RESEARCH ARTICLE

Effect of Diabetes Mellitus on Implant Osseointegration of Titanium Screws: An Animal Experimental Study

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Objective: To explore the effect of diabetes mellitus (DM) on implant osseointegration of titanium screws.

Methods: Sixty rats were randomly divided into a DM group and a control group (each group, $n = 30$). DM group rats were injected with 1% Streptozotocin solution at 65 mg/kg to establish a DM model. Titanium screws were implanted into the rats' distal femurs in both groups. The rats were sacrificed for micro-CT scanning, micro-indentation, biomechanical detection, confocal Raman microspectroscopy, and histological and histomorphometric analysis at 4, 8, and 12 weeks post-implantation, respectively. Messenger RNA (mRNA) expression and protein expression of the related growth factors around the implant were analyzed using real-time polymerase chain reaction and Western blots.

Results: At 4, 8 and 12 weeks, micro-CT scanning, hematoxylin-eosin (HE) staining, Gieson's acid-magenta staining, and fluorescent labeled staining showed disorder in the bone tissue arrangement, a lack of new bone tissue, poor maturity and continuity, and poor trabecular bone parameters around the implant in the DM group. At 4, 8, and 12 weeks, the interfacial bone binding rate in the DM group was significantly lower (16.2% ± 4.8%, 25.7% ± 5.7%, 42.5% ± 5.8%, respectively) than that in the control group (23.6% ± 5.2%, 40.8% ± 6.3%, 64.2% ± 7.3%, respectively; $P < 0.05$). At 8 and 12 weeks, the elastic modulus (17.0 ± 1.8 and 15.1 ± 1.5 GPa, respectively) and trabecular bone hardness (571 ± 39 and 401 ± 37 MPa, respectively) in the DM group were significantly lower than the elastic modulus (23.4 ± 2.3 and 23.8 ± 1.8 GPa, respectively) and trabecular bone hardness (711 ± 45 and 719 ± 46 MPa, respectively) in the control group ($P < 0.05$). The maximum load required for the prosthesis pull-out experiment in the DM group at 4, 8, and 12 weeks (55.14 ± 6.74 N, 73.34 ± 8.43 N, and 83.45 ± 8.32 N, respectively) was significantly lower than that in the control group (77.45 ± 7.48 N, 93.28 ± 8.29 N, and 123.62 ± 9.43 N, respectively, $P < 0.05$). At 8 and 12 weeks, the mineral-to-collagen ratio in the DM group (6.56 % ± 1.35% and 4.45%± 1.25%, respectively) was significantly higher than that in the control group (5.31% ± 1.42% and 3.62% ± 1.33%, respectively, $P < 0.05$). At 12 weeks, mRNA and protein expression levels of bone morphogenetic protein 2, transforming growth factor-β1, vascular endothelial growth factor, osteopontin, osteocalcin, and runt-related transcription factor 2 in the DM group were significantly lower than that in the control group.

Conclusions: DM can negatively affect bone osseointegration, manifesting as disorder in bone tissue arrangement around the implant, a lack of new bone tissue, poor maturity and continuity, poor trabecular bone parameters and lower expression of the related growth factors.

Key words: Biomechanics; Diabetes mellitus; Growth factors; Orthopedic implants; Osseointegration

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Funding informationThis work was supported by Science and Technology Program of Guangzhou, China (202102010079); the Fundamental Research Funds for the Central Universities (21620452); National Natural Science Foundation of China (81601219).

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Received 19 March 2021; accepted 21 March 2022

Introduction

Approximately 800 million people in the world currently are aged ≥60 years, representing approximately 11% of the world's population. By 2050, this number is expected to increase to >2 billion people, representing 22% of the global population.[1–3] Recent International Diabetes Federation data indicate that an estimated 415 million adults worldwide aged between 20 and 79 years had a diagnosis of diabetes mellitus (DM) in 2015, with an expected projected increase to 642 million in 2040 and the prevalence rate increasing from 8.8% to 10.4%.[4] With an aging and rapidly growing population with DM, a large number of patients with DM are likely to undergo orthopedic surgery.[5–11] Observational studies have shown that DM is associated with a higher rate of perioperative complications,[12–14] such as surgical site infection, implant loosening, and fracture non-union. Among the reported complications, implant loosening commonly leads to orthopedic surgical revision and remains the major factor limiting the survival of orthopedic implants. A number of clinical trials have suggested that DM might result in a higher rate of implant loosening.[15–17] The reasons for these findings are unknown, but they may potentially include adverse effects of DM on the osteointegration process of orthopedic implants.

Osseointegration refers to a direct bone-to-metal interface without interposition of non-bone tissue. Branemark described osseointegration as consisting of highly differentiated tissue involving "a direct structural and functional connection between ordered, living bone and the surface of a load-carrying implant."[18,19] Osseointegration is the most ideal combination formed between orthopedic implants and bone tissue,[19] and Branemark demonstrated how titanium (Ti) implants could become permanently incorporated within bone. Ti and Ti-based alloy materials have exhibited advantages in manufactured load-bearing applications as orthopedic implants, due to their excellent mechanical strength and favorable biocompatibilities. Currently, most orthopedic metal implants used in orthopedic surgery are made from Ti and Ti-based alloy materials. The strong implant osseointegration of Ti implants is the basis for the success of orthopedic surgery. Therefore, to understand the effect of DM on implant osseointegration, it is necessary to evaluate the effect of DM on Ti implant osseointegration. Some animal studies have reported adverse effects of DM on Ti implants. However, in these animal studies, histological and histomorphometric analyses were used as the main or only detection method, and few observations were made at molecular and microbiomechanical levels. Based on previous research, a variety of measurement methods ranging across microscopic, micro- and macro-biomechanical, and molecular levels were used in this study, employing continuous detection at three different time points. Compared with previous studies, the effect of DM on implant osseointegration of Ti screws was observed using multiple detection methods at different levels, including the use of some methods for the first time, to obtain more comprehensive results.

This study aimed to: (i) confirm whether DM had an adverse effect on bone osseointegration using Ti screws; (ii) evaluate the microarchitecture, biomechanical properties, and chemical composition around the implant as a consequence of damage induced due to DM; and (iii) evaluate expression of bone-related growth factors around the implant due to the adverse effect of DM on bone osseointegration using Ti screws.

Materials and Methods

Experimental Animals and DM Induction

Sixty healthy male Sprague Dawley rats (age, 2 months; weight 200–230 g) were purchased from Southern Medical University Experimental Animal Technology Development Co., Ltd. The experimental animals were kept at the experimental animal center of Jinan University at a room temperature of 22°C (humidity, 50%–60%) with light and dark alternation at 12h/12h. After 1 week of adaptive feeding, they were randomly divided into a DM group ($n = 30$) and a control group ($n = 30$).

