Research Article

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PL/Vancomycin/Nano-hydroxyapatite Sustained-release Material to Treat Infectious Bone Defect

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Abstract: Objective: To evaluate the therapeutic effect of platelet lysate (PL)/vancomycin/nano-hydroxyapatite sustained-release material on treating staphylococcus aureus-induced infectious bone defects. Methods: 40 New Zealand white rabbits were inoculated with staphylococcus aureus to construct the chronic tibial infectious bone defect model. After incision, debridement and washing, control group 1 was not given any filling, control group 2 was filled with PL/nano-hydroxyapatite sustained release material, control group 3 was filled with vancomycin/nano-hydroxyapatite sustained release material, and the treatment group was filled with PL/vancomycin/nano-hydroxyapatite sustained-release material. Afterwards, the drug release profiles were determined in vitro and in vivo. Then, X-rays and bone specimens were used to evaluate the efficacy of the treatments. Results: TGF- β and PDGF were effectively released for 28 days in vitro. In addition, results of the inhibition zone experiment of the composite material proved that vancomycin had favorable antibacterial activity, which effectively suppressed bacteria for as long as 43 days, thus achieving the sustained-release antibacterial effect. The drug release profiles in vitro also demonstrated that the vancomycin concentration within the lesion region was the highest in composite material, and the infection in experimental rabbits was markedly alleviated. The original backbone deformity regained the normal shape, the normal bone marrow structure began to recover 6 weeks later, and the nano-hydroxyapatite transformed into the trabecula structure. By contrast, the inflammation in the control

group still existed, with no obvious new bone formation. Conclusion: The PL/vancomycin/nano-hydroxyapatite sustained-release material effectively treats chronic infectious bone defects.

Keywords: PL, Nano-hydroxyapatite, Vancomycin, Chronic infectious bone defect, Biomaterial

1 Introduction

At present, the incidence of severe bone or soft tissue damage resulting from high energy injuries, such as traffic accidents and natural disasters, is increasing which may induce infection, giving rise to chronic osteomyelitis [1]. For severe trauma patients, bone and soft tissue defects occur simultaneously with infection, and bacteria are colonized in the tissue space. As a result, the pathogenic bacteria cannot be thoroughly killed, even though early debridement is carried out, and as many as 20% of open bone defect patients will develop postoperative infection [2]. Typically, Gram-positive staphylococcus aureus accounts for the most common pathogenic bacterium leading to osteomyelitis. Vancomycin is the currently approved drug in clinics to treat staphylococcus aureus, including MRSA [3, 4]. However, there are numerous limitations in the current usage, as shown below: 1. the large systemic dosage; 2. low blood concentration in local infection; 3. short half-life of local medication, with no sustained-release effect; and 4. the incapability to induce bone regeneration. To sum up, an effective long-term infection control effect cannot be achieved, which adds to the difficulty in clinical treatment. In 1980, Klemm had first successfully applied gentamicin/polymethacrylate (PMMA) topically to prevent and treat bone and soft tissue infections, which attracted wide attention [5]. However, PMMA is also linked with numerous drawbacks; for instance, 1. it produces a large amount of heat during polymerization, which inevitably exerts great influence on the activity of the polymerized drug; 2. it cannot be degraded in vivo, which should be taken out through

