Degradation and osteogenic induction of a SrHPO$_4$-coated Mg–Nd–Zn–Zr alloy intramedullary nail in a rat femoral shaft fracture model

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**HIGHLIGHTS**

- SrHPO$_4$ reduces the in vivo corrosion rate of Mg–Nd–Zn–Zr implants.
- Release of Sr$^{2+}$ and Mg$^{2+}$ ions induces a biochemical response in rat femur fracture model.
- Osteogenic response is stimulated by the TLR4/PI3K/Akt signaling pathway.
- Improved mechanical stability and osteogenic induction enhances fracture healing.

**ABSTRACT**

Magnesium and Mg-based alloys are promising biomaterials for orthopedic implants because of their degradability, osteogenic effects, and biocompatibility. However, the drawbacks of these materials include high hydrogen gas production, unexpected corrosion resistance, and insufficient mechanical strength duration. Surface modification can protect these biomaterials and induce osteogenesis. In this work, a SrHPO$_4$ coating was developed for our patented biodegradable Mg–Nd–Zn–Zr alloy (abbr. JDBM) through a chemical deposition method. The coating was characterized by in vitro immersion, ion release, and cytotoxicity tests, which showed a slower corrosion behavior and excellent cell viability. RNA sequencing of MC3T3E1 cells treated with SrHPO$_4$-coated JDBM ion release test extract showed increased Tlr4, followed by the activation of the downstream PI3K/Akt signaling pathway, causing proliferation and growth of pre-osteoblasts. An intramedullary nail (IMN) was implanted in a femoral fracture rat model. Mechanical test, radiological and histological analysis suggested that SrHPO$_4$-coated JDBM has superior mechanical properties, induces more bone formation, and decreases the degradation rate compared with uncoated JDBM and the administration of TLR4 inhibitor attenuated the new bone formation for fracture healing. SrHPO$_4$ is a promising coating for JDBM implants, particularly for long-bone fractures.
1. Introduction

The biomechanical properties, osteogenic induction, and corrosion reactions of Mg and Mg-based alloys in both human and animal physiological environments have been thoroughly investigated [1–10], with these studies revealing that released Mg2+ ions and residual corrosion products are metabolized through the urinary and digestive systems. As a result, with these materials, implant removal surgery is no longer necessary, because of their degradative elastic modulus [11,12]. In addition, Mg2+ has osteogenic properties and accelerates the fracture healing process, as has been reported previously [3,13–17]. However, for Mg-based materials to meet the requirements for clinical implantation, several problems, including achieving a balance between degradation/corrosion rate and other mechanical properties before the final bony union of the fracture site, controlling the over-production of hydrogen gas, and providing a pathway for exploiting the beneficial effects of Mg ions on the musculoskeletal system, must be addressed. Although in previous studies, our biodegradable Mg alloy, Mg–Nd–Zn–Zr (JDBM), demonstrated excellent mechanical properties, good biocompatibility, and a uniform corrosion pattern [18–23] compared with alloys such as WZ43 and AZ21, we still observed a high rate of degradation, and corrosion behavior that resulted in implant dislocation or failure [18].

Although many techniques for addressing the problems of Mg-based alloys have been reported, continuous improvement is still required before these are universally adopted [10,20,24–30]. For instance, strontium and strontium ranelate have been used for clinical osteoporosis therapy for more than a decade, and are approved in over 70 countries. Strontium ranelate increases the bioactivity of osteoblasts, inhibits osteoclast formation [31,32], and stimulates pre-osteoblast proliferation, osteoblast differentiation, and mineralization [33]. Clinical trials in osteoporotic patients also showed that strontium ranelate increases bone quality and strength, based on bone mineral density and serum biomarkers of bone metabolism [34,35]. However, in spite of these effects on osteogenesis, the US Food and Drug Administration [36] has not approved the use of strontium ranelate because of the risks strontium poses to patients with cardiovascular disease or chronic renal dysfunction [37,38]. Local delivery of strontium would mitigate these concerns, which has inspired the development of coatings containing Sr2+, to improve corrosion resistance and promote osteogenic inductivity. While there has been intensive in vitro and in vivo study of cytotoxicity and new bone formation with Sr-apatite cements and ceramics [39–43], most Sr-apatite or Sr-related coatings have been applied onto non-biodegradable metallic implants, in particular titanium [36,44]. For instance, in Ref. [45] a strontium-hypoxyapatite (SrHA) film was plasma-sprayed onto Ti–6Al–4V, with the titanium alloy reportedly exhibiting satisfactory mechanical properties and in vitro biocompatibility, subsequently. Similarly, Capuccini et al. [46] deposited a Sr-HA coating on a Ti-alloy using a pulsed-laser technique, and illustrated that the presence of Sr in the coating reinforced the positive influence of HA on osteointegration and bone regeneration.

While the benefits of Sr on bone regeneration and osteogenesis are clear, studies investigating the effect of Sr-coatings on Mg-based biomaterials, with regard to their protection from physical corrosion and osteogenesis induction, are still rare. In the present study, we investigate the properties of a JDBM substrate coated with biocompatible SrHPO4 by chemical deposition. The degradation and bioactivity of this Mg-based implant are comprehensively evaluated using a combination of in vivo and in vitro studies. Here, the coating morphology is identified using scanning electron microscopy (SEM), while in vitro corrosion resistance is determined based on immersion and ion release tests. We also perform bioinformatic studies to explore new signaling pathways, to examine the synergic effect of Sr2+ and controlled Mg2+ release on osteogenic differentiation. We postulate that Sr-coated JDBM will exhibit improved corrosion resistance and good biocompatibility, consequently enhancing the formation of new bone in the fracture healing process.

2. Materials and methods

2.1. Preparation of JDBM intramedullary nail specimens and SrHPO4 coating

Intramedullary nail (IMN) specimens were fabricated using JBDM, with the composition as reported in our previous research (Table 1) [19,47]. Specimens with lengths of 25 mm and diameters of 1.5 mm were prepared using a combination of laser cutting, annealing, acid washing, and electropolishing. Three 0.3 mm diameter holes were drilled through the body of each specimen 8 mm, 13 mm, and 18 mm from the conical apex (Fig. 1A).

To achieve a high bonding strength between the coating and the alloy, SrHPO4 was deposited on the IMNs using the following procedure. All twenty specimens were immersed in 0.1 M HF for 24 h, and then incubated at room temperature (20 °C) in a solution of 0.2 M NH4H2PO4 (75 mL) and 0.2 M Sr(NO3)2 (75 mL) at pH 5.0 (Fig. 1B), for either 24 or 48 h, to obtain SrHPO4. All specimens were subsequently washed with deionized water, dried for 5 min at 37 °C, and then sterilized with 29 kGy of 60Co radiation to complete the coating process. The coating’s morphology was investigated using a combination of SEM (S-4800, Hitachi, Tokyo, Japan) and energy dispersive spectrometry (EDS), to determine its composition. A three-dimensional model of SrHPO4-coated JDBM was reconstructed using micro-computed tomography (micro-CT; SkyScan1172, Bruker, Billerica, MA, USA). The coating’s phase structure was identified using X-ray diffractometry (XRD; D8 Advance, Bruker).

2.2. Ion release in extract medium

Extract medium was prepared according to ISO 10993-5:2009 [1], for in vitro tests of different specimens' effects on cell viability. Briefly, Ti–6Al–4V, JDBM, or SrHPO4-coated JDBM specimens were placed in α-modified Eagle’s medium (α-MEM, Hyclone, Logan, USA), supplemented with 10% (v/v) fetal bovine serum (FBS; Invitrogen, Carlsbad, CA, USA), 100 U/mL penicillin, and 100 mg/mL streptomycin (Gibco BRL, MD, USA), in a 1.25 cm2/mL ratio of sample surface area to culture medium. Specimens were incubated at 37 °C in 5% CO2-humidified atmosphere for 0 h, 6 h, 12 h, 24 h, 48 h, 72 h, 96 h and 120 h, to obtain the extract media, which was then collected and refrigerated at 4 °C for subsequent cell culturing. The Mg2+ and Sr2+ concentrations in the extract media were analyzed using inductively coupled plasma-atomic emission spectroscopy (ICP-AES; VISTAPRO, Agilent, Santa Clara, CA, USA).

