Release characteristics of bone-like hydroxyapatite/poly amino acid loaded with rifapentine microspheres *in vivo*

YUWU LIU, JIMING ZHU and DIANMING JIANG

Department of Orthopedic Surgery, The First Affiliated Hospital of Chongqing Medical University,
Chongqing 400016, P.R. China

Abstract. Bone-like hydroxyapatite/poly amino acid (BHA/PAA) is a potential bone repair material. Rifapentine-loaded poly(lactic-co-glycolic acid) microspheres (RPMs) are bioactive and efficient controlled-release delivery systems used *in vitro*. The aim of the present study was to investigate the *in vivo* drug release characteristics of RPM-loaded BHA/PAA on a rabbit model of bone defect. RPM was combined with BHA/PAA to obtain the drug-loaded, slow-releasing bioactive material. Bone defects were generated in New Zealand white rabbits and the rabbits were then implanted with RPM-loaded BHA/PAA. High-performance liquid chromatography (HPLC) was used to determine the concentrations of rifapentine in the plasma and the local muscle tissues of the treated rabbits. Hematoxylin and eosin (H&E) staining and biochemical analyses were performed to elucidate potential side effects of RPM-loaded BHA/PAA on the heart, liver and kidney histopathology and functions of the treated rabbits. The biocompatibility and osteogenic ability of RPM-loaded BHA/PAA was evaluated by H&E staining. The results demonstrated that the material was completely degraded and absorbed at 12 weeks following implantation and new trabecular bone and cartilage tissues had formed. The *in vivo* release tests revealed that RPM-loaded BHA/PAA exhibited sustained release profiles of rifapentine and the drug concentration in the muscle tissues remained higher than the minimum inhibitory concentration of rifapentine against *Mycobacterium tuberculosis* for as long as 12 weeks. In addition, RPM-loaded BHA/PAA had no long-term side effects to the heart, liver and kidney of the treated rabbits. In conclusion, the present study demonstrated that RPM-loaded BHA/PAA

slowly and continuously released rifapentine *in vivo* and exhibited no side effects on heart, liver and kidney tissues and function. Furthermore, RPM-loaded BHA/PAA promoted new bone formation, while it was gradually degraded and absorbed. The present study provided a theoretical basis for the potential advancement in developing novel treatments for osteoarticular tuberculosis.

Introduction

Tuberculosis is a chronic, transmittable disease caused by *Mycobacterium tuberculosis*. Approximately one-third of the world's population is estimated to be infected with tuberculosis (1-3). Osteoarticular tuberculosis is recognized as the most common site of extrapulmonary tuberculosis, accounting for ~35-50% of extrapulmonary tuberculosis and 3-5% of the total tuberculosis (4,5). The traditional oral anti-tuberculosis agents do not result in satisfactory treatment of osteoarticular tuberculosis, due to the low drug concentration achieved locally in the tuberculosis lesion and a series of side effects (6,7).

The combination of drug-delivery systems and bone tissue repair appears to be the most promising option for advancement in osteoarticular tuberculosis treatment (8). At present, the reconstruction implants used for the treatment of bone defects include autogenous bone, allograft bone and artificial bone. However, autogenous bone and allograft bone have clinical limitations in the treatment of osteoarticular tuberculosis (9). For example, they exhibit low drug loading capacity and have limited tendency to release the drugs in a sustained manner. To overcome these disadvantages, researchers developed artificial bone materials, which have become commonly used carriers for anti-tuberculosis drugs in the treatment of osteoarticular tuberculosis (9).

Poly(lactic-co-glycolic acid) (PLGA) is widely used as a carrier material for controlled drug release (10). It exhibits good biocompatibility and biodegradability, and delays the release of drugs. Rifapentine is a highly effective, first-line anti-tuberculosis drug. It is synthesized in one step from rifampin. Rifapentine has an antibacterial spectrum similar to that of rifampin; however, it has a stronger antibacterial activity to *M. tuberculosis*, a milder adverse reaction and its drug elimination half-life is longer than rifampin (11-14). Rifapentine is able to effectively penetrate into infected bone, dead bone, inflammatory cells and biofilms formed by bacteria

Correspondence to: Professor Dianming Jiang, Department of Orthopedic Surgery, The First Affiliated Hospital of Chongqing Medical University, 1 Youyi Road, Chongqing 400016, P.R. China
E-mail: dmj571026@126.com

to eradicate bacterial infections (15). Furthermore, rifapentine prevents drug resistance (15). An *in vitro* study from Wu *et al* (16) reported that rifapentine-loaded PLGA microspheres (RPMs) are bioactive and efficient controlled-release delivery systems, with great promise towards the treatment of osteoarticular tuberculosis (16).

