Enhanced Bone Remodeling Effects of Low-Modulus Ti–5Zr–3Sn–5Mo–25Nb Alloy Implanted in the Mandible of Beagle Dogs under Delayed Loading

Jing Hu, Xiaobo Zhong, and Xiaoming Fu

ABSTRACT: Titanium (Ti) and its alloys are widely used in the dental and prosthetic implant fields due to their favorable biocompatibility. In this study, porous surface coatings incorporated with nanoscale hydroxyapatite particles on the surface of Ti and Ti–5Zr–3Sn–5Mo–25Nb (TLM) alloy were fabricated by microarc oxidation followed by hydrothermal treatment; the surface roughness and hydrophilicity were obviously enhanced by the surface modification procedure. In vivo, four adult male beagle dogs were selected for an implantation procedure and restored with full metal crowns after healing for 3 months. The bone responses were evaluated via histomorphological observation. Raman spectral analysis and nanoindentation experiments were used to quantitatively and qualitatively estimate the characteristics of the bone formed around the implants. Compared to the Ti group, the TLM titanium alloy group showed a significant increase in the percentage of bone–implant interface contact, bone inside the thread, mineralization, crystallinity, modulus of elasticity, and hardness of the integrated bone after delayed loading in the TLM group. Therefore, the TLM titanium alloy is considered a candidate implant material with desirable biomechanical compatibility, especially under applied stress.

1. INTRODUCTION

For more than half a century, titanium and its alloys have been widely used in the medical field as dental implants and prosthetic materials because of their better biocompatibility, mechanical properties, and corrosion resistance to other metallic biomaterials. Implants are expected to combine with bone tissue and successfully withstand the forces of chewing over the lifetime of a patient. To achieve this goal, biomaterials are required to have ideal bioactivity and biomechanical compatibility.

β-Type titanium alloys have attracted particular attention in recent years and benefit from their intrinsic low elastic modulus that is favorable for homogeneous stress transmission between the implant and bone. These alloys show better mechanical properties to reduce stress shielding effect comparable to commercial pure Ti or Ti–6Al–4V. They are being developed and trialed continuously. Modulus mismatch is responsible for complications, such as peri-implant bone disuse atrophy and implant loosening or fracture as a result of stress shielding and concentration. Moreover, modulus mismatch leads to micromotion at the bone–implant interface and excessive micromotion inhibits bone formation, leading to fibrous tissue ingrowth and implant loosening. This paper focused on the Ti–5Zr–3Sn–5Mo–25Nb (TLM) alloy consists of nontoxic, nonallergenic, and nonvanadium elements. A large number of new β-type Ti alloys have been developed via the addition of alloying elements such as Mo, Nb, Ta, Zr, Mn, Sn, etc. to suppress the elastic modulus and enhance the strength of alloys. They also exhibited superior corrosion resistance and superior biocompatibility to Ti and other alloys. The Ti–5Zr–3Sn–5Mo–25Nb (TLM) alloy has an elastic modulus of approximately 50 GPa, closer to the elastic modulus of cortical bone (~30 GPa), which also exhibits a balance between high strength and low modulus. Niinomi and colleagues developed a β-type titanium Ti–29Nb–13Ta–4.6Zr (TNTZ) alloy which displayed superior application as load-bearing biomaterial. The alloy composites and elastic modulus were similar to those of the TLM alloy. Furthermore, Sn and Mo were proposed to reduce the consumption of high-cost elements such as Ta of TNTZ and additionally contribute to the enhancement of corrosion resistance and mechanical properties. TLM alloy may be more suitable to be used as a metal for dental casting to TNTZ alloy, which has considerably high melting point.
Osseointegration is strongly influenced by the surface morphology and the surface chemistry of dental implants. Microarc oxidation (MAO) is a simple, controllable, and cost-effective surface modification method for producing a stable porous titanium oxide layer doped with calcium, phosphate, and iron in situ. Microarc oxidation (MAO) is a simple, controllable, and cost-effective surface modification method for producing a stable porous titanium oxide layer doped with calcium, phosphate, and iron in situ. Microarc oxidation (MAO) is a simple, controllable, and cost-effective surface modification method for producing a stable porous titanium oxide layer doped with calcium, phosphate, and iron in situ. Titanium oxide coatings created by the
MAO process show good biofunction and tribocorrosion resistance.20–22 Previously, an MAO coating on titanium was broadly found to have excellent effects on cell responses and early osteointegration.23,24 In the work of Zhou et al.,25 implants with MAO coating were implanted into the femoral condyles of New Zealand rabbits to evaluate their performance in vivo. Their results suggested that MAO coating possesses an excellent bone apposition capability in the bone/implant interface. Meanwhile, porous structure created by MAO can provide good biological fixation through bone tissue ingrowth into the porous network as well as further reduce elastic modulus of Ti-based alloys.26 On the other hand, hydrothermal treatment (Htt) is the optimal method to incorporate hydroxyapatite (HA) on a titanium oxide layer.27 The composition and structure of HA are similar to human bone to promote biological compatibility and osteoconductivity.28 In a previous in vivo test, the TLM alloy with an active porous/HA microstructure showed favorable tribology and corrosion behavior, biocompatibility, and osteoconductivity to promote osteointegration.29,30

