 hours.

Human umbilical vascular endothelial cells were placed in 6-well plates and grown to 70-80% confluence, then treated with 1 μM Alprostadil (900100P-1MG, Cayman, Tallinn, Estonia) dissolved in DMSO for 48 hours.

MTT Determination

Human umbilical vascular endothelial cells were put in 96-well plates (2.5×10^3 cells/well, 0.1 mL/well). Five time points from 1 to 5 days were set for the assay, respectively. The prepared MTT solution (20 μL, 5 mg/mL; Sigma-Aldrich) was added to each well and incubated for 4 hours at room temperature, after which the MTT solution was removed. Next, 200 μL of DMSO (Sigma-Aldrich) was added to each well, and the optical density (OD) value was detected at 570 nm using a microplate reader (NYW-96M, Beijing Nuoyawei Instrument, Beijing, China).

Flow Cytometry

The Annexin V-FITC apoptosis detection kit (Thermo Fisher Scientific) was used to identify apoptotic cells. Human umbilical vascular endothelial cells were placed in 6-well plates, each containing 5×10^4 cells, and incubated overnight at ambient temperature. Human umbilical vascular endothelial cells underwent digestion using EDTA-free trypsin (0.25%, Beyotime) and were spun at 1200 rpm for 5 minutes to produce cellular precipitates. Cell precipitates were delicately reconstituted in 500 μL of 1 × Annexin V binding buffer and combined with 10 μL of Annexin V-FITC and 5 μL of propidium iodide for half an hour at ambient temperature, shielded from illumination. Using a FACS Calibur flow cytometer (BD Biosciences, NJ, USA), apoptotic (Annexin V-positive and PI-negative) and necrotic (Annexin V- and PI-positive) cells were identified.

Measurement of Oxidative Stress

Malondialdehyde (MDA) and superoxide dismutase (SOD) activity in HUVECs and patient blood were measured by commercial enzyme-linked immunosorbent assay (ELISA) kits (Abcam). The kits allow rapid detection and quantification of MDA and SOD conjugates to protein. Quantification of MDA and SOD in protein samples is done by comparing the absorbance in the protein sample to the known absorbance of MDA and SOD.

RT-qPCR Assay

Total RNA was isolated from HUVECs using TRIzol reagent (Invitrogen), and RT-qPCR was performed using the SYBR PrimeScript RT-PCR kit (TaKaRa) and the ABI 7500 real-time fluorescent quantitative PCR system (Agilent Technologies, USA). RNA expression levels were calculated by the $2^{-\Delta\Delta Ct}$ method, and *GAPDH* normalized each gene expression level. Specific primers were designed as shown below (Table 2).

Western Blot

The collected HUVECs were washed twice with frozen PBS (Beyotime) and lysed in RIPA lysis buffer (Beyotime) for 30 minutes to extract the total proteins. The BCA Protein Assay kit (Beyotime) was applied to measure the total protein concentration. Equal concentrations of total proteins were separated on 10% SDS-PAGE and transferred to PVDF membranes (Millipore, MA, USA), which were blocked with 5% skimmed milk in PBST (Beyotime) for 2 hours. The cells were incubated at 4°C with specific primary antibodies GAPDH (Abcam; ab37168), transforming growth factor-β1 (TGF-β1; MedChemExpress; HY-P70543), Smad3 (Abcam; ab40854), p-Smad3 (Cell Signaling; 9520S), Smad7 (Proteintech; 66478-1-IG), and cleaved caspase-3 (Abcam; ab2302) overnight and horseradish peroxidase-coupled secondary antibody for 1 hour. The blots were imaged using a Bio-Rad imaging system (Bio-Rad, USA).

Enzyme-Linked Immunosorbent Assay

Tumor necrosis factor alpha, IL-6, and IL-8 in the blood of patients and HUVECs were determined using ELISA kits (R&D Systems). C-reactive protein human ELISA kit (ThermoFisher; KHA0031), endothelin-1 (ET-1) human ELISA kit, and human Nitric Oxide (NO) ELISA kit were utilized to measure CRP, ET-1, and NO, respectively.