Streptozotocin (STZ) was dissolved in a 0.1 mol/L citrate-sodium citrate buffer (0.1 mol/L citrate solution 54 ml + 0.1 mol/L sodium citrate solution 46 ml, pH = 4.4) and prepared into a 1% solution. Rats in the DM group were fed a high-fat and high-sugar diet for 4 weeks, then fasted for 12 h, their body weight was measured, and then intraperitoneally injected with a 1% STZ solution at 65 mg/kg. Three days following the STZ injection, the rats were monitored daily for blood glucose levels, body weight, 24 h food intake, and 24 h water intake. Blood samples were taken from the tail vein and the glucose level was analyzed using a glucose meter (OneTouch, Johnson & Johnson, New Brunswick, NJ, USA). Only rats with a basal blood glucose level above 16.7 mmol/L for two consecutive days were considered to have DM, and the remaining rats in the DM group were then excluded from the study. We monitored and recorded blood glucose levels until 12 weeks post-operatively.

Implantation Procedures

After cleaning, the surgical instruments and drill parts were sterilized in a high-temperature high-pressure steam pot and dried for later use. In addition, the customized Ti screws (diameter, 2.0 mm) were also sterilized in a similar manner and sterile packaged for use after drying. Rats in the DM and control groups were weighed and anesthetized (1.5% pentobarbital sodium solution was intraperitoneally injected at 2 ml/kg). After the effect of anesthesia, the rats were fixed in a supine position on a bespoke operating table, the skin of the bilateral knee joints was prepared, and routine disinfection and drape laying were performed. A median approximate 1.0 cm long slightly medial incision was made in the knee joint. Skin, subcutaneous tissue, and periosteum were incised layer by layer to expose the distal femur. A sharp knife was used to create a preliminary hole convenient for drilling. Using an electric drill, a 2.0 mm diameter drill bit

was then used to drill at low speed in the direction of the coronal plane of the knee joint and perpendicular to the distal end of the femur. After drilling, the bone passage was flushed and screwed in the Ti screws (2.0 mm longer than the bone passage) and trimmed the screws leaving 1 mm exposed on both ends of the screws. Appropriate hemostasis was performed and the incision was sutured layer by layer. Three days post-implantation, an intraperitoneal injection of cefazolin sodium (500,000 units) was administered once a day (Fig. 1).

Methods

Histological and Histomorphometric Analysis

At 4, 8, and 12 weeks post-implantation, the specimens (distal femurs) were divided into soft and hard tissue sections. Soft tissue sections were sliced into 4 μm sections for hematoxylin-eosin (HE) staining. Non-decalcified hard tissue sections were sliced into 50 μm sections for Gieson's acid-magenta staining and fluorescent labeled staining, then the interfacial bone binding rate between the Ti screw thread and bone was measured using computer-assisted software (Leica Qwin Image Processing and Analysis Software, Version 2.4, Amsterdam, the Netherlands).

Micro-Indentation

At 4, 8, and 12 weeks post-implantation, the specimens were fixed with 70% alcohol, dehydrated and fixed, prepared for embedding and sectioning, and used for microscopic indentation mechanical detection of trabecular bone. The polymerized specimens were fixed on a hard tissue slicer, the resin material on the surface of the joint specimens was removed,

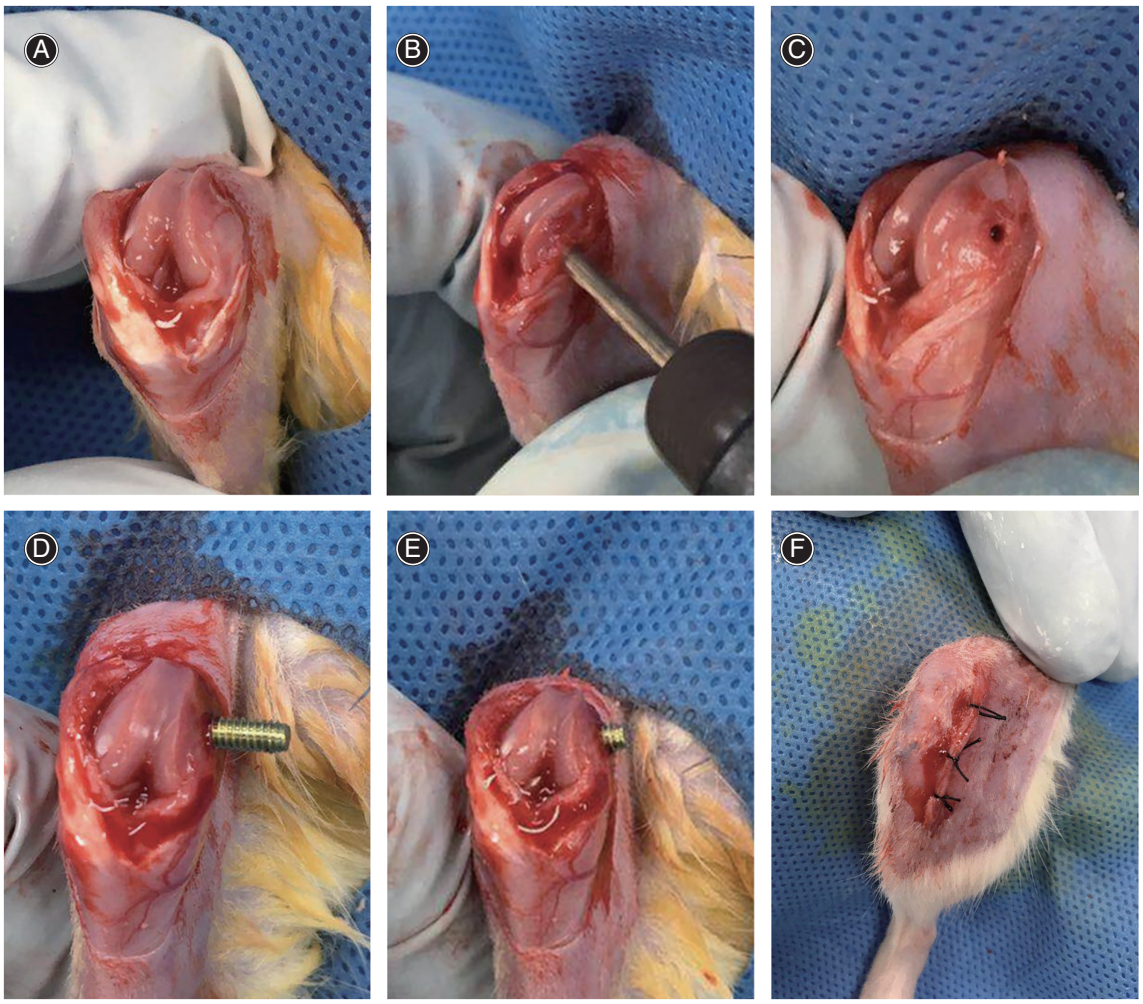

Fig. 1 Implantation procedures. (A) Skin, subcutaneous tissue and periosteum were incised layer by layer to expose the distal femur. (B) Drilling at low speed by an electric drill in the direction of the coronal plane of the knee joint and perpendicular to the distal end of the femur. (C) The bone tunnel in the femur after drilling. (D) Screwing the titanium screws. (E) Trimming them so that 1–2 mm is exposed on both ends of the screws. (F) Appropriate hemostasis was performed and the incision was sutured layer by layer