<table>
<thead>
<tr>
<th>Table 1 Composition of the JDBM IMNs.</th>
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<tbody>
<tr>
<td>Mg</td>
</tr>
<tr>
<td>Wt %</td>
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<td>2.1</td>
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ZW, XYW and YT carried out the conception and design of the research, participated in the acquisition of data, and drafted the manuscript. JZ, CJ and JMH carried out the analysis and interpretation of data. JP, ZYP, YWC and KHW participated in the statistical analysis. HH, YGY and YZQ conceived of the study, participated in its design and coordination, and helped to draft the manuscript. All authors read and approved the final manuscript.
A. Diagram showing the configuration of a device with labeled components.

B. Chemical reaction equations:

\[
\text{NH}_4\text{H}_2\text{PO}_4 + \text{Sr(NO}_3\text{)}_2
\]

\[
0.2\text{M} 75\text{ml} \quad \text{pH} \rightarrow 5.0
\]

\[
\text{Sr(NO}_3\text{)}_2 + \text{NH}_4\text{H}_2\text{PO}_4
\]

\[
0.1\text{M} 150\text{ml} \quad \text{pH} \rightarrow 5.0
\]

\[
\text{Coat: SrHPO}_4
\]

C. Images showing samples at 48 hours and 24 hours.

D. Microscopic images of the coated surface under different conditions.

E. Diagram illustrating a process with blue arrows indicating movement.

F. SEM images showing detailed structures, with measurements indicated in micrometers.

(caption on next page)
were collected and expanded for Non-adherent cells were separated from the adherent rBMSCs, which

2.3. Immersion test

A. (Top) Schematic of the IMN for the femoral shaft fracture model. (Bottom) Photograph of 1. Ti6Al4V, 2. JDBM, and 3. SrHPO4-coated JDBM IMNs. B. Schematic of SrHPO4 coating preparation by chemical deposition reactions. C. SEM image of SrHPO4 coatings on JDBM alloy after chemical deposition for 24 and 48 h. D. SEM images of the coating grains by different magnification, with length of 200–300 μm in red line. E. Micro-CT image of the uniform coverage of the SrHPO4 coating on the JDBM substrate and internal wall of tunnels. F. SEM images of the SrHPO4 coating on the JDBM substrate with thickness of 35–45 μm in red dotted lines(bottom) and internal wall of tunnels (top).

2.4. Tension test for coating

Tension tests were performed for the SrHPO4 coatings on the JDBM substrates according to ASTM F1147-05. Sr-coated JDBM samples (25.4 mm in diameter and 6.35 mm in height, Supplementary Figs. 3A1 and 2A2) were each glued to two sample holders (Supplementary Fig. 3B1 and 3B2) with an SrHPO4-coated surface and an uncoated surface using 0.25 mm thick FM 1000 film. The adhesive film was cured at 176 °C for 2 h at a pressure of 0.138 MPa, then cooled to room temperature. Experiments were performed on a Servo hydraulic test rig (Supplementary Fig. 3B3) at a speed of 2.5 mm/min, to determine whether fractures occurred at the joint between the coatings and the substrates, or inside the coatings, and thus quantify the magnitude of the adhesion of the coatings to substrates and the internal cohesion of the coatings (Supplementary Fig. 3C1 and 3C2).

2.5. In vitro test

2.5.1. Cell model

Rat bone marrow mesenchymal stem cells (rBMSCs) were isolated as previously reported [50]. Briefly, samples of rat bone marrow were diluted in phosphate-buffered saline (PBS) solution at pH 7.4, then layered onto Histopaque (Sigma-Aldrich) for centrifugation, to separate mononuclear cells (MNCs) from erythrocytes and granulocytes. After this, MNCs were collected and washed twice in PBS, then cultured at 37 °C in α-MEM containing 10% FBS, 100 U/mL of penicillin, and 100 μg/mL of streptomycin, for 1 day. Following this, the culture medium was changed to OSM without and with TLR4 inhibitor CLI-095 (3 μmol/L; Invivogen, San Diego, CA, USA) operating in the rapid-run mode. The sequencing procedures were performed to identify the differentially expressed genes (DEGs) in MC3T3-E1 cells, and the differences between possible signaling pathways in JDBM and SrHPO4-coated JDBM osteogenic extract medium.

2.5.2. Cytotoxicity (cell counting Kit-8 [CCK-8])

Cytotoxicity tests were performed using the indirect contact method. MC3T3-E1 and rBMSCs were incubated for 24 h in two 96-well plates (5 × 10^4 cells/mL, 100 μL per well), to ensure their attachment. For Ti–6Al–4V, JDBM, and SrHPO4-coated JDBM, culture medium was replaced with 100 μL of extract medium prepared as described in 2.2, while SrHPO4 (Sigma-Aldrich, USA) was added to α-MEM containing 10% FBS, 100 μL of penicillin, and 100 μg/mL of streptomycin, for Sr2+ extract. Here, 48 μg/mL, 72 μg/mL, 93 μg/mL, and 111 μg/mL of SrHPO4 was included in the culture medium, according to the results of ion release tests, to represent 12 h, 24 h, 48 h, and 72 h, of alloy incubation, respectively. Following 24 h, 48 h, and 72 h of cell incubation, 10 μL of a working solution (KeyGen Biotech, Nanjing, China) consisting of a 1:10 ratio of serum-free culture medium to CCK-8 (cell counting kit-8) solution was added to the wells. The cells were incubated for an additional 3 h, based on the manufacturer’s guidelines. The optical density at 450 nm (OD450) was determined with a microplate reader (Thermo Fisher Scientific, Waltham, MA, USA). These experiments were repeated three times.

2.5.3. RNA sequencing of MC3T3E1 cells

To explore the osteogenic effects of Ti–6Al–4V, JDBM, SrHPO4-coated JDBM, and Sr2+ extract, MC3T3-E1 cells were incubated on a six-well tissue culture plate at 37 °C in 5% CO2 for 1 day. These experiments were repeated three times at a density of 4000 cells/cm². Following initial incubation, the culture medium was replaced with OSM supplemented with the different ionic extracts (n = 3), and the osteogenic-induction extract medium was refreshed every 24 h. After 5 days of incubation, total RNA was isolated using an Rneasy micro kit (Qiagen, Valencia, CA, USA) and treated with a Turbo DNA-Free kit (Life Technologies, Grand Island, NY, USA) to remove contaminating DNA that could lead to false-positive amplification. RNA quality and purity were confirmed with a spectrophotometer (NanoDrop, Thermo Fisher Scientific). Intact RNA was used either for RNA sequencing or real-time quantitative PCR (RT-qPCR) analyses, with the integrity of the extracted RNA verified using an electrophoresis system (4200 TapeStation System, Agilent) prior to cDNA library preparation. The library was sequenced using the HiSeq 2500 tool (Illumina, San Diego, CA, USA) operating in the rapid-run mode. The sequencing procedures were performed to identify the differentially expressed genes (DEGs) in MC3T3-E1 cells, and the differences between possible signaling pathways in JDBM and SrHPO4-coated JDBM osteogenic extract medium.