Hydroxyapatite (HA) is a widely-used replacement material for bone tissue repairing (17,18). Bone-like hydroxyapatite (BHA) is a type of carbonate HA, that is similar to the composition and structure of normal bone. BHA exhibits good biocompatibility, safety and osteogenic activity (19-21). Compared with HA, BHA has stronger biological activity and faster degradability (19). However, pure BHA is not conducive to processing. When BHA is combined with poly amino acid (PAA), which has a similar molecular structure to collagen, its strength and mechanical properties are enhanced. A previous study from our group has demonstrated that BHA/PAA is effective in osteogenesis and reconstruction of long segmental bone defects both *in vivo* and *in vitro* (22). Recently, Yan *et al* (23) reported that RPM-loaded BHA/PAA is effective in the treatment of rabbit chronic osteomyelitis induced by *Staphylococcus aureus*. However, the effects of RPM-loaded BHA/PAA on osteoarticular tuberculosis have not been examined to date.

In the present study, the *in vivo* drug release characteristics of RPM-loaded BHA/PAA were evaluated on rabbit model of bone defects. Furthermore, the osteogenesis induction ability and the side effects of RPM-loaded BHA/PAA were investigated *in vivo*. The present study may provide a novel approach for the clinical treatment of osteoarticular tuberculosis.

Materials and methods

Preparation of materials. RPMs were prepared through an oil-in-water emulsion solvent evaporation method, as previously described (16,23). Briefly, 50 mg of rifapentine (Jinan Mingxin Pharmaceutical Co., Ltd., Sichuan, China) was dissolved into the polymer solution and 200 mg of PLGA (Jinan Daigang Biomaterial Co., Ltd., Jinan, China) was dissolved in 10 ml of dichloromethane. RPMs were prepared with an entrapment efficiency of 85.78±2.00%, an actual drug loading of 17.16±0.40% and a mean diameter of 25.267 μm. The BHA/PAA materials (National Nanomaterial Company of Sichuan University, Chengdu, China) were prepared using the standard atmospheric pressure solution method (24). RPMs were composited with BHA/PAA to make RPM-loaded BHA/PAA, with a size of 15x5x5 mm³, a weight of 750.50±8.54 mg and an aperture size of 100-500 μm.

Rabbit model with bone defects. The animal experiments were approved by the Animal Care Committee of Chongqing Medical University (Chongqing, China). A total of 66 male New Zealand white rabbits at the age of 4 months and weighing 2.5-3 kg were obtained from the Experimental Animal Center of Chongqing Medical University. All rabbits were housed at 17-21°C, with a 12-h light/dark cycle and 30-70% relative humidity. They had free access to water and food. The rabbits were anesthetized by intravenous injection of pentobarbital (30 mg/kg) in the ear margin. The bilateral femoral condyle was shaved and disinfected. An ~5 mm-depth hole was made

by a 4 mm drilling bit from the femoral lateral condyle to the interior condyle. Rabbits in the BHA/PAA group (n=6) or RPM-loaded BHA/PAA group (n=48) were implanted with BHA/PAA or RPM-loaded BHA/PAA materials in the bilateral bone holes, while rabbits in the blank group (n=6) were not treated with any materials (Fig. 1). Rabbits in the normal group (n=6) did not receive any treatment. Following washing with saline, the wounds were closed with sutures layer by layer.

Hematoxylin and eosin (H&E) staining. A total of 6 rabbits from each group was sacrificed at the indicated times. Then, the bone, heart, liver and kidney tissues were collected and fixed in 10% paraformaldehyde overnight, followed by embedding in paraffin. The paraffin-embedded tissues were cut into 5 μm thick sections and immersed in xylene followed by a graded series of ethanol to remove the paraffin. The sections were then stained with H&E (Beyotime Institute of Biotechnology, Shanghai, China) and observed under a light microscope (Nikon Corporation, Tokyo, Japan). Three sections were prepared and viewed for each tissue sample.

