Multifunctions of dual Zn/Mg ion co-implanted titanium on osteogenesis, angiogenesis and bacteria inhibition for dental implants

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ABSTRACT:

In order to improve the osseointegration and long-term survival of dental implants, it is urgent to develop a multifunctional titanium surface which would simultaneously have osteogenic, angiogenic and antibacterial properties. In this study, a potential dental implant material-dual Zn/Mg ion co-implanted titanium (Zn/Mg-PIII) was developed via plasma immersion ion implantation (PIII). The Zn/Mg-PIII surfaces were found to promote initial adhesion and spreading of rat bone marrow mesenchymal stem cells (rBMSCs) via the upregulation of the gene expression of integrin $\alpha 1$ and integrin $\beta 1$. More importantly, it was revealed that Zn/Mg-PIII could increase Zn^{2+} and Mg^{2+} concentrations in rBMSCs by promoting the influx of Zn^{2+}

and Mg^{2+} and inhibiting the outflow of Zn^{2+} , and then could enhance the transcription of Runx2 and the expression of ALP and OCN. Meanwhile, Mg^{2+} ions from Zn/Mg-PIII increased Mg^{2+} influx by promoting the expression of MagT1 transporter in human umbilical vein endothelial cells (HUVECs), and then stimulated the transcription of VEGF and KDR via activation of hypoxia inducing factor (HIF)-1 α , thus inducing angiogenesis. In addition to this, it was discovered that zinc in Zn/Mg-PIII had certain inhibitory effects on oral anaerobic bacteria (*Pg*, *Fn* and *Sm*). Finally, the Zn/Mg-PIII implants were implanted in rabbit femurs for 4 and 12 weeks with Zn-PIII, Mg-PIII and pure titanium as controls. Micro-CT evaluation, sequential fluorescent labeling, histological analysis and push-out test consistently demonstrated that Zn/Mg-PIII implants exhibit superior capacities for enhancing bone formation, angiogenesis and osseointegration, while consequently increasing the bonding strength at bone-implant interfaces. All these results suggest that due to the multiple functions co-produced by zinc and magnesium, rapid osseointegration and sustained biomechanical stability are enhanced by the novel Zn/Mg-PIII implants, which have the potential application in dental implantation in the future.

Keywords: zinc; magnesium; ion implantation; multifunctions; dental implant

1. Introduction

Despite the good biocompatibility of titanium, dental implants still suffer from delayed osseointegration and complicated bacterial infection due to their insufficient osteogenic activity and antibacterial effect [1,2]. In order to resolve this problem, various works on titanium surface modification, such as incorporation of bioactive

trace elements or/and topographical modification, have been carried out for improved bioactivity or antibacterial property [3-6]. However, it is difficult for these two performances only to ensure both rapid osseointegration and sufficient early stability of dental implants. Active angiogenesis is another crucial factor of superior bone integration, because abundant blood vessels are indispensable guarantees for favorable functions of osteoblasts and rapid generation of bone tissue. Therefore, we assumed that titanium surfaces modified by selective ion co-implantation could achieve osteoinductivity, angiogenic activity and antibacterial effect simultaneously. These multiple effects may greatly promote rapid osseointegration and biomechanical stability of dental implants.

An ideal bone-implant interface depends largely on the osteoconductivity and osteoinductivity of the implant surface. Bioactive chemical ingredients, such as key metal ions are favorable to the cellular functions of osteoblasts and the osteogenic differentiation of bone marrow mesenchymal stem cells (BMSCs). It is well known that zinc (Zn) is an important essential trace element for bone metabolism and generation. Furthermore, magnesium (Mg) plays an important role in both osteogenesis and adhesion of osteoblasts to orthopedic implants [7], with bone and mineral metabolism disruption caused by dietary magnesium reduction [8]. Although Zn or Mg, have individually been shown to be able to promote initial adhesion, proliferation and ALP activity of BMSCs or osteoblast [7, 9-11], it is crucial to know whether and how co-implanted zinc and magnesium ions would have synergistic effects on improved osteoinductivity. This interesting idea may have a potential to

become a new strategy to enhance survival rates of dental implants.

In addition to osteogenic activity, formation of new blood vessels in peri-implant bone tissue, which is actually the process by which vascular endothelial cells proliferate from the pre-existing vessels [12], is also vital for rapid osteointegration and success rate of implants, and this behavior relies on both biological and chemical regulation. On the one hand, vascular endothelial growth factor (VEGF) is one of the most important pro-angiogenic factors, and also hypoxia inducing factor (HIF) is a key regulatory factor of VEGF. On the other hand, magnesium plays an important role in vessel formation. Mg^{2+} induces endothelial cells to produce nitric oxide, which is the key fact that VEGF uses to induce angiogenesis [13, 14]. Recently, an *in vitro* study showed that 10mM $MgSO_4$ enhanced protein expression of VEGF in hBMSCs by stimulating HIF-1 α and HIF-2 α [15]. However, it is necessary to investigate whether Mg^{2+} ions implanted on the titanium surface can be released from the material and absorbed by cells to stimulate angiogenesis.

Peri-implantitis remains a fairly intractable problem for the long-term “survival” rate of dental implants because oral cavity is really an open ecological system with abundant microorganisms. Furthermore, surgical trauma and trans-gingival abutment of the implants both tend to create a favorable portal for bacterial invasion. Previous studies have reported that zinc ions show antibacterial effect against both *Staphylococcus aureus* (*S. aureus*) and *Escherichia coli* (*E. coli*) [9,11,16]. However, dental implants are usually confronted with anaerobes or facultative anaerobes that may be different microbial strains from the two above-mentioned ones [17].

