**A lasting anti-bacterial, pro-angiogenic and pro-osteogenic zirconium-based bulk metallic glass for dental implants**

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**Abstract**

Bacterial infection and mismatched mechanical properties are important factors that increase the risk of dental implant failure. However, zirconium (Zr)-based bulk metallic glasses (BMG) can have both high strength and low modulus, as well as good biocompatibility, due to their unique atomic arrangement structure. Based on these common characteristics, different elemental compositions can endow zirconium-based amorphous alloys with different properties. Here we present a Zr-based BMG containing silver (Ag) with good amorphous process ability, exhibiting lasting antibacterial, pro-angiogenic and pro-osteogenic properties. This newly developed Zr61Cu23Al12Ti2Ag2 (at.%) BMG has higher strength and lower modulus than pure Titanium (Ti). Furthermore, it could exert antibacterial effects through both contact inhibition and metal ion sterilization. And this antibacterial property could last over 3 months. The systematically *in vitro* and *in vivo* results thus demonstrate the advantages and application potentials of Zr-based BMG as a highly promising oral implant material for dental implantation.

**Keywords:** dental implants, zirconium-based metallic glasses, antibacterial, pro-angiogenesis, pro-osteogenesis

1. Introduction

Implant treatment has been widely accepted as a predictable option for most clinical situations seen in routine clinical practice[1]. Titanium (Ti) has already been widely used as implant materials due to its moderate mechanical characteristics and good biocompatibility[2-4]. However, a significant mismatch between the elastic modulus of Ti implants and that of natural bone can lead to stress shielding, a notable issue impacting implant stability[5]. And the lower strength of Ti compared to other biomaterials such as Co-Cr alloys and stainless steel[6], has been linked to an increased risk of implant fractures in patients with narrow diameter implants (NDIs)[2]. Meanwhile, titanium lacks of bone-inducing capabilities, which can possibly result in suboptimal osseointegration, particularly in cases with low bone density or osteoporosis[7], leading to concerns about the prolonged treatment duration necessary for successful implant integration. More importantly, Ti lacks intrinsic antimicrobial properties, raising the risk of post-implantation infections. Previous reports indicate that the incidence rate of peri-implantitis has reached as high as 51%[8]. Recently, the surface modification methods of Ti, such as plasma spraying, deposition, and sol-gel processes, have been utilized to enhance its antibacterial activity. However, due to coating peeling and dissolution, the antibacterial effectiveness of these modifications may be disrupted during application. Therefore, it remains challenging to develop the next generation of dental implants with lasting antibacterial ability, high osseointegration capacity and suitable mechanical properties.

Bulk metallic glasses (BMGs), due to their atomically disordered structure and lack of defects like grain boundaries and dislocations, combine the high strength of metals with the low elastic modulus of bioactive glasses[9]. Among them, zirconium (Zr)-based bulk metallic glass (BMG) has emerged as the widely studied metallic biomaterials due to its good biocompatibility, corrosion resistance and adjustable mechanical properties[10]. For example, Ida et al. reported that the Zr70Ni16Cu6Al8 BMG was conducive to adhesion, proliferation of rat bone marrow mesenchymal cells (BMSCs), and had shown better stability, bone integration rate, and bone integration area compared to Ti and Ti alloys[11]. Furthermore, nickel (Ni)-free Zr50+xCu35-xAl7Nb5Pd3 (x=0, 5) BMG had been designed and fabricated, and the results demonstrated that the Zr-Cu-Al-Nb-Pd BMGs had good *in vitro* biosafe and high corrosion resistance in simulated body fluid environments[12]. Further, Zr-based BMGs possess the unique capability to tailor their properties by adjusting their elemental composition[13], yet, the presence of elements like Ni, Be in Zr-based BMGs may cause allergic reaction or other adverse effect[11, 14-16]. Hence, the current challenge lies in identifying Zr-based BMGs compositions with biocompatible elements that possess good amorphous processability, matched mechanical properties and high osseointegrated capacity required for dental implants. Recent studies on the antibacterial properties of Zr-based BMGs have shown promising developments. Du et al. found that Zr-based BMG samples displayed notable antibacterial activity against both *Escherichia coli* and *Staphylococcus aureus* via laser-induced periodic surface structures (LIPSS)[17]. However, there is currently a lack of antibacterial experiments targeting oral pathogens, and continuous monitoring of the antibacterial properties of Zr-based BMG is lacking.

Silver (Ag) is currently the most potent metal element with antibacterial properties[18] for widely biomedical applications. Liu et al. reported that titanium implants coated with Ag nanoparticles displayed considerable antibacterial effectiveness against common oral pathogens, such as *Streptococcus mutans*[19]. Moreover, some researchers have proposed enhancing the antibacterial performance of titanium alloys by incorporating antibacterial elements such as Cu and Ag. Shirai demonstrated the antibacterial activity of Ti-Cu alloy containing 1 wt% Cu[20], and Zhang proved that the precipitation of the Ti2Ag phase in Ti-Ag alloy was the main reason for its antibacterial performance[21].

Yang et al. investigated the impact of Ag substitution for Ti in Zr56Cu24Al9Ni7Ti4-xAgx (x = 0, 1, 2, 3, and 4 at. %) BMGs on their glass-forming ability, thermal stability, and mechanical properties. They found that the alloys with x=2 exhibited the highest ultimate fracture strength and plastic strain[22]. Meanwhile, previous studies had shown that Zr-Ti-Cu-Al BMGs had low elastic modulus and excellent corrosion resistance, making them highly promising for biomedical implants and medical devices[23-25]. Based on the above research, it is possible that introducing a portion of Ag into Zr-Ti-Cu-Al system may lead to the development of a promising new material for dental implant applications. Moreover, it remains unknown whether the blending Ag can also contribute to lasting antibacterial effects while keeping the appropriately mechanical properties.

Here, we had developed a novel amorphous alloy system Zr61Cu23Al12Ti2Ag2 (at.%) (Zr-BMG)and evaluated its physicochemical properties, biocompatibility, antibacterial activity, osteogenic potential, and angiogenic capabilities, aiming to explore its potential as a material for oral implant applications. This formulation intentionally excluded elements (such as Ni, Be, Fe) that were considered unsuitable for use in oral implant systems. In ensuring both excellent amorphous forming ability and system stability, Ag had been incorporated to endow the material with antimicrobial properties. Concurrently, experiments had been meticulously designed in alignment with the specific requirements of dental implants to validate the performance of this new material.

1. **Material and methods**

**2.1 Materials preparation and characterization**

Zr61Cu23Al12Ti2Ag2 was prepared by arc-melting (Institute of Physics CAS, China) of pure elements with a minimum 99.95% purity in an argon gas protective atmosphere, forming cylindrical samples with a diameter of 5mm and rectangular samples with a width of 16mm. Grade IV Ti was used for comparison substrate and all metals were purchased from Beijing Jiaming Platinum Industry Nonferrous Metals Co., Ltd.

The phase composition of the sample was verified using an X-ray diffraction (XRD, D8 Advance, Bruker, Germany) instrument and a transmission electron microscope (TEM, JEM 2100PLUS, JEOL, Japan). Then they were tested using a differential scanning calorimetry (DSC, DSC 404 F3, NETZSCH, Germany) instrument. Chemical compositions of material surfaces of the samples were analyzed using scanning electron microscopy (SEM, S-4800, HITACHI, Japan) and energy dispersive X-ray spectroscopy (EDS, S-4800, HITACHI, Japan). The surface microstructure was analyzed by X-ray photoelectron spectroscopy (XPS, ESCALAB 250X, ThermoFisher Scientific, USA). Monochromated Al Kα X-ray source of 1486.6 eV was used. The narrow spectra were further deconvoluted with Thermo Advantage software.

Material density was measured by micromeritics (AccuPyc II 1345, USA). Compression test was carried out using a universal testing machine (Instron 5967, USA) with a strain rate of 1×10-3 s-1 at room temperature. Ti and Zr-BMG samples (Ø5 mm) were cut and tested for mechanical properties using nanoindentation with a triangular diamond indenter (TI 950, Hysitron, USA). Ti and Zr-BMG samples were uniformly polished to a 5000 grit and their wettability was characterized using static contact angle measurements with a surface contact angle meter (DSA 100E, KRÜSS, Germany). Tests were conducted at 20 ℃ using stimulated body fluid (SBF, Beyotime, China) as the reference fluid. For the above experiments, three parallel samples were set for each group.

The bio-corrosion behaviors of the Zr-BMG and Ti were analyzed in an electrochemical workstation (Versa STAT 3F, Princeton, USA) in the SBF at 37 ℃. After cleaning and air drying, samples were then immersed in 200 μL SBF for varying durations: 1 day, 1 week, 2 weeks, 4 weeks, 8 weeks, and 12 weeks. A blank SBF control was also maintained. The ion concentrations in the SBF were analyzed using inductively coupled plasma mass spectrometry (ICP-MS, PerkinElmer, USA). For the above experiments, three parallel samples were set for each group.

