Original Article

Biosafety of the Novel Vancomycin-loaded Bone-like Hydroxyapatite/Poly-amino Acid Bony Scaffold

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Abstract

Background: Recently, local sustained-release antibiotics systems have been developed because they can increase local foci of concentrated antibiotics without increasing the plasma concentration, and thereby effectively decrease any systemic toxicity and side effects. A vancomycin-loaded bone-like hydroxyapatite/poly-amino acid (V-BHA/PAA) bony scaffold was successfully fabricated with vancomycin-loaded poly lactic-co-glycolic acid microspheres and BHA/PAA, which was demonstrated to exhibit both porosity and perfect biodegradability. The aim of this study was to systematically evaluate the biosafety of this novel scaffold by conducting toxicity tests *in vitro* and *in vivo*.

Methods: According to the ISO rules for medical implant biosafety, for *in vitro* tests, the scaffold was incubated with L929 fibroblasts or rabbit noncoagulant blood, with simultaneous creation of positive control and negative control groups. The growth condition of L929 cells and hemolytic ratio were respectively evaluated after various incubation periods. For *in vivo* tests, a chronic osteomyelitis model involving the right proximal tibia of New Zealand white rabbits was established. After bacterial identification, the drug-loaded scaffold, drug-unloaded BHA/PAA, and poly (methyl methacrylate) were implanted, and a blank control group was also set up. Subsequently, the *in vivo* blood drug concentrations were measured, and the kidney and liver functions were evaluated.

Results: In the *in vitro* tests, the cytotoxicity grades of V-BHA/PAA and BHA/PAA-based on the relative growth rate were all below 1. The hemolysis ratios of V-BHA/PAA and BHA/PAA were 2.27% and 1.42%, respectively, both below 5%. In the *in vivo* tests, the blood concentration of vancomycin after implantation of V-BHA/PAA was measured at far below its toxic concentration (60 mg/L), and the function and histomorphology of the liver and kidney were all normal.

Conclusion: According to ISO standards, the V-BHA/PAA scaffold is considered to have sufficient safety for clinical utilization.

Key words: Biosafety; Bone-like Hydroxyapatite/Poly-amino Acid; Chronic Osteomyelitis; Drug Release; Scaffold

Introduction

Recently, a vancomycin-loaded bone-like hydroxyapatite/poly-amino acid (V-BHA/PAA) bony scaffold was fabricated by a homogeneous system method involving a diffusion control system, by which a porous BHA/PAA scaffold was incorporated with vancomycin-loaded poly lactic-co-glycolic acid (PLGA) microspheres prepared by an emulsification-solvent evaporation method. A previous study verified that the biostructure of this scaffold was very similar to natural human bone with both porosity and perfect biodegradability, which should make it possible for the scaffold to become an ideal antibiotics carrier for the repair of infected bony defects.[1] Although it was theoretically demonstrated that all of the scaffold monomers and degradation products had no significant toxicity or side effects to the human body,[2,3] the scaffold would undergo changes and reactions during procedures including fabrication, storage, and sterilization. Furthermore, the blood concentration of the loaded antibiotics could reach toxic levels at certain times, especially in the early stages after implantation. Therefore, according to the ISO rules for

Access this article online

Quick Response Code:

Website: www.cmj.org

DOI: 10.4103/0366-6999.173489

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Received: 15-10-2015 **Edited by:** Li-Shao Guo
How to cite this article: Cao ZD, Jiang DM, Yan L, Wu J. Biosafety of the Novel Vancomycin-loaded Bone-like Hydroxyapatite/Poly-amino Acid Bony Scaffold. Chin Med J 2016;129:194-9.

medical implant biosafety, the biosafety of the scaffold was systematically evaluated by toxicity tests *in vitro* and *in vivo* in the present study, to provide a reference for subsequent clinical applications.