Porphyromonas gingivalis (Pg) and *Fusobacterium nucleatum* (Fn) are two typical kinds of subgingival anaerobes resulting in chronic periodontitis and have close relationship with peri-implantitis. *Streptococcus mutans* (Sm), one of the most important supragingival bacteria may create an acidic condition that impairs the corrosion resistance of titanium [18]. Nevertheless, it still remains unknown whether zinc has inhibitory effects on these oral pathogenic bacteria. Therefore, in addition to osteogenic activity of zinc-doped titanium, it also has important application value in investigating its inhibitory properties against oral microbes.

In order to integrate the osteogenic, angiogenic and antibacterial properties of zinc and magnesium, in this study plasma immersion ion implantation (PIII) was used to simultaneously incorporate Zn and Mg into titanium (Zn/Mg-PIII), with Zn-PIII, Mg-PIII and pure titanium being used as comparison. The osteoinductivity, pro-angiogenic ability and inhibitory effects on oral microbes of Zn/Mg-PIII were investigated systematically in vitro, with focus on the synergistic effects of Zn and Mg. Furthermore, the in vivo osseointegration, angiogenesis and bonding strength at bone-implant interfaces were also evaluated.

2. Materials and methods

2.1. Materials preparation and characterization

2.1.1. Fabrication of Zn/Mg co-implanted titanium (Zn/Mg-PIII)

Titanium plates (Cp Ti, Grade 2, Xi'an Saite Metal Materials Development Co. Ltd, China) with dimensions of 10 mm × 10 mm × 1 mm and 20 mm × 20 mm × 1 mm and cylindrical implants (Φ2mm × 7 mm) were used. The ultrasonically cleaned

119 samples were chemically etched for 5 min using a solution of H₂O, HF, and HNO₃
120 with a volume ratio of 5:1:4, followed by ultrasonic cleaning using deionized water,
121 ethanol and ultrapure water sequentially. Zn and Mg were then respectively implanted
122 into the pretreated samples at -30 kV for 90 min. The obtained samples were designed
123 as Zn-PIII and Mg-PIII respectively. Zn/Mg co-implantation was carried out using
124 pulsed Zn and Mg cathodic arc sources, and the obtained samples were represented as
125 Zn/Mg-PIII. During PIII treatment, circulating water was used to cool the sample
126 stage. Detailed PIII parameters are listed in Table S1.

127 2.1.2. Surface characterization

128 The surface topography and chemical compositions of different specimens (10
129 mm × 10 mm × 1 mm) were examined using a scanning electron microscope (SEM,
130 Hitachi S-4800, Japan) and X-ray photoelectron spectroscopy (XPS, Physical
131 electronics PHI 5802) respectively.

132 2.1.3. Measurement of zinc and magnesium ions release

133 The different specimens were immersed in 10ml PBS at 37 °C without stirring for
134 7 d, 14 d and 21 d. The amounts of released Zn and Mg ions in the obtained solutions
135 were measured using an inductively coupled plasma-atomic emission spectrometry
136 (ICP-AES, Varian Liberty 150, USA).

137 2.1.4. Water contact angles detection

138 The water contact angles of different surfaces were determined with an optical
139 contact angle instrument (JC2000D3, POWEREACH Inc., China) as detailed in
140 previous study [19].

142 2.2. Cell isolation and culture

143 The experimental protocols were approved by the Animal Care and Medical
144 Ethics Committee of Ninth People's Hospital affiliated to School of Medicine,
145 Shanghai Jiao Tong University. rBMSCs were isolated from the femoral bone marrow
146 of SD rats as was described before [20]. Briefly, femoral marrow was extracted and
147 suspended in Dulbecco's modified Eagle's medium (DMEM, Hyclone, USA) which
148 was refreshed every 3 days. Cells were subcultured when they reached 80-90%

confluence. rBMSCs at the second or third passage were used for biological experiments.

Human umbilical vein endothelial cells (HUVECs) were extracted and cultured as detailed in our previous work with written informed consent of the donors [21]. Briefly, HUVECs were collected from an umbilical vein which was digested with 0.1% collagenase I (Sigma, USA), and then they were cultured in ECM (Sciencell, USA). Endothelial cells at passage 3-6 were chosen to use in experiments.

2.3. Cell adhesion activity assay

Firstly, rBMSCs (5×10^4 cells/well) were seeded on different specimens ($10 \text{ mm} \times 10 \text{ mm} \times 1 \text{ mm}$) in 24 well plates and were cultured for 1 and 4 h. After being washed with PBS for three times, the cells on each specimen were fixed in 4% paraformaldehyde (PFA), permeabilized with Triton X-100 and blocked in bovine serum albumin (BSA) successively. Finally, their cytoskeleton and cell nuclei were stained by rhodamine-Phalloidin and DAPI in dark respectively and observed with a fluorescence microscopy (Leica, Germany).

For SEM examination, after 24 h of cell culture, rBMSCs (5×10^4 cells/well) on other samples ($10 \text{ mm} \times 10 \text{ mm} \times 1 \text{ mm}$) were rinsed with PBS and fixed with glutaraldehyde. After sequential dehydration in gradient ethanol solutions, the samples with cells were coated with platinum and then observed using SEM.

2.4. Cell proliferation measurement

rBMSCs and HUVECs (both 2×10^4 cells/well) were respectively cultured on various specimens ($10 \text{ mm} \times 10 \text{ mm} \times 1 \text{ mm}$) for 1, 4 and 7 days. Cell viability was examined by measuring optical density (OD) value using a Cell Counting Kit-8 (CCK-8, Beyotime, China) assay as we previously described [22].