In this study, we estimated the bone–implant interface contact (BIC) and percentage of bone inside the thread (BIT) and detected the bone quality through Raman spectrum analysis. Moreover, a nanoindentation test was used to evaluate the mechanical properties of integrated bone. Importantly, we assumed that a β titanium-based alloy with porous coating shows better bone remodeling than pure titanium under functional condition. The aim of this study was to investigate the biomechanical behavior, biocompatibility, and bioactivity of a Ti–5Zr–3Sn–5Mo–25Nb (TLM) alloy as a dental implant material and to compare it with commercially pure Ti.

2. RESULTS

2.1. Surface Properties. The surface morphology differed drastically among the groups. As observed via field emission scanning electron microscopy (FE-SEM) (Figure 1), the control Ti-S surface exhibited a relatively smooth surface with machining stripes, while a microporous microstructure layer was observed on the Ti-MAO sample, similar to dentin tubules; additional nano-HA structures covered the porous microstructure in the Ti-MAO-Htt group. The Rₐ was 0.253 ± 0.028, 1.674 ± 0.032, 1.671 ± 0.025, and 1.668 ± 0.022 μm for the TLM-S, TLM-MAO, TLM-MAO-Htt, and Ti-MAO-Htt surfaces, respectively (Figure 2A), indicating that the MAO treatment increased the surface roughness. The contact angle (CA) was 13.8 ± 1.7, 14.1 ± 1.5, 35.6 ± 2.2, 35.3 ± 1.8, 78.4 ± 4.1, and 77.8 ± 3.6° for the TLM-MAO-Htt, Ti-MAO-Htt, TLM-MAO, Ti-MAO, TLM-S, and Ti-S surfaces, respectively (Figure 2B), which showed that the MAO and Htt processes enhanced the surface coating hydrophilicity. Spectrum analysis of surface elements (Figure 2C) showed that the Ca/P ratio in the TLM-MAO-Htt coating was 1.662, which was similar to that of bone tissue (1.67), while the Ca/P ratio in the Ti-MAO-Htt coating was 1.557.

2.2. Bone Responses after Delayed Loading. All of the animals recovered quickly from the implant surgery, and neither clinical signs of inflammation nor infections were observed.

After loading for 3 months in the mandibles of dogs, the TLM-MAO-Htt implant showed greater BIC to BIT ratios than the Ti-MAO-Htt implant (Table 1). As the histological sections (Figure 3) illustrate, bone absorption appeared on the superior margin of the first thread of the Ti-MAO-Htt implant and on the inferior margin of the first thread of the Ti-MAO-Htt implant. More osteocytes and fewer osteoclasts were observed than on the TLM-MAO-Htt implant, while a limited number of osteocytes and no osteoclasts were seen around the Ti-MAO-Htt implants. Comparison of the Raman analysis at the cortical level is shown in Figure 4, and compared to the data obtained without loading, the degree of mineralization, carbonate content, and crystallinity increased in the two groups. The carbonate content and crystallinity of the TLM-MAO-Htt integrated bone were significantly higher than those of the Ti-MAO-Htt integrated bone (Table 2).

After loading for 3 months, the hardness and elastic modulus of the integrated bone in the cortical bone area around the TLM-MAO-Htt implant were significantly greater than those of the integrated bone around the Ti-MAO-Htt implant (Table 3). These results indicate that the quality of the integrated bone remodeling surrounding the TLM implants was better than that around the Ti implants. No obvious space was observed via SEM between the bone and the implants in...
both groups. However, the shadow on the bone side was more obvious and less bone tissue appeared in the Ti-MAO-Htt group (Figure 5).