High-performance liquid chromatography (HPLC). Concentrations of rifapentine in the plasma and the local muscle tissues were determined using HPLC. All 48 rabbits in the RPM-loaded BHA/PAA group were subjected to this assay. A total of 4 ml blood was collected from the ear margin vein and centrifuged at 10,000 x g for 5 min at room temperature. The supernatant (100 μl) was mixed with 1 ml of methanol, followed by centrifugation at 10,000 x g for 10 min at 4°C. Finally, 20 μl of the supernatant was collected for the drug concentration assay. Muscle tissue (~1 g) around the material implanting site was collected and homogenized, and 500 μl of the homogenate was mixed with 1.5 ml formaldehyde and vortexed for 2 min. Following centrifugation at 10,000 x g for 10 min at 4°C, 20 μl of the supernatant was collected for the drug concentration assay. The internal standard rifapentine was obtained from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). HPLC analysis was performed using a C18 column (250x4.6 mm; Waters Corporation, Milford, MA, USA) with the mobile phase of methanol-acetonitrile (5:4 v/v) on a D-2000 HPLC system (Hitachi, Ltd., Tokyo, Japan). The temperature was set at 40°C, the flow rate was 0.6 ml/min and the wavelength was 268 nm.

Biochemical analyses. The blood samples (n=6 for each group) were centrifuged at 1,000 x g and 4°C for 10 min, and the serum was extracted and stored at -20°C. The concentrations of alanine aminotransferase (ALT), aspartate aminotransferase (AST), blood urea nitrogen (BUN) and creatinine (Cr) in the serum were measured using an Olympus AU2700 Automated Chemistry Analyzer (Olympus Corporation, Tokyo, Japan).

Statistical analysis. Statistical analysis was performed using SPSS version 19.0 statistical software (IBM Corp., Armonk, NY, USA) and the data were expressed as the mean ± standard deviation. The significance of differences between two groups was analyzed using Student's t-test. P<0.05 was considered to indicate a statistically significant difference.

Table I. The weight of rabbits following surgery, kg.

	1 week	2 weeks	3 weeks	4 weeks	8 weeks	12 weeks
Normal	2.9±0.1	3.1±0.1	3.3±0.2	3.6±0.3	4.3±0.3	4.7±0.5
Blank	2.4±0.2[a]	2.7±0.1	3.1±0.2	3.3±0.3	3.8±0.3	4.4±0.2
BHA-PAA	2.3±0.3[a]	2.9±0.2	3.1±0.1	3.2±0.2	3.6±0.3	4.5±0.3
RPM-loaded BHA/PAA	2.3±0.2[a]	2.8±0.1	3±0.1	3.3±0.2	3.9±0.4	4.2±0.3

[a]P<0.05 compared with the normal group. BHA/PAA, bone-like hydroxyapatite/poly amino acid; RPM, rifapentine-loaded poly(lactic-co-glycolic acid) microsphere.
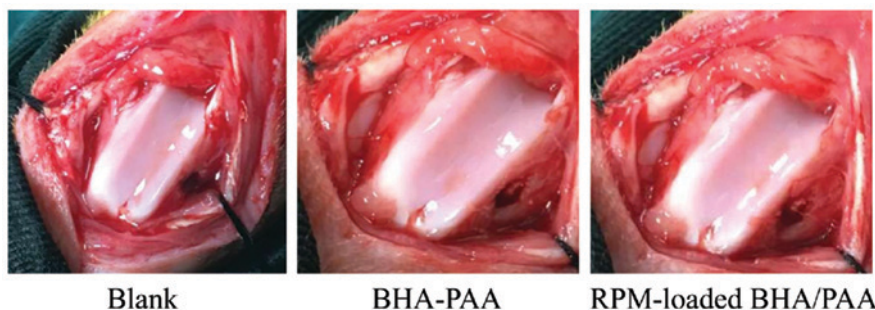