**2.2 Biocompatibility measurements**

Approved by the Medical Ethical Commission of Peking University School and Hospital of Stomatology (PKUSSIRB-202169161), human jawbone marrow mesenchymal stem cells (hJBMMSCs) were extracted from non-smoking patients aged 18-45 undergoing orthognathic surgery and cultured as described in our previous research[45].

The cell counting kit-8 (CCK-8, Dojindo Laboratories, Japan) were used to assess cell viability according to manufacturer’s instruction. Optical density (OD) values were then measured at 450 nm using an Infinite F50 microplate reader (Elx808, BioTek, USA). The cytotoxicity of Zr-BMG and Ti samples on hJBMMSCs was assessed using a LIVE/DEAD cell kit (KeyGen Biotech, China) following manufacturer’s instruction. Samples were photographed under a fluorescence microscope (TCS-SP8 STED 3X, Leica, Germany).

hJBMMSCs were cultured on material samples in 96-well plates for 1, 4, and 24 h. Adherent cells were fixed with 4% paraformaldehyde (PFA, Solarbio, China) for 15 min at room temperature and then stained with DAPI (Sigma, USA). Immunofluorescent images were acquired using a laser scanning confocal microscope (Eclipse Ti2, Nikon, Japan). Cell counts were conducted in 5 randomly selected fields and analyzed using Image J software (National Institutes of Health, USA).

After incubation on the surface of material samples for 24 h, cells were fixed in 4% PFA. Subsequently, they were exposed to a solution of 0.1% Triton-X100 (Sigma) for 10 min at room temperature. Staining was performed using TRITC Phalloidin (Yeasen, China) to visualize F-actin and DAPI (Sigma) for nuclear staining. Samples were examined under a fluorescence microscope (TCS-SP8 STED 3X, Leica, Germany) using a Z-axis layer. Three random fields per material were imaged across three replicates.

After incubation on the surface of material samples for 24 h, cells were fixed in 2.5% glutaraldehyde solution (Solarbio) overnight. After graded dehydration and drying, the specimens were gold sputter-coated for 35 seconds before examination under a scanning electron microscope (SEM, JSM-7900F, JEOL, Japan).

**2.3 Antibacterial evaluation**

In a sterile environment, *Streptococcus mutans* (*S. mutans*, UA159) was streak-plated on Mannitol Salt Agar (MSA) solid medium and cultured at 37 ℃ with 5% CO2 for 48 h, while *Porphyromonas gingivalis* (*P. gingivalis*, ATCC 33277) was streak-plated on Tryptic Soy Agar (TSA) solid medium and incubated in an anaerobic environment (80% N2, 10% CO2, 10% H2) at 37 ℃ for the same duration. 107 CFU/ml bacterial suspensions were used for following experiments.

After sterilization, the materials were placed in a 96-well plate and exposed to *S. mutans* and *P. gingivalis* suspensions for 24 h. Dual staining with SYTO 9 and Propidium iodide (15 min, dark) allowed examination under a laser confocal microscope (TCS-SP8 STED 3X) to differentiate live and dead bacteria using 488 nm and 560 nm excitation wavelengths.

Sterilized materials were placed in a 96-well plate and inoculated with 100 µL bacterial suspensions of *S.mutans* and *P.gingivalis*. The xenon tetrafluoride tetramethylammonium (XTT, KeyGEN) assay was used to assess the viability of bacterial according to manufacturer’s instruction.

Inoculated materials with bacterial suspensions of *S.mutans* and *P.gingivalis* at 107 CFU/mL and culturing for 24 h. After washing and detaching the bacteria, the solution was serially diluted and plated on MSA (for *S.mutans*) and TSA (for *P.gingivalis*) plates. Plates were incubated under appropriate conditions for 48-72 h. The antibacterial rate was calculated by counting colony-forming units, using the formula:

Anti-bacterial rate (%) = (CFUTi-CFUZr-BMG)/CFUTi × 100%

Materials were processed as before, with bacterial culture in a medium containing 1% sucrose (Solarbio) for 24 h, followed by fixation with 99% methanol, staining with 0.1% crystal violet, and dissolution of the stained biofilm in 95% ethanol for absorbance measurement at 570 nm by microplate reader (Elx808, BioTek); For assessing the material's sustained anti-biofilm efficacy, first soaked the material in PBS for varying durations (2, 4, 6, 8, 12 weeks), then co-cultured with bacteria for 24 h as previously described. The formula for calculating the material's biofilm clearance rate was:

Anti-biofilm rate (%) = (OD570Ti-OD570Zr-BMG)/OD570Ti × 100%

After 24 h co-culture with *S. mutans* and *P. gingivalis*, the materials were washed, fixed in 2.5% glutaraldehyde at 4℃ overnight, dehydrated with ethanol, air-dried, affixed to the SEM stage, gold-coated, and examined under SEM (JSM-7900F) at 10 kV to observe bacterial morphology and distribution.

After 24 h of co-culture, the material was washed, incubated with 2',7'-Dichlorodihydrofluorescein diacetate (DCFH-DA, Beyotime) for 30 min in the dark, and observed under a laser confocal microscope (TCS-SP8 STED 3X) at 488 nm. The collected culture medium was also incubated with DCFH-DA (1:500 final concentration), transferred to a microplate, and fluorescence intensity at 522 nm was measured using a microplate reader.

After co-culturing the material with bacteria for 24 h, the original culture medium was collected in a sterile centrifuge tube and centrifuge at 5000 rpm for 10 min. The protein content released by the bacteria in the supernatant was tested with a bicinchoninic acid (BCA, Beyotime) assay kit and measured at 562 nm.

**2.4 *In vitro* cell angiogenesis and osteogenic measurements**

To investigate *in vitro* angiogenesis, HUVECs (Cellverse Bioscience Technology Co., Ltd., China) were cultured in ECM complete culture medium with 5% FBS, 1% endothelial cell growth factor, 100 U/mL penicillin, and 100 U/mL streptomycin. Extracts were prepared following ISO 10993-12-2021: autoclaved materials were soaked in ECM medium at 200 mg/mL for 72 h at 37 ℃, filtered through a 0.22 µm membrane, and stored at 4 ℃.

HUVECs were pretreated with material extracts for 24 h and then seeded on growth factor-depleted Matrigel (BD Bioscience) in 48-well plates. Tube formation assays were conducted using optical microscopy (Olympus, Japan) at 2 h, 4 h, 8 h and 12 h. At 2 h, cells had not yet formed tubular structures, whereas at 12 h, tubular structures began to break down (**Figure S1**). Subsequently, the images of 4 h and 8 h were chosen for quantitative analysis using Image J software.

HUVECs (1×105 cells/well) were seeded in 12-well plates and cultured in extracts with serum until 100% confluency. Linear scratches were made, rinsed with PBS, and images captured at 0 h and 12 h post-cultivation in serum-free extracts to assess wound healing via optical microscopy (Olympus, Japan) and Image J software.

For osteogenic induction, hJBMMSCs were treated with osteogenic medium containing 50 μg/ml ascorbic acid (Sigma), 2 mM β-glycerophosphate (Sigma), and 100 nM dexamethasone (Sigma) until the cell confluence reached 70-80%.

hJBMMSCs were cultured with material samples for 7 days under osteogenic induction, fixed with 4% PFA, and stained with BCIP/NBT solution (Beyotime) to assess ALP activity. For ARS staining, cells were fixed after 21 days, stained with alizarin red S (Cyagen), destained with 10% cetylpyridinium chloride (Sigma), and the calcium concentration was measured using a spectrophotometer (BioTek) at 562 nm.

After fixation with 4% PFA and permeabilization with 0.3% Triton X-100, cells were blocked with 5% BSA (Solarbio). HUVECs were immunostained at day 3 with anti-VEGF primary antibody (1:200, Abclonal) overnight at 4 ℃, followed by TRITC fluorescent secondary antibody (1:400, Bioss) for 1 h at room temperature; hJBMMSCs at 7 days post-osteogenic induction were stained with anti-BGLAP primary antibody (1:200, Abclonal) overnight at 4 ℃, followed by FITC fluorescent secondary antibody (1:400, Bioss) for 1 h at room temperature.

HUVECs nurtured for 3 days and hJBMMSCs after 7-days osteogenic induction were collected for qRT-PCR. Total RNA was extracted from the samples using Trizol (Invitrogen, USA) and cDNA was synthesized from 1 μg of total RNA using reverse transcriptase (Superscript II Preamplification System; Invitrogen). All the reactions were performed in triplicate and were normalized to the reference gene (glyceraldehyde 3-phosphate dehydrogenase, GAPDH). The specific primer sets used for this analysis were listed in **Table S2**.

**2.5 *In vivo* measurements**

The animal experiments conducted in this study received approval from Peking University's Ethics Review Board (PUIRB-LA2023268). All methods are reported in accordance with ARRIVE guidelines for animal experiments[46].