Methods

Fabrication of experimental materials

Vancomycin (Eli Lilly and Company, Indianapolis, Indiana, USA)-loaded PLGA microspheres were prepared by an emulsification-solvent evaporation method, and then incorporated into the BHA/PAA scaffold using a homogeneous system method involving a diffusion control system. After curing molding, the scaffold was finally molded into cuboid granules with a size of 1.5 cm × 0.5 cm × 0.5 cm, containing 8 wt% of vancomycin. For preparation of vancomycin-loaded poly (methyl methacrylate) (V-PMMA), 80 mg of vancomycin was mixed with 920 mg of bone cement powder (DePuy Synthes Company, New Brunswick, New Jersey, USA), before 1 ml of MMA monomer solution (DePuy Synthes Company, New Brunswick) was added and stirred carefully. Before curing, V-PMMA was molded into the same size and shape as V-BHA/PAA. The final content of vancomycin was also equal to 8 wt%.

Groups of experiments

The *in vitro* experiments included a V-BHA/PAA group (VC group), vancomycin-unloaded BHA/PAA group (VUC group), negative control group (NC group), and positive control group (PC group). The *in vivo* tests involved a VC group, VUC group, blank control (BC) group (only debridement without implants), and PC group (V-PMMA). In serum biochemical tests, the PC group was not included, while in blood drug concentration measurements, the VUC group was excluded. Fifteen New Zealand white rabbits (Animal Experimental Center, Chongqing Medical University, China) were involved in each group.

Experimental procedures

Cytotoxicity assay *in vitro*

Leaching solutions for the V-BHA/PAA and BHA/PAA scaffolds were obtained following ISO 10993-5 standards by immersing the respective scaffolds in 1640 culture solution at 37°C for 3 days. L929 fibroblasts (National Engineering Research Center of Ultrasound Medicine, Chongqing Medical University, China) were cultured for 2 days, and then digested with 0.25% trypsin to prepare a cell suspension at 6×10^4 cells/ml. The suspension was inoculated into a 96-well plate at 100 μl/well, with 8 wells per group, and the plate was incubated in a 5% CO_2 constant temperature incubator (LRB MODEL 2300; Sheldon Company, USA) at 37°C for 24 h to allow the cells attach to the wells. The culture medium was then replaced, and the cells were cultured for a further 24 h. After the culture medium was discarded, the cells were washed with phosphate-buffered saline, before 100 μl of leaching solution for the VC and VUC groups, 100 μl of 0.64% phenol solution for the PC group, or 100 μl of fresh culture medium for the NC group was added. After culture for 2, 4, and 7 days, respectively, the cellular morphology was observed under an inverted microscope (CKX41-A32PH, Olympus, Japan). MTT solution was then added to each well and incubated for 6 h, followed by addition of 150 μl of dimethylsulfoxide. After shaking for 10 min, the absorbance (A) values of the wells were detected using an immune enzyme-labeled instrument (Elx-808; Bio-Tek, USA) at a wavelength of 470 nm. The relative growth rate (RGR) of L929 cells was calculated according to the formula shown below, and the cytotoxicity of the leaching solutions was evaluated by the average RGR according to the grading criteria listed in Table 1.[4]

$$\text{RGR} = \frac{\text{A value of exprimental group}}{\text{A value of NC group}} \times 100\%$$

Acute hemolysis test

Eluates from the V-BHA/PAA and BHA/PAA scaffolds were obtained by immersing the scaffolds in normal saline (10 ml/3 cm^2 surface area) and incubation at 37°C for 1 day. The eluates were disinfected using a microporous filter before being assayed. Blood collection was performed by heart puncture of New Zealand white rabbits, followed by immediate addition of 0.5 ml of 20 g/L potassium oxalate to prepare noncoagulant blood. The noncoagulant blood was diluted with normal saline (10 ml of normal saline per 8 ml of blood), before addition of leaching solution, normal saline (NC group), or double-distilled water (PC group) according to the groups. After gentle shaking and centrifugation at 2500 r/min for 5 min, the A value of each supernatant was measured using a spectrophotometer (Perkin Elmer-Lamar II; PerkinElmer, Germany) at a wavelength of 545 nm. The hemolysis ratio was calculated using the formula shown below, by which the biosafety of V-BHA/PAA and BHA/PAA for hemolysis was determined according to the ISO standard (eligible if hemolysis ratio was <5%).

$$\text{Hemolytic ratio} = \frac{\text{A value of exprimental group} - \text{A value of NC group}}{\text{A value of PC group} - \text{A value of NC group}} \times 100\%$$

