Preparation, antibacterial properties and biocompatibility studies on vancomycin-poly(D,L)-lactic loaded plates

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Abstract In the clinic, implant-associated infections are the chief risk facing orthopaedics and traumatology departments. Vancomycin-poly(D,L)-lactic acid (PDLLA) loaded plates were prepared by using solvent casting technology. The bacteriostatic activity to *Staphylococcus aureus* was evaluated in vitro. The biocompatibility of the plates was investigated by acute systemic toxicity test, haemolytic test, cytotoxicity test, etc. The in vitro inhibition test of *Staphylococcus aureus* showed that the plates had an inhibitory effect on *Staphylococcus aureus*. There was no toxicity for the vancomycin-PDLLA loaded plates. There was a good biocompatibility for the vancomycin-PDLLA loaded plates. These findings indicate that the plate maybe used to prevent or cure infection.

Introduction

One complication arising after the internal fixation of tibial fractures is postoperative infection. Patients show a higher susceptibility to infection due to bacterial contamination and soft tissue damage, particularly in open fractures [1]. As a report in 2006 suggests [1], the rate of

infections associating with closed fractures is much lower than that of open ones (1.5% and 3–40%, respectively). Up to 60% of open fractures are contaminated with bacteria at the time of injury [1]. Maurer et al. reported the rate of infection in open tibia fractures increased to greater than 30% when internal fixation was used [2].

During the past 30 years, a drug-delivery system has been gradually developed on the basis of a series of original, fine biocompatibility inorganic material and organ polymers. This system has a broad application perspective in the treatment of osteomyelitis, bone tuberculosis, and bone defects due to infection. As reported earlier, poly(D,L)-lactic acid (PDLLA) has excellent features with respect to implant coating including high mechanical stability [3], good osteoinductive potential, and excellent biocompatibility in vivo [4]. The PDLLA coating is degraded by hydrolysis three to six months after implantation. The products of degradation are metabolised in the citric acid cycle [4–7]. Because of these characteristics, PDLLA can be used in orthopaedic internal fixation.

Common causes of implant-associated infections are *Staphylococcus aureus* and *Staphylococcus epidermidis* [8, 9]. *S. aureus* is a common cause of metal biomaterial, bone joint, and soft tissue infections [8, 10]. Vancomycin (VCM) belongs to a family known as the glycopeptide antibiotics. It has a broad antimicrobial spectrum that covers most bacteria commonly involved in osteomyelitis. Its bactericidal effects on even non-proliferating microorganisms make it favourable for local application. It is currently the top choice for curing severe infections caused by methicillin resistant *Staphylococcus aureus* (MRSA) or methicillin resistant *Staphylococcus epidermidis* (MRSE) [3].

Considering these points, we developed a local drug delivery system for VCM application. Implants were coated with biodegradable PDLLA using a solvent casting tech-

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nique, and VCM was delivered from a PDLA coating on a titanium alloy plate. The purpose of this study was to determine the anti-infective properties and biocompatibility of the plates for medical implants.

Materials and methods

Preparation

Titanium alloy plates were cut into 1.5 cm×1.0 cm×0.5 cm ($N=10$) and 7 mm×5 mm titanium discs ($N=10$); vancomycin was purchased from ELI Lilly K.K Seishin Laboratories of Japan (0.5 g/ampul). The molecular weight of PDLA was 58,000. The test strain was *Staphylococcus aureus* (ATCC 25923) and was susceptible to VCM. Bacterial cells were resuspended in normal saline and adjusted to 1.5×10^8 cfu/ml. All reagents in use (e.g. acetic ether, acetone, and alcohol) were of analytical grade.

All of the titanium alloy plates were cleaned ultrasonically in acetone for ten minutes, a 70% alcohol solution for ten minutes, and distilled water for ten minutes. All samples were open-air dried and then were immersed into a mixture solution of 98% H_2SO_4 and 30% H_2O_2 (v/v,7:3) for one hour. This step was followed by thorough cleaning in distilled water, dislodging, oven drying at 40°C, and autoclave sterilisation.