2.5. Real-time PCR analysis

rBMSCs and HUVECs at a certain density were respectively cultured on various sample surfaces ($20 \text{ mm} \times 20 \text{ mm} \times 1 \text{ mm}$) in 6 well plates. Cellular total RNA was

isolated using an RNeasy Mini Kit (Qiagen, Germany) and reversely transcribed to cDNA with a PrimeScript cDNA synthesis kit (TaKaRa, Japan). The gene expression of integrin $\alpha 1$, integrin $\beta 1$, RUNX2, ALP, OPN, OCN, ZIP1, ZnT1, MagT1 and VEGF in rBMSCs and the expression of VEGF, KDR and MagT1 in HUVECs were quantified by a Bio-Rad sequence detection system (MyiQ2, USA). The gene expression level of GAPDH was used as normalization. The detailed primer sequences of the above-mentioned genes are listed in Table S2.

2.6. ALP staining and ALP activity assay

The rBMSCs (2×10^4 cells/well and 1×10^4 cells/well) were cultured on various samples ($10 \text{ mm} \times 10 \text{ mm} \times 1 \text{ mm}$). At each time point (day 7 and day 14), rBMSCs were fixed with PFA and stained using a BCIP/NBT ALP kit (Beyotime, China). For ALP activity assay, the total protein of cells were extracted and further incubated with p-nitrophenyl phosphate (pNPP) (Beyotime, China) at 37°C . After measuring optical density values, the ALP activity levels were calculated according to a standard curve and normalized to the total protein content [22].

2.7. Immunofluorescence analysis of OCN and HIF-1 α

The rBMSCs and HUVECs (1×10^4 cells/well) were cultured on the specimens ($10 \text{ mm} \times 10 \text{ mm} \times 1 \text{ mm}$) for 14 and 10 days respectively. After fixation in 4% PFA and permeabilization with Triton X-100, the cells were blocked with BSA (Amresco, USA). Afterwards, rBMSCs and HUVECs were incubated overnight at 4°C with primary antibody of anti-osteocalcin and anti-HIF-1 α (Abcam, UK), respectively. Then the washed cells on samples were stained with a fluorochrome-conjugated secondary antibody and DAPI successively. Finally, the specimens were observed using a fluorescence microscope (Leica, Germany).

2.8. Intracellular zinc and magnesium ions detection

rBMSCs and HUVECs (1×10^4 cells/ml) were cultured on various samples ($20 \text{ mm} \times 20 \text{ mm} \times 1 \text{ mm}$). After being isolated from sample surfaces, these cells were

resuspended with PBS and counted using an automatic cell counting analyzer. The concentrations of Zn and Mg ions in rBMSCs and HUVECs were then measured using an **inductively coupled plasma mass spectrometry** (ICP-MS, ELANDRC-e, PE, USA).

2.9. *In vitro* antibacterial assay against oral anaerobe

Porphyromonas gingivalis (Pg, ATCC33277, G-), *Fusobacterium nucleatum* (Fn, ATCC25586, G-) and *S.mutans* (Sm, UA159, G+) were selected to evaluate the antibacterial activity of ion implanted titanium. Firstly, the bacteria were all incubated in 3.7% brain heart infusion broth solution under standard anaerobic conditions (80% N₂, 10% H₂, 10% CO₂, 37°C) [17]. After being cultured with the bacterial suspension for 24 h, each sample (20 mm × 20 mm × 1 mm) was put into a centrifuge tube with 10 ml BHI and then was **vibrated using a Vortex Genie 2 (3000 rpm, 1 min) before ultrasonically oscillated (50 Hz, 5 min)** to dissociate the bacteria. After centrifugation for 10 min, the resuspended bacterial suspension was serially diluted with BHI, and 100 µl of suspension was seeded onto an agar plate for further incubation. The number of active bacteria colony forming unit (CFU) was counted using an automatic colony counter, and the antibacterial ratio was calculated as we previously described [17, 23].

2.10. *In vivo* osseointegration assessment

2.10.1 Animal experiment

The animal experiment was approved by the Animal Care and Experiment Committee of Ninth People's Hospital affiliated to School of Medicine, Shanghai Jiao Tong University. **Twenty 3-month-old** healthy New Zealand rabbits (2.5-3.0 kg) were used in the work. **Four kinds of implants were implanted into the bilateral mid-shaft and condyle of femurs in each animal.** The detailed surgical procedure was shown in Fig. S1.

At 3, 6 and 9 weeks after operation, multiple fluorochromes were injected intraperitoneally **into the 10 rabbits to be raised for 12w** at a sequence of 30 mg/kg

alizarin red S (Sigma, USA), 25 mg/kg tetracycline hydrochloride and 20 mg/kg calcein (Amresco, USA), respectively [24].

10 rabbits were euthanatized at each time point (4 w and 12 w) after operation. Each harvested femur was trimmed into a mid-shaft sample and a condyle sample. At each time point, 5 mid-shaft samples for each group were obtained for push-out test before which a randomly selected sample (12 w) was assayed using micro-CT. The other 5 condyle samples were prepared for histological analysis.

2.10.2 Micro-CT assay

For each group at 12 weeks post-operation, a randomly selected femoral mid-shaft sample was analyzed using a high resolution micro-CT scanner (SkyScan1076, Belgium) to observe the new bone formation around implants as we previously described [22]. A region of interest (ROI) with a radius of 1 mm from implant surface was selected for analysis.

2.10.3 Histological analysis

The bone samples with implants were fixed in formalin and then were dehydrated using gradient ethanol. After being embedded in PMMA and hardened, they were cut (perpendicular to the long axis of the implants) into 150-200 μ m thick sections. After polish treatment, the sections were observed for fluorescent images by a confocal laser scanning microscopy (Leica, Germany). The excitation/emission wavelengths for each fluorochrome were used as 543/617 nm (alizarin red S), 405/580 nm (tetracycline hydrochloride) and 488/517 nm (calcein), respectively [4]. Afterwards, these sections were stained with Van Gieson's picric fuchsine and evaluated with a microscopy for histological examination and histomorphometric analysis as previously described [25-27]. Percentages of bone-implant contact and newly-formed bone area were calculated.