Toxicity assay *in vivo*

A chronic osteomyelitis model in the right proximal tibia of New Zealand white rabbits was established based on a previously reported method.[5] All of the rabbits were

Table 1: Grading criteria for cytotoxicity

Toxicity grade	RGR (%)
0	≥100
1	75–99
2	50–74
3	25–49
4	1–24
5	0

RGR: Relative growth rate.

classified according to gross observation and X-ray detection criteria.[5,6] Only the rabbits with class 2 and above were chosen to perform bacterial culture and identification using affected bone marrow. Only the rabbits with infected bacteria confirmed to coincide with the initially seeded bacteria ultimately became the experimental models. After general anesthesia by intravenous injection of 3% pentobarbital (1 ml/kg body weight), the infected tibial metaphysis was exposed through a primary incision approach to debride all of the dead bone and septic tissues. Finally, a 2.0 cm × 1.0 cm bone defect was made, debrided thoroughly and lavaged 3 times, and implanted with 3 g of V-BHA/PAA, BHA/PAA, or V-PMMA according to the groups. The NC group did not receive an implant. The general conditions, motion conditions, and deaths were recorded on each postoperative day. Blood aspartate transaminase (AST), alanine aminotransferase (ALT), blood urea nitrogen (BUN), and creatinine (Cr) levels were checked preoperatively and at postoperative week 1, week 2, and month 1 to evaluate the influence of the scaffolds on the functions of the liver and kidney. Furthermore, the blood concentrations of vancomycin were assayed by high-performance liquid chromatography (Model 1200; Agilent, Japan) at postoperative day 3, week 1, week 2, and month 1. In addition, the liver and kidney tissues were harvested on the last experimental day to observe the histopathological changes under microscopy after hematoxylin and eosin staining.

Statistical analysis

Statistical analysis was conducted by SPSS (version 21, IBM, USA). At first, *F*-test was used to test variance homogeneity of every group and then least significant difference (LSD) method was used to analyze the results of cytotoxicity assay and acute hemolytic test *in vitro*, while two-way analysis of variance (ANOVA) was applied to analyze the results of toxicity assay *in vivo* meanwhile LSD test was also applied to compare any two groups. Values of $P < 0.05$ were regarded as significant.

Results

Cytotoxicity assay *in vitro*

As shown in Figure 1, the RGR of L929 cells in the VC and VUC groups was above 85% on every experimental day. Therefore, the cytotoxicity grades of V-BHA/PAA and BHA/PAA-based on the RGR values were all below 1. When compared with the PC group, the RGR of L929 cells in the VC and VUC groups was significantly higher on each experimental day. However, when compared with the NC group, the RGR became relative lower at day 2, and thereafter increased until it became equal to the NC group on day 7. Under the inverted phase-contrast microscope, as showed in Figure 2, the morphocytology of L929 cells in the VC and VUC groups was as normal as that in the NC group.

Acute hemolysis test

As shown in Table 2, the hemolysis ratios of the V-BHA/PAA and BHA/PAA scaffolds were 2.27% and 1.42%, respectively. As both values were below 5%, the hemolysis safety of V-BHA/PAA and BHA/PAA was considered to meet the standard of ISO 10993-5.

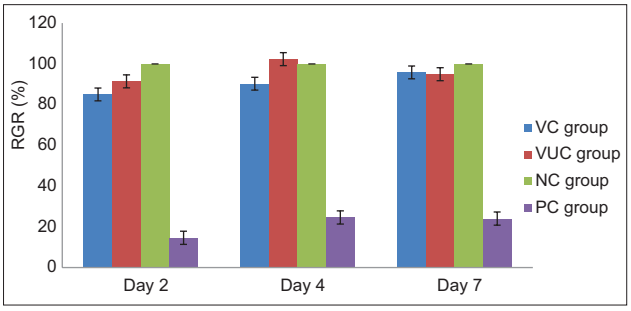

Figure 1: Relative growth rate (RGR) of L929 exposure to media containing leaching solution. Compared with the negative control group: at day 2: *P* (VC group) = 0.032, *P* (VUC group) = 0.039; at day 4: *P* (VC group) = 0.042, *P* (VUC group) = 0.164; at day 7: *P* (VC group) = 0.084, *P* (VUC group) = 0.079. Compared with the positive control group: at day 2: *P* (VC group) = 0.008, *P* (VUC group) = 0.005; at day 4: *P* (VC group) = 0.006, *P* (VUC group) = 0.003; at day 7: *P* (VC group) = 0.004, *P* (VUC group) = 0.004.