and the surface of the specimen prediction test was exposed. The thickness of the sections was set at 150 μm to obtain pathological sections that fully exposed trabecular bone around the screw. The embedded trabecular bone in the samples was polished successively with 400, 600, 800 and 1200 mesh sandpaper, with the thickness of the sections as high as 100 μm. The surface was polished with gradient aluminum powder (0.05 μm), and the trabecular bone indentation experiment was conducted after ultrasonic cleaning with double-steaming water for 10 min.

Biomechanical Detection

At 4, 8, and 12 weeks post-implantation, the specimens were trimmed to an appropriate length, and residual soft tissue and bone debris around the Ti screw were carefully removed. The specimens were fixed in customized aluminum alloy tubes with denture powder and denture water to ensure that the Ti screw was perpendicular to ground level. After the denture powder had hardened, the machine fixture clamped one end of the aluminum alloy tube and the other end of the screw, and the pull-out experiment was calculated at the speed of 0.1 mm/s. Data were collected by the High-precision Biomaterials Experiment system (BOSCH, ElectroForce® 3510) and WinTest® software (Version 1.2, Stuttgart, Germany).

Confocal Raman Microspectroscopy

The chemical composition of the trabecular bone around the Ti screw (mineral-to-collagen ratio) was evaluated using confocal Raman microspectroscopy (CRM) (Jobin Yvon, Horiba, France). At 4, 8, and 12 weeks post-implantation, the specimens were dehydrated in a graded alcohol series and embedded in PMMA. The non-decalcified hard tissue sections were sliced. The section surface was ground on successive grits of silicon carbide paper and polished with 1-mm alumina slurry. A ×50 objective focused the laser (785-nm laser diode source) on a 3 μm region within the trabecular bone, and inelastic light was acquired using a spectrograph with 0.5/cm spectral resolution. The measured spectra consisted of three accumulations with an integration time of 20 s each. Using vender-supplied software (Jobin Yvon), background fluorescence in the spectra was subtracted using a modified polynomial fitting algorithm. Spectra were collected from three trabecular bone locations around the Ti screw. The mineral-to-collagen ratio was calculated as the $\nu 1$ phosphate peak intensity (962/cm) per proline peak intensity (856/cm) and averaged per bone.

Micro-CT

At 4, 8, and 12 weeks post-implantation, the specimens were placed flat in a micro-CT examination slot and fastened to prevent them from moving. The scanning parameters were selected as follows: voltage, 80 kV; current, 0.5 mA; scanning mode, 360° rotation; scanning time, 20 min; exposure time, 350 ms; and resolution, 80 μm. The region of interest (ROI) was defined as bone tissues from 2.0 mm above the growth plate to the proximal 50 slices, extending with a radius of 200 μm from the implant surface. ROI endobone trabeculae were selected for three-dimensional (3D) visualization. MicView V2.1.2 3D reconstruction processing software and ABA special bone analysis software were used for quantitative analysis. The specific measurement parameters were: (i) trabecular thickness (Tb.Th, mm); (ii) trabecular number (Tb.N, 1/mm); (iii) trabecular spacing (Tb.Sp, mm); (iv) bone surface/bone volume (BS/BV, %); and (v) bone volume/total volume (BV/TV, %).

RNA Isolation and Quantitative Real-Time PCR

At 12 weeks post-implantation, total RNA of the rat femurs (3 mm around the implant) was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Reverse transcription was performed using a PrimeScript™ RT reagent kit with gDNA Eraser (Perfect Real Time, Takara, Japan) according to the manufacturer's protocol. A real-time quantification polymerase chain reaction test (RT-qPCR)was carried out on an ABI 7900 system using TB Green™ Premix Ex Taq™ II (Tli RNaseH Plus, Takara, Japan). The relative messenger RNA (mRNA) level was normalized to GAPDH as the reference gene. The relative changes in mRNA expression were analyzed using the $2^{-\Delta\Delta Ct}$ method. Bone morphogenetic protein 2 (BMP-2), transforming growth factor-β1 (TGF-β1), vascular endothelial growth factor (VEGF), osteopontin (OPN), osteocalcin (OCN), and runt-related transcription factor 2 (RUNX2) were selected for real-time PCR. The primer sequences are shown in Table 1.

Western Blots

At 12 weeks post-implantation, the rat femurs (3 mm around the implant) were scraped in lysis buffer and centrifuged at 12,000 g/min at 4 °C for 10 min. The protein concentration was measured using a BCA kit (Beyotime, Shanghai, China). Proteins were separated using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), electrophoresed, and blotted onto a polyvinylidene difluoride (PVDF)

TABLE 1 Sequences of the primers used for qPCR

	Sequences	
Gene	Forward(5′-3′)	Reverse(3′-5′)
BMP-2	ACATCCACTCCACAAACGAG	GTCATTCCACCCCACATCAC
TGF-β1	CTTCTCCACCAACTACTGCTTC	GGGTCCCAGGCAGAAGTT
VEGF	GAGTACCCAGAGCCTCCTCA	AGCACACCAACTCGGTGA
OPN	TCCAAGGAGTATAAGCAGCGGGCCA	CTCTTAGGGTCTAGGACTAGCTTCT
OCN	TGGAGACCGAACGACACAA	CAGCTGATCTTATGGAACC
RUNX-2	CCCACGGCCCTCCCTGAACT	CGTGTGGAAGACAGCGGCGT
GAPDH	CATGAGAAGTATGACAACAGCCT	AGTCCTTCCACGATACCAAAGT

BMP-2, bone morphogenetic protein 2; OCN, osteocalcin; OPN, osteopontin; qPCR, quantification polymerase chain reaction; RUNX2, runt-related transcription factor 2; TGF-β1, transforming growth factor-β1; VEGF, vascular endothelial growth factor.

membrane. After being blocked with 5% BSA/TBST for 1 h, the membrane was incubated with primary antibody at 4 °C overnight. The next day, the membrane was washed with TBST three times and then incubated with secondary antibody (1:5000 dilution) (SAB, Baltimore, MD, USA) for 1 h. The membrane was then washed again with TBST three times. The bands were exposed and imaged with Quantity One software (Bio-Rad, Richmond, CA, USA). BMP-2, TGF-β1, VEGF, OPN, OCN, and RUNX2 were selected for Western blots. The primary antibodies used in this study were as follows: BMP-2 (1:1000, Abcam), TGF-β1 (1:5000, Abcam), VEGF (1:500, Abcam), OPN (1:500, Abcam), OCN (1:500, Abcam), RUNX2 (1:1000, Abcam) and GAPDH (1:10000, Abcam).