2.10.4 Push-out test

The biomechanical bonding strength of bone-implant interfaces were measured via push-out test using a universal material testing system (Instron, UK) as was previously described [3, 28]. The detailed procedure of push-out test was illustrated in Fig. S2.

2.11. Statistical analysis

Each *in vitro* experiment was conducted in triplicate for three times. Based on the normal distribution and equal variance assumption test, the data were expressed as mean $\pm$ standard deviation and analyzed via one-way analysis of variance (ANOVA) with Student Newman-Keuls (SNK) post hoc test. Statistical analysis was performed using SPSS 17.0 software. The differences were considered statistically significant at $p < 0.05$ (*) and $p < 0.01$ (**).

3. Results and discussion

To answer whether the novel Zn/Mg-P111 has multiple and superior biological effects compared to Zn-P111 and Mg-P111, BMSCs, HUVECs, oral bacteria and a rabbit femur implantation model were all used as the breakthrough points. Two main aspects, including the synergistic effects of Zn and Mg and the multifunctions of Zn/Mg-P111 in osteoinduction, pro-angiogenesis, antibacterial ability and rapid osseointegration, were focused on and investigated, thus providing important scientific basis for the dental application of Zn/Mg-P111 implants.

3.1. Surface characterization

The physico-chemical characteristics of the material may largely affect its bioactivity and biofunctions, and play a significant role in regulating cellular behaviors. Zn species play important roles in biological functions, such as DNA synthesis, bone formation and biomineralization [29,30], and they also have excellent antibacterial property [16]. Mg^{2+} ions can stimulate bone regeneration by enhancing protein expression of collagen type X (COL10A1) and vascular endothelial growth

factor (VEGF) in hBMSCs [15]. In view of the versatile functions of the two essential elements, Zn^{2+} and Mg^{2+} were co-implanted into etched titanium to promote its bioactivity and antibacterial property in this study.

It was shown by SEM images that after ion implantation, the rough micron-grade structures remained essentially unchanged (Fig. 1A-1D), because ion implantation treatment forms a thin Zn/Mg containing film on material surface and does not destroy the original microstructure [11]. According to the Zn2p and Mg2s high resolution spectra of ion implanted titanium, only Zn2p or Mg2s peaks were respectively detected from Zn-PIII or Mg-PIII sample (Fig. 2A, 2B), nevertheless two zinc peaks (1021.9 eV and 1021.0 eV corresponding to the binding energy of zinc in oxidized and metallic state respectively) and two Mg peaks (89.0 eV and 88.1 eV corresponding to metallic and oxidized magnesium) were detected from Zn/Mg-PIII surface (Fig. 2C, 2D). The surface elemental compositions of Zn/Mg-PIII further indicated the co-existence of Zn and Mg (Table S3). These results suggest that Zn and Mg are simultaneously incorporated into titanium by PIII treatment, and they both exist in the oxidized state (ZnO/MgO) at the surface and in their elemental metallic form (Zn/Mg) below the surface. In addition, the concentration of Zn or Mg ions implanted in Zn/Mg-PIII may be equal to that of Zn in Zn-PIII or Mg in Mg-PIII respectively, because the same processing parameters in PIII for each group were used (Table S1). The changes of chemical composition may cause Zn/Mg-PIII to have different biological effects from Zn-PIII and Mg-PIII.

Figure 1. Surface topography of the acid etched titanium before and after ion implantation. (A) Pure Ti, (B) Zn-PIII, (C) Mg-PIII, and (D) Zn/Mg-PIII.

Bioactive ions released from biomaterials may greatly influence local biological reaction. To further probe into the relationship between the Zn and Mg ions in Zn/Mg-PIII and its biological effects, the amount of ions released from different materials in PBS were measured using ICP-AES (Varian Liberty 150, USA). The results showed that similar amounts of Zn and Mg ions were released from Zn/Mg-PIII simultaneously and continuously during the immersion time of 21 days (Fig. 2E, 2F). Furthermore, the cumulative Zn^{2+} and Mg^{2+} concentrations leached from Zn/Mg-PIII are comparable to those of Zn^{2+} from Zn-PIII and Mg^{2+} from Mg-PIII respectively (Fig. 2E, 2F). These results indicate that Zn^{2+} and Mg^{2+} can be released synchronously from Zn/Mg-PIII without any influence of their interaction. This particular characteristic may play a positive role in regulating peri-material microenvironment and have long-term multiple effects on cells and bone tissue at implant interfaces.

Figure 2. High-resolution spectra detected from the ion implanted titanium surfaces: (A) Zn2p from Zn-PIII, (B) Mg2s from Mg-PIII, (C) Zn2p and (D) Mg2s from Zn/Mg-PIII. (E) Zn and (F) Mg concentrations in PBS solution after 7, 14 and 21 days of immersion. (n=3)

3.2. Adhesion activity and *proliferation* of rBMSCs on Zn/Mg-PIII surface

As the precursor of osteoblasts, BMSCs play an important role in bone regeneration. The initial adhesion and spreading of BMSCs may directly affect subsequent cellular functions and osseointegration of implants [31]. It was clearly

shown by fluorescent and SEM images that compared to Zn-PIII, Mg-PIII and Ti groups, rBMSCs cultured on Zn/Mg-PIII surface exhibited an increased cell density and more obvious filopodia extension (Fig. 3A, 3B). These results indicate that Zn/Mg-PIII can promote cellular initial adhesion and spreading. This effect may be related to the surface property and chemical composition of Zn/Mg-PIII.

