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BASIC RESEARCH

Isoniazid Could Be Used for Antibiotic-loaded Bone Cement for Musculoskeletal Tuberculosis: An In Vitro Study

Chang Dong Han MD, PhD, Taegwon Oh RPH, PhD, Sang-Nae Cho DVM, PhD, Jae Ho Yang MD, Kwan Kyu Park MD, PhD

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Abstract

Background Antibiotic-loaded bone cement (ALBC) has been used in serious cases of musculoskeletal tuberculosis, but the type and amount of antibiotic that should be used in ALBC have not been determined.

Questions/purposes We therefore determined the (1) elution characteristics and (2) antimycobacterial activity of isoniazid- and rifampicin-loaded bone cement.

Methods A total of 240 elution samples of each of three discs from 40 g bone cement mixed with one of eight dosages: 1 g, 2 g, and 4 g isoniazid, 1 g, 2 g, and 4 g rifampicin, and a combination of 1 + 1 g or 2 + 2 g of isoniazid and rifampicin. The polymerization of rifampicin-loaded bone cement was delayed to mean 122.5 ± 31.1 minutes. We measured the quantity of isoniazid and rifampicin and the antimycobacterial activity on Days 1, 3, 7, 14, and 30.

Results Isoniazid eluted in almost all the samples while rifampicin was detected only on Day 1 with 2 g (0.7 ± 0.4 ug/mL/day), and until Day 14 with 4 g (0.1 ± 0.0u g/mL/day). Most of the samples containing isoniazid showed antimycobacterial activity while the samples containing rifampicin showed antimycobacterial activity only on Day 1 with 1 g (0.52 ± 0.18 ug/mL), until Day 14 with 2 g (0.03 ± 0.00 ug/mL), and until Day 30 with 4 g (1.84 ± 1.90 ug/mL).

Conclusion Rifampicin was unsuitable for ALBC because of its delayed polymerization. Isoniazid eluted and showed antimycobacterial activity for 30 days.

Clinical Relevance The data suggest isoniazid could be considered for use in ALBC for musculoskeletal tuberculosis if used with systemic treatment. For preventing resistance and systemic toxicity, a combination with a second-line drug and an in vivo study would be needed.

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C. D. Han, J. H. Yang, K. K. Park (✉)
Department of Orthopaedic Surgery, Yonsei University College of Medicine, 134 Shinchon-dong, Seodaemun-gu, Seoul 120-752, Korea
e-mail: kkpark@yuhs.ac

T. Oh, S.-N. Cho
Department of Microbiology and Institute for Immunology and Immunological Diseases, Yonsei University College of Medicine, Seoul, Korea

Introduction

Tuberculosis is still the most common cause of death from infectious disease worldwide [44]. The incidence of tuberculosis gradually decreased through most of the 20th century [45], but dramatically increased in the 1980 s and 1990 s, parallel to the spread of AIDS [38]. Musculoskeletal tuberculosis accounts for approximately 10% to 15% of all cases of tuberculosis in the nonindustrialized world [32]. The most common site for skeletal involvement is the spine (50% of cases) [27], followed by the pelvis (12%), hip and femur (10%), knee and tibia (10%), and ribs (7%) [42, 45]. Bone tuberculosis has nonspecific clinical symptoms and a long treatment period lasting for 6 to 18 months [38]; it is not easily cured and has recurrence rates ranging from 0.0 % to 26.4 % [41]. If drug-resistant tuberculosis occurs, extensive chemotherapy for as much as 2 years is

required [38]. Musculoskeletal tuberculosis may be accompanied by such serious complications as deformities resulting from joint destruction [38].

Although antibiotic therapy remains the cornerstone of treatment for musculoskeletal tuberculosis [31], surgical treatment such as débridement and synovectomy [40, 46, 47], arthrodesis [6, 8, 21, 23], amputation [9, 18, 25, 35], resection arthroplasty [4, 15, 17, 29, 37], or prosthetic joint replacement [33] are required in serious cases. Several studies have reported treating serious cases of musculoskeletal tuberculosis with antibiotic-loaded bone cement (ALBC) [26, 28, 39] to control the infection and to sterilize the joint [26]. These case series reported streptomycin- and tobramycin-loaded bone cement treatment [26, 28, 39].