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a second operation; and 3. gentamicin is associated with an extremely high clinical resistance and poor anti-bacteria capacity due to the non-standardized antibiotic application. The above-mentioned drawbacks have severely restricted its application. Given the above drawbacks, the biocompatibility and degradability should be taken into account when selecting the drug vector. To obtain platelet lysate (PL), the blood is subjected to density gradient centrifugation to obtain the concentrated platelet, followed by repeated freezing and thawing for at least 3 cycles, so that the platelets are split to obtain the liquid component [6]. Platelets possess different granules with different materials. Dense granules contain serotonin, epinephrine, histamine, Ca^{++} , adenosine diphosphate, and adenosine triphosphate. Lysosomes are other granules containing elastase, collagenase, cathepsin α -arabinoxidase, β -galactosidase, β -glucuronidase, and n-acetylglucosaminidase. A-Granules are other types of granules and carry wide ranges of growth factors, cytokines, and chemokines. Growth factors and chemokines include transforming growth factor β (TGF- β), brain-derived neurotrophic factor (BDNF), insulin-like growth factor 1 (IGF-1), vascular endothelial growth factor (VEGF), epidermal growth factor (EGF), platelet-derived growth factor AA (PDGF-AA), -AB and -BB, basic fibroblast growth factor (bFGF or FGF-2), bone morphogenetic protein 2,4,6 (BMP-2,-4,-6), hepatocyte growth factor (HGF), connective tissue growth factor (CTGF), chemokine (CXC) ligand12 (SDF-1), CXCL10, CXCL5, CXCL4 (PF4), CXCL3, CXCL2, CXCL1, and inflammatory cytokines such as: interferon γ (IFN- γ), tumor necrosis factor α (TNF- α), granulocyte-macrophage colony-stimulating factor (GM-CSF), granulocyte-colony-stimulating factor (G-CSF), interleukin8 (IL-8), IL-7, IL-6, IL-1 α , macrophage inflammatory protein 1 α (MIP-1 α), and MIP-1 β . All are involved in differentiation, proliferation, angiogenesis, ulcer closure, and repair [7-11]. Leotot et al. (2013), in an animal model, found that the mesenchymal stromal cells seeded on PL, coated with the hydroxyapatite/b-tricalcium phosphate, enhanced vascularization and bone formation in mice [12]. Babo, Centeno et al. (2016) also reported that PL integrated with calcium phosphate (CaP) cement increased cement degradation and bone formation [13]. Chakar et al. evaluated the effects of PL on rabbit's skull bone regeneration. It was reported that PL-induced vertical bone regeneration and promoted bone formation [14, 15]. In recent years, PL has been applied in research on bone tissue engineering, which apparently promotes bone regeneration and repair. This study aimed to preliminarily explore whether PL/vancomycin/nano-hydroxyapatite promoted bone regeneration and resisted

infection by integrating the respective advantages of these three through experiment in vitro as well as the animal osteomyelitis and bone defect models.

2 Materials and methods

2.1 Materials

Vancomycin hydrochloride (Eli Lilly, Japan K.K.); the Cem-Ostetic™ hydroxyapatite bone cement powder (Berkeley Advanced Biomaterials, Inc. San Leandro, CA) was used as the nano-hydroxyapatite; and 5% sodium morrhuate injection was provided by Shanghai Donghai Pharmaceutical Factory. 40 3-4-month-old healthy New Zealand male rabbits weighing about 2 kg were provided by the Laboratory Animal Center of the Affiliated First Hospital of Harbin Medical University (with licence number). The staphylococcus aureus strain was provided by the Microbiology Laboratory of the Affiliated First Hospital of Harbin Medical University.

2.2 PL/vancomycin/nano-hydroxyapatite Preparation and Characterization

Whole blood was collected from the rabbit's central dorsal auricular artery, and centrifuged in the 15 ml centrifuge tube to collect the platelet-rich plasma (PRP). Gradient centrifugation was carried out based on the different sedimentation coefficients of the blood components in whole blood. Thus, a white thin layer (namely, the platelet-rich layer) was obtained at the junction between the red blood cells (with the greatest sedimentation coefficient) and the uppermost layer of supernatant, which was hardly distinguished under naked eyes. Afterwards, a small portion of red blood cells were collected from the supernatant and below the junction for centrifugation at a higher rate, which was the sediment with high concentration of platelet. Then, the platelet sediment was obtained and re-suspended with PBS. Later, the obtained platelet suspension was subjected to 3 cycles of continuous repeated freezing-thawing ($-80^{\circ}\text{C}/37^{\circ}\text{C}$) at a time interval of about 10 min. Subsequently, the platelet membrane and other cell fragments were removed through low temperature high speed centrifugation, and the supernatant was collected, which was the PL. At last, it was preserved in the sterilized freezing tube at -80°C [16].