3. DISCUSSION

An appropriate surface modification is widely accepted as necessary to improve the biocompatibility, bioactivity, and corrosion resistance of biomaterials. In this study, MAO was used to provide a good combination of porous oxide layers on β-type TLM, and then, nanoscale HA particles were successfully incorporated over the porous coating via Htt. We analyzed the surface physicochemical characteristics in vitro, and a considerable number of homogeneous pores were observed on the Ti-MAO and TLM-MAO surfaces, which was similar to the surface appearance reported in a previous study. Htt did not change the porous structure induced by MAO, and produced a nanosized crystalline HA coating on the porous oxide layer, which was in agreement with previous reports. Compared to the Ti-S implants, the TLM-MAO and Ti-MAO implants exhibited significantly rougher and more hydrophilic surfaces. The Ra decreased slightly after Htt, which might be attributed to pits formed by the precipitation of nano-HA occupying the mainly porous structure. The hydrophilicity was further enhanced after the Htt process, and the hydrophilicity of the fabricated coating on pure Ti was similar to that on the TLM surface. The surface morphology of biomaterials has been shown to affect their osteoconductivity in terms of osteocalcin production and ALPase activity. Bai et al. reported that both the proliferation and ALP activity of MC3T3-E1 cells were higher on MAO-treated substrates than on pure Ti substrates, and these results were consistent with those on a TLM surface. Osteoblasts that adhered to an MAO surface had been shown to exhibit a more flattened morphology, with cytoplasmatic extensions that penetrated the pores and bridged a network bound to the porous surface, which could provide a pathway for cells to communicate and mature to a differentiated phenotype. An increase in surface roughness was known to enhance the mechanical interlocking between an implant and bone to improve the implant–bone bonding strength. Hydrophilic surfaces have substantial potential for promoting protein adsorption and cellular responses, especially bone cell differentiation and maturation, which are closely associated with direct bonding and early stabilization of dental implants. HA has excellent osteoconductivity, osteoinductivity, and angiogenic effects and stable chemical properties, all of which contribute to osteointegration. The presence of an HA layer was suggested to verify chemical bonding between bone and the biomaterials.

In the present study, TLM and Ti implants were subjected to the same surface treatment and showed similar surface morphology properties. The difference in the bone remodeling effects between the TLM and Ti groups was primarily attributed to the difference in actual load amount on the bone, which depended on the elastic modulus of the implant material.

In vivo, histomorphological results showed active bone remodeling around the Ti implants, while relatively stable bone formation surrounded the TLM implants, which was further revealed through the visible decrease in the BIC and BIT values in the Ti-MAO-Htt group. The BIC% is defined as the percentage of the length of newly mineralized bone in direct contact with the surface of the implant threads. The BIT% is defined as the percentage of newly mineralized bone volume inside all of the threads, which is similar to the BIC% measurement. The higher BIC and BIT values observed in this study indicated that the bone bonding and the amount of mineralized bone deposited around the implants in the TLM-MAO-Htt group were significantly greater than those in the Ti-MAO-Htt group. These previous findings might explain the
results in the present study indicating that the β TLM alloy was beneficial to reducing stress shielding and that the stress was not completely transferred along the implant but homogeneously dispersed to the peri-implant bone, which could stimulate bone regeneration, reshaping and strengthening and avoid bone resorption back into the body.\textsuperscript{44,45} It showed that osteolysis in the neck of TLM implants was more seriously destroyed than that of Ti ones. The stress almost concentrated on the neck of implant, which induces overloading and bone resorption in this area.\textsuperscript{4,45,46} The fact was very important since the load transfer from the implants to bone was considered optimum to reduce the cervical bone resorption caused by the stress shielding and overloading when the TLM implants consisting of a β-titanium phase are characterized by a low Young's modulus closer to natural bone.\textsuperscript{47,48}