Male Sprague-Dawley rats (8 weeks old, 200-250 g, 6 rats in each group, Charles River, Beijing, China) were randomly assigned to Ti and Zr-BMG groups. Under pentobarbital-induced anesthesia, a medial parapatellar arthrotomy was performed to create a bone tunnel in the intercondylar fossa of the right femur, where a 12 mm long, 1.5 mm diameter cylindrical implant was inserted (**Figure S2**). After 8 weeks, rats were euthanized, blood was collected for biochemical tests, and femurs with implants along with liver, kidney, and spleen were fixed in 4% PFA for further processing.

Femurs with implanted samples underwent graded dehydration, embedding in light-curing epoxy resin, and preparation of thin sections using an grinding machine (Exakt, Germany). These sections were stained with Stevenel’s blue and Van Gieson’s picrofuchsin stain, followed by analysis using a light microscope (Leica, Germany).

Blood sample was then drawn from each rat and subjected to analysis using a Clinical Analyzer (BK-280, Biobase, Shandong, China). Liver, kidney and spleen tissues from rats underwent H&E staining after deparaffinization and rehydration. The sections were examined using a light microscope (BX53, Olympus).

**2.6 Statistical analysis**

The analysis of statistical data was executed using Graph Prism 8.0.1. The presentation of data encompassed mean values along with their corresponding standard deviations (SD). The Shapiro-Wilk test was employed to verify the normal distribution of the original dataset, and for the evaluation of significance relied on both the two-tailed independent Student's t test. Differences were considered statistically significant at: \* for *P* < 0.05, \*\* for *P* < 0.01, and \*\*\* for *P* < 0.001.

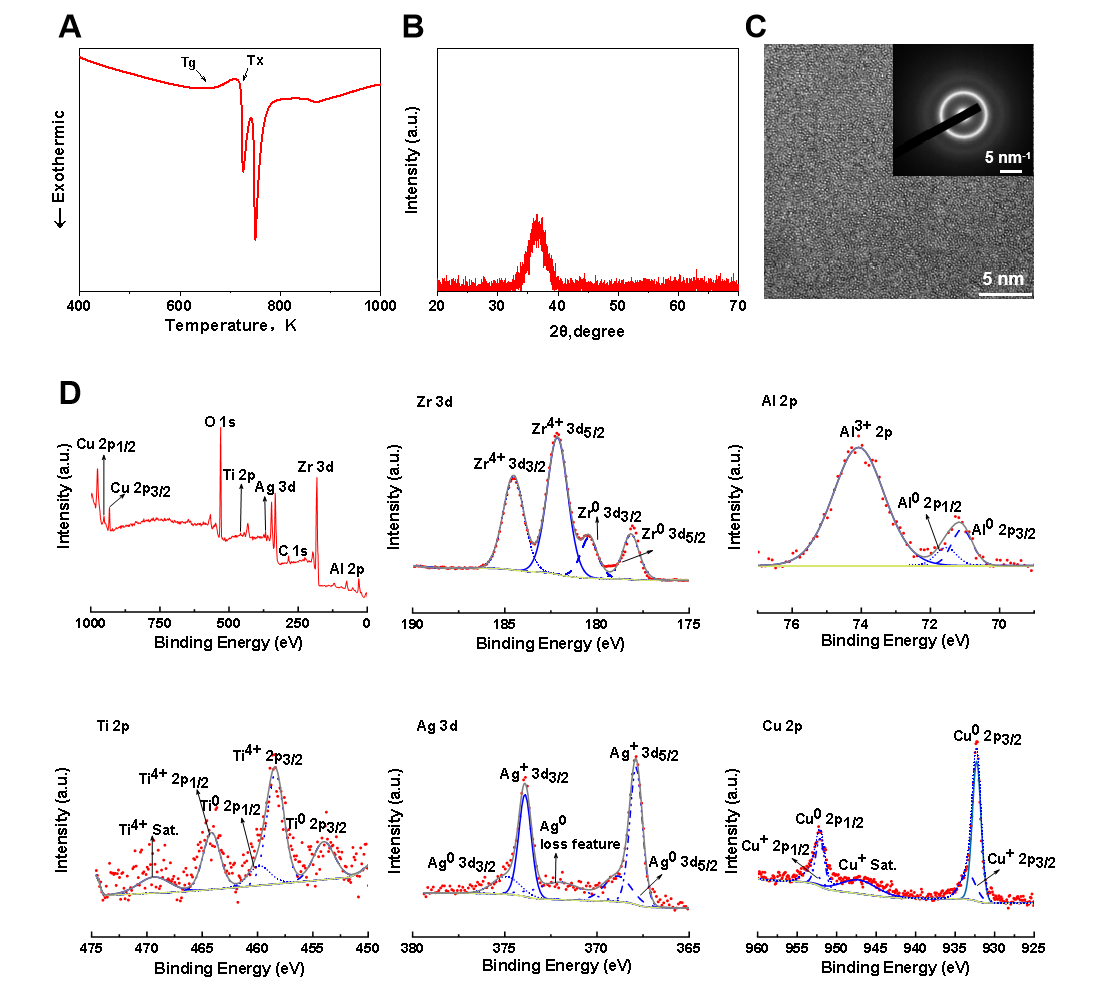
1. Results

3.1 Structural properties, thermal parameters and surface chemical compositions

The new amorphous alloy, Zr61Cu23Al12Ti2Ag2 (at.%) (Zr-BMG), demonstrated its critical molding dimensions in Supplementary Materials (**Figure S3**): a 5 mm diameter cylinder (i) and 16 mm wide rectangles (ii). **Figure 1A** displayed the DSC curve of the novel Zr-BMG, indicating a glass transition temperature (Tg) of 662.01 K and a crystallization temperature (Tx) of 720.30 K, resulting in a supercooled liquid range (∆T) of 58.29 K. The results demonstrated the system's superior glass-forming ability and thermal stability[9].

The XRD diagram shown in **Figure 1B** displayed a characteristic wide scattering peak, indicating the amorphous nature of the Zr-BMG material. Microstructural homogeneity was carefully scrutinized by transmission electron microscope (TEM) and two diffuse halos featuring the short-range order of BMGs confirmed the amorphous state of the alloys (**Figure 1C**).

Scanning electron microscope (SEM) and energy dispersive X-ray spectroscopy (EDS) identified the presence of Zr, Cu, Al, Ti, Ag, O in bulk metallic glasses (**Figure S4**), confirming our elemental composition. X-ray photoelectron spectroscopy (XPS) image (**Figure 1D**) and fine peak analysis of zirconium elements revealed that zirconium in the sample primarily existed in the Zr4+ state (182.15/184.53 eV), indicating that the tetravalent zirconium originates from zirconium oxide. Similarly, aluminum, titanium and silver were also enriched in oxide forms, detected as Al3+, Ti4+ and Ag+, respectively. In contrast, Cu mainly existed in the zero-valent form. The detailed information of XPS peak analysis could be found in Supplementary Materials (**Table S1**). Based on the XPS results, it could be inferred that the predominant Zr element in Zr-BMG primarily existed on the material surface in the form of oxides, indicating the formation of an oxide layer on its surface. Additionally, Cu primarily existed on the material surface in the form of metallic Cu0.



**Figure 1.** Structural properties, thermal parameters and surface chemical compositions.

A) Representative differential scanning calorimetry (DSC) data of Zr-BMG, showing their transition temperature (Tg) and crystallization temperature (Tx). B) Representative X-ray diffraction (XRD) curve illustrated a distinctive "bread-loaf" peak, characteristic of the non-crystalline or amorphous structure inherent to the Zr-BMG material. C) Representative transmission electron microscopy (TEM) image showed microstructural homogeneity and the amorphous ring. D) Representative X-ray photoelectron spectroscopy (XPS) curve presented both the survey spectrum and elemental sub-spectra, affirming the compositional consistency of the Zr-BMG sample with the intended elements. Zirconium in the sample primarily existed in the Zr4+ state,while aluminum, titanium and silver were also enriched in oxide forms, detected as Al3+, Ti4+ and Ag+, respectively. In contrast, copper mainly existed in the zero-valent form.