Vancomycin was added into the Cem-Ostetic™ nano-hydroxyapatite bone cement at a ratio of 160 mg/g, the

mixture was sufficiently mixed on the super-clean bench [17]. The platelet lysate and distilled water were added at a mass ratio of 1:1:0.5, and the resultant mixture was placed in the magnetic stirrer to stir for 3 min at 30°C. Then, the mixture was placed into the molding instrument where it began to solidify. The sample was prepared into the cylinder (with a diameter of 6 mm and a length of about 20 mm), freeze-dried, sterilized with ethylene oxide, and packaged for subsequent use. Similarly, the PL/nano-hydroxyapatite sustained-release material, and the vancomycin/nano-hydroxyapatite sustained-release material were also prepared into the cylinders at the same dimensions for later use.

A scanning electron microscope (SEM, JSM-6500LV, Jeol, Japan) was employed to observe the morphologic structure of the composite material.

An X-ray diffractometer (XRD, DX-2000, Danton Fangyuan Company, China) was utilized to analyze the crystal phase of the composite material.

Ethical approval: The research related to animals use has been complied with all the relevant national regulations and institutional policies for the care and use of animals.

2.3 *In vitro* release experiment of the PL/vancomycin/nano-hydroxyapatite sustained-release material

The PL/nano-hydroxyapatite sustained-release material, and the PL/vancomycin/nano-hydroxyapatite sustained-release material, were cut into standard pieces with a diameter of 4.2 mm and a height of 10 mm. Then, 6 pieces from each group were immersed into 2 mL phosphate buffer and preserved in a water bath at 37°C. The liquid was replaced at 24 h and periodically thereafter. On days 1, 2, 4, 5, 7, 14, 21 and 28, the eluent was absorbed, and the concentrations of growth factors TGF- β and PDGF released *in vitro* by the PL/nano-hydroxyapatite sustained-release material, and the PL/vancomycin/nano-hydroxyapatite sustained-release materials were detected, respectively, through competitive ELISA assay, so as to determine whether vancomycin affected the release of the osteogenic growth factor. Subsequently, the osteogenic growth factor release curve and the release rate were plotted; while the standard curve and the release concentration curve were also drawn.

The PL/nano-hydroxyapatite sustained-release material, and the PL/vancomycin/nano-hydroxyapatite sustained-release material pieces were placed in the agar medium covered with 0.5 McFarland (MCF) (determined

using the standard turbidity meter) ATCC25923 staphylococcus aureus, and 6 pieces were collected from each group. These pieces were then incubated in the incubator at 37°C for 24 h, the inhibition zone was measured, later the test specimens were taken out and further cultured in the fresh medium inoculated with staphylococcus aureus. Thereafter, the inhibition zone was measured once every 3 days, the medium was replaced, and the process was repeated until the inhibition zone disappeared. Subsequently, the inhibition zone diameter variation curve was plotted to compare the release effect.

2.4 Construction of the infectious bone defect model

A bone defect with the diameter of 6 mm was prepared from the highest point of the tibial tuberosity to the vertical midpoint of the posterior tibial cortex; 1.5 MCF (about 4.5×10^8 /ml bacteria) staphylococcus aureus (about 0.3 ml) was implanted into the bone marrow cavity, then, an appropriate amount of 5% sodium morrhuate was injected through the drill hole, and the drill hole was sealed with bone wax, followed by full-thickness suture. Afterwards, the animals were raised in individual cages and fed according to the uniform standard, without any form of antibiotics [18].

2.5 Design of animal study

After successful unilateral tibial modeling in 40 male adult experimental rabbits, the animals were randomly divided into 4 groups, including treatment group (n=10) and 3 control groups (n=10, respectively). The tibiae were then cut open, debrided and washed.

Control group 1: with no filling

Control group 2: filled with PL/nano-hydroxyapatite sustained-release material

Control group 3: filled with vancomycin/nano-hydroxyapatite sustained-release material

Treatment group: filled with PL/vancomycin/nano-hydroxyapatite sustained-release material in the cavity

2.6 Drug release profile *in vivo*

An appropriate amount of vancomycin was added into the blank rabbit plasma to prepare the 20 ug/ml plasma, which was then gradually diluted at two-fold to 0.5 ug/ml, so as to prepare the gradient concentration standard

solutions. Later, the processed samples were detected through high-performance liquid chromatography (HPLC) and the standard equation of a curve was obtained.

