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Original Article

Preparation of compound ornidazole/pefloxacin PLGA microspheres and evaluation of the pharmacological effect on chronic periodontitis

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Abstract: The aim of this work was to preparation of ornidazole/pefloxacin-loaded PLGA sustained microspheres with optimized characteristics and in vivo evaluation of the pharmacological effect on chronic periodontitis. Double-emulsion solvent evaporation technique was used to prepare the microspheres. After optimization through orthogonal, the microspheres were smooth, in good balling index and in less adhesion state, with particle sizes in normal distribution (14~30 μm). The encapsulation efficiency of ornidazole and pefloxacin was 68.15 ± 0.40% and 64.07 ± 0.37% respectively, drug loadings was 6.18 ± 0.15% and 3.95 ± 0.21% respectively. Over time, the drug release rate remained stable. At 20 d, ornidazole in vitro released about 95% by degrees, pefloxacin released above 97%. These results indicated that microspheres prepared were suitable for local injection treatment of periodontitis. Subsequently, the microspheres exerted no obvious influence on the proliferation of periodontal ligament fibroblasts by MTT assay. In the treatment of rats with periodontitis mode, the local sustained release microspheres could significantly reduce the numbers of inflammatory cell infiltration and inhibit destruction of cementum. In conclusion, these microspheres prepared by this method were adjuvant to periodontal instruments with certain advantages and potential values.

Keywords: Ornidazole, pefloxacin, PLGA, sustained released microspheres, optimization of formulation, chronic periodontitis

Introduction

Periodontitis is one of the most common infections characterized by the formation of periodontal pockets and is a major cause of tooth loss. According to recent WHO reports even up to 20% of adults suffer severe periodontitis, and most children and adolescents showed signs of gingivitis [1]. In recent years, several studies had shown that periodontal diseases were associated with a number of systemic diseases such as cardiovascular-disease [2], diabetes mellitus [3], adverse pregnancy outcomes [4, 5] and Alzheimer's disease [6]. These caused more attention to prevention and treatment of periodontal diseases. Though the exact mechanism underlying this disease is not yet fully understood, polymicrobial infection played important roles in the initiation and progression of periodontitis, including anaerobes and facultative anaerobes, and so on [7]. Therefore, effective control and elimination of the pathogens, is key to periodontitis treatment. Currently, the main therapy was nonsurgical scaling and root planning [8]. However, some of periodontal pathogens exist deeply in periodontal tissue and internal teeth, where scaling instruments are not get to. Hence, antimicrobial drug treatment is also required to adjuvant therapy, which can effectively inhibit bacteria growth and promote periodontal tissue regeneration.

Drug therapy includes two routes: local and systemic administration. Compared with systemic administration, an important advantage of local administration is the higher therapeutic concentration in the lesion location, decreasing bacterial resistance and side effect. It is applicable for periodontitis treated with local administration for delivering the drug directly at specific site. In order to achieve the more predictability and reproducibility to control the drug release, the oral sustain drug delivery system is aimed to design. The therapeutic effect of a drug is controlled in the lesion sites with lower and less frequent dose. Up to now, drug carrier system may take many forms: liposomes, niosomes, NPs and microspheres [9]. Poly(lactic-co-glycolic acid) (PLGA) as a biodegradable polymer in microsphere production has been utilized extensively to develop formulations with a sustained release of one therapeutic agent, due to its attractive properties, including the availability of various co-polymer compositions and molecular weights, which makes the manufacture of microspheres with tailored characteristics accessible [10].

Ornidazole is a member of nitromidazoles and is widely used in treatment of oral diseases, which has better activity against anaerobes. Pefloxacin mesylate is a member of quinolones, with better activity against facultative anaerobes. As previously mentioned, anaerobe is dominant microbial population, but facultative anaerobe also plays a part role in deep periodontal tissues [11]. To generate significant antimicrobial activity, we designed the compound ornidazole and pefloxacin mesylate sustained microspheres. When examining the physical and chemical properties of drugs, we found that pefloxacin mesylate dissolved easily in water and was insoluble in organic solvent. This pattern is opposite of ornidazole. Hence, the microspheres were prepared by putting pefloxacin mesylate in inner water phase, ornidazole in oil phase, using a water-in-oil-in-water (W1/O/W2) double-emulsion solvent evaporation technique.

In this study, we aimed to preparation of ornidazole/pefloxacin-loaded PLGA microspheres with optimized characteristics and *in vivo* evaluation of the pharmacological effect on chronic periodontitis. For optimizing properties of microspheres, the influence of PLGA concentration in the organic solvent (mg/mL), the proportion of organic solvents (V/V) and the speed of shear (rpm) were investigated, according to the L9 (34) orthogonal experiment design. Then, the encapsulation efficiency, drug loading and drug release *in vitro* were measured to evaluate the quality of microspheres. In order to estimate the drug safety of ornidazole/pefloxacin-loaded PLGA microspheres, an *in vivo* study was set forth to assess effects of the microspheres on rats with periodontitis mode.

Material and methods

Materials

Poly (d, l-lactide-co-glycolide, PLGA) (MW 2500-0) were pursed from Boehringer Ingelheim, Germany. Polyvinyl alcohol (PVA) and 3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2-H-tetrazolium bromide (MTT) were supplied by Sigma, China. Ornidazole were obtained from Bodyguard Pharmaceutical Co., Ltd., China. Pefloxacin mesylate were pursed from North China Pharmaceutical Group Co., Ltd., China. YAKANGR (metronidazole) was pursed from Xicheng Pharmaceutical Co., Ltd., China. HPLC-grade methanol was provided by Merck, Germany. All the other reagents were of analytical grade.