On the one hand, surface hydrophilicity plays a significant role in cellular adhesion. From the water contact angle values of four kinds of material surfaces (Fig. S3), it can be inferred that Zn/Mg co-implantation improves the surface hydrophilicity of titanium to a certain extent, which may facilitate rBMSCs adhesion and spreading. On the other hand, active chemicals can provide favorable conditions for cell adhesion behavior, while Zn^{2+} and Mg^{2+} ions can both be released from the Zn/Mg-PIII surface (Fig. 2E, 2F). Mg ions can enhance cell attachment by promoting the binding interactions between the integrin family (transmembrane proteins that mediate cell adhesion) of cell surface receptors and their ligand proteins.⁷ Meanwhile, it is believed that the extraordinarily stretched behavior of rBMSCs is mainly attributed to Zn ions [11, 32], which facilitates cellular spreading. Furthermore, real-time PCR analysis showed that Zn/Mg-PIII can more significantly upregulate the gene expression of integrin $\alpha 1$ and integrin $\beta 1$ than Zn-PIII and Mg-PIII (Fig. 3C, 3D). These results suggest that the synergistic effects of zinc and magnesium in Zn/Mg-PIII can enhance the expression of integrin and improve the adhesion ability of rBMSCs.

Figure 3. Adhesion activity and viability of rBMSCs on Zn/Mg-PIII surface. (A)

Fluorescent images and (B) SEM morphology of rBMSCs cultured on different samples (n=3). (C) Integrin $\alpha 1$ and (D) integrin $\beta 1$ gene expression of rBMSCs cultured for 3 and 10 days. (E) Cell proliferation of rBMSCs cultured for 1, 4 and 7 days on various samples. n=9, *p < 0.05, **p < 0.01.

However, good initial cellular adhesion only cannot ensure subsequent desirable proliferation of cells, because high ion concentration may have an adverse impact on cell growth. Therefore, cell proliferation on material surfaces is also an important indicator. The results of CCK-8 assay showed that rBMSCs on Zn/Mg-PIII surfaces exhibit higher viability than those on the other three surfaces (Fig. 3E). This effect may be due to a certain amount of Zn^{2+} and Mg^{2+} released concurrently and continuously from Zn/Mg-PIII, which both promote BMSCs growth [9, 10]. In addition to this, improved cell adhesion and spreading activity of Zn/Mg-PIII surface also enhance intercellular transmission of nutrition and signals, which is conducive to cell proliferation. In conclusion, the above-mentioned advantages of Zn/Mg-PIII in rBMSCs adhesion and growth may create favorable conditions for their physiological function such as differentiation.

3.3. Synergistic effects of Zn/Mg ions on osteogenic differentiation of rBMSCs

The present study is aimed at developing multifunctional titanium for dental implants. Therefore, besides cell adhesion and proliferation, we also focused on whether Zn^{2+} and Mg^{2+} have synergistic promotion effects on osteogenic differentiation of rBMSCs, although single zinc/magnesium-incorporated titanium surface has, respectively, been reported to be able to promote osteogenic activity of

BMSCs [9, 10, 24, 33]. Via qRT-PCR assay it was found that mRNA expression levels of main osteoblast markers (Runx2, ALP, OPN and OCN) were more significantly promoted by Zn/Mg-PIII at day 3 and day 10 (Fig. 4A-4D). These interesting phenomena demonstrate that Zn/Mg-PIII has a greater ability to induce osteogenic differentiation of rBMSCs even more than Zn-PIII and Mg-PIII, probably owing to the synergistic effects of co-implanted Zn^{2+} and Mg^{2+} . Nevertheless, the activation of gene transcription is just the initial step of cell differentiation. The proteins that are successfully translated from mRNA can directly exert cellular physiological functions [34]. In order to further confirm the osteoinductivity of Zn/Mg-PIII, the protein expression of two key osteoblast markers at either early stages (ALP) or late stages (OCN) were analyzed from the perspective of cell functions. An interesting finding is that both ALP activity and the expression of OCN protein are significantly enhanced by Zn/Mg-PIII as compared to the other three materials (Fig. 4E, 4F, 5A). These results reveal that the Zn/Mg-PIII can, not only, induce osteogenic differentiation of rBMSCs but can also exert synergistic physiological effects of Zn^{2+} and Mg^{2+} .

Figure 4. Promotion effects of Zn/Mg-PIII on osteogenic differentiation of rBMSCs. (A-D) The expression of osteogenic differentiation related genes in rBMSCs cultured on various materials for 3 and 10 days: (A) Runx2, (B) ALP, (C) OCN, (D) OPN. (E) ALP staining and (F) ALP activity of rBMSCs incubated on various surfaces for 7 and 14 days. $n=9$, $*p<0.05$, $**p<0.01$.

However, it is essential to know the detailed mechanism of how osteoblast differentiation is enhanced by the effects of Zn^{2+} and Mg^{2+} . Although the total ions concentration released from Zn/Mg-PIII is higher than that from single Zn or Mg

implanted titanium, however, this does not mean that the each effect of Zn and Mg ions may add. The extracellular ions usually do not directly produce biological effects, whereas the intracellular ions with a certain concentration, which are accumulated through ionic uptake by membrane transporters, can effectively result in biological functions [24]. However, it is still unknown that which influence the co-implanted Zn and Mg ions may have on the transmembrane transport system for each other: mutual noninterference, mutual antagonistic or synergistic interactions? This may be the key to investigate the biological effects of Zn/Mg-PIII.