Collection of peri-lesion soft tissues: 1, 2, 3, 6 and 12 weeks after implantation, air was injected into the blood vessel to induce animal death. Then, about 20 mg soft tissue was taken from the subcutaneous soft tissue at 5 mm away from the medial tibial cortical opening at the implantation site and put into the glass homogenizer. 1 ml normal saline was added for homogenization, followed by 5 min of centrifugation at 12000 rpm to collect the supernatant, which was preserved at -70°C until detection.

Collection of peri-lesion bone tissues: 1, 2, 3, 6 and 12 weeks after the implantation, air was injected into the blood vessel to induce animal death. Then, the lesion bone tissues were collected, and the implant and bone marrow in the bone marrow cavity were removed. Later, the bone tissues were cut into pieces using the 5 mm rongeur, ground into powder with the ceramic mortar, and filtered with a 0.45 mm sieve to make sure that all powders passed through the sieve. Subsequently, 200 mg powders were weighed, 6 ml normal saline was added, and the mixture was stirred for 2 h by a constant temperature stirrer at 100 rpm and 37°C, followed by 5 min of centrifugation at 12000 rpm to collect the supernatant, which was preserved at -70°C until detection.

An appropriate amount of perchloric acid was added into each sample for precipitation, followed by 5 min of vortexing, and 5 min of centrifugation at 12000 rpm to collect the supernatant, and then the sample was loaded onto the HPLC. The liquid phase conditions were as follows: mobile phase methanol: pH 3.2 10 mmol/L, KH₂P0₄ 79:21 and detection wavelength of 236 nm.

2.7 X-ray and histological analysis

At 4, 6 and 12 weeks after surgery, radiographic examination was carried out to observe the lesion changes at the projection distance of 70 cm under the exposure conditions of 50 KV, 0.08 s and 120 MA. Efforts were mainly made to observe the density change in the lesion region, callus growth and medullary cavity reconstruction. The scores were rated by one radiologist blind to the grouping according to the Lane-sandhu standard and the modified Norden osteomyelitis scoring method.

At 6 and 12 weeks after surgery, specimens from bone-defect sites were fixed using 10% paraformaldehyde and embedded with paraffin. Histological examinations (HE staining and Masson staining) were evaluated in decalcified cross-sections of bone (5 mm).

2.8 Statistical analysis

All data in this experiment were statistically analyzed using the SPSS software. The quantitative indexes were expressed as means± standard deviation and analyzed by t-test. A difference of $P < 0.05$ was deemed as statistically significant.

3 Results

3.1 Characterization

The morphology of the composite material was observed under a scanning electron microscope (SEM, JSM-6500LV, Jeol, Japan) (**Figure 1**). The granularity of antibacterial composite material was apparently smaller than that of pure nano-hydroxyapatite, which might be ascribed to the fact that the vancomycin particles filled in the n-HA pore. The porous material had a coarse surface, on which numerous micropores with various diameters were distributed. Microscopic findings revealed that crystals were tightly bound to each other, and the porosity and gap of composite material were apparently increased than those of nHA. However, there was no new crystalline phase, indicating that the added vancomycin and PL existed independently in the amorphous form, without degeneration. Therefore, they did not affect the effects of vancomycin and PL.

In the X-ray diffraction (XRD) spectrum of the composite material (**Figure 2**), the $2\theta = 31.8^\circ$ (300) and $2\theta = 25.9^\circ$ (002) represented the major diffraction peaks of the nano-hydroxyapatite crystal [19]. Besides this, the nHA crystal maintained the original hexagonal crystal form before and after polymerization, but the major diffraction peak width became wider, and the back bottom became higher. The addition of organic matter in the composite product elevated the back bottom, suggesting that the addition of organic matter increased the amorphous phase in the composite product. In addition, the increased colloid component also resulted in the poor crystalline of the composite product, compared with that of the pure nano-hydroxyapatite, but the composite product maintained the original hydroxyapatite morphology on the whole.