Table 2: Hemolytic ratio of each group (mean ± SD, *n* = 5)

Groups	Average values	Hemolysis ratio (%)
VC	0.031 ± 0.003	2.27
VUC	0.022 ± 0.003	1.42
NC	0.007 ± 0.002	0
PC	1.062 ± 0.047	100

VC group versus VUC group: No statistical difference; VC group versus NC group: Had statistical difference; VC group versus PC group: Had statistical difference; SD: Standard deviation.

Toxicity assay *in vivo*

General conditions

Within the 1st postoperative week, all animals showed inactivity with limping and wound swelling without significant chills, cyanosis, or dyspnea. Thereafter, the activity of most animals in the VC and PC groups gradually recovered with weight increases. Two animals in the VUC group and three animals in the BC group died because of severe infection verified by autopsy. No animals died in the VC and PC groups.

Serum biochemical test

As shown in Tables 3 and 4, there were no significant differences between the experimental groups (VC group and VUC group) and the control group for not only liver function (AST and ALT levels), but also kidney function (BUN and Cr levels) at each time point examined.

Blood concentration of vancomycin test

As shown in Table 5, the peak values of the blood drug concentration in both the VC and PC groups were found in postoperative week 1, and both were far below the toxic concentration of vancomycin (60 mg/L). At the early postoperative stages (weeks 1 and 2), there were no differences in the blood drug concentration between the VC and PC groups.

Table 3: Serum AST and ALT level (mean ± SD, U/L, n = 5)

Groups	AST				ALT			
	Preoperation	Postoperative week 1	Postoperative week 2	Postoperative month 1	Preoperation	Postoperative week 1	Postoperative week 2	Postoperative month 1
VC	31.17 ± 3.42	29.92 ± 4.24	30.83 ± 3.43	27.34 ± 2.67	55.82 ± 4.69	51.67 ± 9.52	53.57 ± 6.49	54.68 ± 8.37
VUC	33.91 ± 3.27	29.48 ± 3.02	32.65 ± 3.62	32.27 ± 3.95	49.48 ± 7.35	50.35 ± 9.46	49.93 ± 9.49	49.78 ± 9.68
BC	33.73 ± 4.02	31.32 ± 3.62	32.35 ± 2.56	33.52 ± 3.63	53.83 ± 9.69	50.58 ± 6.47	53.24 ± 8.23	52.53 ± 8.29

There was no significant difference between each pair of experimental and control groups at each time point (AST: F = 2.13, P = 0.28; ALT: F = 5.15, P = 0.15), and also no obvious difference between each pair of time points at VC group (AST: F = 5.89, P = 0.16; ALT: F = 0.51, P = 0.66). SD: Standard deviation; AST: Aspartate transaminase; ALT: Alanine aminotransferase.

Table 4: Serum BUN and Cr level (mean ± SD, mmol/L, n = 5)

Groups	BUN				Cr			
	Preoperation	Postoperative week 1	Postoperative week 2	Postoperative month 1	Preoperation	Postoperative week 1	Postoperative week 2	Postoperative month 1
VC	5.29 ± 4.52	8.25 ± 4.52	7.92 ± 3.32	6.45 ± 2.55	87.92 ± 14.16	88.27 ± 10.67	89.89 ± 15.48	85.48 ± 12.78
VUC	7.39 ± 3.74	6.94 ± 4.64	6.93 ± 3.58	7.59 ± 2.48	87.64 ± 13.58	90.40 ± 14.58	89.52 ± 15.24	89.53 ± 12.94
BC	8.29 ± 3.37	7.96 ± 2.51	8.08 ± 3.54	7.39 ± 2.22	92.45 ± 12.53	92.67 ± 15.42	91.56 ± 12.52	89.92 ± 20.11

No significant difference between each pair of experimental and control groups at each time point (BUN: F = 2.33, P = 0.27; Cr: F = 7.00, P = 0.12), and also no obvious difference between each pair of time points (BUN: F = 0.01, P = 0.99; Cr: F = 3.13, P = 0.24). Cr: Creatinine; BUN: Blood urea nitrogen; SD: Standard deviation.