Raman spectroscopy\textsuperscript{49} and nanoindentation tests\textsuperscript{50} were carried out to further assess the bone quality and mechanical properties of bone remodeling, respectively. The quality of the bone tissue adjacent to implants plays an important role in determining mechanical stability because the adjacent bone tissue performs mechanical functions in implant systems.\textsuperscript{51,52} The mineral-to-matrix ratio increased in the TLM and Ti groups, and the increase was more obvious in the TLM group than in the Ti group, which was similar to the carbonate-to-phosphate ratio results, showing that the extent of tissue mineral content and carbonate substitution increased.\textsuperscript{53,54} These results suggest that bone formed around the TLM-MAO-Htt implants was stronger than bone formed around the Ti-MAO-Htt implants after 3 months of loading.\textsuperscript{35} The FWHM peak of the phosphate band was used to indicate the mineral crystallinity, which was significantly higher in the TLM-MAO-Htt group than in the Ti-MAO-Htt group. Crystallinity is related to the mechanical properties of bone tissue, including the modulus, yield stress, and fracture stress.\textsuperscript{56} Hardness and elastic modulus of bone tissue adjacent to the interface were calculated by producing a loading–unloading curve as a result of indentation.\textsuperscript{5,57} Nanoindentation is an experimental technique widely used to test the mechanical properties of bone specimens in vitro.\textsuperscript{51} For each sample, three

| Table 2. Comparison of Raman Spectrum between TLM-MAO-Htt and Ti-MAO-Htt after 3 Months of Loading (X ± SD) |
|---------------------------------|------------------|------------------|------------------|------------------|
| groups | PO\textsubscript{4}\textsuperscript{3−} / amide I | CO\textsubscript{2}\textsuperscript{−} / PO\textsubscript{4}\textsuperscript{3−} | 1/PO\textsubscript{4}\textsuperscript{3−} (FWHM) |
| TLM-MAO-Htt | 9.583 ± 0.114 | 0.186 ± 0.003 | 0.0547 ± 0.0004 |
| Ti-MAO-Htt | 9.292 ± 0.136 | 0.183 ± 0.004 | 0.0559 ± 0.0007 |
| P value | 0.017 | 0.275 | 0.025 |

\textsuperscript{*}P < 0.05; TLM-MAO-Htt vs Ti-MAO-Htt.

| Table 3. Comparison of Hardness and Elastic Module of Integrated Bone among TLM-MAO-Htt and Ti-MAO-Htt Implants |
|---------------------------------|------------------|------------------|
| groups | hardness (X ± SD) (GPa) | elastic module (X ± SD) (GPa) |
| TLM-MAO-Htt | 0.58 ± 0.08 | 18.56 ± 0.23 |
| Ti-MAO-Htt | 0.48 ± 0.10 | 16.82 ± 0.33 |
| P value | 0.000 | 0.000 |

\textsuperscript{****}P < 0.0001; TLM-MAO-Htt vs Ti-MAO-Htt.
different bone were measured to reduce random errors and five indentations were made for each region in the longitudinal direction of bone tissue. The sites and directions of indentation were also randomly selected. There are totally 15 indentations in the cross section of per sample to get the average results. After loading for 3 months, the hardness and elastic modulus of bone integrated with the TLM-MAO-Htt implant were significantly greater than those of bone integrated with the Ti-MAO-Htt implant ($P \leq 0.05$). The mechanical properties of bone around the TLM implants are significantly better than those of bone around the Ti implant. It suggested that the TLM alloy implants with lower elastic modulus enhanced the effect of bone remodeling in bone/implant interface, which was consistent with the result observed by Zacchetti et al., who showed that the quality of the trabecular bone midway between two implants was increased when moderate mechanical stimulation was applied to the tibiae of rats. Homogeneous transmission of the functional stress was beneficial for bone formation and remodeling.44,59 A large mismatch of the modulus between the bone and implant can also lead to micromotion and negatively affect the stress distribution at the bone--implant interface, which induces the bone atrophy and mineralization and decreases implant stability.4,60,61

Our study has several limitations. We supposed that the sample was an isotropic solid in this study, but it did not correspond to the anisotropy and heterogeneity of bone. We also lack considerations that may affect the results, such as hydration testing condition of sample and changeable Poisson’s ratio of different bone samples and orientations. Further studies looking specifically at aspects of the comparability and reliability of this technique are required to establish optimal method for the measurement of bone mechanical property. Poor tribology and corrosion resistance of biomedical coating also limit its clinical application in implants. To improve these properties, a Ca--P-containing coating were prepared on metal surface via MAO processes in recent work.20,21,28,62 Sobolev et al. reported that corrosion resistance of the protective oxide coating on MAO-treated Ti-6Al-4V specimens was 20 times higher than the untreated sample by a potentiodynamic polarization test in NaCl solution.63 It is of great significance to study the delamination of biomedical coating, but an experiment involving this aspect was not carried out. Similar conclusions were reported commonly that the ceramic MAO and HA layer linked tightly with the metal substrates due to the physical interaction (e.g., surface roughness and small grain of the substrate) and chemical bonding between titanium and coating.51,64–66 Further studies are necessary to verify the interfacial bonding strength between coating and substrate and to investigate the long-term friction together with tribocorrosion behavior of the biomaterial under severe exposed conditions and loading for implant applications.