3.2 *In vitro* release experiment

This experiment quantitatively analyzed the release of TGF- β and PDGF between the PL/nano-hydroxyapatite

Figure 1. A scanning electron microscope was employed to observe the morphologic structure of the composite material: **a** the pure nano-hydroxyapatite; and **b** PL/vancomycin/nano-hydroxyapatite sustained-release material.

Figure 2. The X-ray diffractometer was utilized to analyze the crystal phase of the composite material. **a** PL/vancomycin/nano-hydroxyapatite sustained-release material; and **b** the pure nano-hydroxyapatite.

sustained-release material and the PL/vancomycin/nano-hydroxyapatite sustained-release material and discovered that the two had similar release curves (**Figure 3-4**). In other words, the addition of vancomycin did not have an obvious influence on the release of growth factors by PL. The PL/vancomycin/nano-hydroxyapatite sustained-release material peaked on the second day, and the growth factor concentration was gradually reduced with the increase in time. Meanwhile, stable release concentration was obtained, and the concentrations of TGF- β and PDGF reached 14.224 pg/ml and 16.623 pg/ml on day 14.

Ji-Le Jiang et al. proved that, the vancomycin-loaded nano-hydroxyapatite pellets were able to kill the methicillin-resistant staphylococcus [20]. Moreover, Michela Mori et al. discovered that calcium alginate particles for the combined delivery of platelet lysate and vancomycin hydrochloride were capable of killing *Staphylococcus aureus* cells in chronic skin ulcers [21]. To further determine whether the vancomycin anti-bacterial activity in the composite material was affected, the inhibition zones of the PL/nano-hydroxyapatite sustained-release material and the PL/vancomycin/nano-hydroxyapatite sustained-release material were determined. Statistical analysis revealed no obvious differences in the *in vitro* release anti-bacterial activities between the vancomycin/nano-hydroxyapatite sustained-release material and the PL/vancomycin/nano-hydroxyapatite sustained-release material. The American Clinical Laboratory Standards Institute (CLSI) has reached the global standard of use (the standards are formulated by the CLSI Susceptibility Testing Sub-committee) through consensus assessment based on the staphylococcus M2-disk diffusion method that, the effective inhibition zone diameter of vancomycin is > 14 mm. The effective anti-bacterial duration of vancomycin can be as long as 43 days, which attains the sustained-release anti-bacterial effect (**Figure 5**).

4 weeks after surgery, the anteroposterior and lateral position X-ray films of tibia were taken, and the modified Norden Osteomyelitis score [22, 23] was (5.89 ± 1.11) points. Meanwhile, all models were successfully constructed, and no death was reported. No animals died during this study, and no local or systemic side effect of PL or nHA or vancomycin was observed either.

3.3 Drug release profile in vivo

The CLSI has reached the global standard of use (the standards are formulated by the CLSI Susceptibility Testing Sub-committee) through consensus assessment

Figure 3. TGF- β release curve of nanocomposite sustained-release materials in vitro

Figure 4. PDGF release curve of nanocomposite sustained-release materials in vitro

Figure 5. 2-D Point-Line Map of Median and Time Relation of the Inhibition Zone

that, the minimum inhibitory concentration (MIC) of vancomycin on the staphylococcus is ≤ 4 $\mu\text{g/ml}$. Our experimental results indicated that, after the composite material was implanted into the body, the highest average vancomycin concentration in the surrounding tissues at 5 mm away from the implant was 32.661 $\mu\text{g/ml}$, and that at week 6 was as high as 3.087 $\mu\text{g/ml}$. In addition, the highest average vancomycin concentration in the lesion bone tissues was 501.665 $\mu\text{g/ml}$, and that at 12 weeks was 4.188 $\mu\text{g/ml}$, always higher than the MIC of pathogenic bacteria. The effective anti-bacterial concentration was maintained for 12 weeks, which satisfied the requirements of course of treatment for chronic osteomyelitis (**Figure 6, 7**).