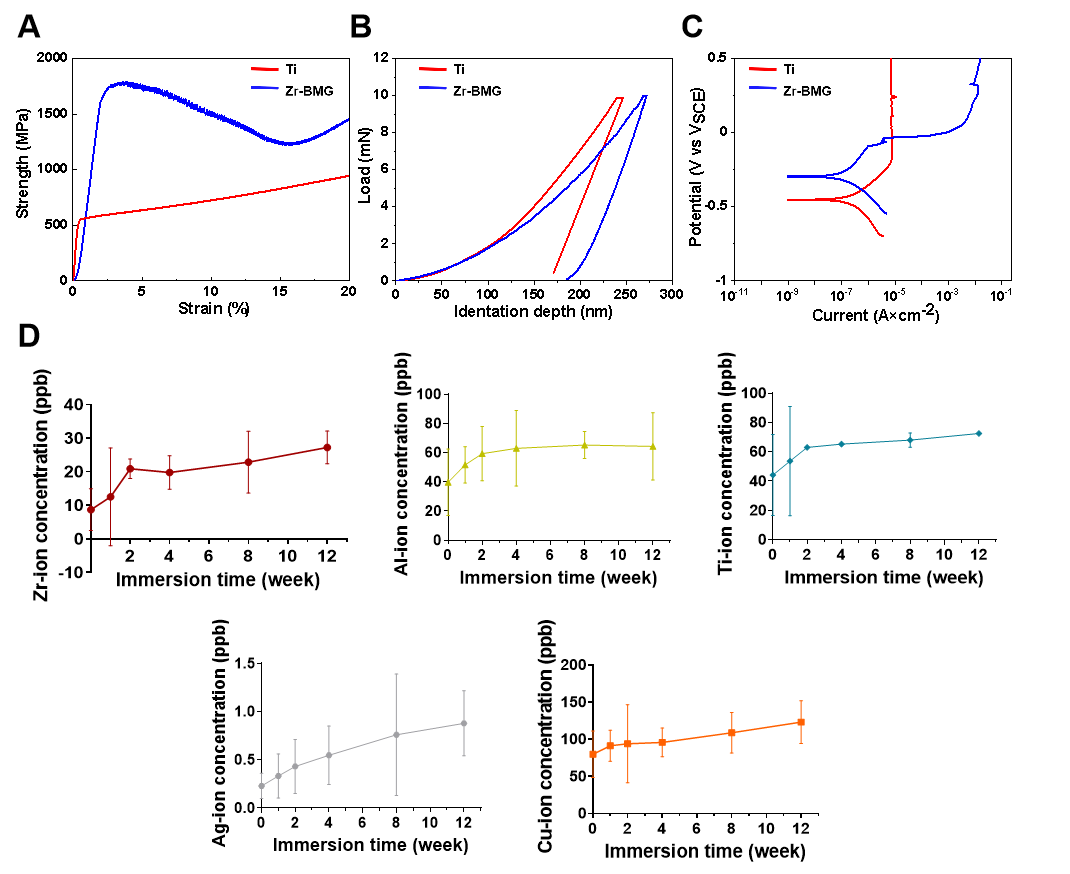
**3.2 The mechanical properties, hydrophilic characterization and corrosion resistance**

Compressive stress-strain curves and load-displacement curves of all samples were shown in **Figure 2A, B**. The compressive strength of Zr-BMG (1603.67±98.80 MPa) was about 3 times that of Ti (550.33±37.00 MPa) (**Table 1**). The reduced Young’s modulus of Zr-BMG was 94.20±7.16 GPa (Table 1), which was closer to the reported cortical bone modulus (30 GPa) than Ti. Meanwhile, the microhardness of Zr-BMG was 5.30 GPa, which was greater than that of Ti (4.02 GPa). The mass density of the Zr-BMG, measured by the Archimedean technique with an accuracy of 0.1%, was approximately 6.27 g/cm³ (Table 1). Calculations revealed that the Zr-BMG exhibited a specific strength of 237.64 MPa cm³/g, which was higher than that of Ti (115.13 MPa cm³/g).

**Figure 2C** displayed the polarization curves of Ti and Zr-BMG, the pitting corrosion potential of Zr-BMG was -0.06V versus saturated calomel electrode (VSCE), the curve also clearly illustrated the formation of a passive region for Zr-BMG, with a width of approximately 0.03V versus VSCE, indicating the development of a passive film on the material's surface. **Table 2** summarized essential electrochemical parameters obtained from the potentiodynamic polarization curves, Zr-BMG exhibited a higher self-corrosion potential and lower self-corrosion current density compared to Ti. Consequently, in SBF environment, Zr-BMG demonstrated superior corrosion resistance to Ti, with the formation of a protective passive film on its surface. Considering the XPS results, the main component of the formed passive film was ZrO2.

The cumulative ionic concentrations released by the Zr-BMG in the SBF solution over time were depicted in **Figure 2D**. During the initial two weeks, the concentrations of all metal ions released increased rapidly with prolonged immersion time. However, from week 2 to 12, the rate of increase in metal ion release concentrations notably decreased, gradually reaching a stable level. This slow and sustained metal ion release from Zr-BMG avoided the adverse effects associated with rapid increases in metal ion concentrations over a short period.

The surface contact angle results with SBF for the two materials showed no significant differences (**Figure S5**).

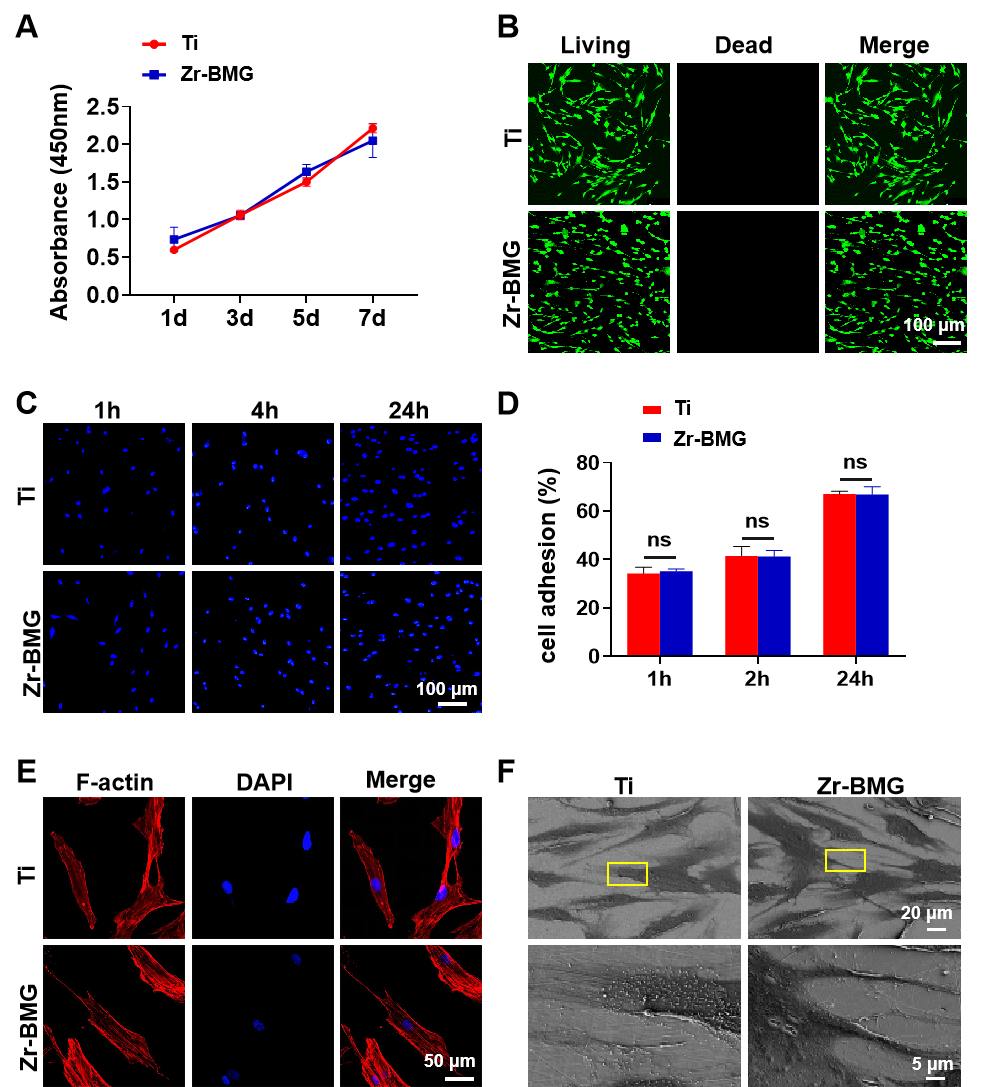


**Figure 2.** Mechanical properties and electrochemical results. A) Compression stress-strain curves showed that the mechanical strength of Zr-BMG significantly surpassed Ti. B) Load-displacement curves of two samples at indentation load of 10 mN, showing that the reduced Young’s modulus of Zr-BMG was closer to the reported cortical bone modulus than Ti. C) Potentiodynamic-polarization curves and corrosion parameters of two samples in simulated body fluid (SBF), indicating a slower corrosion rate of Zr-BMG compared to Ti. D) The release of different metal ions in SBF at 37℃. The release of various metal ions from Zr-BMG stabilized after 2 weeks in the SBF environment.