In clinic, the ultimate goal of dental implants is to perform masticatory function. It is a key to exhibit stable and reliable osseointegration between the implant materials and surrounding bone under load-bearing condition. This can be expectedly achieved through the use of low-elastic-modulus biomedical materials, for example, the approximate $\beta$-type TLM alloy coated with multiporous layer by MAO technique.

4. CONCLUSIONS

MAO and Htt generated a hybrid, rough, hydrophilic coating, which achieved osteointegration after implantation into the mandible of Beagles. In a loading experiment, the effect of bone remodeling around the TLM implants was significantly improved compared to that around the Ti implants. Within the limitations of this study, the following conclusions can be drawn:

1. The microarc oxidation combination with hydrothermal process is an excellent surface modification technique to enhance the surface bioactivity on Ti--$5Zr--3Sn--5Mo--25Nb$ (TLM) alloys.
2. Low-intrinsic-elasti-modulus TLM alloy exhibited desirable biomechanical compatibility in vivo. Therefore, it is promising to be an appropriate candidate biomaterial applied to load-bearing dental implants.

5. MATERIALS AND METHODS

5.1. Sample Preparation. All of the titanium and TLM alloys were provided by Northwest Nonferrous Metal Research Institute (Northwest Institute for Non-ferrous Metal Research, China). A TLM titanium alloy, which was approximate to a $\beta$-type or titanium alloy, was used as the substrate material and
base material for the implants. Prior to the MAO process, the specimens were increasingly ground with 200–2000 grit abrasive paper and then dried for further use. The following three groups of plates were prepared and characterized: (1) TLM-MAO-Htt: TLM titanium alloy subjected to MAO treatment that has been described in previous studies,\textsuperscript{67} followed by Htt at 200°C for 4 h to prepare MAO-Htt-treated samples, as described in previous studies;\textsuperscript{68} (2) Ti-MAO-Htt: pure titanium treated via MAO and Htt; and (3) Ti-S: smooth, pure titanium without surface coating treatment. Pure Ti and TLM plates (⌀15 mm × 1 mm) were cut with a lining-cutting machine and used for surface characterization and in vitro cell studies. Cylindrical implants with a diameter of 4.1 mm and a length of 8 mm were designed for the in vivo experiment.

### 5.2. Physicochemical Characterization of the Coatings

The surface morphology and elemental composition of the coatings were examined by a field emission scanning electron microscope (Nova Nano-SEM 230) equipped with an energy-dispersive X-ray spectrometer. The surface average roughness ($R_a$) and contact angle (CA) of the specimens were analyzed using a surface profiler (SURFCOM C2800E) and a video contact angle measurement system (Phoenix-300), respectively. The phase compositions of the samples were analyzed by X-ray diffractometry (X'Pert Pro MPD).

### 5.3. Surgical Procedure

All animal procedures were approved by the IACUC of Chongqing Medical University, China, and conducted in accordance with the principles of medical ethics.

Four adult male beagles (weight of 13.87 ± 2.33 kg, 1 year old) were used in the mandibular dental implantation experiments. Animals were housed in individual cages in an air-conditioned room with free access to water and food and an artificial 12/12 h day/night cycle. The animals were allowed to acclimate for 1 week prior to surgery. The dogs were randomly numbered. Before all operations, the animals were intramuscularly anesthetized with ketamine (40 mg/kg) and locally anesthetized with articaine. Animals were then placed on a sterile drape to provide sterile conditions during surgery. Supra- and subgingival scalings were performed 2 weeks prior to surgery. A dentition defect model was established through bilateral extraction of four premolars from the mandible of the dogs. After a 3 months wound-healing period, three-dimensional computed tomography (CT) images of the jaws with the dentition defects were used to ensure the implant conditions. Three implant holes (⌀4.1 mm × 8 mm) were drilled at each lateral edentulous area, followed by dental implantation. The implant site obeyed the Leiten distribution.\textsuperscript{69} The implants were placed in a direction that allowed occlusion with the upper dentition, and two groups of implants (three TLM-MAO-Htt on the left and three Ti-MAO-Htt on the right) were placed in the defect region. All of the implants were restored by placement of a full metal crown 3 months after implantation that formed light occlusion with the upper dentition to simulate an intraoral load-bearing environment for 3 months. Then, the dogs were sacrificed by external carotid vein exsanguination and the bilateral mandibles with implants were harvested.