Microspheres preparation by optimization condition

Compound ornidazole/pefloxacin PLGA microspheres were prepared and optimized using a water-in-oil-in-water (W1/O/W2) double-emulsion solvent evaporation technique according to previous studies [10, 12]. Firstly, an aqueous solution of pefloxacin was prepared as inner water phase (W1). A certain quantity of PLGA and ornidazole powder (10:1, w/w) dissolved in methylene chloride/ethyl acetate (1:1, v/v), as oil phase (O). Secondly, the oil phase was added to internal water phase, then by ultrasonic emulsification under ice bath, to obtain initial emulsion. Thirdly, the initial emulsion were quickly dropped into 3% PVA solution (W2) under mechanical stirring for 1.5 min at 15000 rpm, getting second emulsions. Then, the second emulsions were magnetically stirred (300 rpm) for 3 hours at room temperature to volatile organic reagent completely. Finally, the formed microparticles were collected by centrifugation (4500 rpm, 15 min) and washed with deionized water. The microspheres were freeze-dried at -70°C for 10-12 h, by adding 2% mannitol (protective agent) and sequentially freeze-dried at -45°C overnight, and then dried in 0°C for 2 h and sequentially following dried in 25°C for 2 h, to obtain the microspheres. Then, the microspheres were sterilized by 2 KGy 60 Co.

Table 1. Factors level table of orthogonal experimental design

Level	Factor		
	A (mg/mL)	B (V/V)	C (rpm)
1	80	4:1	15000
2	160	1:2	20000
3	200	1:4	25000

Here, we optimized the preparation technology by investigating the influence of PLGA concentration in the organic solvent (A, mg/mL), the proportion of organic solvents (B, V/V) and the speed of shear (C, rpm), according to the L9 (34) orthogonal experiment design (**Table 1**).

Powder properties

The spherical degree of drug carrier microspheres (α) was determined by Tablet critical state stability [13]. The hourglass method was used to detect angle of repose of microspheres (β) [13]. The density of microspheres (ρ) was also investigated by graduated cylinder. Each batch was repeated examined six times and the average was obtained [13].

Surface morphology

The morphological characteristics of microspheres were determined by scanning electron microscope (SEM) (JXA-840, JEOL, Japan) and the particle size and distribution were measured by LS 230 laser granularity analyzer (Beckman Coulter, Inc., USA) according to the method by previous studies [9, 14].

Drug loading and entrapment efficiency

To determine loading percentage of ornidazole/pefloxacin in the microspheres, 5 mg of the dried microspheres were dissolved in 1 mL of methylene chloride. The resulting solution was then diluted with methane to 50 mL, ultrasonic for 1 d, filtered through a 0.25 μm type membrane filter (Fisher Scientific) and injected into a high pressure liquid chromatography (HPLC) system for determination of the concentration as well as the amount of ornidazole/pefloxacin [15]. The HPLC system consisted of a Waters 600 pump and a Waters 2487 Dual Absorbance Detector (Waters Corp., USA) set at 277 nm. Agilent XDB C18 column (4.6 mm×250 mm, 5 μm, Agilent Technologies, Inc., Santa Clara, USA) was utilized for drug separation, while a 0.1 mol/L potassium dihydrogen phosphate dissolved in methanol solution was used as the mobile phase. The flow rate was set at 1 mL/min. The chromatography was carried out at 30°C and the injection volume was 20 μL. The calibration curve of ornidazole and pefloxacin were measured by preparing standard solution of ornidazole and pefloxacin at a series of concentration of 1, 5, 10, 20, 25, and 30 μg/mL, respectively.

The drug loading percentage and encapsulation efficiency were calculated. Encapsulation efficiency(%)=(amounts of drugs in microspheres)/(theoretical amounts of drugs in microspheres)×100 Drug loading(%)=(amounts of drugs in microspheres)/(amounts of microspheres)×100.

Drug release in vitro

The in vitro release of microspheres was measured in phosphate buffered saline (PBS, pH 7.4) containing 1% sodium lauryl sulfate at 37.5°C. In a 5 mL centrifuge tube, approximately 100 mg of microspheres were suspended in 2 mL of PBS and shaken horizontally at 100 rpm in a shaking bath maintained at 37.5°C. Samples of 4 mL were removed from the tubes at sampling times of 0.5 h, l h, 2 h, 3 h, 4 h, 5 h, 6 h, 12 h, 24 h, 2 d, 3 d, 4 d, 5 d, 6 d, 8 d, 10 d, 12 d, 14 d, 16 d, 18 d and 20 d, after centrifugation at 4000 rpm for 5 min. The medium removed from the tubes was replaced with the same amount of fresh buffer solution. The collected samples were filtered through a 0.25 μL filter and subjected to further HPLC analysis described above.

Influence on of proliferation of the periodontal ligament fibroblasts

Human PDL fibroblasts were prepared from extracted teeth for orthodontic treatment as described previously [16]. Periodontal ligament fibroblasts (PDL) were cultured from human periodontal tissues. Using blades, PDL tissue fragments were removed from the middle one third of the extracted tooth roots and washed three times with DMEM medium (Gibco BRL, San Diego, CA, USA) containing antibiotics of 300 mg/mL streptomycin, 300 unit/mL penicillin, and 0.75 mg/mL amphotericin-B (Gibco BRL). Then, PDL tissue fragments were placed in 100-mm dishes and cultured in DMEM medi-

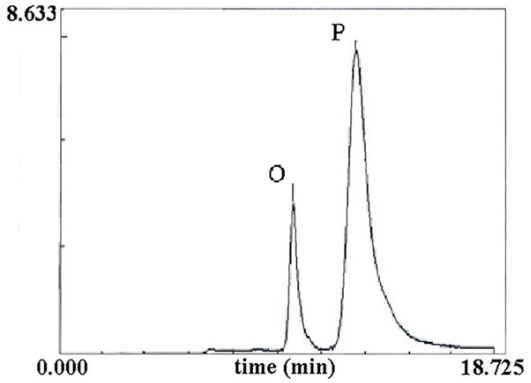

Figure 1. HPLC chromatographic data of ornidazole and pefloxacin mesylate. O: the signal of ornidazole, P: the signal of pefloxacin mesylate.

um with 10% fetal bovine serum (FBS, Gibco BRL) and antibiotics (100 mg/mL streptomycin, 100 unit/mL penicillin). The culture media was replaced every 2 days until cells grew from the fragments, after which the cultures were maintained with DMEM containing antibiotics and 10% FBS. Upon reaching 80% confluence, the cells were removed with 0.25% trypsin and passaged to 100-mm dishes. The cells used in this study had been passaged 4 times.