**3.3 *In vitro* measurements**

3.3.1 Biocompatibility

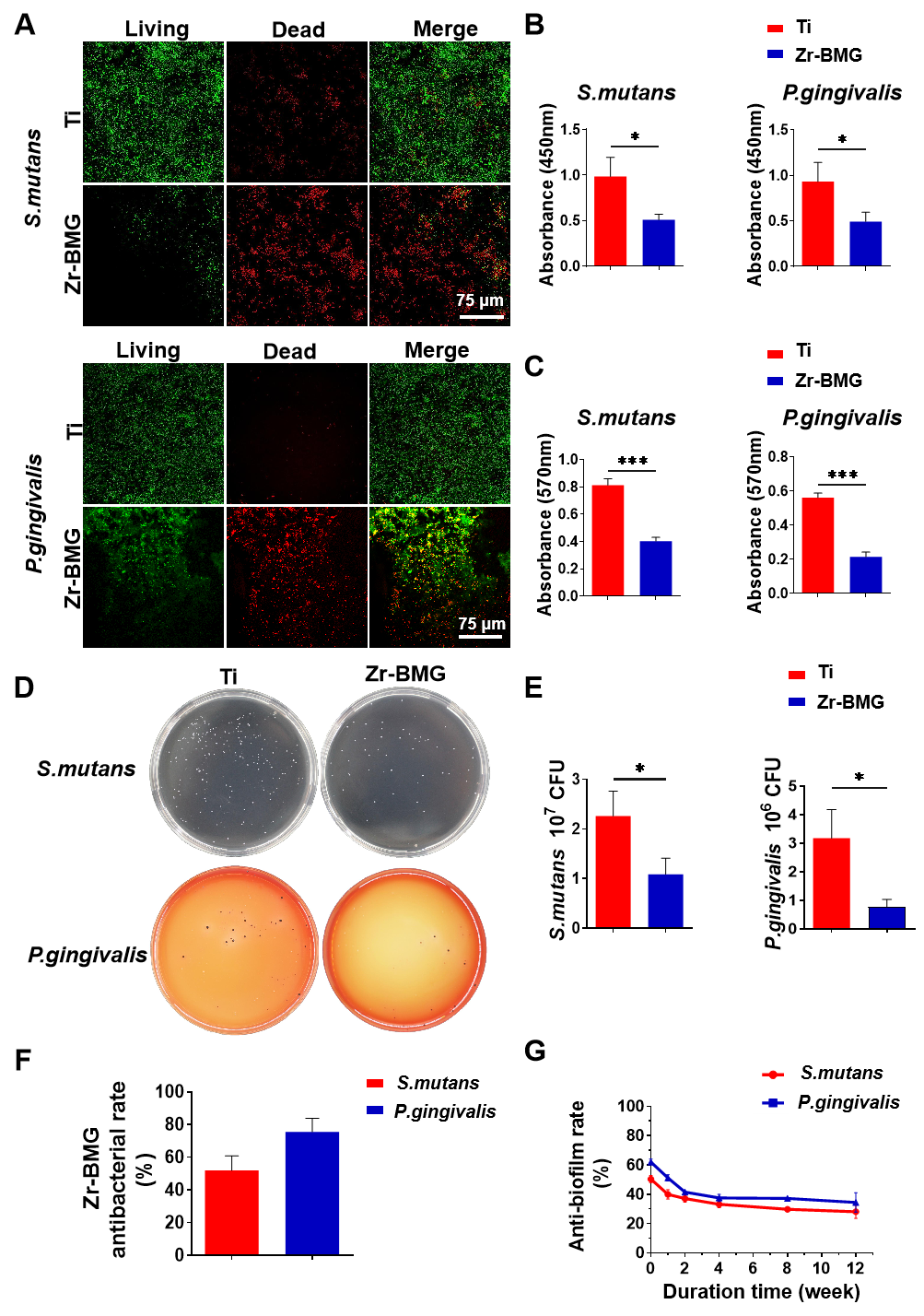
Cell adhesion and spreading on biomaterial surfaces are crucial, as cells are highly sensitive to the physical and chemical properties of materials. **Figure 3A** demonstrated the CCK-8 assay results, revealing similar cell proliferation rates on Zr-BMG and Ti surfaces at intervals of 1, 3, 5, and 7 days. **Figure 3B** presented the results of cell viability of hJBMMSCs culturing on Zr-BMG or Ti for 24 h, indicating no acute cytotoxicity. This suggested that both materials had a comparable effect on the proliferation of hJBMMSCs in this experimental context. As depicted in **Figure 3C**, hJBMMSCs were evenly distributed on the surfaces of all groups. Statistical analysis confirmed no significant difference between the two groups (**Figure 3D**). Cellular cytoskeleton staining (**Figure 3E**) revealed similar morphologies of cells adhered to both Zr-BMG and Ti surfaces. Scanning electron microscopy (SEM) images (**Figure 3F**) demonstrated comparable attachment and growth morphologies of cells on both materials.



**Figure 3** Cell viability,cell adhesion and cell morphology features. A) The CCK-8 assay results, revealing similar cell proliferation rates on Zr-BMG and Ti surfaces at intervals of 1, 3, 5, and 7 days. B) Fluorescent staining images presenting the results of cell viability after 1, 4, and 24 hours of cell culture, indicating minimal cytotoxicity differences between Zr-BMG and Ti. C) Representative cell adhesion figures and cell number counts at 1 h, 4 h, and 24 h. D) Statistical analysis of the cell number confirmed no significant difference between the two materials. E) Phalloidin staining of hJBMMSCs F-actin at 24 h. F) Scanning electron microscopy (SEM) images of hJBMMSCs cell morphology on material surface at 24 h. Data were presented as mean ± SD. ns: *P*>0.05.

3.3.2 Antibacterial properties assessment

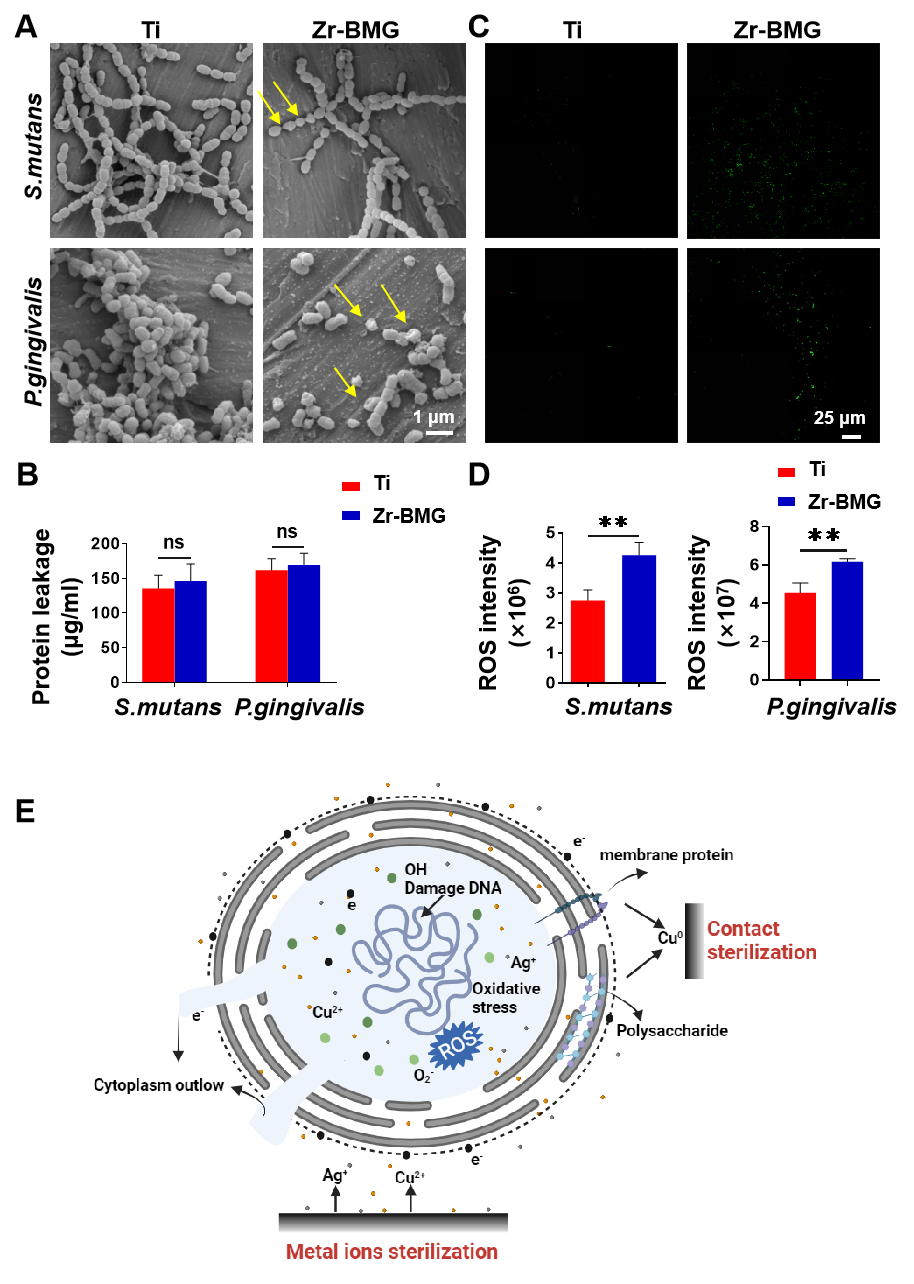
Implant-related infections (IRIs) are common post-implantation complications, often due to bacterial adhesion and subsequent biofilm formation on material surfaces[19]. In this study, we selected two common oral pathogens, *Porphyromonas gingivalis* (*P.gingivalis*) and *Streptococcus mutans* (*S.mutans*), to evaluate the antibacterial performance of the developed biomaterials after co-culturing with the materials. **Figure 4A** displayed live/dead staining of bacteria on Ti and Zr-BMG samples. In contrast to the widespread presence of living bacteria on Ti surface, less bacteria were found adhered to the Zr-BMG surface, among which largely were dead colonies. The result of XTT assay demonstrated the bacterial proliferation on Zr-BMG surface were significantly inhibited, indicating its superior ability to suppress bacterial activity of *P.gingivalis* and *S.mutans* (**Figure 4B**). **Figure 4C** demonstrated that bacterial biofilms on Zr-BMG surface were significantly inhibited. To further assess the anti-bacterial effect of Zr-BMG on *S.mutans* and *P.gingivalis*, adhered bacteria were ultrasonically removed from the material surface and counted for colony formation (**Figure 4D**). The results depicted in Figure 4E demonstrated a significant decrease in colony numbers for the Zr-BMG group, with approximately 52% of *S. mutans* and 76% of *P. gingivalis* strains being eliminated compared to the Ti control, as shown in **Figure 4F**. This indicated superior antibacterial effectiveness of the Zr-BMG material. Using crystal violet staining for quantification, we assessed the long-term anti-bacterial biofilm formation on Zr-BMG surfaces. **Figure 4G** illustrated an initial rapid decline in Zr-BMG's biofilm inhibition efficacy, stabilizing from week 2 to 4, maintaining consistent bactericidal activity. Even after 3 months, Zr-BMG retained a biofilm inhibition rate of 36.42% for *S.mutans.* and 44.00% for *P.gingivalis*.