To probe further into the abovementioned question, the transporting pathways of Zinc and magnesium were investigated. Zinc transporters of the ZnT (cation diffusion facilitator, SLC30) family and ZIP (Zrt- or Irt-like protein, SLC39) family play significant roles in zinc homeostasis and specific cell functions [35]. ZIP1 is a ubiquitous zinc transporter for zinc influx through the plasma membrane in both MSCs and in osteoblastic cells, and overexpression of ZIP1 in MSCs can promote gene expression of Runx2 and osteopontin [36]. On the contrary, the ZnT family (ubiquitous zinc exporters predominantly locating in the plasma membrane) decreases cytoplasmic zinc in BMSCs, and its overexpression in BMSCs inhibits the expression of ALP, type I collagen, as well as calcium deposition [37]. In addition, the only known selective plasma membrane Mg^{2+} transporter is MagT1, which is essential for vertebrate Mg^{2+} influx and plays an essential role in cellular growth and survival [38, 39]. In a word, ZIP1, ZnT1 and MagT1 may greatly influence the flow direction and intracellular amount of Zn^{2+} and Mg^{2+} .

Therefore, in order to confirm the synergistic effects of Zn^{2+} and Mg^{2+} in osteoinduction and to better understand its possible mechanism, the influences of different materials on gene expression of ZIP1, ZnT1 and MagT1 in rBMSCs and intracellular $\text{Zn}^{2+}/\text{Mg}^{2+}$ concentrations were systematically analyzed. It was found that Zn/Mg-PIII and Zn-PIII with implanted zinc can upregulate expressions of ZIP1. Meanwhile, significant trends towards both promoted expression of MagT1 and decreased expression of ZnT1 were observed in Zn/Mg-PIII and Mg-PIII groups with Mg^{2+} incorporation (Fig. 5B, 5C). This means zinc does not influence Mg^{2+} uptake, whereas magnesium may reduce the outflow of Zn^{2+} . Furthermore, ICP results indicated that there were significantly higher concentrations of Zn^{2+} in rBMSCs for Zn/Mg-PIII group than that for Zn-PIII group, and the intracellular Mg^{2+} concentrations for Zn/Mg-PIII group and Mg-PIII group were both similarly increased. (Fig. 5D, 5E). Judging from all these results, it can be concluded that due to the synergistic effects of zinc and magnesium, Zn/Mg-PIII promote the influx of Zn^{2+} and Mg^{2+} into rBMSCs and meanwhile suppress the outflow of Zn^{2+} , thus increasing intracellular Zn^{2+} and Mg^{2+} concentrations. As a result, the increased Zn^{2+} and Mg^{2+} effectively activate Runx2 (a key osteoblast transcription factors), and then enhance the expression of ALP and OCN protein, thus inducing the osteogenic differentiation of BMSCs.

Figure 5. Synergistic effects of Zn/Mg ions on osteogenic induction. (A) Immunofluorescent images of OCN in rBMSCs on various surfaces for 14 days: OCN protein stained by Dylight488 (green). (B, C) ZIP1, ZnT1 and MagT1 gene expression in rBMSCs on various samples at day 7 and day 14. (D) Zn^{2+} and (E) Mg^{2+} ions concentration in rBMSCs cultured on different materials for 14 days. $n=9$, $*p$

<0.05, ** p <0.01.

3.4. Pro-angiogenic effects of Zn/Mg-PIII

Angiogenesis plays a critical role in the rapid growth and maturation process of bone tissue. In this perspective, our strategy of Zn/Mg co-implantation is not only aimed at better osteogenic activity but also aims at improving angiogenesis. So as to confirm this effect, the influences of Zn/Mg-PIII on angiogenic ability of vascular endothelial cells, whose proliferation along blood capillary is the initial step of angiogenesis, were studied. Firstly, Zn/Mg-PIII and Mg-PIII surfaces were both found to significantly improve the viability of HUVECs at day 4 and day 7 compared to pure titanium, while Zn-PIII did not have this effect (Fig. 6A), suggesting that magnesium ion implantation can effectively facilitate the growth of HUVECs. Then, the protein expression of hypoxia-inducible factor (HIF)-1 α in HUVECs was analyzed via immunofluorescence staining, because HIF-1 α promotes angiogenesis by mimicking hypoxic conditions and helps in the transcription of VEGF which is a major proangiogenic factors and angiogenic signaling protein [15]. As is shown in Fig.6C, the expression of HIF-1 α in HUVECs on Zn/Mg-PIII and Mg-PIII surfaces is much more intensive than that on pure Ti, indicating that there is stimulation of HIF-1 α by Mg²⁺. This result conforms to the known viewpoint that Mg²⁺ plays a crucial role in angiogenesis by aiding the transcriptional activity of HIF-1 α [15, 40]. The enhanced expression of HIF-1 α means that the endogenic VEGF of HUVECs may be induced by released Mg²⁺. Further results confirm that both Zn/Mg-PIII and Mg-PIII surfaces upregulate the gene expression of VEGF and KDR (the receptor of

VEGF) in HUVECs and also induce VEGF expression in rBMSCs (Fig. 6B). All these results fully substantiate the fact that magnesium of Zn/Mg-PIII can stimulate the expression of VEGF in HUVECs (endogenous) and rBMSCs (exogenous) via activation of HIF-1 α , thus directly and indirectly promoting the angiogenic activity.

Figure 6. Pro-angiogenic effects of Zn/Mg-PIII. (A) Cell viability of HUVECs cultured for 1, 4 and 7 days on various samples. (B) Gene expression of VEGF and KDR in HUVECs and VEGF expression in rBMSCs cultured on various surfaces for 10 days. (C) Immunofluorescent images of HIF-1 α , (D) MagT1 gene expression and (E) Mg²⁺ ions concentration in HUVECs cultured on various surfaces for 10 days. n=9, * p < 0.05, ** p < 0.01.