2.5.4. RT-qPCR

Alkaline phosphatase (ALP) and osteocalcin (OCN) expression (i.e., genes related to toll-like receptor-4 (TLR4) and osteogenic differentiation) was analyzed using RT-qPCR, with GAPDH as the housekeeping gene. rBMSCs and MC3T3-E1 cells were seeded in 96-well plates at a density of 1 × 10^4 cells/ml, and incubated in a humidified incubator for 1 day. Following this, the culture medium was changed to OSM supplemented with Ti–6Al–4V, JDBM, or SrHPO4-coated JDBM extract, without and with TLR4 inhibitor CLI-095 (3 μmol/L; Invivogen, San Diego, CA, USA) [51]. The cells were incubated for seven and fourteen additional days (for MC3T3-E1 and rBMSCs, respectively), with the extract culture medium replaced every 24 h. Samples of MC3T3-E1 were harvested on days 3 and 7 of their incubation, while rBMSCs were harvested on days 7 and 14. Total RNA was isolated using TRIzol...
(Invitrogen) and cDNA synthesis was performed according to the protocol provided by the manufacturer (Takara bio, Osaka, Japan). RT-qPCR analysis was conducted using the Bio-Rad C1000 tool (Bio-Rad, Hercules, CA, USA) and SYBR Premix ExTaq II (Takara bio, Osaka, Japan). The primer sequences used in these experiments are listed in Table 2. The expression levels of osteogenic differentiation related genes were evaluated and normalized to those of GAPDH.

2.5.5. Western blotting

To verify the molecular mechanism for osteogenic differentiation with SrHPO4-coated JDBM, protein was harvested from MC3T3-E1 cells and rBMSCs seeded in six-well plates. Following one initial day of incubation, culture medium was replaced with OSM medium supplemented with Ti–6Al–4V, JDBM, Sr2+, or SrHPO4-coated JDBM extract with and without CLI-095 (3 μmol/L, Invivogen, San Diego, CA, USA) [51]. Cells were incubated as described in 2.5.4, with OSM extract medium refreshed every 24 h. The samples of MC3T3-E1 were harvested on day 7 of incubation, and rBMSCs were harvested on days 7 and 14. Collected samples were lysed in radioimmunoprecipitation assay buffer (Beyotime Bioscience, Shanghai, China) containing phenylmethylsulphonyl fluoride, with lysates quantified using a BCA assay kit (Thermo Fisher Scientific) prior to denaturing for 10 min at 99 °C. Collected protein was first resolved using sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) performed at 80 V for 30 min and 120 V for 1 h. SDS-PAGE was conducted on precast gels, followed by transfer to a methanol-soaked polyvinylidene fluoride membrane for 2.5 h at 250 mA using a blocking solution consisting of Tris-buffered saline, 0.05% Tween, and 5% nonfat milk powder. The membranes were labelled with primary antibodies to mouse ALP (1/1000), OCN (1/1000), TLR4 (1/500), GAPDH (1/1000) (Abcam, Cambridge, UK), PI3K, p-PI3K, p-Akt, and pan-Akt (Cell Signaling Technology, Danvers, MA, USA) overnight at 4 °C, followed by incubation for 2 h at room temperature with fluorescent secondary antibodies. An Odyssey infrared imaging system (LI-COR Biosciences, Lincoln, NE, USA) was used to measure the levels of specific proteins after the treatments.

2.5.6. Osteogenic differentiation and alizarin red assay

To induce osteogenic differentiation, rBMSCs and MC3T3-E1 cells were seeded in six-well tissue culture plates at a density of 4000 cells/cm² and incubated in humidified atmosphere for 1 day. Following this, the culture medium was changed to OSM medium supplemented with Ti–6Al–4V (control), JDBM, or SrHPO4-coated JDBM extract with and without CLI-095 (3 μmol/L), which was refreshed every 2 days. Mineralization was detected by alizarin red staining after 14 days of culture.

For alizarin red s staining, rBMSCs and MC3T3-E1 cells were washed in 1 × PBS and fixed in 70% ethyl alcohol for 20 min at room temperature. The cells were then rinsed with water, and stained with alizarin red s (40 mM, pH 4.2) for 10 min. Following this, cells were washed five times in water to remove non-specific staining. The stained dye was extracted with 10% cetylpyridinium chloride in 0.1 M phosphate buffer (pH 7.0), and its absorbance was determined at 560 nm. Imaging evaluation was performed with a photo scanner (Scanjet G3110, HP, Palo Alto, CA, USA) [13].

2.6. In vivo studies

2.6.1. Animal surgery

All animal surgery procedures conducted in this study complied with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Animal Ethics Committee of Zhongshan Hospital, Fudan University (Shanghai, China) (Animal Committee Approval No. 2018-12). To explore the therapeutic effect of SrHPO4 and its degradation behavior in vivo, coated and uncoated JDBM IMNs, as well as Ti–6Al–4V implants, were inserted into the right femoral medullary cavities in 128 twelve-week-old male Sprague–Dawley rats (Charles River, Beijing, China) after fracturing their right femoral shaft. The rats were first anesthetized by intraperitoneal injection with ketamine (75 mg/kg) and xylazine (10 mg/kg). The right knee and femur were then exposed (Fig. 6A1 and 2), and a 1.5 mm wide and 25 mm long tunnel was drilled from the patellofemoral groove of the distal femur along the femoral shaft axis (Fig. 6A3). Finally, a fracture gap (Fig. 6A4) was created in the middle of the femoral shaft using a 0.3-mm-diameter string saw, where the implants were inserted (Ti–6Al–4V (control); n = 32, JDBM; n = 32, and SrHPO4-coated JDBM; n = 64)).) Implants were allowed to heal for 1 month.

2.6.2. Triple fluorescent labeling

Fluorescent labeling to observe new bone formation and mineralization was performed by intraperitoneal injection of the following fluorochromes: 25 mg/kg of oxytetracycline hydrochloride (Sigma-Aldrich) at 1 week after implantation, 30 mg/kg of alizarin red s (Sigma-Aldrich) 4 weeks after implantation, and 20 mg/kg of calcein (Sigma-Aldrich) 8 weeks after implantation. All animals were sacrificed by an intraperitoneal injection of sodium pentobarbital 3 days after the final labeling treatment.

2.6.3. X-ray imaging

X-ray images of the rat femur in the anteroposterior position were obtained 24 h, 72 h, 4 weeks, 8 weeks and 16 weeks after surgery, using

<table>
<thead>
<tr>
<th>Grade</th>
<th>Description</th>
<th>Score</th>
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<tr>
<td>1</td>
<td>No calcification</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>Patchy calcification</td>
<td>2</td>
</tr>
<tr>
<td>3</td>
<td>Calcification has the appearance of a callus</td>
<td>3</td>
</tr>
<tr>
<td>4</td>
<td>Callus bridging the fracture gap</td>
<td>4</td>
</tr>
<tr>
<td>5</td>
<td>Continuity of bone trabeculae</td>
<td>5</td>
</tr>
<tr>
<td>6</td>
<td>Remodeling to normal bone</td>
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</tr>
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</table>
Fig. 2. SrHPO4 coating characterization. A. EDS results and SEM image of the SrHPO4 coating. B. XRD pattern of the SrHPO4-coated JDBM implant. C. (Left) Production of hydrogen gas and (right) pH of the medium for uncoated and SrHPO4-coated JDBM alloys immersed in Hank’s solution. D. SEM images of the surface morphology of uncoated and SrHPO4-coated JDBM implants after 10 days immersion (white arrows). E. (Upper) Released Mg$^{2+}$ concentration for SrHPO4-coated and uncoated JDBM and (Lower) released Sr$^{2+}$ concentration from SrHPO4-coated JDBM after 10 days immersion in α-MEM measured by ICP-AES. F. Cell viability of MC3T3E1 cells treated with Ti6Al4V, JDBM, SrHPO4-coated JDBM, and Sr$^{2+}$ α-MEM extracts for 24 h, 48 h, and 72 h measured with a CCK-8 assay.
an X-ray machine (Digital Diagnost, Philips, Amsterdam, Netherlands) with exposure settings configured as 52 kV, 3.2 mA s, and 10.9 ms. The X-ray radiographs reflected the host reaction to hydrogen release in the femur, fracture healing, bone callus formation, implant failure (bending, breakage, and dislocation), and non-union or delayed union. Tomographic evaluation of fracture healing was performed by three independent observers [53] using the grading system shown in Table 3. Callus widths for each implantation group were measured 4 weeks, 8 weeks, and 16 weeks post operation, to determine bone callus formation.