**Figure 4** Antibacterial properties of the materials A) The live/dead staining images of *S.mutans* and *P.gingivalis* seeded on the surfaces of the two materials. Ti surfaces had lots of live bacteria, but Zr-BMG surfaces had fewer, mostly dead ones. B) XTT assay of the bacterial activity on the surfaces of the two materials. The bacterial proliferation on Zr-BMG surface was significantly lower than Ti. C) The result of crystal violet staining. The bacterial biofilm formation on Zr-BMG surface was significantly inhibited. D) The images of the dilution plate after co-culturing bacteria with the two materials (black colonies for *P.gingivalis* and yellow-white colonies for *S.mutans* ). E) Quantitative analysis of colonies. Compared to Ti, the quantity of both live bacteria adhering to Zr-BMG was significantly reduced. F) The bactericidal rate of Zr-BMG against *P.gingivalis* was (75.56±4.74)%, and for *S.mutans*, the bactericidal rate was (51.96±5.13)%, indicating that Zr-BMG had a stronger killing ability against *P.gingivalis*. G) The removal efficiency of Zr-BMG on bacterial biofilm initially decreased rapidly, gradually stabilizing from the 2nd to 4th week. Data were presented as mean ± SD. \**P* < 0.05 and \*\*\**P* < 0.001.

3.3.3 Antibacterial mechanism

This study utilized scanning electron microscopy (SEM) to observe bacterial morphology and distribution on materials. **Figure 5A** showed bacteria inoculated on Ti exhibited spherical shapes with smooth surfaces. In contrast, those on Zr-BMG displayed extensive cell wall ruptures and membrane disruptions, with observable twisted and completely detached membrane structures. Concurrently, this study measured the bacterial protein release on different materials. **Figure 5B** illustrated that bacteria inoculated on Zr-BMG exhibited a slightly higher protein release compared to those on other materials, yet the difference was not statistically significant (*P* > 0.05). ROS are integral to cellular physiological processes, with excessive ROS leading to oxidative stress and cell death[26, 27]. As depicted in **Figure 5C**, higher ROS production was observed on bacteria adhered on Zr-BMG surface. In the meantime, significant ROS signals were detected in the bacterial suspension in the Zr-BMG group (**Figure 5D**). Therefore, we speculated that the antibacterial mechanism of this material might include both contact sterilization and ion sterilization (**Figure 5E**). When bacteria came into contact with the material, the Cu on the surface could kill the bacteria by disrupting structures such as the bacterial cell membrane. Simultaneously, the material could release copper and silver ions in the SBF solution, inducing substantial ROS production in bacteria, elevating intracellular ROS levels, causing oxidative stress damage, and leading to bacterial cell death.

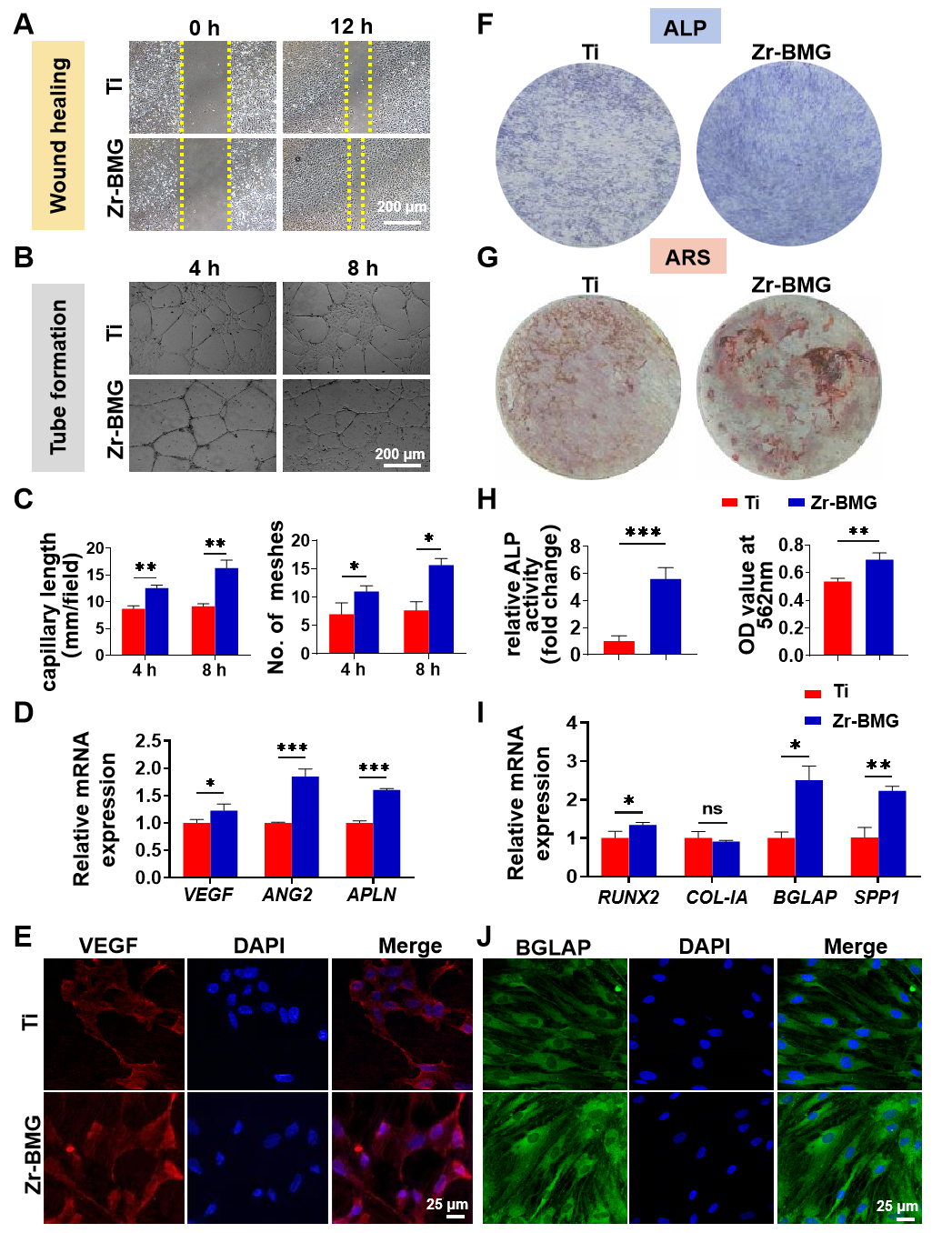


**Figure 5** Antibacterial mechanism A) Representative scanning electron microscope (SEM) images of *S.mutans* and *P.gingivalis* inoculated on the surfaces of the two materials. The bacterial cells inoculated on the surface of Zr-BMG exhibited a large number of cell wall ruptures, membrane fractures, and blurred or unclear cytoplasm with overflow. B) The bacterial protein release levels after inoculation with *S.mutans* and *P.gingivalis* on two materials. The bacterial protein release levels on Zr-BMG were slightly higher, but there was no significant difference between the two. C) Images of the bacterial reactive oxygen species (ROS) staining on the material surface after inoculation with *S.mutans* and *P.gingivalis*.D) Fluorescence intensity of free-released ROS. The Zr-BMG induced substantial ROS production in bacteria, elevating extracellular ROS levels. E) Schematic diagram of the antibacterial mechanism of Zr-BMG. Data were presented as mean ± SD. ns: *P* > 0.05 and \*\**P* < 0.01.