Since Mg²⁺ ions released from Zn/Mg-PIII have been proved to promote angiogenesis, it is critical to study how Mg²⁺ ions enter into cells and exert this function. MagT1 transporter is the essential cellular structure for vertebrate Mg²⁺ influx [41], therefore we further focused on the influences of Zn/Mg-PIII on the expression of MagT1 and Mg²⁺ content in HUVECs. The results from real-time PCR assay evidently showed that the Zn/Mg-PIII surfaces significantly upregulated the gene expression of MagT1 (Fig. 6D), and ICP analysis further confirmed that the intracellular Mg²⁺ concentrations were increased (Fig. 6E), indicating an acceleration of Mg²⁺ inflow by Zn/Mg-PIII. In general, Mg²⁺ ions from Zn/Mg-PIII increase Mg²⁺ influx by promoting the expression of MagT1 transporter in HUVECs, and then stimulate the transcription of VEGF and KDR via activation of HIF-1 α , thus inducing angiogenesis.

3.5. Inhibitory effects of Zn/Mg-PIII against oral anaerobes

Since there is a need for developing multifunctional dental implant, another important goal of Zn/Mg-PIII titanium is to exert the antibacterial effects of zinc. Due to the fact that the oral cavity is an open ecological environment, despite having desirable osteogenic and angiogenic activity, dental implants without necessary antibacterial effects could still be infected by oral pathogens. So as to deal with this problem, our strategy was to incorporate a certain amount of zinc (a trace element with antibacterial effects) onto the titanium surface, thus attempting to produce a certain degree of inhibitory effects on oral bacteria. Although zinc-incorporated biomaterials have been reported to exhibit antibacterial ability against oxybiontic bacteria (*S. aureus* and *E. coli*) [9,11], so far it has not been reported whether Zn ion implanted titanium has some inhibitory effects on oral anaerobes, which are closely related to bacterial peri-implantitis. Therefore, from the view of the expected clinical application, we selected three kinds of typical oral anaerobic bacteria (*Pg*, *Fn* and *Sm*) and studied antibacterial effects of ion-implanted titanium against them, respectively. The results from bacteria counting method show that inhibitory rates of Zn/Mg-PIII and Zn-PIII surfaces on *Pg*, *Fn* and *Sm* are all significantly higher than those of Mg-PIII (Fig. 7), suggesting the two Zinc-containing titanium surfaces have comparable inhibitory properties against oral pathogens. These effects may be attributed to the implanted zinc.

Figure 7. Inhibitory effects of Zn/Mg-PIII against oral anaerobes. Re-cultured oral bacterial colonies and the corresponding colony forming unit (CFU) number of *Sm* (A), *Fn* (B), *Pg* (C) on BHI agar plates after dissociation from the various surfaces: . The inhibitory rates of ion implanted samples on *Sm* (D), *Fn* (E) and *Pg* (F). $n=9$, $**p<0.01$.

534

535 The bacteriostatic action of biomaterial is mainly produced via two ways. One is
536 the release of antibiotic ingredients from the materials. The other is the direct contact
537 and interaction between pathogens and antibacterial substances on the material
538 surface. It is known that most of antimicrobial implant coatings depend on quick
539 biodegradation and the release of antibiotics [42], which may cause safety concerns
540 such as release rates and antibiotic resistance [17]. Therefore, the development of
541 inorganic antibacterial agents with bacteriostatic ability and biosecurity is highly
542 valuable.

543 In this study, ZnO exists on the Zn/Mg-PHII surface, and this zinc-containing
544 surface can, gradually and continuously, release a certain amount of Zn^{2+} ions in
545 physiological medium for a long period of time (Fig. 2), without resulting in any
546 cytotoxicity. Furthermore, Zn-incorporated coatings was believed to well inhibit
547 bacterial adhesion and growth by releasing zinc ions and generating reactive oxygen
548 species (ROS) from ZnO [11]. Therefore, it can be inferred that Zn/Mg-PHII with its
549 good biosafety can not only inhibit adhesion of oral anaerobic bacteria, but can also
550 have some bacteriostatic action on the surrounding environment. These effects may
551 help dental implants resist the potential oral microbial infection *in vivo*.

552 In addition to this, it is worth mentioning that Zn/Mg-PHII have comparable
553 bacteriostatic ability (inhibitory rate of 40-50%) against Gram-negative anaerobes (*Pg*
554 and *Fn*) and Gram-positive one (*Sm*), suggesting that its bacteriostatic effects may
555 have a broad spectrum. This feature may be better adapted to withstand the complex

oral microbial system.

3.6. Accelerating effects of Zn/Mg-PIII on osteogenesis and osseointegration *in vivo*

The above series of *in vitro* studies have proved that Zn/Mg co-implanted titanium has multifunctions in osteogenesis, angiogenesis and anaerobic inhibition. However, it is really important to evaluate the *in vivo* osteogenic ability and osseointegration effect of Zn/Mg-PIII implant, because rapid peri-implant bone formation and integration are critical factors for the success rate of implantation [43]. Therefore, the peri-implant osteogenesis in rabbit femurs was investigated from the point of space and time. On the one hand, the 3D images from Micro-CT assay clearly showed that obvious new bone was formed around the Zn/Mg-PIII implant (Fig. 8A), indicating that Zn/Mg co-implantation can effectively promote the *in vivo* osteogenic activity of implants. On the other hand, the spatial and temporal evolution of new bone formation and mineralization was assessed by a multicolor sequential fluorescent labeling assay. As shown in the CLSM images, the most intense and widely distributed polychrome fluorescence was found to expand around Zn/Mg-PIII implant surface (Fig. 8B). These interesting findings provide powerful evidences to substantiate the fact that Zn/Mg-PIII implant can both stimulate rapid bone formation and maintain sustained osteogenesis. These critical functions may be mainly attributed to the synergistic effects of Zn/Mg ions in enhancing osteogenic differentiation of BMSCs which plays an essential role in bone formation [4].