Cell viability was determined by MTT assay. Briefly, PDL fibroblasts were cultured in a 96-well plate at the density of 4.0×10^7 cells/well for 24 h before be treated with drug carrying microsphere, blank microspheres, ornidazole and pefloxacin standard (3:2, w/w) or negative control group for another 12 h or 24 h, respectively. Then 10 μL of 5 mg/mL MTT was added to each well and incubated with the cells at 37°C for further 4 h. After removing the cell medium, the formed formazan was dissolved with 100 μL DMSO and the optical density was measured with a spectrophotometer (Thermo Multiskan MK3, German) at 490 nm.

Evaluation on drug efficacy in vivo

Periodontitis was induced in male Sprague-Dawley rats (180 ± 20 g), by placing a thin steel ligature around the upper first molars and inoculating them with porphyromonas *gingivalis* 381. The use of animals, ethical clearance, and the study protocols were in strict accordance with the Guide for the Care and Use of Laboratory Animals as adopted by the China National Institutes of Health.

Firstly, the rats were lightly anaesthetized with surgical doses of 3% sodium pentobarbitone (35 $mg\cdot kg^{-1}$). A sterile, thin steel ligature was placed around the cervix of the maxillary right first molar. Once the rats recovered from the anesthetic, they were allowed to eat a high-sugar diet and drink 10% water with sucrose *ad libitum*. About 1 week after induction, 0.1 mL P. *gingivalis* 381 (10^{10} cells/mL) (standard strain purchased from the R&D Department of P&G Co.) was injected into the gingival crevice every other day, 5 times in all, to improve the efficiency [11, 17]. After 8 weeks, sulus bleeding index (SBI), plaque index (PI), probing pocket depth (PPD), aspartate aminotransferase (ABL), alveolar bone loss (AST-GCF) and histopathology were used to evaluate the success of the rat ECP.

A total of 84 rats were used in the current study. Of these, ECP was induced to 72 rats, while the remaining 12 rats were used in the normal group.

Group A, SRP without drug administration. Group B, systemic administration of ornidazole (SO): the rats subjected to ECP received ornidazole (100 $mg\cdot kg^{-1}$ body weight/day in drinking water for 2 weeks) after SRP. Group C, systemic administration of compound ornidazole and pefloxacin (SOPM): the rats subjected to ECP received compound ornidazole and pefloxacin (60 mg·kg-1 ornidazole with 40 $mg\cdot kg^{-1}$ pefloxacin, body weight/day in drinking water for 2 weeks) after SRP. Group D, local drug delivery of YAK-ANGR (metronidazole) (LYAK): the rats subjected to ECP received a local application of YAK-ANGR (YAKANGR is held by forceps and gently administrated into the periodontal pocket, every other day for 2 weeks) after SRP. Group E, local drug delivery of compound ornidazole/pefloxacin PLGA microspheres (LOPM): the rats subjected to ECP received a local application of microspheres (The microspheres is locally injected into periodontal tissue in 0.1 mL, every other day for 2 weeks) after SRP.

Control Groups: the normal rats and the rats subjected to ECP used for the identification of ECP comprised the negative control group and positive control group, respectively.

After 2 weeks, the following end-points of the inflammatory process were evaluated: sulus bleeding index (SBI), plaque index (PI), probing

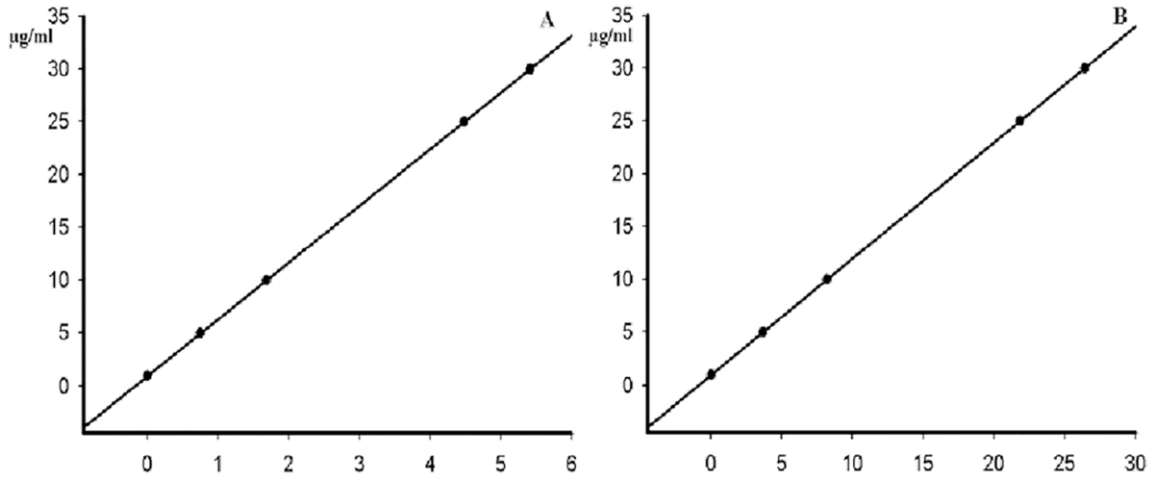

Figure 2. The standard curve. A: The standard curve of ornidazole, Y=5.3625X+0.9628, r=0.9999, B: The standard curve of pefloxacin mesylate, Y=1.0984X+0.9831, r=0.9998.