3.4 X-ray analysis

Lane-sandhu X-ray scoring results at 4, 6 and 12 weeks after surgery: differences between treatment group and control groups 1, 2, 3 were statistically significant ($P < 0.05$); differences in control groups 2 and 3 were statistically significant compared with control group 1 ($P < 0.05$) (**Figure 11**).

Modified Norden osteomyelitis scoring results at 4, 6 and 12 weeks after surgery: differences between the treatment group and control groups 1, 2, 3 were statistically

significant ($P < 0.05$); differences in control group 3 were of statistical significance compared with control groups 1 and 2 ($P < 0.05$) (**Figure 12**).

3.5 Histological analysis

At 6 and 12 weeks after surgery, the rabbits were sacrificed to collect the lesion tissues for pathological sectioning, followed by HE staining and Masson staining. (Solid arrow: implant material; Hollow arrow: fibrous tissue and bone matrix; Star: trabecular bone and lamellar bone structure) The percentage of new bone in the total section area after surgery was analyzed. As observed from the results in **Figure 17**, the new bone areas in 6- and 12-week after surgery groups had increased with time, and the mean new bone area in treatment group was higher than that in the control group. The data in each group at different time points were compared using t-test, and the results indicated that, the percentages of new bone area in treatment group at 6 and 12 weeks after surgery were higher than those in control group, and the differences were statistically significant ($P < 0.05$) (**Figure 17**).

Figure 6. Vancomycin concentrations in local soft tissues measured through HPLC

Figure 7. Vancomycin concentrations in bone tissues measured through HPLC assay

Figure 8. At 4 weeks after surgery, **a** (control group 1): the osteosclerotic region was enlarged, and an ectosteal abscess was clearly observed; **b** (control group 2): the osteosclerotic zone was obscure, some materials fused with the bone tissues, and an ectosteal abscess was seen; **c** (control group 3): bone regeneration and bone fusion were not obvious, and no abscess was seen; **d** (treatment group): the infection lesion became smaller, and the composite materials partially fused with the bone tissues.

Figure 9. At 6 weeks after surgery, **a** (control group 1): the osteosclerotic zone was obscure, with an enlarged ectosteal abscess; **b** (control group 2): the materials partially fused with bone tissues, and an ectosteal abscess was observed; **c** (control group 3): the material margin was ill-defined with the bone tissue boundary; **d** (treatment group): the materials favorably fused with bone tissues, the bone trabecular structure was slightly disordered, and the new bone was under reconstruction.

Figure 10. At 12 weeks after surgery, **a** (control group 1): the bone trabecular structure completely disappeared, and the medullary cavity was blocked; **b** (control group 2): the abscess faded away, and the osteosclerotic zone shrunk; **c** (control group 3): the material implantation zone gradually became obscure, and some new bone was generated; **d** (treatment group): the new bone completely fused with the materials, the new bone displayed a favorable shape, the normal bone trabecular structure was restored, and the medullary cavity was recanalized.

Figure 11. Lane-sandhu X-ray scores in each group at various time periods after surgery

Figure 12. Modified Norden osteomyelitis X-ray scores in each group at various time periods

Figure 13. **a** At 6 weeks after surgery, HE staining for histological observation suggested that, a large number of inflammatory cells were seen in control group (2x100), along with partial tissue necrosis with loose structure; **b** for control group 3x400: the implanted vancomycin/nano-hydroxyapatite sustained-released materials were partially degraded, along with some fibrous tissue ingrowth, and no inflammatory cell or abscess substance was observed; **c** for the treatment group x400: the implanted PL/vancomycin/nano-hydroxyapatite sustained-released materials were partially degraded, along with certain fibrous tissue ingrowth, and original bone trabecular structure was seen locally.

Figure 14. **a** At 12 weeks after surgery, HE staining for histological observation suggested that, **b** for control group 2x400, the implanted PL/nano-hydroxyapatite sustained-released materials were degraded, and the defect region was filled with inflammatory fibrous tissues; **c** for control group 3x400: a large number of bone matrix structures were seen and immature bone formation was also detected; for treatment group x400: the implanted PL/vancomycin/nano-hydroxyapatite sustained-released materials were almost completely absorbed and replaced by the newly generated bone tissues in the host to form the bone plate.