### 5.3.1. Sample Preparation

Specimens were longitudinally sectioned from the major axis with a hard tissue microtome (Leitz 1600, Germany). The mesial half of every implant was used to obtain hard tissue slices with a thickness of 30 μm for bone histological and histomorphological analyses. Additionally, the sections were stained with methylene blue and basic fuchsin stain to quantitatively assess new bone formation and peri-implant osseointegration. Meanwhile, the distal half of sections was ground and polished to obtain a smooth surface and then ultrasonically cleaned for Raman analysis (Renishaw, U.K.) and the nanoindentation experiment (Agilent). Four samples were randomly selected from each group and 8–10 tissue sites per specimen were selected for Raman analysis of the cortical bone adjacent to the implant threads; five sites in three regions per sample (the same samples used for Raman analysis) were selected for nanoindentation analysis.

### 5.3.2. Histological Examination

Histological examination was performed under a light microscope (OLYMPUS, Japan) equipped with a camera (Canon550D, Japan). BIC was defined as the sum ratio of the implant length in direct contact with new bone tissue and the total length of the implant adjacent to native bone. BIT was defined as the percentage of newly formed bone volume inside all of the threads between the implant and the cancellous bone. Image-Pro Plus.6.0 (IPP6.0) microscopy image analysis software was used to analyze the data.

### 5.3.3. Raman Spectrum Analysis

Raman spectra were obtained using a standard confocal Raman microscope (Renishaw, U.K.) with a 785 nm semiconductor laser at 50 mW for 10 s, a frequency range of 200–2000 cm$^{-1}$, and a spectral resolution of 2 cm$^{-1}$ through a 20× long working distance objective. Multiple spectra of the same sample were integrated to represent relative Raman spectrum signal intensities in a mapped sample region. The featured baseline was subtracted from all spectra, which were then deconvolved via Lorentzian functions to obtain specific parameters, such as the amplitude, area, and wavenumber. The raw peak intensities for the three different bone minerals were calculated as follows: the PO$_4^{3−}$ν1 (960 cm$^{-1}$)/amide I (1667 cm$^{-1}$) peak area ratio; the carbonate CO$_3^{2−}$ (1070 cm$^{-1}$)/PO$_4^{3−}$ν1 (960 cm$^{-1}$) ratio; and the full width at half-maximum (FWHM) PO$_4$ν1 peak.

### 5.3.4. Nanoindentation Test

Nanoindentation tests were performed using a G200 Nano Tester (Agilent) with a displacement resolution below 0.01 nm and a maximum indentation depth of 500 m. Diamond Berkovich indenters were employed based on a continuous stiffness-measuring technology. A maximal load of 500 mN was applied on the surfaces for 50 s during the indentation load nanoindentation tests. The slope of the unloading force–displacement curve was recorded and analyzed, after which the average hardness and modulus were determined from the depth region of the unloading portion of the curve between 200 and 2000 nm. The assumed Poisson's ratio for bone is 0.3. The Young's modulus E and hardness H of the specimen were then computed through the relationship described by Oliver and Pharr.\textsuperscript{70} The E and H values in the cortical bone region were used to characterize the mechanical properties of the peri-implant integrated bone.

### 5.4. Statistical Analysis

Statistical analyses were conducted with SPSS 18.0. The quantitative data were expressed as mean ± standard deviation (X ± SD). Statistical analysis was performed with one-way analysis of variance. The Kruskal–Wallis $H$-test was used to compare the average values of multiple independent parameters. Values of $p < 0.05$ were considered statistically significant.
AUTHOR INFORMATION

Corresponding Author
*E-mail: 500808@hospital.cqmu.edu.cn. Mobile: 1398386306. Stomatological Hospital of Chongqing Medical University, No. 426, North Songshi Road, Yubei District, Chongqing 401147, China.

ORCID

Jing Hu: 0000-0002-4940-436X

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