Outcome Measures

Section Staining and the Interfacial Bone Binding Rate. HE staining of the soft tissue sections, Gieson's acid-magenta staining, and fluorescent labeled staining of hard tissue sections shows the amount of new bone tissue, and the order and maturity of trabecular bone. After a between-group comparison, any differences in the amount of new bone tissue, and in the order and maturity of trabecular bone around the Ti screw, could be observed. The interfacial bone binding rate was calculated from hard tissue sections using Leica Qwin Image Processing and Analysis Software (Version 2.4), which reflects the degree of interface connection between the prosthesis and bone, and is a commonly used quantitative index to measure the degree of bone osseointegration.

A 3D Reconstruction Image and Trabecular Bone Parameters. A micro-CT reconstruction image can indicate reconstruction of trabecular bone around the Ti screw. Compared with histological and histomorphometric analysis, the amount of new bone tissue and the order and maturity of trabecular bone around the Ti screw could be observed more clearly, and the DM group and the control group could also be compared more clearly. Trabecular bone parameters included Tb.N, Tb.Sp, BS/BV, BV/TV, and Tb.Th. Compared with the interfacial bone binding rate, these parameters provide various trabecular bone parameters in detail, which are more reliable and provide a detailed quantitative index.

The Pull-Out Experiment. The pull-out experiment is the most basic measure of biomechanical properties. The maximum load required for the prosthesis pull-out experiment reflected biomechanical differences between the two groups, with a higher load indicating better biomechanical properties.

The Elastic Modulus and Trabecular Bonehardness. The elastic modulus and trabecular bonehardness around the Ti screw are influenced by intrinsic bone material properties including mineralization and collagen integrity. Through

TABLE 2 Blood glucose levels, body weight, 24 h food intake, and 24 h water intake for rats in the DM and control groups after injection ($\bar{x} \pm s$)

Monitoring indicators	Groups	Time points						
		Pre-injection	3 days	4 days	5 days	6 days	1 week	2 weeks
Bloodglucose (mmol/L)	DM	6.6 ± 2.2*	25.2 ± 3.2*	24.7 ± 4.1*	24.5 ± 3.5*	23.9 ± 4.4*	24.3 ± 4.0*	23.7 ± 4.2*
	Control	6.5 ± 1.9	6.9 ± 2.4	6.7 ± 2.1	6.6 ± 2.3	6.8 ± 1.8	6.7 ± 2.5	6.5 ± 2.3
Weight (g)	DM	396.7 ± 7.5	365.6 ± 7.1*	338.7 ± 7.4*	312.4 ± 6.8*	305.6 ± 7.8*	301.8 ± 8.0*	297.3 ± 8.5*
	Control	392.4 ± 6.1	391.7 ± 6.5	389.4 ± 6.7	390.3 ± 7.3	393.5 ± 7.6	396.4 ± 7.8	395.8 ± 6.8
24 h food intake (g)	DM	27.6 ± 4.5	35.6 ± 7.8*	46.1 ± 9.9*	56.4 ± 10.8*	53.6 ± 11.7*	55.8 ± 10.4*	57.6 ± 9.7*
	Control	26.5 ± 3.7	25.6 ± 4.6	27.7 ± 5.4	24.5 ± 4.8	26.6 ± 5.9	25.8 ± 3.6	27.2 ± 4.5
24 h water intake (g)	DM	35.1 ± 5.8	125.6 ± 34.1*	138.7 ± 44.4*	142.4 ± 46.8*	151.6 ± 35.6*	150.7 ± 45.1*	152.3 ± 48.3*
	Control	37.9 ± 6.7	35.6 ± 7.8	36.7 ± 6.4	35.4 ± 6.6	38.6 ± 5.9	37.8 ± 8.7	36.3 ± 7.5

*Indicates $P < 0.05$ compared with the control group.; DM, diabetes mellitus.

TABLE 3 Blood glucose levels and body weight for rats in the DM and control groups post-operatively ($\bar{\chi} \pm s$)

Monitoring indicators	Groups	Time points				
		pre-operatively	1 week	2 weeks	3 weeks	4 weeks
Bloodglucose (mmol/L)	DM	23.7 ± 4.2*	24.1 ± 5.8*	25.1 ± 6.7*	24.5 ± 4.9*	26.3 ± 7.3*
	Control	6.8 ± 2.3	6.5 ± 3.2	6.7 ± 3.7	6.4 ± 2.1	6.3 ± 3.6
Weight (g)	DM	297.3 ± 8.5*	284.4 ± 7.3*	279.6 ± 8.3*	286.6 ± 6.4*	290.3 ± 7.9*
	Control	395.8 ± 6.8	396.7 ± 8.4	403.6 ± 7.6	410.5 ± 8.7	404.2 ± 7.7

* Indicates $P < 0.05$ compared with the control group.; DM, diabetes mellitus.

assessing micro-indentations, we could observe the influence of DM on implant osseointegration at a micro-biomechanical level, with higher results indicating better biomechanical properties.

The Expression Levels of Bone-Related Growth Factors. BMP-2, TGF-β1, VEGF, OPN, OCN, and RUNX2 are all factors associated with bone growth, with an increase in levels indicating increased growth and a decrease indicating decreased growth. Using Western blots, RNA isolation, and RT-qPCR, we could obtain mRNA and protein expression levels of BMP-2, TGF-β1, VEGF, OPN, OCN, and RUNX2. Through comparing the two groups, we determined the influence of DM on bone growth around the prosthesis.

The Mineral-to-Collagen Ratio. According to the Raman scattering effect, a CRM test analyzes the scattering spectra with different frequencies of incident light to obtain information on molecular vibration, rotation, and other aspects, and to obtain information on the molecular composition of organic materials and inorganic samples. An increase in the mineral-to-collagen ratio represents an increase in bone quality, characterized as increased remodeling and mineral crystallinity.

Statistical analysis

All data, presented as mean and standard deviation (SD), were analyzed using Statistical Package for Social Sciences (SPSS, Chicago, IL, USA; version 18.0) software. Descriptive statistics were used to describe demographic and measurement variables concerning all the femurs. Categorical variables are expressed as frequencies and percentages for each variable. Continuous variables are presented as mean ± SD. Analysis of Variance (ANOVA) and Student Newman–Keuls tests were applied to compare types. A P -value <0.05 was considered statistically significant.