2.6.4. Biomechanical tests

Tests were performed on the implants prior to surgery (0 weeks), and 4 weeks, 8 weeks, and 16 weeks after surgery, to evaluate the biomechanical properties of coated and uncoated JDBM. This consisted of a three-point-bending test conducted on a Zwick Z020 testing machine (ZwickRoell, Ulm, Germany). Here, the implants were positioned on support 15 mm apart and a load was applied at a displacement rate of 2 mm/min. The failure modes of an implant and ultimate load to failure in newtons were recorded according to Refs. [1]. For post-operative testing, the implants were delicately driven out from the implantation entry point in the femur, cleaned by stirring in 10% phosphate-buffered saline (PBS) for several seconds, and air dried for approximately 5 min.

2.6.5. Micro-CT

Fracture healing, new bone formation and implant degradation was evaluated using micro-CT, with scans performed before implantation, and 4 weeks, 8 weeks, and 16 weeks after implantation (n = 5), using a micro-CT scanner (SkyScan1172, Bruker) with a 10 μm voxel size, 79 kV tube potential, and 125 μA tube current. Three-dimensional reconstruction was performed using Recon software (Bruker) based on lateral two-dimensional projections acquired of samples. To analyze fracture healing, the transverse fracture line was defined as the reoriented center of the region of interest using SkyScan DataViewer software (Bruker), with the confined perimeter (600 slides in total) consisting of 300 slides (18 μm/slide) upwards and downwards of this center. For bone mineral density (BMD), trabecular number (Tb.N), and trabecular spacing (Tb.Sp) analysis, the cortical bone was excluded from the 600-slice ROI, to focus on the quality of trabecular bone around the implants. The remaining sections of the uncoated and uncoated JDBM implants were segmented from the surrounding soft and hard tissues based on the absorption coefficient (equivalent to mineral density) using SkyScan CTAn software (version 1.13.5.1, Bruker). After segmentation, implant degradation was evaluated based on volume quantification. Osteogenic induction was analyzed based on new bone formation in the three tunnels in the implants, determined through 3D reconstruction.

2.6.6. Undecalcified sectioning analysis

Hard tissue processing was performed on the implanted femur after micro-CT scanning. All samples were fixed in 10% buffered formalin for 3 days, then stepwise dehydrated in an ethanol gradient for 3 days, and incubated in xylene for 3 days, subsequently. Finally, the samples were embedded in methyl-methacrylate. The embedded samples were cut into 200 μm sections and micro-ground to a thickness of 50–70 μm. Histological analyses of bone-implant contact (BIC) and new bone formation were performed following Van Gieson staining. Here, the region less than 2 mm from the implant [54], was measured using an optical microscope (DM2500, Leica, Wetzler, Germany). The bone area (BA) across the fracture was defined to encompass a 5 mm locus from the middle of fracture gap, and was analyzed using the Image-Pro Plus software package (Media Cybernetics, Rockville, MD, USA). Fluorescence microscopy of oxytetracycline hydrochloride (blue), alizarin red s (red), and calcine (green) was also conducted using a confocal laser scanning microscope (LSM 700, Carl Zeiss, Oberkochen, Germany) at excitation/emission wavelengths of 351/460 nm, 543/617 nm, and 488/517 nm, respectively. The region of measurement for labelled bone area (LBA%) was defined as described in 2.6.5. Mineralization apposition rate was calculated as the change in the distance between green band and blue band over 8 weeks.

2.6.7. Histological and immunohistochemical analysis

Following experiments, the expression of OCN and TLR4 in the fracture gap region was analyzed by immunohistochemical staining. Implanted femurs were harvested and fixed in 4% neutral-buffered formalin for 48 h. The samples were decalcified in 9% formic acid for 4 weeks at room temperature. The implants were then removed gently from this solution, to ensure sample integrity was maintained, dehydrated in an ethanol gradient and embedded in paraffin. After this, the samples were cut into 4-μm-thick slices, with consecutive sections stained chemically with hematoxylin-eosin (HE), Masson’s trichrome, tartrate-resistant acid phosphatase (TRAP), and ALP. The sections were then dewaxed in xylene and hydrated with graded alcohol. Endogenous peroxidase activity was quenched with a 10 min incubation in 0.3% hydrogen peroxide. After blocking with 1% goat serum (1:100 dilution, Sigma-Aldrich), the sections were incubated overnight at 4 °C with primary antibodies for OCN (Servicebio, Wuhan, China) and TLR4 (Servicebio, China). Sections were washed with PBS three times and incubated with the goat anti-rabbit IgG secondary antibody (Invivogen, San Diego, CA, USA) for 1 h at 37 °C. Stained specimens were developed in 3,3’-diaminobenzidine solution (Dako, Hamburg, Germany) using a hematoxylin counterstaining process. The relative area of the fracture gap was calculated using Image-Pro Plus.

2.6.8. In vivo safety evaluation

Rat blood was collected 4 weeks, 8 weeks, and 16 weeks after implantation to measure Mg2+ concentration, and 0 h, 72 h, 4 weeks, 8 weeks, and 16 weeks after implantation to measure alanine aminotransferase (ALT), aspartate transaminase (AST), creatinine (Cr), and blood urea nitrogen (BUN) concentration. Internal organs, including the heart, kidneys, and liver, were harvested at 16 weeks, and fixed in 4% formalin for 48 h. Histological procedures were performed as described above. A series of sections were stained with HE to examine the effect of the implants on internal organs.

2.7. Statistical analysis

All data were analyzed with GraphPad (GraphPad Software Inc., La Jolla, CA, USA) and SPSS 13.0 software (IBM, Armonk, NY, USA). The results of measurements are expressed as mean ± standard deviation.
A

Statistical Analysis of Differentially Expressed Genes

B

Sr-coated JDBM vs JDBM

C

Gene Expression Heatmap

D

Gene Ontology (GO) Terms

E

KEGG Enrichment Analysis

F

Heatmap of Expression Levels
Fig. 3. RNA sequencing of MC3T3E1 cells treated with different extracts. A. DEGs in MC3T3E1 cells treated with JDBM, SrHPO4-coated JDBM, Ti6Al4V, and Sr2+ extracts. B. Venn diagram of DEGs in SrHPO4-coated JDBM vs JDBM and Sr2+ extract vs Ti6Al4V. The 321 DEGs were identified as being upregulated in MC3T3E1 cells by the synergic effect of Sr2+ with Mg2+ released from SrHPO4-coated JDBM. C. Volcano plot of DEGs. The red dots represent the 321 DEGs identified in B. D. Analysis of enriched DEGs by the GO and KEGG pathways. Count represents the number of DEGs enriched in each term. The black trend line represents -log 10 (p-value). BP, biological process; CC, cellular component; MF, molecular function. E. Bubble chart showing top 20 enriched genes in the KEGG pathway. PI3K/Akt is ranked first for its significant p-value and count number. F. Heatmap of DEGs, including TLR4, after treatment with JDBM and SrHPO4-coated JDBM extracts.

and were analyzed using either the Student’s t-test or one-way analysis of variance (ANOVA), followed by the least significant difference (Tukey’s) test for multiple comparisons using SPSS 19.0 software (SPSS Inc., Chicago, IL, USA), with p < 0.05 indicating significant difference between groups.