Data Analysis

Data were statistically analyzed using SPSS 20 statistical software. Count data were expressed as %, and differences between groups were compared using the χ^2 test. Normality was tested by the Shapiro–Wilk method. Information on continuous variables conforming to normal distribution was described by mean ± SD, and differences were compared using the *t* test. Non-normally distributed data are expressed as [*M* (P25, P75)], and comparisons of differences between groups were tested using the Mann–Whitney *U*-test. $P < .05$ meant the difference was statistically significant.

Statement

No artificial intelligence (AI)-assisted technologies were used in the production of the submitted work.

RESULTS

Alprostadil Improves Clinical Indicators in Patients with Diabetes Mellitus Combined with Peripheral Atherosclerosis

As shown in Table 3, the total effective rate of treatment in the Alprostadil group was higher than that in the conventional group ($P = .042$). After Alprostadil treatment, the whole blood viscosity, plasma viscosity, erythrocyte pressure volume, and fibrinogen as well as inflammatory factors of the

Table 2. Primer Sequence

Gene	Forward Primer (5′→3′)	Reverse Primer (5′→3′)
ABCA1	TGCAAGGCTACCAGTTACATT	TTAGTGTTCTCAGGATTGGCT
VEGF	TTGCCTTGCTGCTCTACCTCCA	GATGGCAGTAGCTGCGCTGATA
GAPDH	CCCAGCAAGAGCACAAGAGGAAG	GAGGGGAGATTCAGTGTGGTGGG

Table 3. Comparison of Clinical Efficacy between the 2 Groups (Cases, %)

Groups	n	Obviously Effective (%)	Effective (%)	Ineffective (%)	Deterioration (%)	Total Effective Rate (%)
Conventional group	77	24	31	32	3	55
Alprostadil group	77	39	47	13	1	86
χ^2						3.468
P						0.042

Alprostadil group were lower than those of the conventional group (Figure 1A and B), which indicated that Alprostadil treatment effectively reduced the risk of thrombosis and inflammatory reactions. Alprostadil causes adverse reactions in the digestive system, which are mainly manifested as nausea and vomiting. Neurological and cardiovascular adverse reactions are also more common and usually include dizziness and fatigue. In addition, allergic reactions such as skin rashes can occur.[15] As shown in Table 4, the incidence of adverse reactions with Alprostadil administration was lower than that of the conventional group (*P* = .030).

Alprostadil Inhibits Platelet Aggregation and Promotes Platelet Spreading

Hyperglycemia leads to glycation of the platelet surface, affecting their membrane fluidity and enhancing platelet adhesion, which may worsen diabetic peripheral atherosclerosis.[16,17] The degree of platelet aggregation was determined