Implants were coated with VCM-PDLA using a solvent casting technique by Gollwitzer et al. [11]. A total of 2.0 g PDLA was accurately weighed and thoroughly dissolved in 40 ml of acetoacetate, and then 0.1 g of VCM was added to it. The solution was ultrasonically oscillated to form a 5w% (w/w, VCM/PDLA) uniform scattering mixture solution. The treated plates were immersed in the mixture solution for five minutes. These plates were air dried in ventilation spaces for ten minutes and then were immersed in the mixture solution again. The procedure was repeated three times to achieve a dense and regular polymer coating. All samples were air dried overnight. Finally, coated plates were obtained. All coating steps were carried out under aseptic conditions in laminar air flow.

Antibacterial properties

Bacterial inhibition was performed using the method described by Acar et al. [12]. *S. aureus* bacteria (strain ATCC 25923) were cultured and uniformly distributed over the total area of Mueller-Hinton (MH) culture agar medium in a Petri dish.

All samples (7 mm×5 mm titanium discs) were sterilised with ethylene oxide. VCM-loaded PDLA titanium alloy plates were placed in the Petri dishes with the coating

facing the agar medium ($N=5$). Titanium alloy plates without antibiotic were used as negative controls and placed in the Petri dishes with the coating facing the agar medium ($N=5$). Finally, all agar Petri dishes were incubated in an incubator for 24 h at 37°C. The inhibition zones forming around the discs were measured.

Biocompatibility

This reaction deals with the evaluation of biocompatibility of VCM-PDLA loaded plates by methods that measure acute systemic toxicity, skin sensitisation, as well as haemolytic and cytotoxicity activities. In this study, ISO10993-4, ISO10993-5, ISO10993-10, ISO10993-11 procedures were followed [13–16].

L929 cell lines (rat fibroblasts) were obtained from Cell Laboratory of Daping Hospital, Third Military Medical University (Chongqing, China), and were cultured in Dulbecco's Modified Eagle Medium (DMEM, Gibco, USA) containing 10% foetal calf serum in a incubator at 37°C and 5% CO_2 .

Test animals included ten Kunming mice (five male and five female, six weeks old, body weight 20 ± 3 g) in an acute systemic toxicity test, one healthy adult New Zealand white rabbit (male, 2350 g) in a haemolytic test, and 30 albino guinea pigs (15 male and 15 female, weighing 300–350 g) in a skin sensitisation test. All the animals were obtained from the Animal Centre of Daping Hospital, Third Military Medical University (Chongqing, China) and raised in an SPF laboratory.

Acute systemic toxicity test (systemic injection test) [16]

In the test methods for acute systemic toxicity, ten Kunming mice were divided into two groups. Extracts of VCM-PDLA loaded plates in normal saline prepared at 37°C for 72 hours were used for the test group (sample extracts). For the control group, normal saline samples alone were used. The test material extracts and normal saline were injected (50 ml/kg) into mice via peritoneal injection. The animals were observed immediately, then at 24, 48, and 72 hours after treatment for adverse signs, such as prostration, cyanosis, dyspnea, and were weighed daily.

Haemolytic test [13]

Venous blood (10 ml) from one healthy adult New Zealand white rabbit was taken to which 0.5 ml of 2% potassium oxalate solution (Kelong chemical Reagent Factory, ChengDu, China) was added. Eight millilitres of the blood with potassium oxalate were taken, added to 10 ml normal saline and then treated with the test group (sample extracts), the negative group (normal saline) and the positive group

(double distilled water). These solutions were incubated for 30 min at 37°C and were centrifuged at 2000 r/min for 15 min. The optical density (OD) value of the supernatants was measured at 545 nm using a BioSpec-mini spectrophotometer (Shimadzu, Japan). Haemolytic indexes of the test samples were calculated according to optical density (OD) value.