3.3.4 Angiogenesis and osteogenesis ability

Angiogenesis is a critical process during bone integration, where new blood vessels provide oxygen and nutrients to surrounding cells to promote their proliferation and differentiation. HUVECs cultured in material extracts underwent scratch assays (**Figure 6A**), and quantitative analysis (**Figure S6**) demonstrated that Zr-BMG outperformed Ti in promoting wound healing. Moreover, we closely examined tube formation in HUVECs at 4 and 8-hour intervals through microscopic observation. **Figure 6B** showed that Zr-BMG led to denser and more widely distributed microvascular networks with fewer isolated cells compared to Ti. Statistical analysis in **Figure 6C** revealed that Zr-BMG significantly enhanced angiogenesis, as evidenced by total tubular length (*P* < 0.01) and network count (*P* < 0.05). qRT-PCR analysis demonstrated a significant upregulation of angiogenesis-related markers, including *VEGF*, *ANG2*, and *APLN*, in Zr-BMG after 3 days of co-culturing (**Figure 6D**). Immunofluorescent staining further confirmed the elevated protein expression of *VEGF* in Zr-BMG compared to Ti (**Figure 6E**).

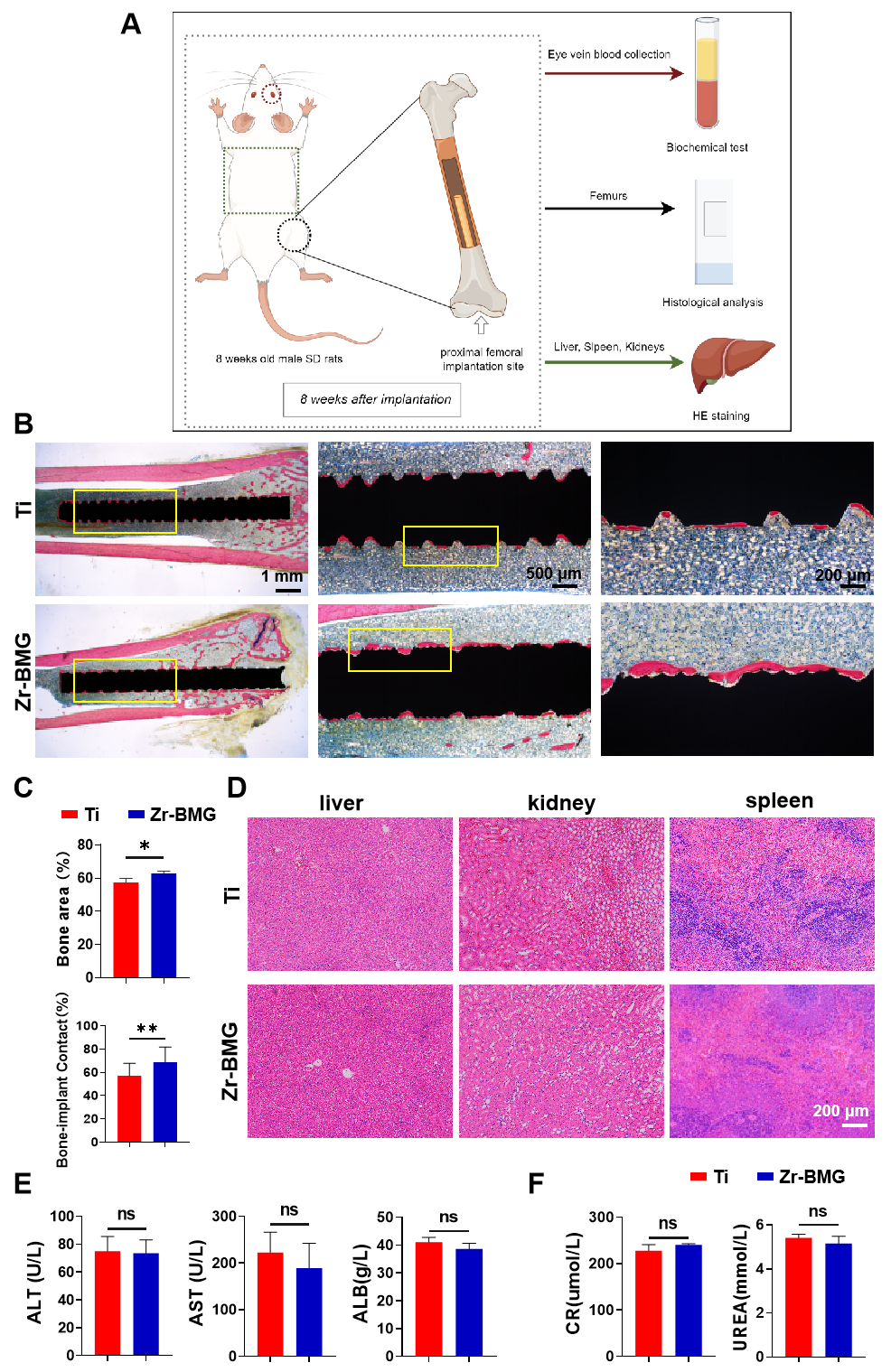
This study explored the osteogenesis capabilities of Zr-BMG by employing a jawbone-specific *in vitro* model, utilizing hJBMMSCs, thereby capturing the unique osteogenic feature of the jaw bone[28] to evaluate the potential of Zr-BMG as a dental implant material. hJBMMSCs were directly cultured on materials’ surface to evaluate their osteogenic potential. ALP is an early marker of osteogenic differentiation in hJBMMSCs[29]. Mineralized nodules are considered as indicators of the mature and late-stage osteogenesis[29]. **Figure 6F** showed a significant increase in ALP activity in hJBMMSCs on Zr-BMG compared to Ti, as revealed by statistical analysis (*P* < 0.001) (**Figure 6H**). Subsequently, the same cell types were induced for osteogenic differentiation for a period of 14 days and then stained with alizarin red S. The results, displayed in **Figure 6G**, showed that cells on Zr-BMG developed considerably larger calcium nodules with more intense staining than those on the Ti substrate. A further quantitative analysis (Figure 6H) of the staining confirmed that Zr-BMG significantly enhanced the extracellular matrix mineralization capabilities of hJBMMSCs post-osteogenic differentiation, demonstrating its superior osteoinductive potential. After 3 days of osteogenic induction of cells on Ti and Zr-BMG, the expression levels of bone-related mRNAs were presented in **Figure 6I**. In this study, we assessed the expression of osteogenesis-associated factors, *RUNX2*, *COL-1A*, *BGLAP*, and *SPP1*. Our analysis revealed a significant upregulation of these factors in cells cultured on the surface of Zr-BMG, with statistically significant differences between the two groups (*P* < 0.05). Immunofluorescence analysis in **Figure 6J** revealed that, when compared to Ti, the expression of OCN in hJBMMSCs cultured on Zr-BMG was higher.



**Figure 6** The angiogenesis and osteogenic reaction of different materials A) Scratch assay of HUVECs cultured in material’s extract. Scratch experiment images showed that compared to Ti, HUVECs treated with Zr-BMG extract exhibit a faster migration speed. B) Representative images of tube formation assay at 4 h and 8 h. C) Quantitative measurement of the tube formation assay, indicating that Zr-BMG could efficiently promote vasculature development. D) qRT-PCR of angiogenesis marker, *VEGF*, *ANG2* and *APLN*. E) Immunofluorescent staining of VEGF, confirming that Zr-BMG up-regulated the protein level of VEGF in comparison with Ti. F) The alkaline phosphatase (ALP) staining images of hJBMMSCs after osteogenic induction on the material surface. G) The alizarin red S (ARS) staining images of hJBMMSCs seeded on the material surfaces after osteogenic induction. H) Quantitative measurement of ALP activity and dissolved ARS OD value measured at 562 nm. Zr-BMG exhibited enhanced ALP activity and better calcium nodules formation. I) qRT-PCR of osteognic markers. Zr-BMG significantly increased the expression of *RUNX2*, *COL-IA*, *BGLAP* and *SPP1*. J) Immunofluorescent staining of BGLAP confirming that Zr-BMG up-regulated the protein level of BGLAP in comparison with Ti. Data were presented as mean ± SD. \**P* < 0.05, \*\**P* < 0.01 and \*\*\**P* < 0.001.