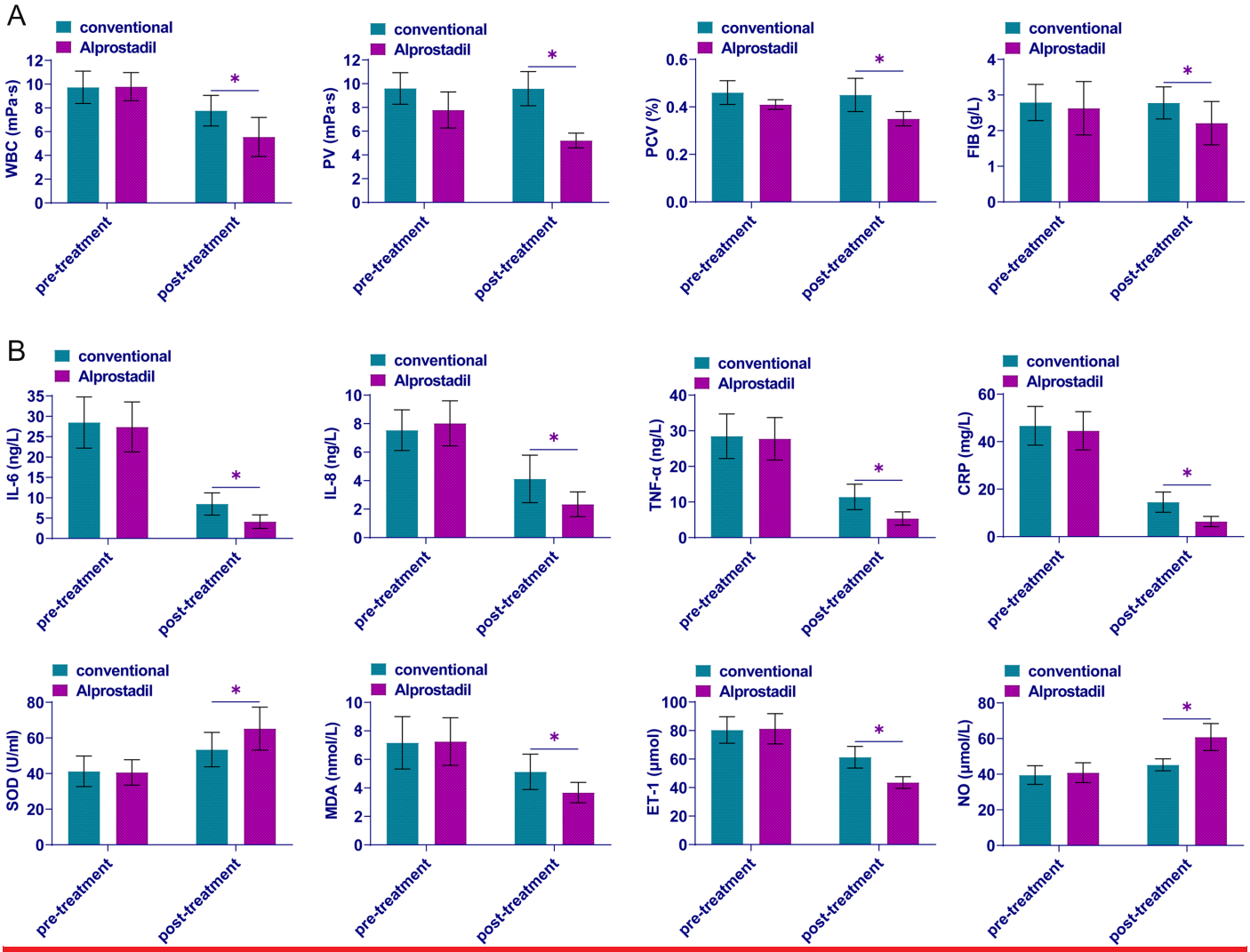

Figure 1. Alprostadil improves clinical indicators in patients with DM combined with peripheral atherosclerosis. (A) Values of whole blood viscosity, plasma viscosity, erythrocyte pressure volume, and fibrinogen before and after treatment in both groups; (B) levels of inflammatory factors detected by ELISA before and after treatment in both groups. Data are expressed as mean ± SD (n = 5). *P < .05 vs. conventional.

Table 4. Comparison of the Incidence of Adverse Reactions between the 2 Groups (Cases, %)

Groups	n	Nausea and Vomiting (%)	Dizziness (%)	Weakness (%)	Rash (%)	Total Incidence
Conventional group	77	3	5	7	3	20
Alprostadil group	77	1	2	1	2	6
χ^2						4.122
P						0.03

by the turbidimetric assay in both groups, and it was found that Alprostadil effectively reduced platelet aggregation, and the proportion of platelet aggregation in the Alprostadil group was lower than that in the conventional group (Figure 2A). Similarly, through the results of platelet spreading experiments, it was found that Alprostadil promoted platelet spreading and significantly elevated the proportion of spreading in the Alprostadil group (Figure 2B).

Alprostadil has Ameliorative Effects on High Glucose and Oxidized Low-density Lipoprotein Cholesterol-Induced Human Umbilical Vascular Endothelial Cells