3. Results

3.1. Characterization of SrHPO4-coated JDBM alloy

SEM images depicting the morphology of the SrHPO4 coating after 24 h and 48 h of chemical deposition are shown in Fig. 1C. We observed granite-shaped coating grains 200–300 μm in size, composed of SrHPO4 (Fig. 1D). With a 48-h deposition process, the coverage of these grains appears to be uniform. In contrast, multiple fissures can be observed with only 24 h of deposition. Moreover, the coating coverage of the internal walls of the three tunnels in the JDBM substrate was the same using either micro-CT or SEM analysis (Fig. 1E and F). Based on hard tissue sectioning (Fig. 1F), the thickness of the coating ranged from 35 μm to 45 μm. In addition, EDS identified Sr at a concentration of 11.26 atom % ± 1.392 atom %, while the ratio of Sr to P was nearly 1.15:1 (Fig. 2A). The XRD patterns of the coated and uncoated alloys are shown in Fig. 2B. From tests conducted to quantify the adhesion/internal cohesion of the SrHPO4 coatings, the average tension strength is 11.26 atom %±1.392 atom %, while the ratio of Sr to P was nearly 1.15:1 (Fig. 2A). The XRD patterns of the coated and uncoated alloys are shown in Fig. 2B. From tests conducted to quantify the adhesion/internal cohesion of the SrHPO4 coatings, the average tension strength was 4.3 MPa ± 1.0 MPa, with a minimum value of 3.0 MPa (Table 5). No bare alloy surface was observed after testing, indicating that the fracture occurred inside the coatings, i.e., the adhesion of the coatings to substrates was stronger than the internal cohesion of the coatings.

3.2. Immersion test

Fig. 2C depicts the results of immersion tests conducted in Hank's solution, indicating that for both the uncoated and SrHPO4-coated JDBM alloy, the rate of hydrogen production increased rapidly in the first 4 days (with the uncoated alloy producing hydrogen faster than the coated alloy in this period), and then decreased. After 10 days, 1.7 mL/cm² ± 0.9 mL/cm² of hydrogen had been produced by the uncoated JDBM, while 0.6 mL/cm² ± 0.3 mL/cm² of hydrogen had been produced by SrHPO4-coated JDBM. Using these volumes as an indicator for corrosion, we surmise that the presence of the coating decreased the corrosion rate (Table 4). After 10 days of immersion, the pH values of the Hank’s solutions were 7.93 ± 0.05 and 7.72 ± 0.05, for uncoated and coated JDBM, respectively (Fig. 2C). Fig. 2D shows SEM images of the surface of coated and uncoated JDBM samples after 10 days of immersion. A decrease in the size of the SrHPO4 coating grains can be noted, falling from 200 to 300 μm (Fig. 1D) to 40–50 μm, indicating that corrosion still occurs on the surface of the coated sample (i.e., the SrHPO4 coating only retards corrosion). Other corrosion products can also be observed on the surface of JDBM, with Mao et al.’s [20] previous report suggesting that these are composed mainly of hydroxyapatite, calcium magnesium phosphate and magnesium hydroxide.

3.3. Ion release concentration

Analysis of the in vitro degradation of coated and uncoated JDBM highlighted that both samples released Mg2+ ions into α-MEM during incubation (Fig. 2E), with a constant concentration of 14.3 μg/mL ± 1.0 μg/mL observed at 120 h with the coated alloy, compared with 55.5 μg/mL ± 2.9 μg/mL for the uncoated alloy, which appeared to still be increasing. This result demonstrates the rapid degradation rate of bare JDBM and the mitigating effect of the SrHPO4 coating on degradation. Finally, a rapid increase in Sr2+ concentration was observed during the first 24 h of incubation, which slowed down at 120 h, yielding a final concentration of 139.0 μg/mL ± 11.0 μg/mL, suggesting that the SrHPO4 coating also degraded.

3.4. In vitro tests

3.4.1. Cytotoxicity of extracts toward MC3T3E1 cells

The viability of MC3T3-E1 cells treated for 24 h, 48 h, and 72 h in Ti–6Al–4V, uncoated JDBM, SrHPO4-coated JDBM, and Sr2+ extract is shown in Fig. 2F. Although there was an increase in OD450 for the cells treated with uncoated JDBM, SrHPO4-coated JDBM, and Sr2+ extract, overall, there were no statistically significant differences (p > 0.05) in the cells after 24 h and 48 h of treatment. However, after 72 h, the viability of cells in the SrHPO4-coated JDBM group was significantly greater than those in the uncoated JDBM and Sr2+ extract groups.

3.4.2. Identification of TLR4/PI3K/Akt signaling pathway activated by SrHPO4-coated JDBM extract

To explore the molecular mechanism dictating the effect of uncoated and coated JDBM on MC3T3-E1 cell cytotoxicity, we conducted a statistical comparison of DEGs produced by cells cultured in Ti–6Al–4V, uncoated JDBM, SrHPO4-coated JDBM, and Sr2+ extract (Fig. 3A). We identified 347 upregulated genes and only 17 downregulated genes with SrHPO4-coated JDBM compared to JDBM, using thresholds of p < 0.05 and |log FC| > 0.585. In addition, 321 genes were activated independently by Sr2++, separate from those activated by the synergic effect of Sr2+ and Mg2+ released by SrHPO4-coated JDBM (Fig. 3B). These genes are visualized as the red dots in the upregulation region of the volcano plot in Fig. 3C.

The differences between gene expression with different extract medium were evaluated by Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis. The top five GO terms and KEGG pathways were selected based on the most significant p-value (Fig. 3D). From this, we identified that the DEGs were significantly enriched in pathways associated with PI3K/Akt signaling, RNA transportation, circadian rhythm, ABC transportation, and p53 signaling. In particular, genes with functions associated with cell proliferation and growth were most enriched in the PI3K/Akt signaling pathway, suggesting that it was activated by SrHPO4-coated JDBM, based on significant count number and p-value (Fig. 3E).

The protein–protein interactions (PPI) of the cells were analyzed using the Search Tool for the Retrieval of Interacting Genes (STRING), based on the DEGs. PPI networks (Supplemented Fig. S1A) were constructed to distinguish the set of upstream genes that were most enriched in activating the PI3K/Akt signaling pathway, from which we identified Bdnf, Hesp90a1, Hesp90b1, Itif, Itil, Lpar4, Pkraa2, and TLR4. Of these, TLR4 (Fig. 3F, supplementary Fig. 1A), located in the plasma membrane, can be stimulated by extracellular changes, and transduces signals to the PI3K/Akt pathway intracellularly; the activation of TLR4 by the SrHPO4-coated JDBM extract upregulates PI3K, which induces
Fig. 4. Osteogenic effects of SrHPO$_4$-coated JDBM extracts on MC3T3E1 cells via the TLR4/PI3K/Akt signaling pathway. A. ALP, OCN, and TLR4 expression in MC3T3E1 cells after treatment with Ti$_6$Al$_4$V, JDBM, SrHPO$_4$-coated JDBM, and SrHPO$_4$-coated JDBM + CLI-095 extracts. B. pan-Akt, p-Akt (Ser 473), p-Akt (Thr 308), p-PI3K (p85), and PI3K expression in MC3T3E1 cells after treatment with Ti$_6$Al$_4$V, JDBM, SrHPO$_4$-coated JDBM, and SrHPO$_4$-coated JDBM + CLI-095 extracts medium. C. MC3T3E1 cells stained with Alizarin Red S after 21 days of incubation with Ti$_6$Al$_4$V, JDBM, SrHPO$_4$-coated JDBM, and SrHPO$_4$-coated JDBM + CLI-095 extracts medium. (*p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001).
the phosphorylation of the Akt system downstream to control cell growth and proliferation.