**3.4 *In vivo* measurements**

Establishing direct contact between bone and implant without the presence of fibrous tissue is crucial for successful osseointegration[30]. To further analyze the osteogenic effects of Zr-BMG on cancellous bone, an alcian blue-acid fuchsin stain was performed to assess the osseointegration of Ti and Zr-BMG implants after 8 weeks (**Figure 7A**). As shown in **Figure 7B**, osteoid tissue appeared blue-green and newly mineralized bone appeared deep red. More bone-like tissues were formed in direct contact with the Zr-BMG, in comparison with Ti control at 8 weeks post-implantation surgery. Histological analysis of hard tissue sections supported a significantly larger bone-implant contact area (*P* < 0.05) and length (*P* < 0.01) around Zr-BMG implants than Ti at 8 weeks post-surgery (**Figure 7C**), indicating a superior osseointegration ability of Zr-BMG compared to Ti. Although Zr-BMG contained complex metallic elements, researchers had not observed significant pathological changes in the liver, kidneys or spleens (**Figure 7D**). Similarly, no signs of pathological features were observed in serum tests of rats implanted with Ti and Zr-BMG after 8 weeks (**Figure 7E, F**).



**Figure 7**. *In vivo* osteogenic potential of materials A) The flow chart of in-vivo measurement. B) Representative images of Stevenel’s blue and Van Gieson’s picrofuchsin staining showing new bone formation around implants. Zr-BMG implants were able to induce more bone formation that was in direct close contact to implant surfaces. C) Quantitative measurement of bone area percentage around implants and relative bone-implant-contact length. Zr-BMG implants achieved better osseointegration thanTi. D) H&E staining of liver, kidney and spleens sections, showing no obvious pathological change. E) Biochemical parameters in animals with the implanted Ti and Zr-BMG. Data were presented as mean ± SD. ns: *P* > 0.05, \**P* < 0.05 and \*\**P* < 0.01.

1. **Discussion**

The addition of Ag enhanced the alloy's glass-forming ability (GFA), meeting the criteria outlined by Takeuchi and Inoue[9], which included three or more elements, atomic size mismatch exceeding 12%, and negative mixing enthalpy between constituent elements. In the Zr-Ti-Cu-Al-Ag system, Ag exhibited negative mixing enthalpies with Zr, Ti, and Al (**Table S2**)[31], fostering chemical short-range ordering in the liquid state, enhancing local packing efficiency, and suppressing long-range atomic diffusion for improved glass-forming ability. From an atomic scale perspective, Ag was incorporated into the Zr-Ti-Cu-Al alloy due to its chemical similarity to Cu but with a different atomic size (RAg/Cu = 0.810), which was expected to enhance the dense atomic packing in the glass, thereby promoting GFA[32].

The compressive strength of the Zr-BMG was 1603.67 MPa, with an elastic modulus of 94.20 GPa, consistent with most reported Zr-Cu-Al metallic glasses (strength ranging from 1600-2000 MPa and modulus from 80-100 GPa)[12, 33]. Studies had indicated a linear relationship between strength and elastic modulus[12, 34]. Despite the development of new β-Ti alloys with an elastic modulus as low as 60 GPa[35], both their strength and hardness decreased. The Zr-BMG exhibited balanced strength and elastic modulus, making it suitable for implants. Meanwhile, the Zr-BMG demonstrated elevated microhardness compared to Ti, implying enhanced resistance to wear and a possible decrease in osteolysis triggered by wear debris, commonly referred to as particle-induced disease[36]. Additionally, Zr-BMG exhibited higher specific strength than Ti, thus allowing it to achieve the same strength with a smaller cross-sectional area as Ti[37], thereby reducing the diameter of the implant itself.

In general, the corrosion resistance is determined by the nature and characteristics of the passivation film on the alloy surface[38]. Analysis of the XPS results revealed that the excellent corrosion resistance of Zr-BMG was attributed to the formation of a highly protective surface film enriched with Zr4+, which was consistent with the findings of Hua et al[39].

Bone integration and vascularization are closely intertwined processes, crucial for successful implantation[40]. ICP analysis showed that Zr-BMG could release Cu2+ in stimulated body fluid, and its concentration could reach 98.70 pbb. It had been reported that Cu was a key element that affected blood vessel growth and could stimulate blood vessel production at the molecular level[23]. Li et al. had demonstrated that the Zr-Ti-Cu-Al BMG significantly enhanced the expression of *VEGF*, thus contributing to the regulation of vasculogenesis[24]. Another study had found that HUVECs cultured on Zr-Ti-Cu-Al BMG showed higher proliferation, evidenced by significantly higher optical density values starting from day 7, and enhanced differentiation with higher gene expression levels of *VEGF* compared to those cultured on Ti at day 7. These findings aligned with our *in vitro* experiments using HUVECs[41]. Therefore, we reasonably speculated that the Cu2+ had contributed to promote angiogenesis.

In our study, we not only confirmed the bactericidal efficacy of Zr-BMG against two common oral pathogens, *P.gingivalis* and *S.mutans*, but also demonstrated its capability to effectively disrupt bacterial biofilm formation over the long term. We conducted preliminary explorations into the mechanisms underlying its antibacterial properties. Two main views on the antibacterial mechanism of Cu- and Ag-containing alloys exist: (1) Contact sterilization, where bacterial functions are disrupted upon contact with metal particles, leading to metabolic disturbances and cell death. (2) Metal ion sterilization. Metal ions dissolve upon contact, producing reactive oxygen species (ROS) that damage DNA, cell membranes, and proteins, thereby impacting antibacterial properties[21, 26, 27, 42-44]. As shown in Figure 6E, we observed the morphology of bacteria in direct contact with the material using scanning electron microscopy. After 24 h of contact, bacteria on the surface of Zr-BMG displayed extensive cell wall ruptures and membrane disruptions, with observable twisted and completely detached membrane structures. Moreover, significant ROS signals were detected in the bacterial suspension in the Zr-BMG group. This might be related to the metal ions released by Zr-BMG. The dissolution of Ag ions led to the production of reactive oxygen species (ROS), thereby influencing antibacterial properties. Similarly, the dissolution of Cu ions resulted in the loss of cell membrane potential and cytoplasm release, leading to cell membrane rupture and Cu ion-induced ROS generation, ultimately damaging bacterial structure. Additionally, ICP-MS experiments demonstrated that the release of Cu ions and Ag ions from Zr-BMG in simulated body fluid was stable, persisting for at least 12 weeks, significantly longer than the previously reported 42 days[44]. This indicated that Zr-BMG could exert a sustained and stable antibacterial effect.

The present study introduced a novel alloy, Zr61Cu23Al12Ti2Ag2 (at.%), which had promising potential for clinical application as a dental implant material. However, this study had several limitations. Firstly, the lack of ductility in Zr-BMG severely limited its machinability using conventional processing methods. In the future, we would explore methods for processing metallic glasses such as additive manufacturing. Secondly, there was still a lack of material science mechanism studies regarding the advantages of the developed Zr-BMG. Future studies would include control groups of Zr-based BMG with different elemental compositions to explore these mechanisms, potentially guiding the development of future materials. Thirdly, we were unable to confirm the intracellular signaling of this study result. To verify the biological mechanism, experiments such as next-generation sequencing or proteomics analysis were required.

1. **Conclusions**

This study presented a novel amorphous alloy, Zr61Cu23Al12Ti2Ag2 (at.%) (Zr-BMG), which offered superior mechanical properties, strong antibacterial effects, and improved osseointegration capabilities for dental implants, making it a favorable alternative to conventional Ti alloy.

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**Conflicts of interests**

The authors declare that they have no competing interests.

**Author contributions**

Feifei Wang, Yunshu Wu, and Fu Zheng contributed equally to this work. The authors thank Yuhao Zeng for his kind assistance in *in vivo* implantation surgery, Zifan Zhao for his support in materials preparation and Yao Huang for acquisition of TEM images. Xu Zhang, Baoan Sun and Yuchun Sun supervised this work, they are listed as co-corresponding authors.

**Data availability statement**

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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| --- | --- | --- | --- | --- | --- |
| **Materials** | **Density**  **(g/cm3)** | **Compressive strength**  **(MPa)** | **Reduced young’s modulus**  **(GPa)** | **Microhardness**  **(GPa)** | **Contact Angle**  **(°)** |
| **Ti** | 4.78 ± 0.02 | 550.33 ± 37.00 | 120.45 ± 17.13 | 4.02 ± 0.91 | 53.63 ± 2.17 |
| **Zr-BMG** | 6.27 ± 0.01 | 1603.67 ± 98.80 | 94.20 ± 7.16 | 5.30 ± 1.27 | 54.14 ± 4.61 |

**Table 1.** Density, compressive strength, reduced young’s modulus and contact angle. Data were presented as mean ± SD.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Materials** | **Ecorr (V VS SCE)** | **Icorr (A/cm2)** | **Passive region**  **(V vs SCE)** | **Pitting corrosion potential**  **(V vs SCE)** |
| **Ti** | -0.45 | 8.53×10-8 | - | - |
| **Zr-BMG** | -0.30 | 1.09×10-7 | 0.03 | -0.06 |

**Table 2.** Corrosion potential (Ecorr), corrosion current density (Icorr), and passive region of materials.