Table 2. Results analysis of orthogonal experiment

Lever	Factors			Mean value of encapsulation efficiency (%)
	A (mg/mL)	B (V/V)	C (rmp)	
1	1	1	1	33.46
2	1	2	2	35.18
3	1	3	3	42.30
4	2	1	2	31.65
5	2	2	3	47.48
6	2	3	1	45.62
7	3	1	3	34.57
8	3	2	1	67.10
9	3	3	2	59.43
K1	36.98	33.23	48.73	
K2	41.58	49.92	42.09	
K3	53.70	49.12	41.45	
R	16.72	16.69	7.28	

Note: K1, K2 and K3 were total average of three levels, respectively; R, reengage value.

pocket depth (PPD), gingival crevicular fluid-aspartate aminotransferase (GCF-AST), alveolar bone loss (ABL), and HE staining.

Fertility toxicity on rats

A total of 100 numbers were randomly divided into negative control, low-medium-high-dose group and intervention group by random digits table on male and female SD rats respectively, with 20 animals in each groups. Those rats of low-medium and high-dose group were fed

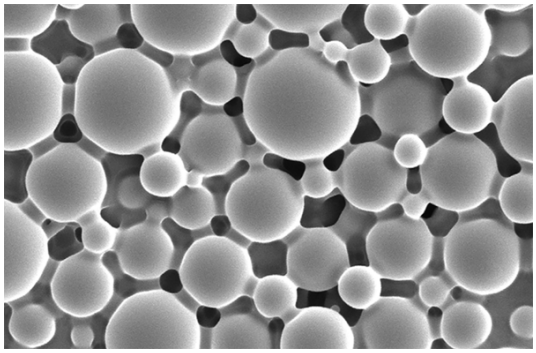

Figure 3. Representative scanning electron microscopy of ornidazole/pefloxacin PLGA sustained-release microspheres (magnification×800).

daily the sustained release microspheres at 1 g/kg, 4 g/kg, and 8 g/kg respectively, those of the negative control group were fed daily distilled water, for continuously 42 days on male SD rats, from 14 days of unmating to the 7th day of pregnancy continuously on female SD rats; and those of the intervention group give cyclophosphamide (CP, 40 mg/kg) by intraperitoneal injection, for continuously 5 days. Then, the male rats were killed after a successful mating; the female rats were sacrificed in on the 15th day in pregnancy. During the period of administration, the appearance, actions, drinking and death situation on rats were observed daily; after those of rats were killed, there are many relative property observation of major organs such as heart, lung, kidney, liver, spleen, should be observed, and anomalies information were recorded. In the period of manage-

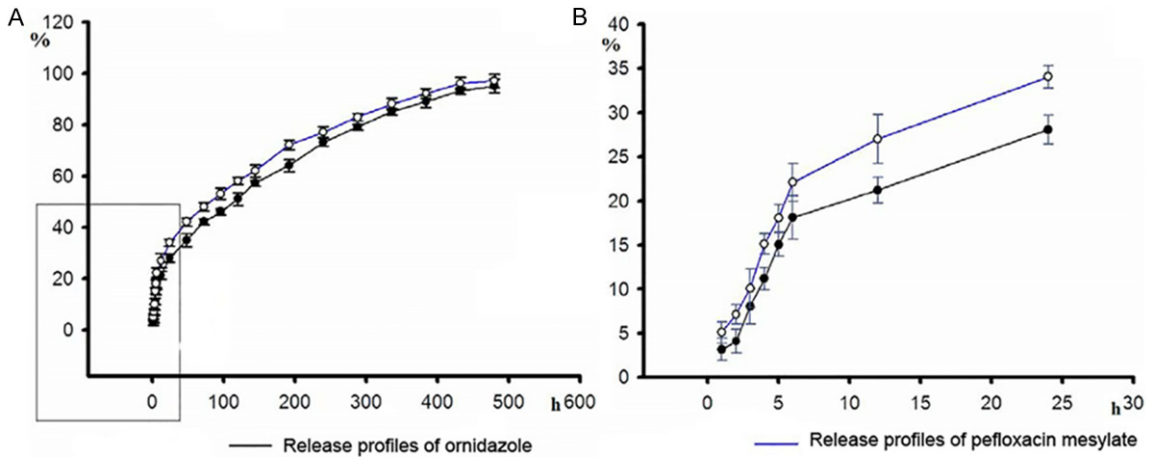

Figure 4. Cumulative drug release curves of ornidazole and pefloxacin *in vitro* study. A. Release profiles of ornidazole and pefloxacin mesylate from microspheres from 0 h-480 h; B. Release profiles of ornidazole and pefloxacin mesylate from microspheres from 0 h-24 h.

Table 3. Particle property of microspheres

Batch	1	2	3	$\bar{x} \pm S$
α°	35.67	37.15	35.16	35.99 ± 1.03
β°	34.30	35.45	38.56	36.10 ± 2.20
ρ°	0.3689	0.4156	0.3868	0.3804 ± 0.0311

ment, the changes of body weight would be recorded weekly on male rats, or be recorded weekly before unmating and per 5 days after mating on female rats. There is mating test on those of rats with 1:1 male and female rats, meanwhile, successful mating time, the fertility, pregnancy force were observed respectively; the coefficient on testis, epididymis, ovary and uterus, were also performed respectively.

Statistical analysis

Data were expressed as mean ± SD. Mean values were compared by single-factor analysis of variance (ANOVA) and a paired t-test using the SPSS13.0 statistical software. The Kruskal-Wallis and Dunn' tests were used for histopathological analysis. $P<0.05$ was considered significant.