Results

General Conditions

Blood glucose test results, which were taken 3 days after STZ injection for up to 12 weeks post-implantation, showed a significant increase of >16.7 mmol/L in the DM group compared with the control group (Tables 2–4). In the DM group, body weight was significantly lighter, and 24 h food and water intake were significantly greater (Tables 2–4). The DM group rats showed no significant fluctuations in blood glucose levels, indicating no significant difference in DM severity in rats in the DM group. Two rats failed to model, three rats died of excessive anesthesia, and six rats died of postoperative infection.

In the control group, the rats had normal blood glucose levels, normal 24 h food and water intake and normal daily activities, while their body weight increased steadily. Two rats died from excessive anesthesia (Tables 2–4).

Histological and Histomorphometric Analysis

HE Staining of the Soft Tissue Sections, Gieson's Acid-Magenta Staining, and Fluorescent Labeled Staining of the Hard Tissue Sections

Over time, new bone tissue formed around the Ti screws in both groups. Compared with the control group at the same period (4, 8, and 12 weeks post-implantation), bone tissue around the Ti screws in the DM group appeared to be disordered, with less new bone tissue, poor maturity and continuity, and more gaps between the Ti screw thread and the bone (Figure 2A–C).

Data Analysis of the Hard Tissue Sections

At 4, 8, and 12 weeks post-implantation, the interfacial bone binding rate between the Ti screw thread and bone in the DM group was significantly lower (16.2% ± 4.8%, 25.7% ± 5.7%, and 42.5% ± 5.8%, respectively) than that in the control group (23.6% ± 5.2%, 40.8% ± 6.3%, and 64.2% ± 7.3%, respectively), P<0.05 (Figure 2D).

Micro-Indentation

At 4 weeks, the elastic modulus and trabecular bonehardness in the two groups showed no statistical significance. At 8 and 12 weeks, the elastic modulus (17.0 ± 1.8 and 15.1 ± 1.5 GPa, respectively) and trabecular bonehardness (571 ± 39 and 401 ± 37 MPa, respectively) in the DM group was significantly lower than the elastic modulus (23.4 ± 2.3

TABLE 4 Blood glucose levels and body weight of rats in the DM and control groups (5–12 weeks post-operatively)

Monitoring Indicators	Groups	Time points							
		5 weeks	6 weeks	7 weeks	8 weeks	9 weeks	10 weeks	11 weeks	12 weeks
Bloodglucose (mmol/L)	DM	25.1 ± 5.3*	24.5 ± 5.6*	25.4 ± 6.2*	24.8 ± 4.9*	25.1 ± 5.5*	25.8 ± 4.7*	24.9 ± 5.9*	25.3 ± 6.1*
	Control	6.7 ± 2.2	6.6 ± 2.9	6.7 ± 2.7	6.5 ± 2.4	6.5 ± 2.2	6.6 ± 2.8	6.4 ± 2.7	6.8 ± 2.1
Weight (g)	DM	295.8 ± 6.9*	288.9 ± 7.5*	289.5 ± 8.2*	296.4 ± 8.1*	287.5 ± 7.2*	288.7 ± 8.3*	291.5 ± 7.7*	291.6 ± 6.9*
	Control	397.2 ± 7.9	406.8 ± 8.2	401.4 ± 8.0	399.7 ± 7.6	395.5 ± 7.8	402.9 ± 7.3	408.6 ± 7.4	402.7 ± 8.3

* Indicates $P < 0.05$ compared with the control group.; DM, diabetes mellitus.

and 23.8 ± 1.8 GPa, respectively) and trabecular bonehardness (711 ± 45 and 719 ± 46 MPa, respectively) in the control group, $P < 0.05$ (Fig. 2E,F).

Biomechanical Test

The maximum load required for the prosthesis pull-out experiment in the DM group at 4, 8, and 12 weeks post-implantation was significantly lower (55.14 ± 6.74 N, 73 ± 8.43 N, and 83.45 ± 8.32 N, respectively) than that in the control group (77.45 ± 7.48 N, 93.28 ± 8.29 N, and 123.62 ± 9.43 N, respectively), $P < 0.05$ (Fig. 2G).

The CRM Test

At 4 weeks, the mineral-to-collagen ratio of the two groups showed no statistical significance. At 8 and 12 weeks, a reduced mineral-to-collagen ratio was found in the DM group compared with the control group and the mineral-to-collagen ratio in the DM group (6.56% ± 1.35% and 4.45% ± 1.25%, respectively) was significantly higher than that in the control group (5.31% ± 1.42% and 3.62% ± 1.33%, respectively), $P < 0.05$ (Figure 2H).

Micro-CT Results

3D Reconstruction

Overtime, new bone tissue had formed around the Ti screws in both groups. At the same time (4, 8, and 12 weeks post-implantation), compared with the control group, bone mass around the Ti screws was lower in the DM group, the bone tissue texture was weaker, and the trabecular bone arrangement appeared to be sparser and more irregular (Fig. 3A).

Trabecular Bone Parameters

At 4 weeks post-implantation, Tb.Th, Tb.N, Tb.Sp, BS/BV, and BV/TV in the two groups showed no statistical significance. At 8 weeks post-implantation, Tb.N, BS/BV and BV/TV in the DM group were found to be significantly lower than those in the control group. Tb.Sp in the DM group was significantly higher than that in the control group, but Tb.Th in the two groups showed no statistical significance. At 12 weeks post-implantation, Tb.Th, Tb.N, BS/BV, and BV/TV in the DM group was found to be significantly lower than that in the control group, Tb.Sp in the DM group was significantly higher than that in the control group (Fig. 3B–F).

mRNA and Protein Expression Levels of BMP-2, TGF-β1, VEGF, OPN, and RUNX2

At 12 weeks post-implantation, mRNA expression of BMP-2, TGF-β1, VEGF, OPN, OCN, and RUNX2 in the DM group was significantly lower than that in the control group on RT-qPCR analysis (Fig. 4A–F). At 12 weeks post-implantation, BMP-2, TGF-β1, VEGF, OPN, OCN, and RUNX2 protein expression levels in the DM group were significantly lower than those in the control group when analyzed using Western blots (Fig. 4G–H).