3.4.3. Osteogenic differentiation of MC3T3-E1 cells and rBMSCs

We verified the expression of osteogenic-related proteins in MC3T3-E1 cells with western blotting, confirming that 7 days of treatment with SrHPO4-coated JDBM extract upregulated TLR4, ALP and OCN, compared with treatment using uncoated JDBM or Ti-6Al-4V extract (Fig. 4A). These proteins were downregulated when TLR4 inhibitor CLI-095 (3 μmol/L) was included with the SrHPO4-coated JDBM extract, further demonstrating TLR4’s role in the cell’s osteogenic response. In contrast, the expression of TLR4 and osteogenic differentiation markers in rBMSCs was upregulated 14 days after incubation in Sr-coated JDBM extract, compared to incubation in JDBM extract (Supplementary Fig. 2A). In addition, ALP and OCN were also upregulated with respect to their expression in Sr2+ extract (Supplementary Fig. 1B), highlighting the synergistic effect of Sr2+ and Mg2+. The results of RT-qPCR (Fig. 5A and B) also showed higher expression of osteogenic-related genes in MC3T3-E1 at the mRNA level after 7 days of treatment in Sr-coated JDBM extract medium that was downregulated by the TLR4 inhibitor, consistent with the findings produced at the protein level. Similarly, with rBMSCs, Sr-coated JDBM extract markedly increased the expression of osteogenic differentiation marker genes, including ALP, OCN, and TLR4, 14 days after incubation (Supplementary Fig. 2B).

We also explored mechanisms affecting MC3T3-E1 cell growth and proliferation downstream of TLR4 expression, finding that while the expression of PI3K and p-Akt (Ser 473 and Thr 308) was elevated in cells treated with SrHPO4-coated JDBM extract, total Akt (pan-Akt) expression remained unchanged (Fig. 4B). However, CLI-095 significantly decreased PI3K and p-Akt expression (Fig. 4B), attenuating the osteogenic differentiation of MC3T3-E1. This suggests that the PI3K/Akt pathway is inhibited by the upstream TLR4 inhibitor, indicating that the SrHPO4-coated JDBM extract medium controls osteogenic differentiation through the TLR4/PI3K/Akt pathway.

Alizarin red s staining conducted after 21 days of incubation showed that treatment with SrHPO4-coated JDBM extract medium improved mineral deposition with both MC3T3-E1 cells and rBMSCs, compared with treatment with JDBM extract or control osteogenic medium. In contrast, including CLI-095 (5 μM) with the SrHPO4-coated JDBM extract inhibited osteogenic differentiation with both cell lines (Fig. 4C and Supplementary Fig. 2B).

3.5. In vivo tests

3.5.1. X-ray imaging

Post-operative X-ray images of the IMN implants are shown in Fig. 6B. With the uncoated JDBM implants, hydrogen gas was released in the 24 h immediately after surgery, and there was a significant
Fig. 6. Implantation of IMN and healing of the femoral shaft fracture in the rat model. A. Photographs of implantation of IMN in the femoral shaft fracture rat model. B. Radiographs of rat femurs containing IMNs at 24 h, 3 days, 4 weeks, 8 weeks, and 16 weeks. Red arrows indicate where hydrogen gas was produced at 24 h and 3 days in the JDBM group and where bone calluses were formed across the fracture gap. C. Fracture healing score and callus width evaluated at 4, 8, and 16 weeks (*p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001).
increase in gas volume produced through the fracture gap into the surrounding tissue compartment after 3 days. In contrast, with the SrHPO4-coated JDBM implants, only a tiny volume of hydrogen gas was released from the fracture site 24 h and 3 days post operation. We also observed that the bone marrow cavities around the coated and uncoated JDBM implants had widened 4 weeks after operation, in contrast to the cavities around the Ti–6Al–4V control. Implant failure rates of 43.8% (14/32; ten breakages, two dislocations, and two bending), 6.3% (2/32; two breakages), and 15.6% (5/32; two breakages, one dislocation, and two bending) were recorded for the JDBM, Ti–6Al–4V, and SrHPO4-coated JDBM implants, respectively, during the observation period. Finally, callus width measurements indicated there was no callus formation across the fracture gap with the TLR4 inhibitor group, even after 16 weeks of incubation, when the implant failures (breakages) had been recorded. The fracture healing evaluation score calculated from X-ray images obtained after 8 weeks of implantation was significantly higher with the SrHPO4-coated JDBM group than the JDBM group. This score was also greater than the value obtained with the Ti–6Al–4V group, although not significantly.

3.5.2. Micro-CT assessment

Fig. 7A shows representative images of 3D reconstruction of a rat femur with implanted IMNs 4 weeks, 8 weeks, and 16 weeks after surgery. The Ti–6Al–4V, JDBM, and SrHPO4-coated JDBM implants remained in their original position, and bone growth was observed in the 600-slice region of interest, as bone callus formation. In contrast, no callus formation was observed with the Sr-coated JDBM + CLI-095 group. The quality and mass of bone around the implant were quantified using the bone volume/tissue volume and BMD of the trabecula (Fig. 7A). A large increase in bone mass was observed with the Sr-coated JDBM group compared with the other three groups. However, from the horizontal 2D images of the region of interest, the BMD was the higher with the SrHPO4-coated JDBM group and lower with the JDBM group, compared with the Ti–6Al–4V group, at each time point, indicating a large bone bridge is formed across the fracture gap with the Sr-coated JDBM group. The BMD level suggests that the trabecular bone was less mature and bone formation was directed towards the JDBM implants in the femoral cavity.

Fig. 7B depicts a comparison of 3D reconstructions of new bone formation induced on the sides of the holes drilled in the IMNs, for further analysis of osteogenesis. At week 8, the bone accumulation volume was significantly higher in the SrHPO4-coated JDBM group, and lower in the JDBM group, compared with the Ti–6Al–4V group, implying a better balance between corrosion behavior and osteogenic induction with SrHPO4-coated JDBM. Such osteogenic activity can be inhibited by CLI-095.

The corrosion morphology in the 3D reconstructions of the IMN implants 0 weeks, 8 weeks, and 16 weeks after surgery (Fig. 7C) was evaluated, to analyze the degradation of coated and uncoated JDBM. While the morphology of the Ti–6Al–4V IMN was unchanged after 16 weeks of implantation, there was clear evidence of corrosion on the surface of both the coated and uncoated JDBM IMNs. This corrosion resulted in a larger decrease in JDBM implant volume compared with SrHPO4-coated JDBM implant volume; the volume of JDBM implants volume decreased from 44.8 mm³ ± 0.6 mm³ at implantation, to 40.5 mm³ ± 3.2 mm³ and 38.28 mm³ ± 3.658 mm³ 8 weeks and 16 weeks after implantation, respectively. Thus, the absence of coating caused the high incidence of implant failure at 16 weeks observed with the JDBM group.

3.5.3. Biomechanical test

The maximum bending load of the coated and uncoated JDBM IMNs was compared 0 weeks, 4 weeks, 8 weeks, and 16 weeks after implantation (Fig. 7D). No significant difference was observed at 0 weeks, indicating that the mechanical properties of both groups of implants were initially comparable. After 4 weeks of implantation, there was a large drop in the maximum bending load of the uncoated JDBM IMNs, due to the rapid release of Mg₂⁺ into the surrounding bone marrow. This rate of decrease became relatively stable 8 weeks and 16 weeks after implantation, reflecting the uniform degradation observed in the in vitro characterization of JDBM. However, the protection provided by the SrHPO4 coating slowed the degradation of JDBM, producing a relatively slow and steady decrease in the biomechanical properties of the coated IMN. These results are consistent with the volume loss calculated from the morphology observed in the micro-CT scans.

3.5.4. Histological and immunohistochemical analysis

For sequential histomorphometry, triple-labelled histological sections of the region around the fracture gap were observed using fluorescent microscopy (Fig. 8A). Three strips labelled blue (Oxytetracycline Hydrochloride), red (Alizarin Red S), and green (Calcein), 1 week, 4 weeks, and 8 weeks after implantation, respectively, showed new bone formation at the edge of the fracture site. According to the LBA%, the SrHPO4-coated JDBM group exhibited the greatest osteogenesis across the gap. Furthermore, significantly faster new bone formation for fracture healing over the 8 weeks was noted, based on the mineralization apposition rate (MAR), compared with the uncoated JDBM group. We note that histomorphometry appears to overstate CLI-095's inhibition of new bone formation. This is because, CLI-095 influences triple fluorescent labeling, an effect that is worsened when longer incubation periods are considered.