Results and discussion

Preparing and optimizing process

Here, emulsification-solvent evaporation method was used to preparation of compound ornidazole/pefloxacin PLGA sustained-release microspheres. Duo to two drugs existing in microspheres, a method of high performance liquid chromatography (HPLC) was performed to detect these two drugs content. In **Figure 1**, the retention time (Rt) of ornidazole was 10.299 min, while the Rt of pefloxacin was 13.107 min. The separation effect was good enough to test signals of these two drugs. And then both of the two drugs had good linearity over a proper range (1~30 μg/mL). The standard curve of each drug was provided in **Figure 2** with the linear equation and correlation coefficient. According to this method, the encapsulation efficiency and drug loading could be measured.

Previous studies had shown that many factors could affect the performance of the microspheres, such as polymer materials, organic solvents, drugs, emulsification time [18]. Here, with the encapsulation efficiency as the main investigation index, the influence of PLGA concentration in the organic solvent (A, mg/mL), the proportion of organic solvents (B, V/V) and the shear speed (C, rpm) were optimized through orthogonal test on 3 factors 3 levels (**Table 1**). These results were summarized in **Table 2**. Range (R) as a measure of variation is the difference between the maximum and minimum values. The greater range represented that the factor had greater influence of the whole system. Here, it showed that R A>R B>R C. These results suggested that the PLGA concentration in the organic solvent had greatest influence of the encapsulation efficiency, following by the proportion of methylene chloride and ethyl acetate, finally for the shear rate. In various factors, optimal levels were A3, B2, C1, respectively. Hence, the best prescription was

Table 4. Drug loading and encapsulation efficiency of microspheres

Batch	Drug loading (%)		Encapsulation efficiency (%)	
	Ornidazole		Pefloxacin mesylate	
1	6.23	4.13	67.75	63.69
2	6.02	3.99	68.54	64.10
3	6.30	3.72	68.17	64.64
$\bar{X} \pm S$	6.18 ± 0.15	3.95 ± 0.21	68.15 ± 0.41	64.07 ± 0.37

selected as the PLGA concentration in the organic solvent at 200 mg/mL, the ratio of methylene chloride/ethyl acetate at 1:2 and the shear rate at 15000 rpm.

Powder properties and surface morphology

After optimization, color of powder was white. Under Light and scanning electron microscopy, the microspheres were smooth, with good balling index and less adhesion state (**Figure 3**). Roundness degree of powder (α), repose angle of powder (β) and bulk density of powder (ρ) were used to evaluate the pelletizing ratio of microsphere, friction liquidity and solubility, respectively. The better α was, the better liquidity was. Usually, as α≤30°, microspheres had best pelletizing ratio. While, when α≤40°, microspheres had better pelletizing ratio to meet the needs of production and use [13]. Similarly, as β≤30 and ρ≤0.5 g/mL, microspheres had good liquidity to meet the demand in the process of production. Here, α, β and ρ of microspheres prepared were 35.99 ± 1.03, 36.1 ± 2.20, 0.3804 ± 0.0311 g/mL, respectively (**Table 3**). According to the evaluation criteria of powder properties, microspheres prepared in this study exhibited a good liquidity to meeting the requirements of Chinese Pharmacopeia. By screening through molecular sieve, the particle sizes of microspheres were in normal distribution (14~30 μm); the mean value was 21.60 ± 2.41 μm, in accordance with the demand of the local administration. All the data above indicated that microspheres prepared by this optimized method with good powder and surface morphological properties were appropriate for local administration to rat via the intramuscular injection.

The encapsulation efficiency, drug loading and in vitro release study

After optimized processing method, the encapsulation efficiency of ornidazole and pefloxacin was 68.15 ± 0.40% and 64.07 ± 0.37% respectively, according to the calculation formula provided above. Similarly, the drug loading was 6.18 ± 0.15% and 3.95 ± 0.21% respectively (**Table 4**). At this point, the drug sum of the two drugs remained at around 10%, meeting the requirements of Chinese Pharmacopeia.

In vitro release study, from the first day to the 20th day, both drugs release rate remained stable, suggesting these drugs were well ent-rapped. At 20 d, ornidazole in vitro released about 95% by degrees and pefloxacin released above 97% (**Figure 4A**). It was shown as **Figure 4B** that ornidazole in vitro released about 25% by degrees and pefloxacin released about 35%, at 24 h. The burst release effects were obs-erved. Up to now, the strategy to removing the "sudden release effect" thoroughly has not been found. Some studies suggested that an outer layer of chitosan outside of PLGA could inhibit burst release effects [19]. However, co-mposite carrier material was not suitable for injection. In another way, to a certain extent, an advisable burst release effects produced a positive role in promoting to antibacterial effect of drugs. It could improve curative effect and control t local toxicity, as long as controlling burst release effects in a reasonable range [20]. Here, the burst release effects of drugs were controlled in 30%, meeting the requirements of Chinese Pharmacopeia.

Effect on periodontal ligament fibroblasts growth

Periodontal ligament fibroblasts (HPLFs) as the most representative type of cell groups in the periodontal tissue, play important roles in forming the main fiber, cementum and rebuilding alveolar bone. Here, HPLFs were cultured by tissue culture method and were identified through morphology and immunohistochemistry. In order to judge cell safety of these microspheres, cell toxicity in 50 mg/mL microspheres, blank microspheres, and 5 mg/mL of ornidazole/pefloxacin were investigated through MTT method in 1~7 d. The results in **Table 5** indicated that no significant effect among different treatment groups was observed on HPLFs proliferation, at different time points. It indicated that microspheres prepared by this method showed no cell toxicity and could be used to evaluate pharmacological effect on chronic periodontitis *in vivo*.