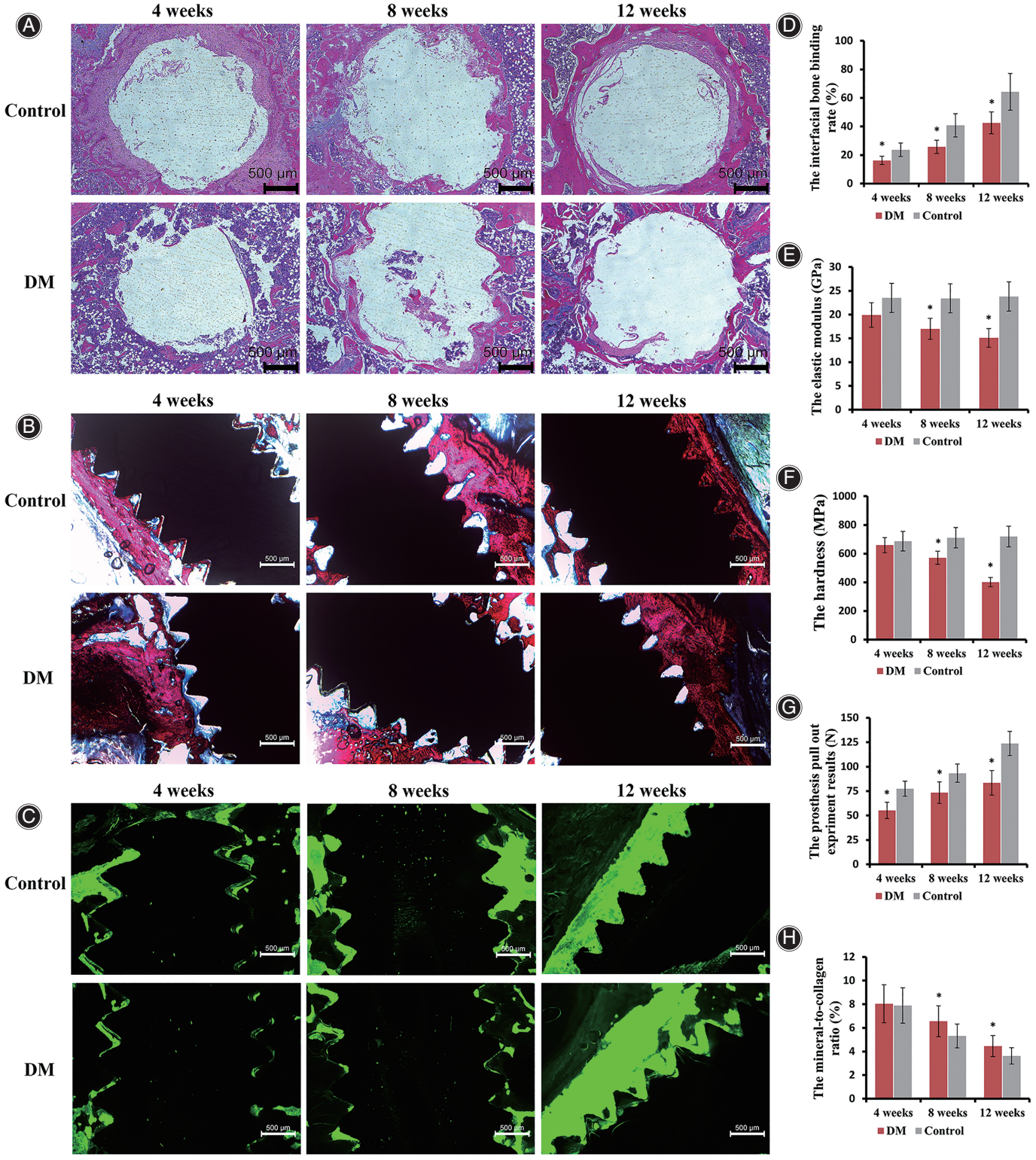

Fig. 2 Histological and histomorphometric analysis, micro-indentation, biomechanical test and confocal Raman microspectroscopy. (A) Hematoxylin-eosin (HE) staining of the soft tissue sections, scale bar = 500 μm. (B) Gieson's acid-Magenta staining of the hard tissue sections, scale bar = 500 μm. (C) Fluorescent labeled staining of the hard tissue sections, scale bar = 500 μm. (D) The interfacial bone binding rate. (E–F) The elastic modulus and hardness of bone trabeculae. (G) The maximum load required for the prosthesis pull out experiment. (H) The mineral-to-collagen ratio. The data are shown as the mean ± SEM, *indicates $P < 0.05$. DM, diabetes mellitus