As shown by the results of the MTT assay, 50 mM high glucose (HG) and 50 μg/mL ox-LDL treatment of HUVECs for 24 hours resulted in a significant decrease in cell proliferation capacity. In the presence of Alprostadil, HUVEC proliferation capacity was significantly increased (Figure 3A). The flow cytometry assay showed that Alprostadil significantly inhibited HG- and ox-LDL-induced apoptosis in HUVECs (Figure 3B). Western blot results indicated that HG- and ox-LDL promoted cleaved caspase-3 in HUVECs, and Alprostadil reversed this effect (Figure 3C). In addition, Alprostadil promoted SOD production, inhibited MDA levels, and attenuated the degree of oxidative stress (Figure 3D). ABCA1 is critical for maintaining intracellular cholesterol homeostasis and plays a key role in inhibiting vascular endothelial cell formation.[18,19] VEGF is a specific vascular endothelial cell mitogen that plays a primary role in pathological processes related to the proliferation of vascular endothelial-producing cells.[20] The RT-qPCR results showed that Alprostadil significantly elevated ABCA1 and decreased VEGF in HG- and ox-LDL-induced HUVECs (Figure 3E). ELISA assays showed that the production of TNF-α, IL-6, and IL-8 was significantly elevated in HG- and ox-LDL-induced HUVECs, whereas the production of TNF-α was significantly elevated in HG- and ox-LDL-induced HUVECs. Alprostadil reversed this effect (Figure 3F). In addition, CRP enhanced the impairment of endothelial cell function and the inflammatory effects of ox-LDL. ET-1 regulates vasoconstriction, inflammation, and endothelial cell proliferation by interacting with NO.[21] ELISA assay showed that Alprostadil significantly reduced CRP and ET-1 and enhanced NO (Figure 3G).

Alprostadil Modulates the Transforming Growth Factor-β1/SMAD Pathway

As downstream of the TGF-β1 pathway, SMAD proteins play important roles in diabetes, of which Smad3/7 have been widely studied. Western blot assay showed that in HG- and ox-LDL-induced HUVECs, TGF-β1 and p-Smad3 were elevated, and Smad7 was reduced; Alprostadil reversed the effect, significantly elevating the expression level of Smad7 and promoting the down-regulation of TGF-β1 and p-Smad3 expression (Figure 4). This suggests that inhibition of Smad3 and activation of Smad7 may be an effective means of slowing down peripheral atherosclerosis in DM combined.

DISCUSSION

There is long-standing evidence that diabetes promotes atherosclerosis, but its mechanisms have yet to be fully clarified. There is evidence that DM combined with peripheral atherosclerosis is closely associated with oxidative stress and inflammatory responses. An HG-induced increase in superoxide may be a key event leading to atherosclerosis. This study investigated the efficacy and mechanism of Alprostadil in the treatment of DM combined with peripheral atherosclerosis through clinical studies and cellular experiments.

Diabetic patients have abnormal blood flow and fibrin deposition. Disease progression can lead to stenosis,

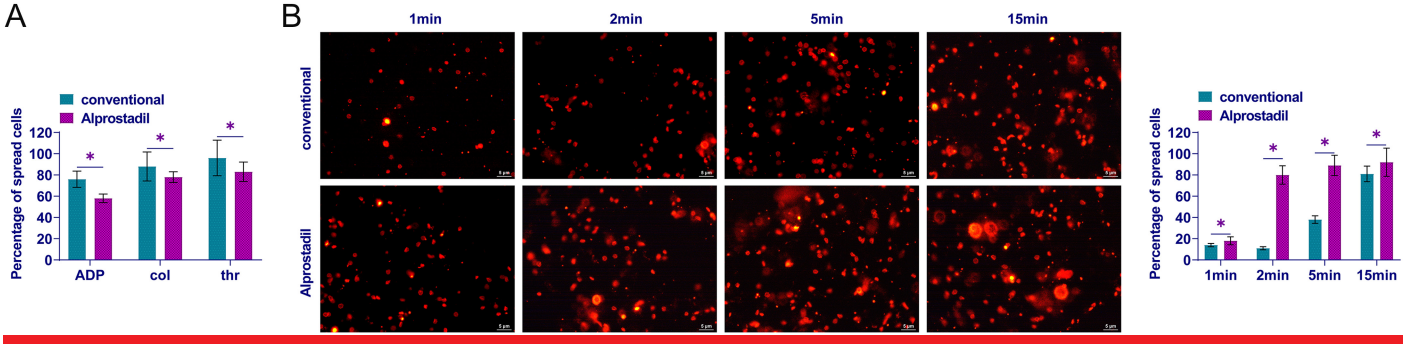

Figure 2. Alprostadil inhibits platelet aggregation and promotes platelet dispersion. (A) Turbidimetric method to determine the degree of platelet aggregation in both groups; (B) fluorescence microscopy to count the proportion of platelet spreading in both groups. Data are expressed as mean ± SD (n = 5). **P* < .05 vs. conventional.