Figure 1. A rabbit model of bone defects following material implantation. BHA/PAA, bone-like hydroxyapatite/poly amino acid; RPM, rifapentine-loaded poly(lactic-co-glycolic acid) microsphere.
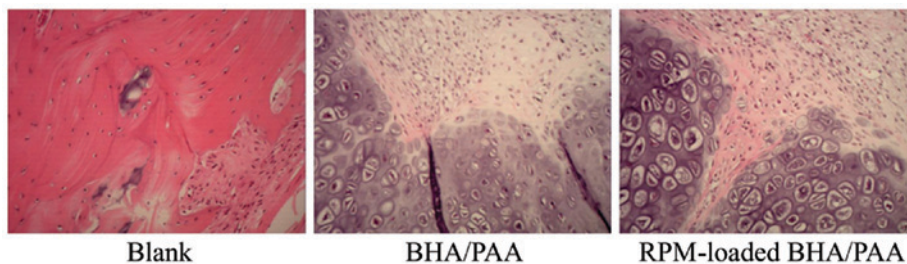

Figure 2. Hematoxylin and eosin staining of bone tissue sections at 12 weeks following implantation. Magnification, x400. BHA/PAA, bone-like hydroxyapatite/poly amino acid; RPM, rifapentine-loaded poly(lactic-co-glycolic acid) microsphere.

Results

General observation after bone defect. Following surgery, all the rabbits suffered from lackluster behavior, lameness and decline in appetite; the wound was red and swollen, and the rabbits lost weight. However, no mortality was observed. One week following implantation, the rabbits gradually recovered. The weight of the rabbits in the RPM-loaded BHA/PAA group increased and returned to the normal weight after two weeks. The weights of the rabbits in the blank and BHA/PAA group also increased, but remained lower than normal (Table I). After 12 weeks, normal gait was restored in the BHA/PAA and RPM-loaded BHA/PAA groups; however, the rabbits in the blank group still suffered from lameness.

H&E staining of bone specimens. At 12 weeks following implantation, bone specimens were collected and stained with H&E. As demonstrated in Fig. 2, a large amount of fibrous tissue was observed in the blank group. In the BHA/PAA and RPM-loaded BHA/PAA groups, the implanted materials were degraded and absorbed, and new bone had formed.

Concentrations of rifapentine in the plasma and the local muscle tissues. The concentrations of rifapentine detected in the plasma and muscle tissues of the RPM-loaded BHA/PAA group are listed in Table II. Rifapentine concentration in the plasma samples reached the peak level at day 1 following implantation; however, it decreased significantly at day 3 and was undetectable at 2 weeks following implantation (Table II). The concentration of rifapentine in the muscle tissues around the material implanting site was also examined. The results revealed that rifapentine concentration in the muscle tissues was higher than the minimum inhibitory concentration of rifapentine against *M. tuberculosis* (0.12-0.25 mg/l) (25) for at least 12 weeks following implantation (Table II).

Side effects of RPM-loaded BHA/PAA on heart, liver and kidney. The heart, liver and kidney tissues were collected

Table II. Concentrations of rifapentine in plasma and local muscle tissues in the RPM-loaded BHA/PAA group.

Sample	Concentration of rifapentine, μg/ml							
	1 day	3 days	1 week	2 weeks	3 weeks	4 weeks	8 weeks	12 weeks
Plasma	5.2	1.1	0.2	0	0	0	0	0
Muscle tissues	12.2	8.5	8	7.5	6.3	5.1	3.8	3.1

BHA/PAA, bone-like hydroxyapatite/poly amino acid; RPM, rifapentine-loaded poly(lactic-co-glycolic acid) microsphere.
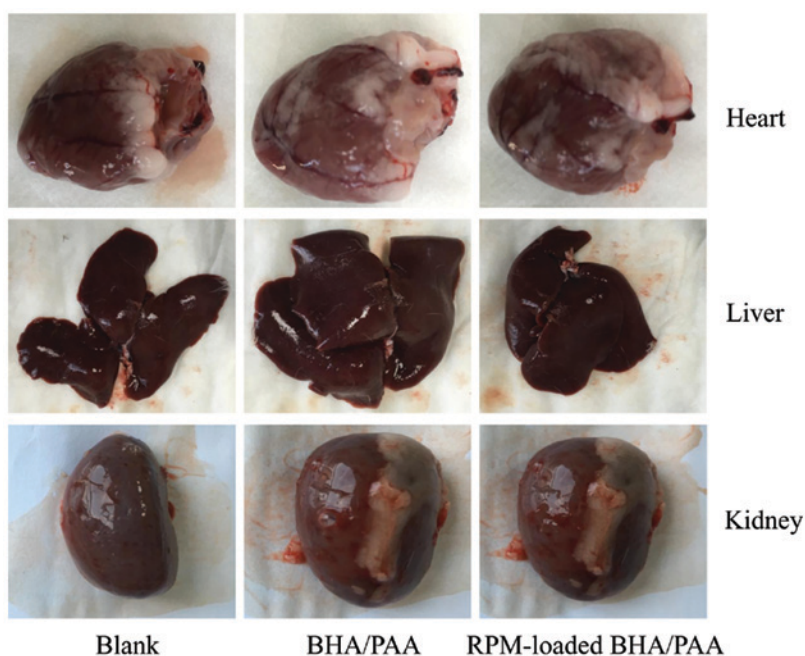