The BA and BIC of the longitudinal undecalified sections showed that significant bone growth was induced at the surface of the SrHPO4-coated JDBM IMN (Fig. 8B). The amount of new bone along the coated JDBM was greater than the amount along the uncoated JDBM, where only a limited amount of new bone formation was observed. A histological comparison of fracture healing with the different implants is shown in Fig. 8C. With the SrHPO4-coated JDBM group, the new lamellar bone formation almost filled the fracture gap 8 weeks after implantation, and there was greater mineralization, more trabeculae, and a mature integrated bone structure at the fracture site. In contrast, while with the JDBM group there were newly formed trabeculae on both sides of the implant, with greater densities than the Ti–6Al–4V group, there was still an obvious fracture gap. However, CLI-095 inhibited evidence of bone growth with the Sr-coated JDBM group, consistent with radiological findings.

Decalcified histological sections were subjected to chemical (HE, Safranin-O, ALP, and TRAP) and immunohistochemical (OCN and TLR4) staining (Figs. 9 and 10), to explore the expression and distribution of osteogenic factors and the TLR4 signaling pathway at the fracture site. As fracture healing requires two cooperating intra-
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membranous and endochondral ossification events, we investigated the chondrogenesis induced in response to Sr-coated JDBM. The growth of cartilage within the callus area was quantified through HE and Safranin-O staining (Figs. 9A and 10A), which also revealed the morphology of the fracture line and bone callus, providing a reference for subsequent immunohistochemical staining. Although the cartilage fractions were drastically elevated 8 weeks post-surgery with the Ti–6Al–4V, JDBM, and SrHPO₄-coated JDBM groups (Figs. 9F and 10F), our results suggest that Sr-coated JDBM implants significantly enhance chondrogenesis late in the fracture healing process. At higher magnifications, TRAP positive stained osteoclasts were observed at the edge of fracture site (Figs. 9E and 10E). However, there were no significant differences in their appearance between the four implant groups, either 4 weeks or 8 weeks after implantation.

OCN, which stains osteoblasts and osteoids specifically, was greatest at the interface of the bone and the fracture gap. The difference in OCN expression (Figs. 9C and 10C) between the groups was significant, and similar to the ALP staining pattern (Figs. 9B and 10B). Our in vitro results indicate that TLR4 in the plasma membrane is stimulated extra-cellularly to activate the canonical PI3K/Akt signaling pathway downstream, which is reflected in cell growth and proliferation. The distribution of TLR4 is consistent with the distribution of OCN in osteoblasts at the fracture site, and TLR4 expression is significantly higher with the SrHPO₄-coated JDBM group. Furthermore, administration of the TLR4 inhibitor attenuated the expression of osteogenic-related markers detected by Safranin-O, ALP, and OCN staining. The results
indicate that SrHPO4-coated JDBM accelerates fracture healing by activating TLR4 to induce the proliferation of osteoblasts.

3.5.5. In vivo safety evaluation

The biosafety of the Mg alloy implants was evaluated by HE staining the vital organs (Fig. 11A). There were barely any pathological signs or histological evidence of deterioration or necrosis in consecutive sections of heart, liver, and kidney that were harvested from each group of rats 8 weeks and 16 weeks after surgery. Interestingly, with the JDBM group, the renal tubule diameter was significantly dilated 8 weeks after surgery, which may be related to compensation of kidney function induced by a biodegradable product or Mg2+ release. However, no further dilatation was observed subsequently, suggesting that ions released from uncoated JDBM may result in a high concentration of Mg2+ in the blood. Hence, we monitored the serum Mg2+ concentration and blood biochemical indexes up to 16 weeks. With the Ti-6Al-4V and SrHPO4-coated JDBM groups, the serum Mg2+ concentration remained steady over the entire incubation period (Fig. 11B), indirectly demonstrating that the SrHPO4 coating prevents the release of Mg2+ due to corrosion. In contrast, with the JDBM group, the serum Mg2+ concentration reached a significant peak 8 weeks after surgery, and had decreased to a value close to the initial concentration at 16 weeks, indicating that physiological adaptation could rebalance the serum Mg2+ concentration elevated by the release of Mg2+ from the JDBM implant. Liver and kidney function, evaluated based on ALT, AST, Cr, and BUN serum levels, were the same in all groups over the observation period.

4. Discussion

Overly rapid corrosion in physiological environments causes the degradation of Mg-based alloys, leading to poor mechanical integrity of IMNs early in the implantation period. Hence, to decrease the degradation rate of our patented JDBM Mg alloy [55], we developed a SrHPO4 coating to protect orthopedic implants made of JDBM, and compared its performance to other surface modifications investigated to promote corrosion resistance in Mg alloys for orthopedic use. Chen et al. [26] first deposited an Sr-P coating on high purity (＞99.9%, ≤40 ppm Fe) Mg specimens in 2014. However, this coating could only be deposited onto pure Mg, not Mg-based alloy, limiting its use in orthopedic applications. With their study, the Mg2+ concentration measured by ICP-AES was 1.6 mM ± 0.7 mM after 10 h of alloy immersion, much higher than the 0.2 mM ± 0.1 mM Mg2+ concentration observed in our study after 12 h of immersion, suggesting that our SrHPO4-coating has a better ability to control the release of Mg2+. With Lee et al.’s [28] nanoparticle coating deposited on a silane-modified Mg surface, the rate of hydrogen generation was 0.45 mL/cm2/day, which was still increasing after 20 days of alloy immersion. In contrast, with our SrHPO4 coating, only 0.6 mL/cm2 ± 0.3 mL/cm2 of hydrogen had been produced after 10 days of immersion, equivalent to a generation rate of 0.06 mL/cm2/day. Moreover, the hydrogen generation rate began to decrease after 4 days of immersion, further highlighting the superiority of the SrHPO4-coated surface with respect to the silane-modified surface. Similarly, from in vitro experiments, the corrosion rate obtained with Niu et al.’s [18] brushite (Ca-P) coating deposited on the surface of a Mg alloy substrate was 0.39 mm/year, more than twice the 0.18 mm/year evolution obtained with our SrHPO4-coated JDBM substrate in this study. While Zhang et al. [18,56] reported an in vitro corrosion rate of 0.149 mm/year using a poly (lactic acid) and brushite coating, this bilayer coating has a complicated preparation process. Furthermore, when used as biomaterials in previous studies, drawbacks of polymers have included the generation of acidic degradation products and corrosive particles that create a locally acidic environment, unsatisfactory osteointegration or osteogenic induction, and stimulation of noninfectious inflammation [17,57], suggesting that they may cause complications in clinical applications. Hence, our Sr-coated JDBM implant is advantageous given its low elemental toxicity, strength, ductility, and degradation behavior [55].

In addition to their generally poorer corrosion performance, with the coatings developed in previous studies, no further investigation was conducted on their effect on new bone formation and biodegradation. While other studies have examined the efficacy of the individual use of strontium, and strontium-based or strontium-incorporated coatings for improving biocompatibility and osteointegration properties, the synergistic effect of Mg and Sr in relation to the proliferation of osteoblasts or any osteo-inductivity was not observed with the studies discussed above. Hence, in investigating the key molecular mechanisms underlying the stimulation of osteogenic differentiation in MC3T3-E1 pre-osteoblasts by SrHPO4-coated JDBM extract (or the synergistic effect of Mg and Sr), alternative studies were considered. Z. Wang et al.’s study of MAO-Sr, a nonporous, Sr-substituted, hardysonite ceramic coating, deposited on a traditional biomedical Ti alloy (Ti-6Al-4V) used in dentistry, found that Sr2+ release may promote osteogenic and angiogenic activities mediated by the MAPK/Erk1/2 and PI3K/Akt signaling pathway in canine bone marrow-derived stem cells [36]. Autefage et al.’s microarray study of hMSC treated with growth medium conditioned with bioactive glass (BG) incorporating strontium ions noted the upregulation of BMP-2 expression in vivo and in vitro, suggesting progressive commitment of hMSC toward osteoblastic lineage in response to SrBG treatment [58,59]. The genome analysis conducted in this study showed that the extract medium upregulated TLR4, which is expressed in most human tissues, and activated the PI3K/Akt signaling pathway.