The selection of the proper antibiotic is important for control of infection in ALBC treatment [11]. With pyogenic osteomyelitis, numerous studies have described the selection of the appropriate type and amount of antibiotic for ALBC treatment [1, 2, 5, 11, 13, 16] since ALBC was introduced by Buchholz and Engelbrecht [7] in 1970. However, there is limited information regarding ALBC for treatment of musculoskeletal tuberculosis [26, 28, 39]. These case reports only document antibiotic-loaded bone cements were used for control of tuberculous osteomyelitis [26], but did not perform or cite systematically designed studies to determine the type and amount of antibiotics for ALBC. Anguita-Alonso et al. showed rifampicin can be used to make beads [1], whereas two other studies [2, 13] reported adding rifampicin with bone cement prevented complete polymerization of the cement. Among the first-line drugs for treatment of tuberculosis, including isoniazid, ethambutol, rifampicin, and pyrazinamide, we used only isoniazid and rifampicin which are available for intravenous administration and could be mixed with bone cement aseptically [41].

We therefore conducted an in vitro study with isoniazid- and rifampicin-loaded bone cement on the (1) elution characteristics and (2) antibacterial activity.

Materials and Methods

We mixed eight different combinations of antibiotics with 40 g customized bone cement powder containing 1 g gentamicin (CMW 3; DePuy, Warsaw, IN, USA) (Fig. 1). The eight different combinations of antibiotics included 1 g, 2 g, or 4 g isoniazid (I3377; Sigma-Aldrich, St Louis, MO, USA); 1 g, 2 g, or 4 g rifampicin (R3501; Sigma-Aldrich); and a combination of 1 g + 1 g or 2 g + 2 g isoniazid and rifampicin. The ALBC was made under sterile conditions in the operating room. The powder mixture was mixed by hand for 2 minutes after the liquid monomer was added. For each of the eight combinations of ALBC powder, three discs were made to measure the elution characteristics and antibacterial activity. The discs were cylinders made from premade molds and were 10 ± 0.8 mm in diameter and 50 ± 1.2 mm in height, with a weight of 4.4 ± 0.26 g (Fig. 2). With all the ALBCs containing rifampicin, including those with combined isoniazid and rifampicin, polymerization was delayed; a clay-like texture persisted until the bone cement was hardened at mean 122.5 ± 31.1 minutes. Each ALBC disc was immersed in a 50-mL test tube that was protected from light and contained 40 mL phosphate buffered saline (PBS). The test tubes were kept at 37 °C. The PBS was replaced daily at a sterile bench. One millimeter of elution sample (eluent) was taken for measurement of elution characteristics and 1 mL for antimycobacterial activity on Days 1, 3, 7, 14, and 30, just before exchanging the PBS. A total of 240 samples (120 samples for elution characteristics and 120 samples for antimycobacterial activity) were obtained. All samples were frozen at −20 °C until measurement.

A liquid chromatography–mass spectrometry (LC/MS) system was used to quantify the amount of antibiotic eluent. The eluents were segregated with high-performance liquid chromatography (HPLC)(Agilent 1100 series; Agilent, Santa Clara, CA, USA) and detected with a mass spectrometer (API3200; AB SCIEX, Framingham, MA, USA). For the HPLC, 0.1% formic acid: acetonitrile (80:20 [v/v]) was used as the mobile phase, and Kinetex C18 (2.6 μm, 4.6 mm φ x 50 mm; Phenomenex, Torrance, CA, USA) was used as the column. We used the positive ion mode and the multiple reaction monitoring mode for the mass spectrograph.

To construct the calibration curve plot, the reference standards of isoniazid and rifampicin were dissolved in 50% methanol to achieve a concentration of 10 μg/mL. We then used this solution to create a reference solution with a concentration of 0.1 to 50 μg/mL. The reference solution was kept in cold storage. To analyze the specimens, each specimen was centrifuged at 3000 rpm for 5 minutes. Then 0.1 L was injected into the LC/MS system and measured.