Figure 3. Heart, liver and kidney specimens at 12 weeks following implantation. BHA/PAA, bone-like hydroxyapatite/poly amino acid; RPM, rifapentine-loaded poly(lactic-co-glycolic acid) microsphere.

at 12 weeks following implantation. As illustrated in Fig. 3, no significant changes were observed in the appearance of heart, liver and kidney amongst the three groups. In addition, H&E staining revealed no histopathological abnormalities in the heart, liver and kidneys of the treated rabbits (Fig. 4). Biochemical analyses for markers of liver and kidney functions revealed that the concentrations of ALT, AST, BUN and Cr in the serum were significantly increased at day 1 and 3 following implantation; however, serum concentrations for all four markers returned to normal levels within 1 week (Table III).

Discussion

BHA/PAA is a polar polymer which has great potential as a bone repair material. It combines the biocompatibility and bone conduction of BHA with the mechanical properties of PAA, to induce osteogenesis. Previous studies have reported that the polar groups in BHA/PAA, including-OH,-NH$_2$ and -COOH, promote the colonization of bone cells on the material (26). In addition, the porous network structure and surface roughness of BHA/PAA provide a biochemical environment to promote osteoblast proliferation, differentiation, migration, adhesion and promote bone repair and regeneration (27-29). A previous study by Yan and Jiang (22) confirmed that BHA/PAA exhibits good osteogenesis activity both *in vitro* and *in vivo*; *in vitro*, BHA/PAA promoted the growth, adhesion and calcium nodule formation of MG63 bone osteosarcoma cells, while in an animal model of radial bone defect, the implanted BHA/PAA material could be fused with the host bone and new bone gradually formed. In the present study, the BHA/PAA material was completely degraded and absorbed at 12 weeks following implantation and new trabecular bone and cartilage had formed. The present results were consistent with previous studies from our group (22,23) and confirmed that BHA/PAA is a good bone graft substitute material with good biological compatibility and capable of osteogenic induction.

RPM was first generated by our research group (16) and preliminary experiments have characterized the physio-chemical properties of RPMs. RPMs were spherical with rough surfaces. The *in vitro* drug release studies revealed that following an initial rapid drug release, rifapentine release gradually decreased and ~80% of the encapsulated rifapentine was released following ~4 weeks of incubation. Furthermore, RPMs were able to eliminate *S. aureus in vitro*. These results indicated that RPMs were bioactive and efficient controlled-release delivery systems, and may exhibit a great

Table III. Concentrations of ALT, AST, BUN and Cr in serum.

	Experimental group	ALT (U/l)	AST (U/l)	BUN (mmol/l)	Cr (μmol/l)
1 day	Blank	48.82±7.05	55.65±9.09	7.78±1.25	131.97±25.62
	BHA/PAA	88.56±6.08[b]	92.37±10.46[a]	15.11±2.05[b]	245.11±25.95[b]
	RPM-loaded BHA/PAA	98.76±11.68[b]	113.67±18.21[b]	15.92±1.84[b]	258.12±30.57[b]
3 days	BHA/PAA	81.58±11.67[a]	82.05±7.05[a]	10.07±2.12	218.91±30.34[a]
	RPM-loaded BHA/PAA	85.05±9.05[b]	85.34±7.02[a]	11.15±2.01	222.14±25.98[a]
1 week	BHA/PAA	60.66±7.25	70.02±7.11	9.21±1.88	200.74±28.79
	RPM-loaded BHA/PAA	65.28±7.88	73.27±8.05	9.35±2.05	198.12±25.67
2 weeks	BHA/PAA	54.25±6.92	54.25±5.88	7.15±1.68	142.28±29.22
	RPM-loaded BHA/PAA	52.43±7.02	60.13±7.67	8.02±1.22	155.28±20.95