Skin sensitisation assay (Buehler test) [15]

In the sensitisation test, 30 albino guinea pigs were divided into three groups. On the day before the test, the fur on the back of the animals was shaved. Sample extracts were used as a test group, 0.9% NaCl as negative control, and 5% formaldehyde (Kelong chemical Reagent Factory, ChengDu, China) as positive control. A 20-mm×20-mm four-ply sterile gauze patch was immersed in each of the three group solutions and then applied directly to the pre-shaved skin under an occlusive dressing for six hours and removed. This procedure was repeated once a week for three weeks. Fourteen days after the last induction application all test and control animals were challenged with the patch by using the same procedure. An evaluation of irritancy was carried out, recorded at 1, 24, 48 and 72 hours after the application. An overall product irritancy value was calculated for each animal. Sensitisation was considered positive when the animals in the group showed an average irritancy value of 2 or more.

Cytotoxicity test [14]

VCM-PDLLA loaded plates were sterilised by ethylene oxide (EO). Plates were extracted with DMEM (0.2 g/ml) for 24 h at 37°C, in a 5% CO₂ incubator (sample extracts). Phenol solution (0.64% v/v, Kelong chemical Reagent Factory, ChengDu, China) represented the positive control. DMEM was chosen as the negative control.

Third passage rat fibroblasts were suspended at the concentration of 1×10^4 cells/ml, and the cell suspensions were inoculated in 96-well plates (200 µl/well). The cells were cultured in an incubator at 37°C and 5% CO₂ for 24 hours. Then the culture medium was removed and replaced by sample extracts, as well as negative and positive control solutions. Cells were thereafter cultured for 24 hours and were subject to MTT (methyl tetrazolium, Sigma, USA.) assay at 24, 48, and 72 hour incubation periods. After selecting eight wells in each group, 20 µl MTT dye (5 mg/ml) was added to each well and incubated for four hours at 37°C and 5% CO₂. Then, 200 µl dimethyl sulphoxide solution (DMSO, Sigma, USA) was added to dissolve the formazan crystals. The OD values of the formazan solutions were measured using an enzyme linked immunosorbent

assay microplate reader at 490 nm (ELx800, Bio-Tek Instruments, Inc.). The proliferation ratio was defined as the increased proportion of cells compared to the control.

Statistical analysis

Data was expressed as the mean ± standard deviation (SD). Statistical differences were analysed using Student's *t* test. A value of *P*<0.05 was considered statistically significant. SPSS software version 10.0 (SPSS, Inc., Chicago, Illinois) was used for statistical analysis.

Results

Bacteriostasis experiments

With regard to the effect of bacteriostasis, the VCM-PDLLA loaded plates had more of a bacteriostatic effect with evident inhibition zones (4.25 mm), compared with the PDLLA plates with no inhibition zones.

Biocompatibility

In this study, the biocompatibility of the VCM-PDLLA loaded plates was examined. The biocompatibility studies were undertaken with respect to acute systemic toxicity, haemolytic testing, sensitisation, irritation and cytotoxicity.

Acute systemic toxicity was performed by qualitative evaluation of toxicology symptoms. The animals were observed for toxicological symptoms or death at 24, 48 and 72 hours following injection. Response to systemic injection was evaluated according to ISO 10993-11 [16]. During the 72 hour observation period, mice treated with normal saline exhibited no adverse physical symptoms such as prostration, cyanosis, dyspnea, abdominal irritation, diarrhea and tremor [16]. None of the animals treated with the test sample extracts showed a different biological reaction from the animals treated with the normal saline. All mice were normal and showed no adverse physical symptoms after injection. There were no differences between the body weights of the control and test animals at 24, 48 and 72 hours post injection. Animals from the two groups gained body weight and appeared healthy during the 72 hour observation period. As a result, the specimens did not cause any toxicological symptoms or death and were considered to have met the requirements of the systemic injection test.

Haemolytic testing was performed according to ISO 10993-4. The VCM-PDLLA loaded plate had a 2.41% haemolytic index and is non-haemolytic; thus it has blood compatibility.