For each eluent, we measured antimycobacterial activity against *Mycobacterium tuberculosis* (Mtb) H37Rv which was modified from American Type Culture Collection (ATCC) 25618 using the green fluorescent protein (GFP) reporter microplate assay [12]. Mtb H37Rv-GFP was grown in 10 mL of Middlebrook 7H9 broth (Difco, Sparks, MD, USA) supplemented with 50 mg/L kanamycin, 0.2% (v/v) glycerol (Sigma), 1.0 g Casitone (Difco) per liter, 10% (v/v) oleic acid, albumin, dextrose, and catalase (OADC, Difco), and 0.05% (v/v) Tween® 80 (Sigma) until its optical density at 600 nm reached 0.4. Twofold serial dilutions of each eluent were made in 7H9 broth in the microplates. The culture was diluted to 1:50 in 7H9 and

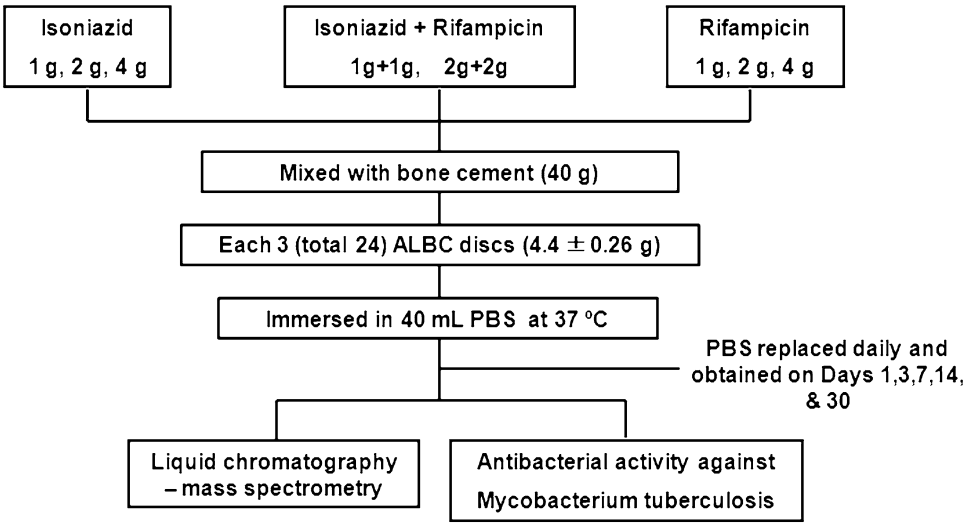

Fig. 1 A chart shows the schematic flow of this study. ALBC = antibiotic-loaded bone cement; PBS = phosphate buffered saline.

Fig. 2 Antibiotic-loaded bone cement discs with 4 g isoniazid (upper cylinder) and 4 g rifampicin (lower cylinder) are shown. The rifampicin-loaded bone cement is bent after delayed polymerization.

was inoculated to yield 2 x 10^5 CFU/mL in plate wells. The plates containing eluent dilutions and Mtb were incubated at 37 °C for 7 days, after which fluorescence was measured in a Fluostar Optima (BMG Labtech GmbH, Ortenberg, Germany) microplate fluorometer in the bottom-reading mode with excitation at 485 nm and emission at 520 nm. The minimal inhibitory concentration (MIC) was defined as the lowest concentration of eluent that inhibited fluorescence by 90% compared with the fluorescence of bacteria-only wells. The MICs of isoniazid and rifampicin were measured at the same time. The actual concentration of each antibiotic in each eluent was calculated by comparing the MIC of each eluent with that of either isoniazid or rifampicin. Antibacterial activity against Mtb was considered effective when the concentration of isoniazid was greater than 0.031 μg/mL [10] and when the concentration of rifampicin was greater than 0.016 μg/mL [10].

All the data are expressed as mean and SDs. Statistical analyses were performed with the SPSS software for Windows (version 20.0; SPSS, Chicago, IL, USA).