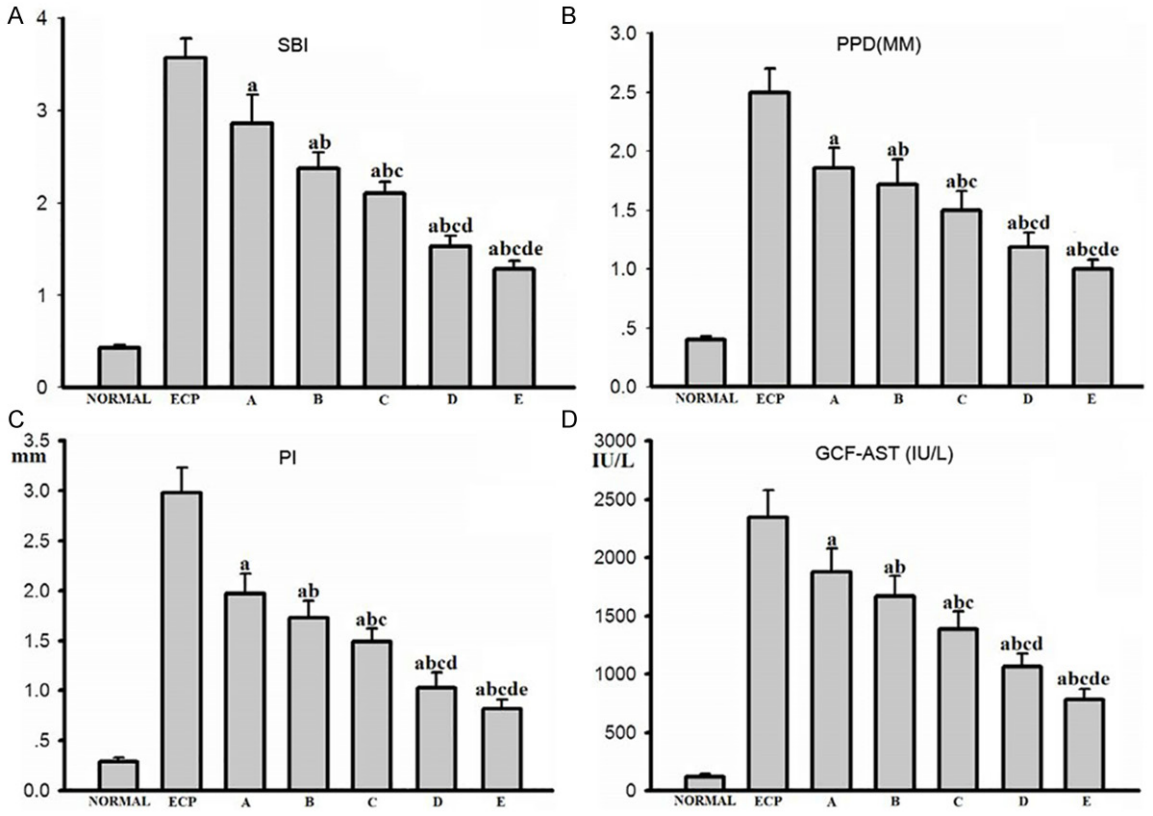

Figure 5. Effects of the SRP+antibiotics on various parameters (live) in rats subjected to ECP. n=12. A: vs. NORMAL P<0.05. B: vs. SRP+SO P<0.05. C: vs. SRP+SOPM P<0.05. D: vs. SRP+LYAK P<0.05, E: SRP+LOPM (ANOVA & Paired t-test).

Table 5. Results of proliferation of the periodontal ligament fibroblasts

Times	Microspheres (%)	Compound of ornidazole and Pefloxacin (%)	Pefloxacin mesylate (%)	Negative group (%)
0	0.289 ± 0.036	0.293 ± 0.042	0.297 ± 0.064	0.284 ± 0.034
1	0.309 ± 0.072	0.313 ± 0.067	0.318 ± 0.026	0.298 ± 0.004
2	0.336 ± 0.008	0.328 ± 0.054	0.325 ± 0.018	0.326 ± 0.026
3	0.386 ± 0.018	0.373 ± 0.087	0.370 ± 0.060	0.372 ± 0.014
4	0.399 ± 0.045	0.391 ± 0.059	0.395 ± 0.036	0.389 ± 0.054
5	0.378 ± 0.087	0.380 ± 0.048	0.376 ± 0.049	0.374 ± 0.064
6	0.356 ± 0.026	0.358 ± 0.086	0.361 ± 0.087	0.357 ± 0.044
7	0.315 ± 0.056	0.299 ± 0.093	0.312 ± 0.009	0.314 ± 0.041

Evaluation of pharmacological effect in vivo

In order to evaluate pharmacological effect of microspheres on chronic periodontitis *in vivo*, SBI, PI, PPD, ABL, AST-GCF and histopathology were determined and were evaluated on a scale: 0 (no inflammation, healthy) to 5 (severe inflammation) at six sites of each tooth.

After 8 weeks of induction, the periodontal health indicators in ECP rats, showed statistically significant differences compared with normal rats. The mean value of SBI in normal rats was 0.43, which increased to 3.57 in ECP rats; after treatment, it was reduced to 2.37, 2.10, 1.53 and 1.28, 2.37 in SO, SOPM, LYAK and LOPM, respectively (**Figure 5A**). The mean value of PI in normal rats was 0.40, which increased to 2.50 in ECP rats; after treatment, it was reduced to 1.72, 1.50, 1.19 and 1.00 in SO, SOPM, LYAK and LOPM, respectively (**Figure 5B**). The mean value of PPD (mm) in normal rats was 0.29, which increased to 2.98 in ECP rats; after treatment, it was reduced to 1.73, 1.49, 1.03 and 0.82 in SO, SOPM, LYAK and LOPM, respectively (**Figure 5C**). The mean value of GCF-AST (IU/L) in normal rats was 121.55, which