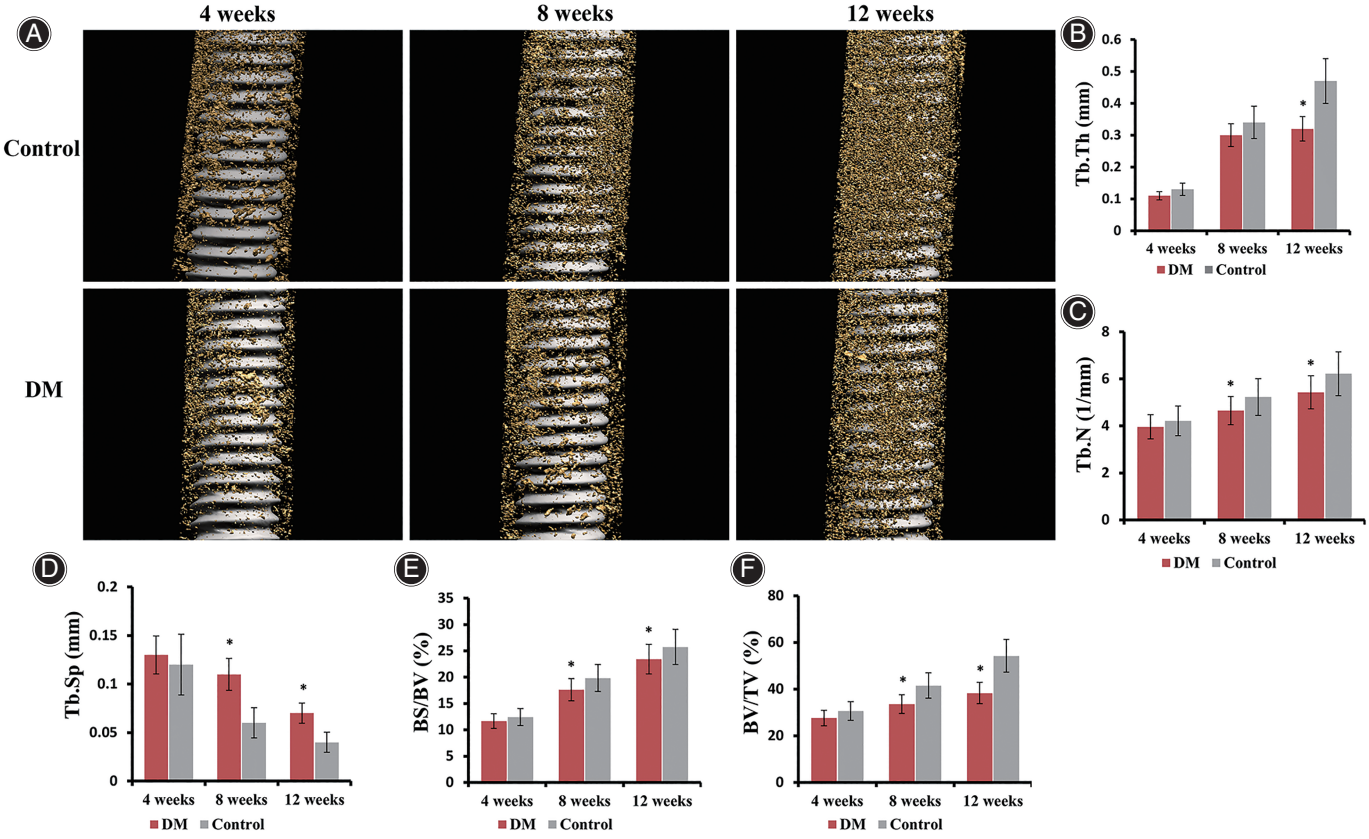

Fig. 3 Micro-CT three-dimensional (3D) reconstruction and trabecular bone parameters. (A) Representative micro-CT 3D reconstruction of peri-implant bone, the yellow areas on the implant surface refers to the bone areas that contact the implant surfaces. (B–F) Quantitative analysis of trabecular bone parameters regarding Tb.Th, Tb.N, Tb.Sp, BS/BV, and BV/TV. The data are shown as the mean ± SEM, *indicates $P < 0.05$. BS/BV, bone surface/bone volume; BV/TV, bone volume/total volume; DM, diabetes mellitus; Tb.N, trabecular number; Tb.Sp, trabecular spacing; Tb.Th, trabecular thickness

Discussion

In this longitudinal study, we adopted a variety of measurement methods at microscopic, micro- and macro-biomechanical, and molecular levels, employing continuous detection at three different time points. First, micro-indentation and CRM were performed to observe the influence of DM on implant osseointegration in a DM rat model, which showed that DM aggravated implant osseointegration at microscopic, micro- and macro-biomechanical, and molecular levels. Furthermore, we observed that mRNA and protein expression levels of the related growth factors around the implant were significantly lower in the DM group.

Histological and Histomorphometric Analysis

A markedly lower bone-implant contact percentage and diminished quantity of newly formed bone in the cortical area around the implant in rats with DM has previously been observed in studies involving histological and histomorphometric analysis.[20–26] For example, Hasegawa *et al.*[20] observed that bone-implant contact in the cortical area was 12% for rats in a DM group and 61% for rats in a control group at 4 weeks, and reported a two-fold difference at 8 weeks following implantation. Similar differences were observed, but the rates are relatively lower in the present study. It may be related to the difference in the implants, where they implanted a rectangular inner chamber and we implanted a Ti screw with a larger surface area. Siqueira *et al.*[21] observed that rats with DM exhibited a 50% reduction in the area of formed bone at 21 days. Although this study reported similar differences, we are not sure if 21 days is long enough to complete the process of bone formation and bone remodeling. Giglio *et al.*[23] observed that the bone binding rates of rats with DM compared with controls were 55% and 91% at 14 days, and 56% and 97% at 30 days, respectively. All these studies observed a reduction of osseointegration capacity in rats with DM; however, most of these studies selected only two detection time points, with the longest time point being 8 weeks post-implantation; therefore, only early changes could be observed. HE staining of the soft tissue sections, Gieson's acid-magenta staining, and fluorescent labeled staining of the hard tissue sections at three different time points were employed to obtain more comprehensive quantitative data. Bone tissue around the implant was observed to be disordered, with less new bone tissue, poor maturity and continuity, and more gaps between the Ti screw thread and the bone in the DM group at