Figure 8. New bone formation around Zn/Mg-PIII implants. (A) Micro-CT 3D

images of new bone formation around various implants in rabbit femur 12 weeks after implantation (n=1). (B) Sequential fluorescent labeling images of newly-formed bone around various implants in rabbit femoral condylar: Alizarin red S (red), tetracycline (yellow), Calcein (green). (n=5)

The evolution of peri-implant bone growth and integration is believed to objectively reflect the process of osseointegration [22]. Although zinc or magnesium containing implants were found to have superior bone contact to pure titanium [24,44,45], it is urgent to know whether Zn/Mg-PIII implant could accelerate osseointegration even more than single ion implanted titanium. Therefore the current study carried out a dynamic histological research, which is acknowledged to be a golden standard in analyzing osseointegration of biomaterials [26, 27]. At 4 weeks after implantation, higher amount of immature newly-formed bone can be observed around Zn/Mg-PIII implants compared to the other three groups (Fig. 9A), and the bone-Zn/Mg-PIII interfaces exhibit some degree of bone integration in direct contact form, with cuboidal-shaped active osteoblasts lined adjacent to the new bone (Fig. 9B). In addition to this, some tube-like tissues which suggest the growth of new blood vessels were formed in the new bone (Fig.9B). These phenomena evidently indicate that Zn/Mg co-implanted titanium can enhance rapid osseointegration and angiogenesis during early stages (4w). Abundant blood vessels can provide sufficient nutrients and renewable autologous cells for superior bone formation and integration. After 12 weeks, an increased amount of woven bone was formed around Zn/Mg-PIII implants with higher bone density and bone contact (Fig. 9C). In short, due to the multifunction of Zn/Mg-PIII, the accelerated osteogenesis and osseointegration at

bone-implant interfaces consequently occur.

Figure 9. Histological and biomechanical characteristics at bone-implant interfaces. (A-C) Histological morphology in undecalcified sections: rounded bone-implant interfaces after implantation for 4 (A) and 12 (C) weeks; (B) Partial magnifications of the rectangular regions in the corresponding sections: NB -new bone, OB -old bone, blue stars -blood vessels (tube like tissue). (D) Percentage of bone-implant contact and (E) new bone area from the histomorphometric measurements. (F) Peak load at bone-implant interfaces from push-out test. $n=5$, $*p<0.05$, $**p<0.01$.

In order to investigate the degree of bone integration and bone formation in an accurate quantitative manner, the bone-implant contact (BIC) rate and new bone area (NBA) rate were measured via histomorphometric analysis. It is well known that BIC index has a close relation to the strength of bone-implant interface [22, 25]. The results showed that both the BIC rate and NBA rate for Zn/Mg-PIII implants were statistically higher than those of the other three kind of implants ($p<0.05$), and increased with time (Fig. 9D, 9E). All these results demonstrate that Zn/Mg-PIII implants have rapid and sustained osseointegration abilities, which may be largely attributed to the synergistic effects of Zn/Mg ions on osteoinduction, and are also related to the promotion effects of Mg on angiogenesis.

The immediate or early loading is the development tendency of dental implants [46], and mechanical retentions of implants greatly determine their ‘right time’ for loading and their ‘survival rate’. Therefore, the bonding strength at various bone-implant interfaces were examined by biomechanical analysis. As shown in Fig. 9F, among the four investigated groups, only Zn/Mg-PIII group exhibited a higher peak load than the Ti group at week 4. After 12 weeks, Zn/Mg-PIII group displayed

higher peak loads than even Zn-PIII and Mg-PIII groups, with their values all being superior to those of the Ti group ($p<0.05$). In general, these results sufficiently suggest that the accelerated and durable osseointegration of multifunctional Zn/Mg-PIII implants can successfully enhance their early and sustained biomechanical stability which are beneficial to the early loading and long-term survival of dental implants. In the future, further animal experiments on alveolar bone implantation of large animals and plenty of pre-clinical studies are needed. After all, the present *in vitro* and *in vivo* study may have some difference from the physiological environment in human body.

4. Conclusions

Zn/Mg ions are co-implanted simultaneously on titanium via plasma immersion ion implantation. The newly-developed Zn/Mg-PIII can facilitate the initial adhesion and proliferation of rBMSCs, and can increase Zn^{2+} and Mg^{2+} concentrations in rBMSCs by influencing the expression of both zinc transporters (ZIP1 and ZnT1) and magnesium transporters (MagT1). As a result, the osteoblast differentiation of rBMSCs is induced by the synergistic effects of the increased Zn^{2+} and Mg^{2+} . Furthermore, Mg^{2+} ions released from Zn/Mg-PIII increase Mg^{2+} influx by promoting the expression of MagT1 in HUVECs, and then activating HIF-1 α which is an essential regulatory factor of VEGF, thus enhancing angiogenesis. In addition to this, owing to the antibacterial property of zinc, Zn/Mg-PIII has a certain inhibitory effects on the growth of oral anaerobes (*Pg*, *Fn* and *Sm*). In *vivo*, the osteogenesis, angiogenesis, osseointegration and the bonding strength at the bone-Zn/Mg-PIII

implant interfaces are all significantly enhanced. In conclusion, the multifunctional Zn/Mg-PIII implants presenting with good osteoinductivity, pro-angiogenic and antibacterial effects can enhance rapid osseointegration and sustained biomechanical fixation, and can potentially have an interesting role in dental implantation.

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In vitro

In vivo

Zn-P111

Zn/Mg-P111

Osseointegration