Table 5: The result of blood concentration of vancomycin test (mean ± SD, mg/L, n = 5)

Groups	Postoperative day 3	Postoperative week 1	Postoperative week 2	Postoperative month 1
VC	<2	5.28 ± 0.29	5.02 ± 1.35	4.94 ± 1.87
BC	<2	<2	<2	<2
PC	<2	6.24 ± 0.82	4.18 ± 1.55	2.04 ± 1.15

From 1 week postoperative on, no significant difference existed between every pair of time points both in VC and PC group (P > 0.05); There was also no obvious difference between VC and PC group both in postoperative week 1 and postoperative week 2 (P > 0.05), while obvious difference was found in postoperative month 1 (P = 0.016). SD: Standard deviation.

At 1 month postoperatively, the blood drug concentration in the VC group was slightly higher than that in the PC group.

Histological observation of the liver and kidney

Under microscopic examination, as shown in Figure 3, the liver and kidney tissues in the experimental groups showed normal appearances without significant differences compared with the control groups.

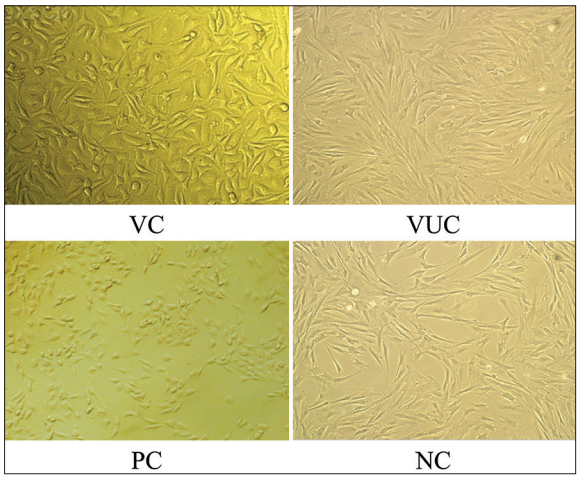

Figure 2: Microscopic observations after incubation of L929 cells with leaching solutions (Original magnification ×200). The morphocytology of L929 cells in the VC and VUC groups is as normal as that in the negative control group, while the cell count in the positive control group is significantly decreased with cellular atrophy, spherical shapes, and cellular death.

Discussion

The BHA/PAA scaffold is a novel curable and biodegradable porous bone restorative material, which was researched and developed by us in association with Sichuan Guona Science and Technology Co., Ltd.[1] Among the active ingredients, BHA is an extremely bone-like hydroxyapatite that has been proven to be nontoxic and show favorable biocompatibility with the human body.[7] PAA is a polymer of six amino acids (6-aminocaproic acid, glycine, L-alanine, L-phenylalanine, L-proline, and L-lysine) that also shows biodegradability and biocompatibility with the human body.[8] The BHA/PAA scaffold is a composite of BHA and PAA materials, which perfectly combines the favorable mechanical property of BHA with the biocompatibility and biodegradability of PAA. By adjusting the proportions of BHA and PAA, the rate and speed of the scaffold degradation can be adjusted, meaning that the scaffold should have great potential for bone repair and as a drug carrier. Preliminary experiments initially demonstrated that the BHA/PAA scaffold had good biosafety and biocompatibility, and could be degraded completely *in vivo* and replaced by bone tissue.[1] The scaffold produced degradation products *in vivo* that included near-neutral oligopeptides and amino acid molecules or underwent further degradation to carbon dioxide and water, which are all nontoxic to the human body.[1,9]

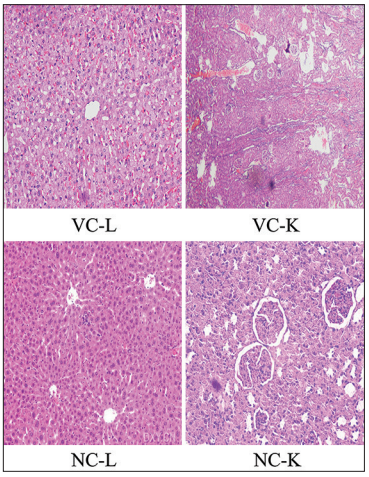

Figure 3: Histological performance of liver and kidney 12 weeks after scaffolds implantation (H and E staining, Original magnification ×200, L: Liver; K: Kidney). Under microscopy, liver and kidney tissues in VC group both showed as normal as negative control group.