Results

Isoniazid was detected in almost all eluent samples whereas rifampicin was only partly detected (Table 1). Generally, the greater the concentration of the mixed antibiotics the greater the concentration of the antibiotics in the eluent and the lower the concentration became with time (Table 1). However, rifampicin was not detected except on Day 1 in the samples that contained 2 g rifampicin, and up to 2 weeks in the samples that contained 4 g rifampicin. The same trend also was observed when isoniazid and rifampicin were mixed (Table 1). The elution concentration of isoniazid decreased considerably to approximately 20% at Day 3, but the concentration was still 5% to 10% at Day 30 compared with Day 1. Like the concentration of isoniazid, the concentration of rifampicin decreased considerably after Day 1, even in the samples that contained 4 g. The samples that contained 1 + 1 g and 2 + 2 g isoniazid showed a trend similar to that of the

Table 1. Results of liquid chromatography–mass spectrometry (ug/mL/day)

Day	Isoniazid			Mean ± SD	Rifampicin			Mean ± SD	Isoniazid + rifampicin			Mean ± SD				Mean ± SD
	1 g				1 g				1 g + 1 g							
									Isoniazid				Rifampicin			
	1*	2*	3*		1*	2*	3*		1*	2*	3*		1*	2*	3*	
1	6.4	6.7	2.8	5.3 ± 2.2	ND	ND	ND	ND	10.2	10.0	4.1	8.1 ± 3.5	ND	ND	ND	ND
3	0.9	1.4	0.9	1.1 ± 0.3	ND	ND	ND	ND	1.2	ND	1.2	1.2 ± 0.0	ND	ND	ND	ND
7	1.6	0.0	1.6	1.1 ± 0.9	ND	ND	ND	ND	0.9	0.7	0.4	0.7 ± 0.3	ND	ND	ND	ND
14	1.2	1.2	1.4	1.3 ± 0.1	ND	ND	ND	ND	0.2	0.3	0.2	0.2 ± 0.1	ND	ND	ND	ND
30	0.6	0.4	0.4	0.5 ± 0.1	ND	ND	ND	ND	0.1	0.1	0.1	0.1 ± 0.0	ND	ND	ND	ND
	2 g				2 g				2 g + 2 g							
1	11.2	18.0	12.6	13.9 ± 3.6	0.3	0.6	1.1	0.7 ± 0.4	23.7	22.8	20.1	22.2 ± 1.9	ND	0.1	0.1	0.1 ± 0.0
3	1.9	2.8	2.3	2.3 ± 0.4	ND	ND	ND	ND	4.0	3.8	2.4	3.4 ± 0.9	ND	ND	ND	ND
7	2.9	2.8	2.8	2.8 ± 0.1	ND	ND	ND	ND	2.2	1.9	1.4	1.8 ± 0.4	ND	ND	ND	ND
14	1.1	2.1	2.4	1.9 ± 0.5	ND	ND	ND	ND	4.4	1.4	1.2	2.3 ± 1.8	ND	ND	ND	ND
30	1.5	0.6	0.7	0.9 ± 0.5	ND	ND	ND	ND	0.6	0.3	0.4	0.4 ± 0.2	ND	ND	ND	ND
	4 g				4 g											
1	30.9	22.5	21.3	24.9 ± 5.2	2.0	4.0	3.9	3.3 ± 1.1								
3	3.6	3.8	3.9	3.8 ± 0.1	0.2	0.3	0.1	0.2 ± 0.1								
7	4.7	5.0	5.2	5.0 ± 0.2	0.1	0.2	0.2	0.2 ± 0.1								
14	3.6	0.1	2.9	2.2 ± 1.8	0.1	ND	0.1	0.1 ± 0.0								
30	2.1	1.7	1.4	1.7 ± 0.3	ND	ND	ND	ND								

* Results from individual discs; ND = not detectable.

samples containing isoniazid alone, but rifampicin was not detected (Table 1).

Most of the samples containing isoniazid showed antimycobacterial activities while the samples containing rifampicin only partly showed antimycobacterial activities (Table 2). All the eluents containing isoniazid were effective against Mtb. Rifampicin maintained its antimycobacterial activity up to Day 30 in the samples containing 4 g rifampicin, but it maintained such activity only on Day 1 in the samples containing 1 g rifampicin, and up to 2 weeks in the samples containing 2 g rifampicin (Table 2). In the samples containing isoniazid and rifampicin, antibacterial activity was present up to Day 30, although it was unclear which drug was responsible for the activity.