The test extracts and the blank negative control did not trigger irritation responses during the skin sensitisation assay. Therefore, the overall irritation response was zero for all test group and negative group animals. However, 100% of the animals in the positive control group responded with a score increase of 2 or more at the end of the challenge period in guinea pigs. Based on these results, the VCM-PDLLA loaded plate test extract was found to be non-sensitising under the conditions of this experiment.

In the cytotoxicity test, the MTT assay revealed that the extracts of the VCM-PDLLA loaded plate did not affected the viability of L929 cells. Following 72 hour incubation periods, the cell viability was measured using the MTT assay, and the cell relative growth rate (RGR) reached 98.61% in the test group. The cytotoxicity of these plates was in grade I according to ISO 10993-5 [14], which means these kinds of materials have very good cytocompatibility.

Discussion

Various local antibiotic delivery techniques are currently used to reduce the rate of infection. Researchers have begun to explore the materials of antibiotic-coated implants. Gollwitzer et al. [11] coated stainless steel and titanium K-wires with PDLLA loaded with gentamicin or teicoplanin and inserted them into an *S. aureus* suspension diluted with normal saline to an inoculum of 2.5×10^5 cfu/ml. The PDLLA-coated implants significantly reduced the adhesion of viable staphylococci in comparison to bare K-wires made from either titanium or stainless steel alloys. The combination of PDLLA with gentamicin, teicoplanin, or both antibiotics on the implant reduced viable counts to almost undetectable levels. Schmidmaier et al. [17] reported that gentamicin-coated intramedullary tibial nails were Communauté Européenne (CE)-certified for Europe and Canada, and several patients have already been treated with them for implant-related infection. Eight patients with open tibia fractures have been treated with PDLLA and gentamicin-coated, unreamed tibial nails (UTNs). None of these patients developed an infection in the first year of follow-up. The results suggest that a local application of gentamicin from PDLLA-coated implants might support systemic antibiotic prophylaxis by preventing implant-associated osteomyelitis. In addition to their established systemic antibiotic prophylaxis, these new methods could reduce the rate of infection in open fracture situations.

In this study, implants were coated with biodegradable PDLLA by a solvent-casting technique. After chemical processing, however, we questioned whether VCM would retain its biological activity. VCM's antibacterial effect was quantified by the inhibition zone. From the results, VCM

incorporated in the PDLLA coating did inhibit the growth of *S. aureus* bacteria in vitro. The control group showed that no inhibition zones around the titanium discs were observed. Thus, this demonstrated the efficacy of VCM incorporation into PDLLA-coated implants against the growth of *S. aureus*.

No toxic symptoms were observed with any of the mice injected with VCM-PDLLA loaded plate extracts during the acute systemic toxicity test. Haemolytic tests indicated it had blood compatibility. The classical skin sensitisation test in guinea pigs revealed no significant skin reactions. Finally, cytotoxicity testing showed the plates had good cytocompatibility. These results clearly demonstrate that VCM-PDLLA loaded plates have good biocompatibility.

In addition, one should be aware of the following problems. Given that resistance to antibiotics will remain an issue every time an antibiotic is used, any antibacterial coating must fulfill two important requirements: the optimal antibacterial substance must be chosen and the release kinetics must be tailored to minimise the time of exposure to antibiotic concentrations below the minimally inhibitory concentration (MIC) of the resident bacteria [18]. Biofilm plays a major role in *S. aureus* infection especially regarding the treatment, even though its role in the development of infection is weak [19]. These problems will be a subject of research in the future.

The weakness of this study is that the number of samples may not be enough and more samples may be needed to confirm the findings of this paper. Improvement is required for the technique in making the surface of plates smoother. It may be possible to improve this by using a different molecular weight of PDLLA or by using the automatic technique to immerse the treated plates in the mixture solution. These methods are the next step for future work.

Conclusion

VCM-PDLLA loaded plates were effective at inhibiting *S. aureus* in an in vitro model. The results of biocompatibility experiments showed that the plate had good biocompatibility.

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