While the mechanism by which TLR4 osteoblasts was activated by Mg and Sr released from implants still needs further exploration, a potential explanation is as follows. As a member of the pattern recognition receptor family (PRR) [60–62], TLR4 can be complexed with MD2 (myeloid differentiation factor 2) or TRIF adaptor, triggering the activation of intracellular signaling, nuclear factors, and the production of inflammatory cytokines. Hence, we suggest that the pattern recognition complex detects the Mg and Sr released from the implant as an exogenous material that induces an inflammatory response from the host.

As well as initiating primary immune responses, the TLR4/P38K/Akt pathway regulates other physiological functions, including the proliferation and differentiation of stem cells. Chen et al. reported that activating TLR4 induced the differentiation of bone marrow-derived stem cells to osteoblasts [63]. In addition, various inflammatory cytokines, such as prostaglandin and tumor necrosis factor, are secreted following the activation of TLR4, which aid the early stage of bone
A. HE & Safranin-O 8W

B. ALP 8W

C. OCN 8W

D. TLR4 8W

E. Trap 8w

F. Graphs showing quantitative analysis of OCN, ALP, and TLR4 expression.
fracture healing [64,65]. In both our in vitro and in vivo studies, the upregulation of TLR4 by the SrHPO4-coated JDBM extract enabled the phosphorylation of the PI3K/Akt system, as illustrated by the higher cell viability of MC3T3-E1 pre-osteoblasts treated with SrHPO4-coated JDBM extract. Our in vivo data (Fig. 9C, D, 10C and 10D) highlights that immunohistochemical staining detected lower amounts of TLR4 at the fracture site 4 weeks post-operation, compared to the amounts observed 8 weeks post-operation, suggesting that TLR4 was upregulated late in the fracture healing process, when new bone formation was at its peak and completely mature osteoblasts were in the fracture gap. This result appears to explain the inconsistency in the in vitro results where expression of TLR4 in MC3T3-E1 was elevated by Sr-coated JDBM extract early in osteogenic differentiation (after 1 week of incubation), but not in rBMSCs. Moreover, the PI3K/Akt pathway was only attenuated, and not completely deactivated by the TLR4 inhibitor, CLI-095, similar to a previous study of the effect of resveratrol on the TLR4/PI3K/Akt system in relieving osteoarthritis progression [66].

Fracture healing and osteogenic induction by the Ti–6Al–4V, JDBM, and SrHPO4-coated JDBM implants were analyzed based on the morphology of the bone callus across the fracture gap, trabeculae formed on the implant surface, and new bone formation inside the holes of IMN. Histological observation was conducted 4 weeks and 8 weeks after surgery, as recommended by previous studies [27,54], since new bone formation around the IMN or at the fracture site stops at this point, and the bone structure begins to remodel in alignment with the mechanical stress. Although many studies have focused on the degradation performance of pure Mg alloy in osteosynthesis, the effect of biomechanical variation in fracture healing under in vivo weight-bearing conditions remains unclear for both coated and uncoated Mg alloys. In spite of the enhanced fracture healing observed by Zhang et al. resulting from the osteogenesis of periosteum-derived stem cells (PDSCs) from the secretion of CGRP to periosteum [13], pure Mg rods did not have adequate mechanical strength to fix femoral shaft fractures within 3 weeks of implantation, with 71.4% of these failures being due to breakage. The dramatic decrease in maximum bending load observed in our study demonstrates that the mechanical stress on the fracture gap in the first 4 weeks of implantation is strongly correlated with the degradation rate of the Mg alloy implants, especially for sections exposed to the bone marrow cavity. According to Marsell et al. [67], the mechanical strength of absorbable biomaterials used as internal fixations should be maintained for at least 4 weeks. The initial bending strength of bare high-purity Mg screws implanted in a rabbit intracondylar fracture model was reduced by 40%–177.3 N [1], which is higher than the bending strength of uncoated JDBM. Consequently, we suggest that direct exposure to liquid bone marrow tissue in the medullary cavity accelerates the corrosion rate of JDBM, highlighting the importance of coating IMN implants. The excessive hydrogen gas produced by corrosion, as observed in the X-ray images (Fig. 6B) accumulated inside the medullary cavity, leading to irregular biomechanical stability. The rapid degradation rate caused the early loss of the mechanical strength of the uncoated JDBM, and transferred more weight-bearing load to the fracture site too early in the healing process, resulting in the high rate of implant failure and fracture re-dislocation in the uncoated JDBM group. In contrast, the residual bending force for the SrHPO4-coated JDBM implants 4 weeks after surgery was nearly 76.5% (196.15 N ± 12.3 N) of its original value (256.5 N ± 17.9 N), and implant failure rates were reduced. Therefore, the SrHPO4 coating not only served as a protective layer to maintain the integrity of the JDBM substrate, it also induced new bone formation across the fracture gap, around IMN, and inside the holes.

Immunohistochemical analysis of the in vivo experiment (i.e., ALP and OCN staining at 8 weeks) demonstrated that uncoated JDBM induced osteogenesis as expected. However, the quality of trabecular bone (BMD) of micro-CT data was unexpected; more newly-formed trabecular bone was induced to the surface of Sr-coated JDBM IMN compared to uncoated JDBM, ensuring stable osteointegration and sufficient mechanical strength to facilitate the fracture healing process. Meanwhile, although the TRAP expression in osteoclasts, observed primarily at the edge of fracture site (as shown in Figs. 9E and 10E), remained low, the expression of OCN steadily increased over the observation period, suggesting that, at this timepoint, new bone formation was predominant at the fracture site, and fracture healing had not yet proceeded to the bone remodeling stage. Although there was no significant difference between the four implantation groups 4 weeks and 8 weeks after surgery, based on the protein expression in TRAP-positive cells in the JDBM and Sr-coated JDBM groups, the synergistic effect of Mg and Sr on osteoclasts needs further exploration. To date, the implantation of Mg-based medical devices in patients or animals with normal kidney function has not resulted in reports of hypermagnesemia-related cases. However, biosafety concerns remain regarding the use of Mg-based implants for the population of people diagnosed with chronic kidney or liver disease, due to their reduced function and structure of internal organs, including the heart, kidney, and liver, suggesting that Sr-coated JDBM is a new corrosion resistant alternative to traditional Mg-alloys, with osteogenic potential.

5. Conclusion

To address the drawbacks associated with Mg-alloy in clinical applications, we investigated the use of SrHPO4-coated JDBM as a biodegradable material for IMNs for internal fixation of femoral shaft fractures. Using a combination of in vitro and in vivo study, we demonstrated that the improved corrosion resistance provided by the coating, and the new bone formation induced, enhanced fracture healing. In addition, by exploring the synergistic effect of Sr-coated JDBM on osteoblasts, we identified the TLR4/PI3K/Akt signaling pathway as the underlying mechanism controlling osteogenic response. Biosafety tests confirmed that the implants had no adverse effect on the function and structure of internal organs, including the heart, kidney, and liver, suggesting that Sr-coated JDBM is a new corrosion resistant alternative to traditional Mg-alloys, with osteogenic potential.
A

8W

Heart

Kidney

Liver

Ti6Al4V

JDBM

Sr-coated JDBM

16W

Heart

Kidney

Liver

Ti6Al4V

JDBM

Sr-coated JDBM

B

Liver Function Levels:

Kidney Function Levels:

(caption on next page)
Fig. 11. Biosafety evaluation results. A. HE-stained sections of heart, liver, and kidney harvested at 8 and 16 weeks to detect pathological changes after implantation. B. Serum concentration of Mg2+.

References


W. Ding, Opportunities and challenges for the biodegradable magnesium alloys as next-generation biomaterials, Regen. Biomater. 3 (2) (2016) 79–86.