Figure 15. At 6 weeks after surgery, Masson staining histological observation indicated that, **a** (control group) x100: **a** large number of inflammatory cells were observed, and a small amount of ossein was generated. **b** (control group) x400: the implanted vancomycin/nano-hydroxyapatite sustained-release material was partially degraded, together with certain osteoid tissues ingrowth, and no inflammatory cell or purulent substance was seen; **c** (treatment group) x100: the implanted PL/vancomycin/nano-hydroxyapatite sustained-release material was partially degraded, along with some fibrous tissue ingrowth, and original trabecular structure occurred locally.

Figure 16. At 12 weeks after surgery, the Masson staining histological observation revealed that, **a** (control group 2) x100: the implanted PL/nano-hydroxyapatite sustained-release material was partially degraded, which was filled with a large amount of immature ossein. **b** (control group 3) x100: a large number of bone matrix structures were observed, along with immature bone formation. **c** (treatment group) x400: the implanted PL/vancomycin/nano-hydroxyapatite sustained-release material was almost completely absorbed and replaced by the newly formed bone tissues in the host, and the bone lamella was formed.

Figure 17. New bone percentage in the total section area of each group at different time periods

4 Discussion

Infectious bone defects remain a major challenge in orthopedics, and conventional antibiotic treatment cannot achieve favorable outcomes due to the special trabecular structure in bone tissue. Some scholars have discovered that the bacteria within the lesions are only temporarily declined, even after the absolutely restrict debridement, and they will rapidly multiply without sufficient local antibiotic concentration. However, systemic medication is associated with great toxic and side effects, which cannot be maintained for a long time, and the blood concentration will decline rapidly [24]. Thus, it remains an urgent problem to be solved at present about how to develop the one-stage treatment with sustained-released anti-inflammatory effect, osteogenic activity, bone conduction, degradability and absorbability through bone tissue engineering technique, so as to alleviate patient sufferings.

Nano-hydroxyapatite has favorable in vivo sustained-release effects, which is an ideal drug sustained-release carrier linked with the superiorities of initial release amount, unapparent burst release and long maintenance of effective concentration [25, 26]. nHA is closer to the human bone tissue composition, which favorably fills the defect, exerts the bone conduction effect, and is gradually

replaced by the bone tissue [27-29]. Nano-hydroxyapatite is an absorbable artificial bone material, which can be moulded under room temperature, solidified within a short time, and will not produce heat in reaction; besides, it is the favorable carrier of vancomycin [30]. Webster TJ et al. [31] first reported the phenomenon of osteoclast aggregation on nano-hydroxyapatite material surface. As a result, they believed that the nano-HA displayed obvious effects on enhancing the osteoclast function in vivo, and the resorption rate increased with the increase in the exposed area on material surface. In addition, the importance of micropore inside the biological ceramic materials has attracted increasing attention from scholars. Research suggests that, when the material porosity is greater than 100 μm , it has better bone growth capacity, since it provides the space that allows for cell migration [32]. Osseointegration can be accelerated through regulating the microporosity inside the material, which thereby promotes osteogenesis [33]. Additionally, the increase in microporosity apparently boosts the exposed area on the material's surface, and increases cell adhesion and protein adsorption, which facilitates angiogenesis, thereby further enhancing the new bone growth rate [34]. Some researchers implant the nano-hydroxyapatite artificial bone with autologous red bone marrow into the abdominal muscles of rabbit and have discovered that an

obvious Haversian system lamellar bone is formed in the center of composite material. Such results suggest that the combined application of two materials shows favorable bone induction effect, besides, the nano-hydroxyapatite artificial bone does not change the induced osteogenesis property of red bone marrow [35].