[a]$P<0.05$ and [b]$P<0.01$ compared with the blank group. ALT, alanine aminotransferase; AST, aspartate aminotransferase; BUN, blood urea nitrogen; Cr, creatinine; BHA/PAA, bone-like hydroxyapatite/poly amino acid; RPM, rifapentine-loaded poly(lactic-co-glycolic acid) microsphere.
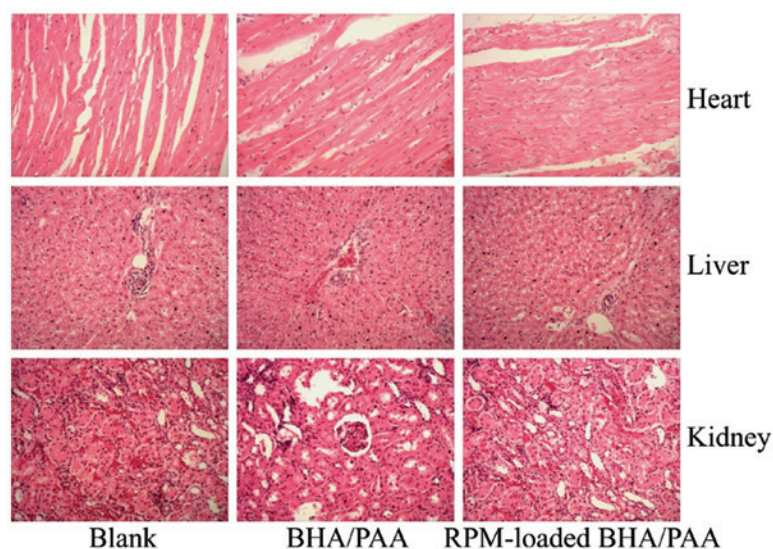

Figure 4. Hematoxylin and eosin staining of heart, liver and kidney tissue sections at 12 weeks following implantation. Magnification, x400. BHA/PAA, bone-like hydroxyapatite/poly amino acid; RPM, rifapentine-loaded poly(lactic-co-glycolic acid) microsphere.

potential in the treatment of osteoarticular tuberculosis (16). In the present study, BHA/PAA was used as a carrier of RPM to prepare a novel sustained-release drug material.

A previous study has demonstrated the curative effect of RPM-loaded BHA/PAA in the treatment of rabbit chronic osteomyelitis induced by *S. aureus* (23). The *in vitro* experiments demonstrated that RPM-loaded BHA/PAA was able to slowly release antibiotics and inhibited bacterial growth effectively for up to 5 weeks. *In vivo*, RPM-loaded BHA/PAA effectively eradicated the bacterial infection, induced osteogenesis and promoted new bone formation while the material was gradually degraded and absorbed. The present study aimed to investigate the curative effect of RPM-loaded BHA/PAA in the treatment of osteoarticular tuberculosis. The *in vivo* release tests demonstrated that RPM-loaded BHA/PAA exhibited sustained release profiles of rifapentine and the drug concentration in the muscle tissues remained higher than the minimum inhibitory concentration of rifapentine against

M. tuberculosis for as long as 12 weeks. Furthermore, the H&E staining and biochemical analyses indicated that RPM-loaded BHA/PAA had no long-term side effects to the heart, liver and kidneys of the treated rabbits.

In conclusion, the present study revealed for the first time that RPM-loaded BHA/PAA may be a useful material for treating osteoarticular tuberculosis. RPM-loaded BHA/PAA promoted new bone formation, while it was gradually degraded and absorbed. Furthermore, rifapentine was released in a sustained manner from this material and no side effects were observed in the heart, liver and kidney of the treated animals. The present results may therefore provide useful new tools towards improving the treatment of osteoarticular tuberculosis.

Acknowledgements

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