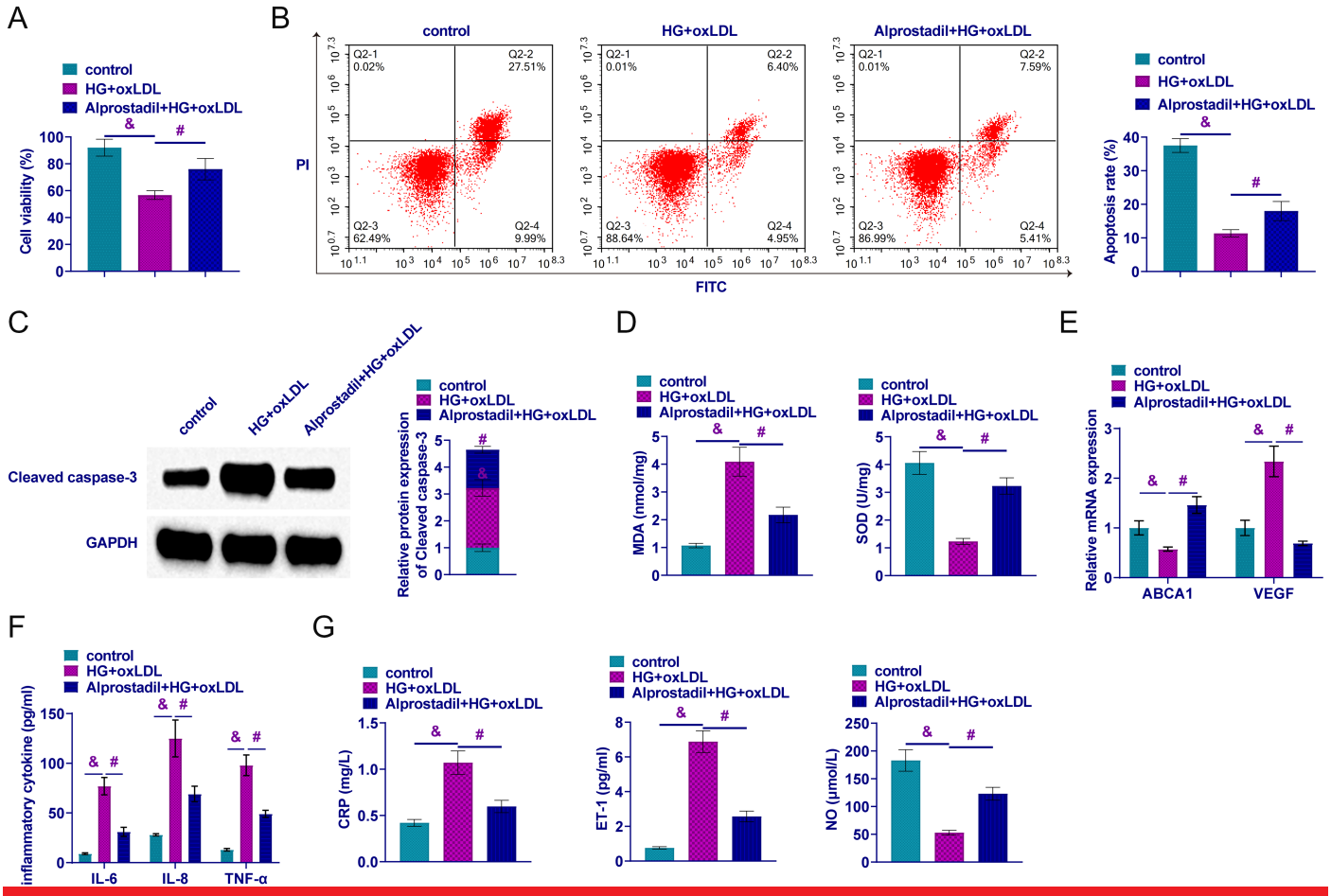

Figure 3. Alprostadil has ameliorative effects on HG- and ox-LDL-induced HUVECs. (A) MTT assay for the effect of Alprostadil on the proliferation of HUVECs; (B) flow cytometry assay for the effect of Alprostadil on apoptosis of HUVECs; (C) Western blot assay for the expression of cleaved caspase-3 in HUVECs; (D) Kit assay for MDA and SOD production; (E) RT-qPCR to determine the expression of *ABCA1* and *VEGF* in HUVECs; (F) ELISA to determine the production of TNF-α, IL-6, and IL-8 in HUVECs; (G) ELISA to determine the levels of CRP, ET-1, and NO in HUVECs. Data are expressed as mean ± SD (n = 3). $^{\&}P < .01$ vs. control; $^{\#}P < .01$ vs. HG + ox-LDL.