Discussion

ALBC treatment has been recommended to control serious musculoskeletal tuberculosis infections [26, 28, 39]. However, the type and amount of antibiotics for ALBC for treatment of musculoskeletal tuberculosis has not been systematically studied with subsequent lack of adequate guidelines. We therefore determined the (1) elution characteristics and (2) antimycobacterial activity of isoniazid- and rifampicin-loaded bone cement.

This study has some limitations. First, as it is an in vitro study, it may not accurately reflect in vivo conditions. In this study, PBS was exchanged daily to reproduce a washout of the joint fluid, and elution fluid was collected just before the exchange of PBS as a measure of the elution concentration per day. Future studies such as those involving animal testing are required to investigate the concentration of antibiotics and their toxicity when oral medications are concomitantly administered in real treatments. Second, although a multidrug modality generally is used to treat tuberculosis and can include the use of isoniazid, ethambutol, rifampicin, and pyrazinamide, we included only isoniazid and rifampicin, which are available in sterilized intravenous form. Furthermore, when rifampicin was used to create ALBC, the polymerization of the bone cement was delayed, and thus we concluded rifampicin cannot be mixed with bone cement. Although Anguita-Alonso et al. used rifampicin to make beads [1], consistent with our study, others [2, 13] have reported the combination of rifampicin with CMW prevents complete polymerization of the cement (Table 3). As it was found

Table 2. Measurement of antimycobacterial activity of effusion sample (eluent) (ug/mL)[†]

Day	Isoniazid			Mean ± SD	Rifampicin			Mean ± SD	Isoniazid + rifampicin			Mean ± SD
	1 g				1 g				1 g + 1 g			
	1*	2*	3*		1*	2*	3*		1*	2*	3*	
1	19.968	9.984	9.984	13.31 ± 5.76	0.625	0.624	0.313	0.52 ± 0.18	39.936	20.001	9.984	23.31 ± 15.25
3	0.624	1.248	0.624	0.83 ± 0.36	ND	ND	ND	ND	0.624	ND	0.312	0.47 ± 0.22
7	1.284	ND	1.248	1.25 ± 0.00	ND	ND	ND	ND	0.312	0.156	0.312	0.26 ± 0.09
14	0.500	1.000	1.000	0.83 ± 0.29	ND	ND	ND	ND	0.250	0.062	0.125	0.15 ± 0.10
30	ND	0.500	0.250	0.38 ± 0.18	ND	ND	ND	ND	0.063	0.063	0.063	0.06 ± 0.00
	2 g				2 g				2 g + 2 g			
1	39.936	19.968	19.968	26.62 ± 11.53	2.500	2.500	25.00	2.50 ± 0.0	39.936	79.872	79.872	66.56 ± 23.06
3	2.496	2.496	2.496	2.50 ± 0.00	0.250	0.250	0.016	0.17 ± 0.14	2.496	2.496	2.496	2.50 ± 0.00
7	2.496	2.496	2.496	2.50 ± 0.00	0.063	0.250	NC	0.16 ± 0.13	1.248	1.248	0.624	1.04 ± 0.36
14	2.000	2.000	2.000	2.00 ± 0.00	0.031	0.031	NC	0.03 ± 0.00	0.499	0.499	0.499	0.50 ± 0.00
30	ND	2.000	1.000	1.50 ± 0.71	ND	ND	ND	ND	0.500	0.250	0.125	0.29 ± 0.19
	4 g				4 g							
1	79.872	79.872	39.936	66.56 ± 23.06	5.000	10.001	5.000	6.67 ± 2.89				
3	4.992	4.992	2.496	4.16 ± 1.44	2.003	1.002	2.003	1.67 ± 0.58				
7	4.992	4.992	4.992	4.99 ± 0.00	1.002	1.002	2.003	1.34 ± 0.58				
14	4.000	8.000	7.992	6.66 ± 2.31	0.500	1.000	1.002	0.83 ± 0.29				
30	4.006	2.000	8.013	4.67 ± 3.06	0.500	1.002	4.006	1.84 ± 1.90				

[†]Eluent was considered effective against *Mycobacterium tuberculosis* when the concentration was greater than 0.031 ug/mL for isoniazid or greater than 0.016 ug/mL for rifampicin; * results from individual discs; ND = not detectable.

that isoniazid can be mixed with bone cement, future studies on second-line drugs such as moxifloxacin, kanamycin, or streptomycin are needed. Third, we tested only one type of bacteria, *Mycobacterium tuberculosis*. Because the rates of infection with nontuberculous mycobacteria and multiresistant tuberculosis have been increasing [44, 45], studies on various strains are needed. Finally, variable types of cement were not investigated in the current study, and different types of cement could reveal different results owing to their variable elution characteristics [1, 5, 11, 16, 24].