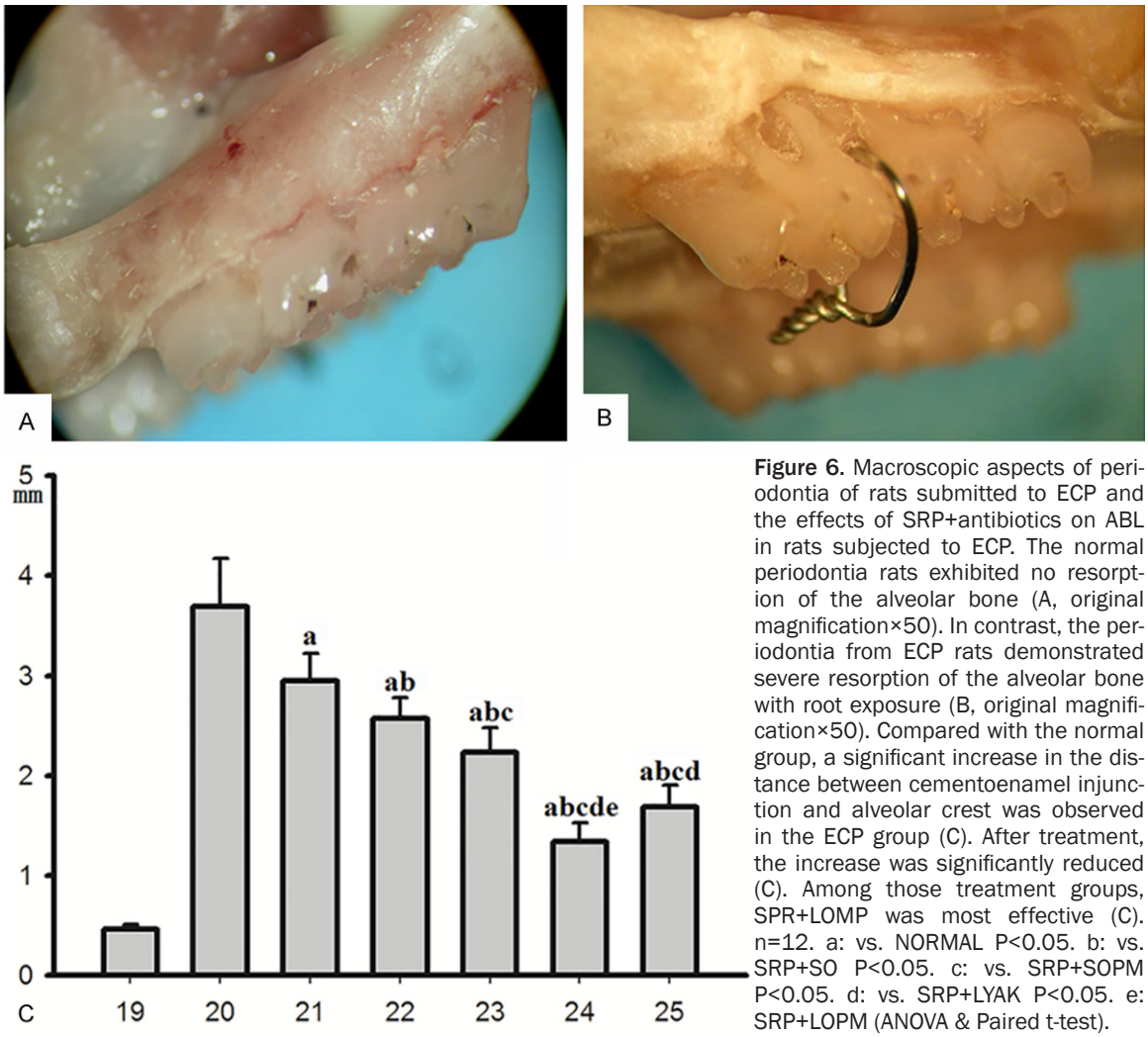

Figure 6. Macroscopic aspects of periodontia of rats submitted to ECP and the effects of SRP+antibiotics on ABL in rats subjected to ECP. The normal periodontia rats exhibited no resorption of the alveolar bone (A, original magnification×50). In contrast, the periodontia from ECP rats demonstrated severe resorption of the alveolar bone with root exposure (B, original magnification×50). Compared with the normal group, a significant increase in the distance between cementoenamel injunction and alveolar crest was observed in the ECP group (C). After treatment, the increase was significantly reduced (C). Among those treatment groups, SPR+LOMP was most effective (C). n=12. a: vs. NORMAL P<0.05. b: vs. SRP+SO P<0.05. c: vs. SRP+SOPM P<0.05. d: vs. SRP+LYAK P<0.05. e: SRP+LOPM (ANOVA & Paired t-test).

increased to 2346.70 in ECP rats; after treatment, it was reduced to 1669.50, 138-5.34, 1067.46 and 788.01 in SO, SOPM, LYAK and LOPM, respectively (**Figure 5D**). A significant ABL between the ECP group and normal group was observed (**Figure 6A**, **6B**). Compared with the ECP group, similar reductions were observed after SO, SOPM and LYAK, whereas further reduction was observed after LOPM. More specifically, the ABL (mm) in normal rats was 0.46, which increased to 3.69 in ECP rats; after treatment, it was reduced to 2.57, 2.23, and 1.69 and 1.34 in SO, SOPM, LYAK and LOPM, respectively (**Figure 6C**).

Compared with tissue sections taken from the normal group (**Figure 7A**, **7B**), histopathology observation in ECP rats revealed inflammatory cell infiltration, apical migration of the junctional epithelium, severe cementum destruction resembling a periodontitis lesion (**Figure 7C**, **7D**). The disorders of these indicators indicated the success of periodontitis mode and were consistent with the results presented in previous studies [21]. After 20 days treatment, the histopathology of the periodontium of the SO (**Figure 7G**, **7H**), SOPM (**Figure 7I**, **7J**), LYAK (**Figure 7K**, **7L**) groups after SRP revealed that all the treatment groups produced beneficial changes in the clinical indicators compared with ECP, whereas a more significant reduction in LOPM (**Figure 7M**, **7N**) was observed. Inflammatory score showed the normal group, ECP group, SO, SOPM, LYAK and LOPM received a median score of 0.0, 2.5, 1.81, 1.62, 1.38 and 0.95 (**Figure 7O**), respectively. Therefore, these results indicated that, all the combined treatment groups responded to therapy with significant resolution of the infection, adjunctive local sustained release microspheres were most effective for periodontitis.