In this study, vancomycin was encapsulated as drug-loaded microspheres synthesized with PLGA, which is a degradable macromolecular organic compound randomly polymerized by lactic acid and glycolic acid. As reported in the literature, PLGA possesses favorable features of biocompatibility, nontoxicity, and ease of creation into sacciform or membrane structures.[10] In addition, if ester bonds in its chemical structure are disrupted, it can be degraded *in vivo* to lactic acid and glycolic acid,[11] which are normal catabolites in the human body. Consequently, PLGA is commonly considered to have excellent biosafety and has been extensively applied in the pharmaceutical, medical engineering, and modern industrial fields. In the USA, the Food and Drug Administration has approved the clinical application of PLGA, and formally included PLGA in the USA pharmacopoeia as a pharmaceutical excipient.[12] Recently, PLGA has been widely used for controlled drug release because of its desirable biocompatibility and biosafety as well as its lack of obvious disintegration phenomenon.

As an antimicrobial glycopeptide, vancomycin is always one of the most common antibiotics for treatment of *Staphylococcus aureus*, especially methicillin-resistant *S. aureus*,[13] and has been used extensively in orthopedics since the late 1950s. However, vancomycin can manifest obvious systemic toxicity toward the human body if applied by intravenous injection for a long period,[14,15] for which nephrotoxicity and hepatotoxicity are the most common effects (incidence of approximately 12–43%).[16] The toxicity of vancomycin shows a significant dose- and time-dependence. Briefly, the toxicity increases as the blood drug concentration and application time increase. Currently, it is generally accepted that the initial blood trough level of a drug is the most important predictive index of toxicity, and the corresponding level of vancomycin is 60 mg/L.[13] Owing to the obvious systemic toxicity by systemic administration, local application of vancomycin is increasingly being adopted to decrease the toxic side effects.[17,18]

Use of an antimicrobial-loaded bone tissue engineering scaffold is the most common controlled release system for local antibiotics to treat an infectious bone defect, because a scaffold can clearly improve the bioavailability of antibiotics and accordingly decrease their absorption into the bloodstream, thereby minimizing the toxicity and side effects of the drugs.[19] However, in general, the procedure for a drug-loaded release system to deliver a drug *in vivo* involves an initial burst release process within 24 h of implantation before stable release, during which time the blood drug concentration can instantly achieve its highest level, and easily result in a toxic response. Furthermore, with prolongation of the drug-release time, the stable release procedure can lead to drug accumulation in the bloodstream, which can also produce toxic effects.[20] Therefore, according to the ISO rules concerning medical implant biosafety, we conducted toxicity tests *in vitro* and *in vivo* to systematically evaluate the biosafety of the V-BHA/PAA scaffold to provide a reference for subsequent clinical applications. Through cytotoxicity tests *in vitro*, we found that the V-BHA/PAA scaffold could influence the growth of L929 fibroblasts initially, but not cause cell death directly, and thus the cellular morphology remained normal. Thereafter, the effect of the scaffold to promote cell proliferation became stronger and stronger as time passed, by which the cell growth inhibition could be compensated effectively. The hemolysis test demonstrated that the V-BHA/PAA scaffold had eligible hemolysis biosafety following ISO standards. Through the toxicity test *in vivo*, we found that the blood concentration of vancomycin after implantation of V-BHA/PAA was far below its toxic concentration (60 mg/L) throughout the test period. Moreover, the function and histomorphology of the liver and kidney all showed normal appearances, just like those in the NC group and PC group (V-PMMA material), which has been used widely in the clinic.

In conclusion, according to ISO standards, the V-BHA/PAA scaffold is considered to have sufficient safety for clinical utilization.

Financial support and sponsorship

This study was supported by a grant from the National Natural Science Foundation of China (No. 81171685).

Conflicts of interest

There are no conflicts of interest.

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