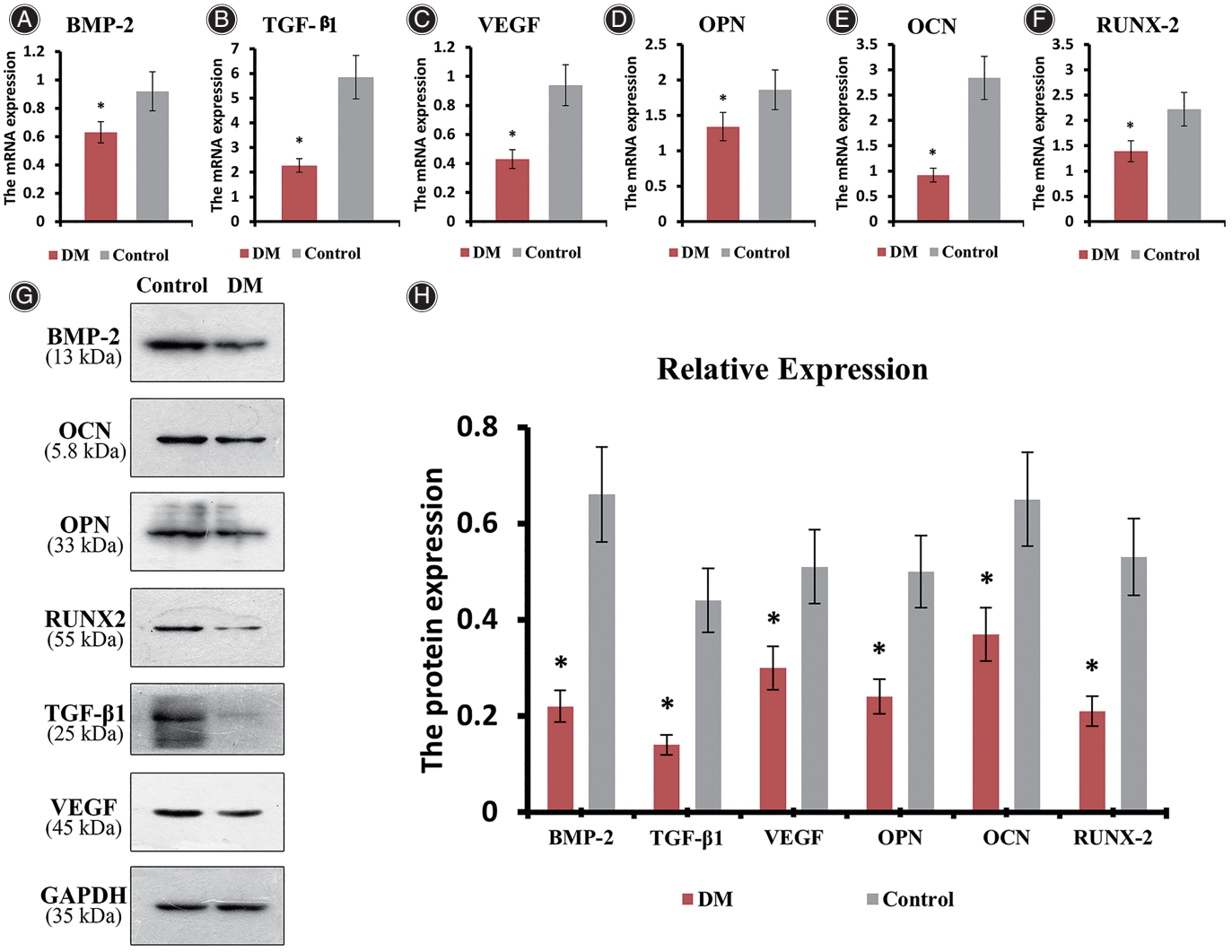

Fig. 4 mRNA expression and protein expression levels of BMP-2, TGF-β1, VEGF, OPN, OCN and RUNX2. (A–F) The mRNA expression levels of BMP-2, TGF-β1, VEGF, OPN, OCN and RUNX2. (G–H) The protein expression levels of BMP-2, TGF-β1, VEGF, OPN, OCN and RUNX2. The data are shown as the mean ± SEM, *indicates $P < 0.05$. BMP-2, Bone morphogenetic protein 2; DM, diabetes mellitus; OCN, osteocalcin; OPN, osteopontin; RUNX2, runt-related transcription factor 2; TGF-β1, transforming growth factor-β1; VEGF, vascular endothelial growth factor

4, 8, and 12 weeks post-implantation. The ratio of the Ti screw bone binding area in the DM group was significantly lower than that in the control group at 4, 8, and 12 weeks post-implantation. Compared with previous studies, we performed histological and histomorphometric analyses, made detailed and comprehensive pathological sections, and accurately quantified and compared implant osseointegration between the two groups.

Micro-CT

Some studies have reported that 3D images of microarchitecture and the quality of trabecular bone provide a clearer perspective on implant osseointegration capacity using micro-CT.[27–29] In those studies, reconstructed 3D images and quantitative analysis showed poor osseointegration and trabecular structure around implants in rats with DM, with the only differences being in the selective indexes and detection times. While these studies reconstructed the bone and implant as a whole, our study only reconstructed the implant and surrounding bone tissue to obtain more visual images that can intuitively show the effect of DM on bone trabeculae. Wu *et al.*[27] analyzed Tb.N, Tb.Sp, BV/TV, Tb.Th, and %OI at 12 weeks, Jia *et al.*[28] analyzed BV/TV, Tb.N, and %OI at 8 weeks, and Hua *et al.*[29] analyzed BV/TV Tb.N, Tb.Sp, and bone-implant-contact at 3 months post-implantation. Although similar differences were observed, these studies were unable to capture dynamic changes in trabecular parameters. The dynamic changes of trabecular parameters in our study can intuitively show the effect of DM on bone trabeculae. Tb.N, Tb.Sp, BS/BV, and BV/TV in the DM group were found to be significantly inferior to those in the control group at 8 and 12 weeks post-implantation. Tb.Th

in the DM group was also found to be significantly inferior to that in the control group at 12 weeks post-implantation. The 3D reconstruction showed disorder of bone tissue arrangement, a lack of new bone tissue, poor maturity and continuity, and poor trabecular bone parameters around the implant in the DM group. Compared with previous studies,[27–29] we used the micro-CT at three different time points to observe dynamic changes in bone tissue around the implant.

The Pull-Out Experiment and Micro-Indentation

Biomechanical properties, another important means to reflect implant osseointegration capacity, were found to be significantly inferior in the DM group. Pull-out (or push-out) tests are the most basic measure of biomechanical properties. Previous studies[27–29]have reported significantly inferior results in the pull-out or push-out tests in DM groups. In this study, biomechanical detection showed the maximum load required for the prosthesis pull-out test in the DM group at 4, 8, and 12 weeks post-implantation was significantly lower than that in the control group. Similar differences were observed in our study, and we innovatively applied another micro-biomechanical test. Micro-biomechanical test can indicate that the composition changes caused by DM causes the changes of macromechanics. Micro-indentation was used to test mechanical properties at a micro-biomechanical level. The elastic modulus and trabecular bonehardness around the implant, which are influenced by intrinsic bone material properties including mineralization and collagen integrity, were found to be significantly reduced in the DM group at 8 and 12 weeks. To our knowledge, this study is the first to adopt micro-indentation to determine the influence of DM on implant osseointegration at a micro-biomechanical level.

The Expression Levels of Osteoblast-Specific Genes and the CRM Test

In addition to an observation of microstructure and biomechanical properties, molecular level studies can help clarify the relevant mechanisms. Hamann *et al.*[30] reported a 40%–80% reduction in expression of the osteoblast-specific genes BMP-2, RUNX2, OCN, and OPN. In their study, the rapid, rather than insidious, onset of type 2 DM with persistently elevated serum glucose levels of 40 mmol/L for 8 weeks represents an extreme metabolic situation that cannot fully translate into clinical medicine. Wang *et al.*[31] found that mRNA and protein expression levels of the osteoblast-specific genes RUNX2, OCN, and Osterix in a DM group were significantly lower than those in a control group. Similar to Hamann's study, Wang *et al.*[31] also detected the related growth factors in bone marrow cells. Hamann *et al.*[30] and Wang *et al.*[31] reported that poor implant osseointegration could be related to the low expression of related growth factors around the implant. In this study, mRNA and protein expression levels of BMP-2, TGF-β1, VEGF, OPN, OCN, and RUNX2 in the DM group were found to be significantly lower at 12 weeks post-implantation. In contrast to previous studies, bone tissues around the implant were used for RT-qPCR and Western blot analyses. Compared with whole bone marrow cells used in previous studies,[30,31] the related growth factors were more highly enriched in bone tissues around the implant. Furthermore, using a CRM test, a significantly reduced mineral-to-collagen ratio of trabecular bone was observed around the implant in the DM group at 8 and 12 weeks post-implantation. Our findings further confirmed reduced mineralization in trabecular bone around the implant, which both complements and is consistent with previous studies. Moreover, this study was the first to adopt CRM tests to observe the influence of DM on implant osseointegration at a molecular level.

Limitation of the Study

This study used diabetic rats model of type I DM induced by STZ. In humans, the incidence of type II DM is significantly higher than that of type I DM. The use of animal models of type II DM will be more convincing.

Conclusions

DM negatively affected bone osseointegration, which manifested as disorder in the arrangement of bone tissue around the Ti implant, a lack of new bone tissue, poor maturity and continuity, poor trabecular bone parameters and lower expression of the related growth factors.

Acknowledgments

The authors are very grateful to Bo-yuan Zheng, Yun-jun Wu, Wen-yi Gan, Sheng-chun Wang and Ji-wen Chen for their selfless efforts in this research. Their help ensured the progress of this research.

Conflict of Interest

All authors declare that they have no conflict of interest with other people or organizations that could inappropriately influence this work.

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