microcirculation disorders leading to ischemic hypoxia, and this vicious cycle leads to increased disease and other complications. Therefore, it is particularly important to strictly control blood glucose while improving microcirculation. Alprostadil exerts its role in vasodilatation and lowering peripheral resistance.[22] According to the literature, there are 2 mechanisms that are more recognized in the treatment of diabetes and its comorbidities with Alprostadil. One is that Alprostadil inhibits platelet aggregation, improves microcirculation, relieves ischemia in neural tissues, promotes the increase of intracellular cAMP concentration by regulating adenylyl cyclase and phosphoric acid activity, and activates a series of cAMP-dependent protein kinases, restoring the lesion damage induced by microangiopathy in diabetic patients. Another pathogenetic mechanism is oxidative stress, and the production of mitochondrial superoxide is a common mechanism leading to diabetes and its chronic complications, and the role of antioxidants in the treatment of diabetes is increasingly being emphasized.[23] Accordingly, the present study explored these 2 mechanisms. In this study, patients with DM combined with peripheral atherosclerosis. received either conventional treatment alone or Alprostadil adjunct as symptomatic therapy. The total effective rate of treatment in the Alprostadil group was higher than that in the conventional group, and the application of Alprostadil lowered whole blood viscosity, plasma viscosity, erythrocyte pressure volume, and fibrinogen, and inhibited platelet aggregation. These results suggest that adjuvant therapy with Alprostadil is more effective in relieving clinical symptoms and reducing fibrinogen and platelet deposition in the blood. Endothelial cells regulate vascular tone and structure by regulating vasodilation and constriction, anti-inflammatory and pro-inflammatory processes, antioxidants and pro-oxidants.[24-26] Not only was Alprostadil shown to be more effective in reducing TNF-α, IL-6, and IL-8 inflammatory factor levels and inhibiting the inflammatory response in patients, but the same results were shown in an in vitro cellular model assay. It is also interesting to note that the overall incidence of adverse effects was significantly higher in conventionally treated patients than in patients treated with adjunctive therapy with Alprostadil, which leads us to speculate whether Alprostadil has a mitigating effect on adverse

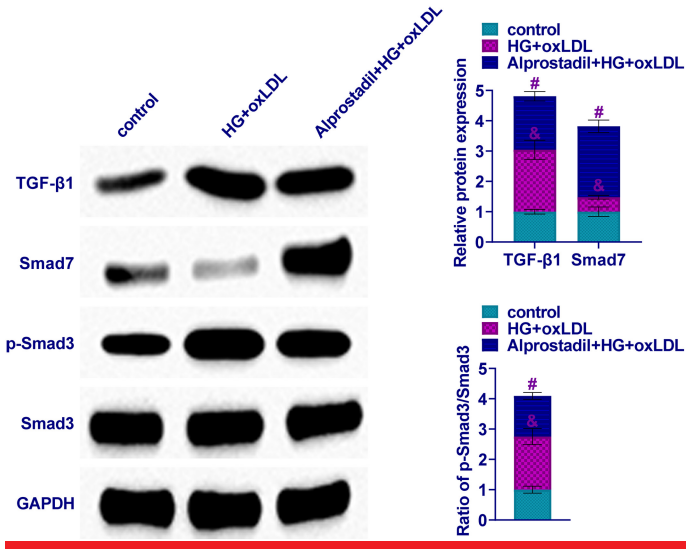

Figure 4. Alprostadil regulates the TGF-β1/SMAD pathway. Western blot to determine TGF-β1, Smad3, p-Smad3, and Smad7 in HUVECs. Data are expressed as mean ± SD (n = 3). $^{\&}P < .05$ vs. control; $^{\#}P < .05$ vs. HG + oxLDL.