PRP is obtained through concentration and isolation of the whole blood, and its platelet concentration is 4-5 times higher than that in the whole blood. PL is obtained through the repeated freezing and thawing cycles based on PRP, so that the growth factors can be sufficiently released, and the platelet membrane and other immunogenic impurities can be removed, thus providing the possibilities of allograft and xenograft [36]. PL contains numerous growth factors, including the platelet-derived growth factor (PDGF), transforming growth factors β_1 , β_2 (TGF- β_1 , β_2), insulin-like growth factor (IGF), vascular endothelial growth factor (VEGF), platelet-derived endothelial cell growth factor, interleukin-1 (IL-1), epidermal growth factor (EGF), fibroblast growth factor (FGF), and platelet activating factor-4. These growth factors adhere to the target cell membrane surface after they are released and activate the cell membrane receptors. Such receptors exist in the membrane of a variety of cells, such as mesenchymal stem cells, fibroblasts, osteoblasts, and endothelial cells. These membrane receptors can further induce the internal signal proteins, form signal transduction, and activate the normal gene sequence expression of cells, such as cell proliferation, osteoid production, and matrix formation. On the other hand, these growth factors promote cell division and proliferation, enhance collagen synthesis, activate blood vessel growth, and induce cell differentiation. As a result, they play important roles in bone induction and bone regeneration repair [37]. Growth factors, such as TGF- β [38], IGF-1 [39], and PDGF [40], exert vital roles in bone reconstruction. For one thing, they activate osteoclasts and induce bone absorption; for another, they activate the chemotaxis, proliferation and differentiation of osteoprogenitor cells to promote osteogenic activity. Typically, these two processes take place simultaneously and accelerate each other, thus completing bone reconstruction. The major molecular signal mechanism is to tightly associate bone adsorption with bone formation through the RANK (receptor activator of NF-kappa B) /RANKL (ligand) /OPG (osteoprotegerin) system, thus maintaining the skeleton system integrity [41, 42]. In the extracted PL, cytokines PDGF and TGF- β have the greatest contents, which are the important coupling factors to maintain the dynamic balance between bone adsorption and bone formation. Therefore, different from the single-factor effect, the interventional pattern of PL

on bone reconstruction is relatively complicated, which is not the isolated effect on promoting osteogenesis or bone resorption. PL is the carrier system for multiple growth factors, and it has complex and diverse biological effects. Nonetheless, its mechanism of action in promoting bone reconstruction, which is different from the simple superposition of single factor effects, remains to be further explored.

Upon histological observation, the mean new bone areas in the treatment group at 6 and 12 weeks after surgery were higher than those in control group ($P < 0.05$). At the early stage, the inflammatory cells around the composite material were remarkably reduced, a large number of osteogenic cells appeared, and osteoblasts together with the secreted osteoid substances were observed in the junction. At the middle stage, a large number of calluses were formed, and the osteon rudiment occurred in the calluses. At the late stage, the local lamellar bone structure was preliminarily formed under the action of material degradation and osteoclasts, and many calluses were formed and reconstructed into the mature osteons. To sum up, the PL/vancomycin/nano-hydroxyapatite sustained-release material achieved a favorable sustained-release effect and displayed anti-infection and osteogenic properties.

5 Conclusions

In this study, PL is prepared through two centrifugation and three freezing-thawing cycles. In brief, vancomycin, PL and nano-hydroxyapatite are mixed at a certain ratio to prepare the novel composite biological stent material. Afterwards, the characterization properties of the composite material are analyzed, and its basic crystal form is not markedly changed. The increased material porosity contributes to body fluid flow, cell migration and adhesion. The porous composite material exhibits the coarse microstructure surface, which has favorable porosity and is suitable as a bone graft substitute material. There is no obvious difference in the release of growth factors between PL/vancomycin/nano-hydroxyapatite sustained-released composite material and PL/nano-hydroxyapatite composite sustained-release material, and the former shows sustained-release action. All in all, the PL/vancomycin/nano-hydroxyapatite sustained-released composite biological material has superior capacity in repairing the infectious bone defect in rabbit, along with favorable anti-bacterial and osteogenic capacities, which provides a new method for treating infectious bone defects in the future.

Conflict of interest: Authors state no conflict of interest.

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