We found only isoniazid and not rifampicin showed proper elution characteristics during the 30-day study period. Even though the elution of isoniazid dramatically decreased after Day 3, it persisted up to Day 30. To the best of our knowledge, there has been no published study on isoniazid-loaded bone cement. The elution characteristics of isoniazid-loaded bone cement were superior to those of rifampicin-loaded bone cement even ignoring the difficulty of mixing rifampicin with bone cement.

Isoniazid showed antimycobacterial activity greater than the MIC in every amount of isoniazid-loaded bone cement for the entire study period and was superior to that for rifampicin-loaded bone cement. Even though isoniazid showed proper elution characteristics and antimycobacterial activity for 30 days in our in vitro study, one should consider using other second-line drugs together with isoniazid for several reasons. First, a minimum period for treatment of musculoskeletal tuberculosis is longer than 30 days. The goal of ALBC is intensive administration of antibiotics to the affected region in addition to systemic chemotherapy, which is the cornerstone treatment of musculoskeletal tuberculosis [38]. For an intensive effect to an affected region, use of combined drugs is needed. Second, isoniazid has the greatest bactericidal activity [22, 30], but it is the most common first-line agent to which resistance is found in *Mycobacterium tuberculosis* [34]. When it is used for ALBC, especially, isoniazid in bone cement could result in variable concentrations which can result in underdosage and development of resistance [3]. Considering the reported high prevalence of multidrug-resistant tuberculosis [43], additional studies are needed with second-line agents that could be used with isoniazid for ALBC. Third, isoniazid is widely distributed in body fluids and tissues [20, 36] and could cause toxicity, especially to the liver [14, 19]. Even though the advantage of ALBC is local administration of antibiotics, isoniazid could increase the hepatotoxicity when combined with systemic

Table 3. Summary of studies evaluating rifampicin-loaded bone cement

Study	Cement type	Amount of antibiotics	Methods	Results	Conclusion
Anquita-Alonso et al. [1]	Simplex P (Stryker, Mahwah, NJ)	2.5%/ 7.5%/ 15.0 %	*Micrococcus luteus* ATCC 9341	5 ± 2/147 ± 15/409 ± 46 ug/mL/hour of area under the curve, 4 ± 1/15 ± 2/31 ± 11 ug/mL of peak concentration	Rifampicin may be suitable for management of orthopaedic infections.
Beeching et al. [2]	CMW1 (Depuy, Blackpool, UK)	2.5 %	*Staphylococcus aureus* ATCC 25923, Escherichia coli ATCC 25922	Not documented	Did not harden for several days.
De Palma et al. [13]	CMW (Depuy, Blackpool, UK)	1.2 g/40 g	Strength test	Not documented	The combination of rifampicin with CMW cement prevented complete polymerization of the cement.
Current study	CMW 3 (Depuy, Blackpool, UK)	Rifampicin 1 g/2 g/4 g	Compressive strength, antibacterial activity	Strength could not be measured; partially effective antibacterial activity	Polymerization was prevented.

ATCC = American Type Culture Collection.

treatment. Because the risk of hepatotoxicity increases with age [20], use in elderly patients needs caution. We are fully aware that for evaluation of toxicity and of the occurrence of resistance, an in vivo study should be conducted, and especially for resistance, second-line drug use with isoniazid should be considered.

We found rifampicin is unsuitable as an antibiotic for bone cement owing to its delayed polymerization, but isoniazid eluted Mtb and showed antimycobacterial activity for 30 days. The findings suggest isoniazid can be considered for use in ALBC if used with systemic treatment but this would need to be confirmed with in vivo experiments. For preventing resistance and considering its systemic toxicity, isoniazid should be considered for second-line drug use.

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