effects, and whether this effect has a long-term nature, which needs to be demonstrated in subsequent experiments. Alprostadil can improve the clinical management of diabetic nephropathy by inhibiting platelet aggregation and thrombosis through dilating renal blood vessels and reducing urinary protein. However, it is difficult to achieve optimal clinical outcomes with Alprostadil alone.[23] Therefore, the combination of Alprostadil is currently a more effective treatment strategy, such as alpha-lipoic acid combined with Alprostadil for the treatment of diabetic nephropathy.[27]

Transforming growth factor-β1 is a cytokine that exerts biological functions through autocrine and paracrine pathways, and the mechanism of TGF-β1 in diabetes is still controversial. As a key regulator of extracellular matrix (ECM) synthesis and degradation in DM, the high glucose status of patients enhances TGF-β signaling, and dysregulation of TGF-β1 tends to lead to diseases.[28,29] In addition, the TGF-β1 signaling pathway can slow down the progression of atherosclerosis by regulating the inflammatory response,[30] which is expected to be a new target of action for slowing down the process of atherosclerosis. Active TGF-β1 regulates Smad2/3 by binding to TGFBR2, which directly transfers TGF-β1 signaling from the cell membrane to the nucleus. In the process of transduction, ECM deposition and cellular fibrosis can occur.[31] Smad3 is required for TGF-β-induced chemotaxis and expression of TGF-β1. Smad7 is an inhibitory Smad that is induced by Smad3 and competitively binds to TGFBR1 or inhibits the phosphorylation of Smad2 and Smad3 through the degradation of TGFBR1.[32,33] In this study, in diabetic cell models and patients' blood, along with p-SMAD3 activation and elevated TGF-β1 expression levels, SMAD7 expression was reduced, consistent with previous findings.[34] The effect was reversed under Alprostadil treatment. Combined with the above findings, the TGF-β1/SMAD pathway plays a crucial role in the onset and progression of DM.[35] Because the TGF-β1 signaling pathway exists in cascade with numerous signaling pathways and has a wide range of physiological roles, single inhibition of one of them may not achieve the desired therapeutic effect. This follows from the fact that clinical inhibition of TGF-β1 alone does not slow and treat diabetes. Therefore, modulation of its downstream key proteins may be a viable option to combat diabetes. Utilizing Alprostadil to target and restore the balance of Smad3/7, which in turn helps to restore the overall homeostasis of the TGF-β1 pathway, may be a novel strategy for the treatment of diabetes.

There are also some limitations to this study. The present study is a single-center study, with a relatively small sample size and a short follow-up time for patients, which resulted in the possible bias of the results of the present study. In the future, we need multi-center studies with increased sample sizes and follow-up times for further testing and validation. This study tested the hypothesis that multiple hypotheses raise issues of multiplicity, which may increase the likelihood of false-positive results. The dose and study duration of Alprostadil were determined based on previous studies, and further experiments are still needed to determine whether this dose and duration had a significant effect on atherosclerotic status.

In conclusion, the clinical efficacy of Alprostadil in the treatment of DM combined with peripheral atherosclerosis is high, and it attenuates clinical symptoms and reduces adverse reactions when used adjunctively with conventional treatment. In addition, Alprostadil was demonstrated to have a significant ameliorative effect on HG- and ox-LDL-induced HUVECs. This finding provides a theoretical basis for the clinical use of Alprostadil in the treatment of DM combined with peripheral atherosclerosis.

Availability of Data and Materials: The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Ethics Committee Approval: The present study was approved by the Ethics Committee of The Affiliated Hospital of Southwest Medical University (Decision date: 2015.02; Decision number: 201502SC83), and written informed consent was provided by all patients prior to the study's start. All procedures were performed in accordance with the ethical standards of the Institutional Review Board, The Declaration of Helsinki, and its later amendments or comparable ethical standards.

Informed Consent: Written informed consent was obtained from the patients who agreed to take part in the study.

Peer-review: Externally peer-reviewed.

Author Contributions: H.Y. designed the research study. H.Y. and Q.W. performed the research. H.Y. and Q.W. provided help and advice on the experiments. H.Y. and Q.W. analyzed the data. H.Y. wrote the manuscript. Q.W. reviewed and edited the manuscript. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript.

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