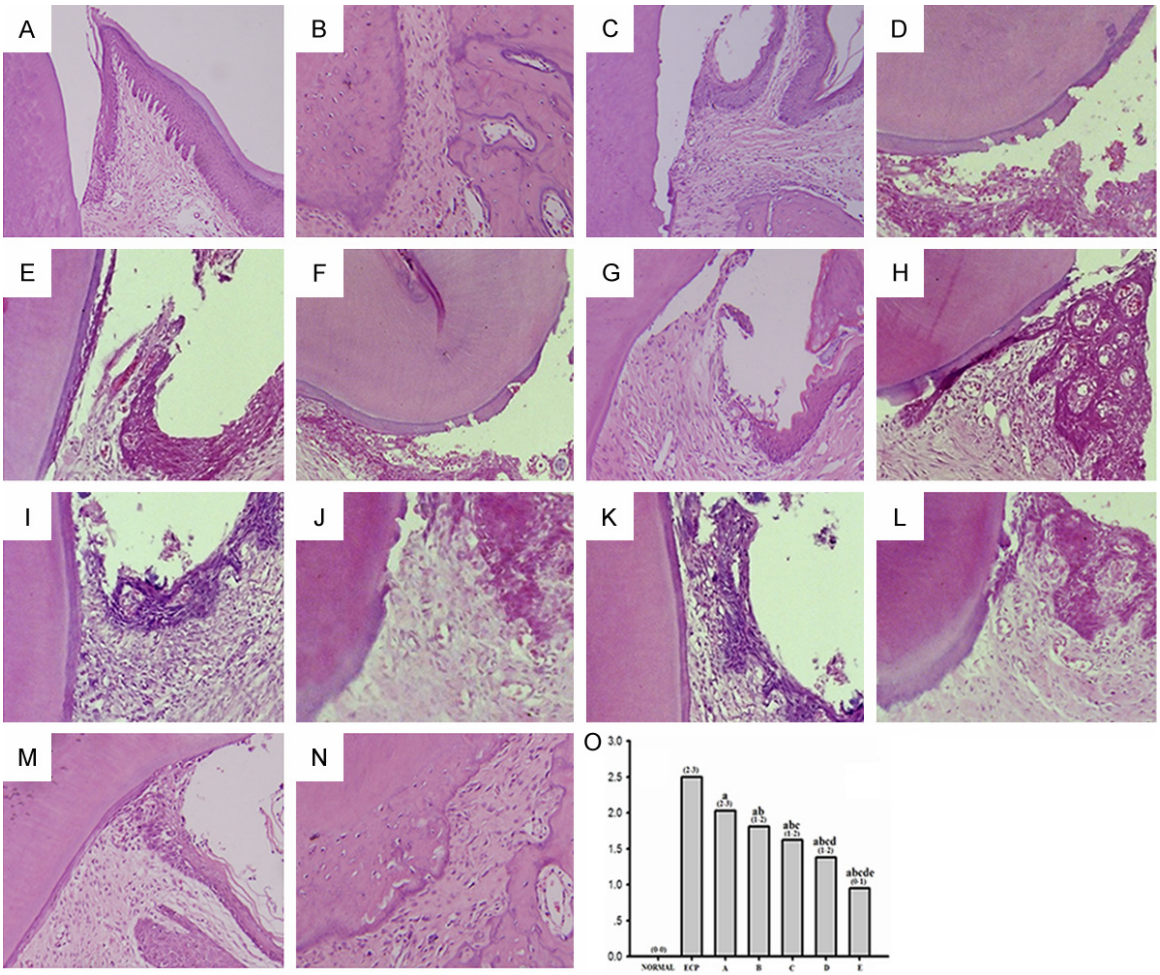

Figure 7. HE staining of the rat periodontia subjected to ECP and treated with SRP+antibiotics. Photomicrographs (A and B) showing the region around the first molars of normal rats; these indicate that the gingival, periodontal ligament, and cementum are normal. Rat periodontia subjected to ECP (C and D) showed inflammatory cell infiltration with apical migration of the junctional epithelium, as well as severe cementum destruction. SRP alone (E and F) could inhibit the destruction of periodontium to some exten. And SRP+SO (G and H), SRP+SOMP (I and J), and SRP+LYAK (K and L) all resulted in a significant reduction in the number of inflammatory cell infiltration and cementum destruction. Among those, SPR+LOPM (M and N) was most effective (HE stain; original magnification×200). According to the evaluation standard, the scores ranged from a scale of 0 to 3. Data are medians with the range within the parentheses (O); n=12. A: vs. NORMAL $P<0.05$. B: vs. SRP+SO $P<0.05$. C: vs. SRP+SOPM $P<0.05$. D: vs. SRP+LYAK $P<0.05$. E: SRP+LOPM (Kruskal-Wallis and Dunn' tests).

In the current study, we designed sustained microspheres consisting of ornidazole and pefloxacin mesylate by double emulsion-solvent evaporation method. After optimization, the microspheres had good powder properties and surface morphology. The encapsulation efficiency, drug loadings and release activity in vitro of the microspheres were suitable for local injection treatment of periodontitis. A safety evaluation of these microspheres was performed *in vitro* and *in vivo* studies. It indicated that these microspheres had no toxicity on periodontal ligament fibroblasts. In the treatment of chronic periodontitis, the pharmaceutical preparation (microspheres) was adjuvant to periodontal instruments and had certain advantages. The ornidazole/pefloxacin-loaded PLGA microspheres used as sustained release delivery systems were potential for the treatment of periodontitis in local administration.

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Disclosure of conflict of interest

None.

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