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Voriconazole into PLGA nanoparticles: Improving agglomeration and antifungal efficacy

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Abstract

This study is concerned with preparing PLGA nanoparticles loaded with voriconazole (PNLV), investigating the burst release and agglomeration of PNLV, and also evaluating antifungal efficacy of PNLV compared with voriconazole (VRC). The emulsion–solvent evaporation technique for nanoparticles and tests against fungi were completed. The amount of VRC in PNLV with sodium hexametaphosphate was 2.01 ± 0.27%, and burst release of PNLV was reduced by about 33% using 20% ethanol solution ($n = 3$). The mean D_{50} of PNLV with or without this salt was 132.8 nm and 6.3 μm, respectively ($n = 5$). *In vitro*; the fungal numbers treated with PNLV (3.5 mg/ml, equal amount calculated by VRC) and VRC (70 μg/ml) in tubes at the day 7 were 5.74 $\log_{10}$ and 6.72 $\log_{10}$, respectively ($P < 0.05$). *In vivo*; the fungal burden treated with PNLV and VRC in tissue from mice kidneys at day 7 after administration was 0.64 $\log_{10}$ and 2.61 $\log_{10}$, respectively (5 mg/kg, $P < 0.001$). The hematoxylin–eosin stain in mice kidney showed that the pathological lesions treated with PNLV were relieved in contrast with those with VRC. These results suggest that the emulsion–solvent evaporation process is feasible in preparing PNLV. Moreover, ethanol solution decreased burst release and Na-HMP inhibited agglomeration. PNLV could improve the VRC antifungal efficacy.

Keywords: Voriconazole; Agglomeration; PNLV; Burst release; *Candida albicans*

1. Introduction

Voriconazole (VRC) is modified from fluconazole and acquires an improving potency and spectrum (Naithani and Kumar, 2005). It can be administered via the oral and intravenous (I.V.) routes (Herbrecht, 2004). Although the oral bioavailability is estimated to be 96% (Leveque et al., 2006), it is likely that most physicians may not opt for oral therapy when initiating VRC due to the indications of the drug. However, beta cyclodextrin (BCD) derivatives containing in I.V. VRC increase the aqueous solubility of VRC but limit the application of the drug for its nephrotoxicity and haemolysis (von Mach et al., 2006).

Poly(lactide-co-glycolide) (PLGA) can be formulated into various carriers for different drug classes due to their favorable properties such as good biocompatibility, biodegradability, and safety (Jiao et al., 2002). PLGA nanoparticles loaded with voriconazole (PNLV) have all qualities of a delivery system of nanoparticles such as decreasing the fluctuation of drug concentration in blood and increasing drug bioavailability and, importantly, are free from BCD. Also, the continuous release of the drug in PNLV gradually degrades in the body fluid and avoids simultaneous breakdown of VRC. Therefore, the drawback of drug instability can be improved by the PNLV drug delivery system.

Abbreviations: BCD, beta cyclodextrin; *C. albicans*, *Candida albican*; CFU, clonal formation unit; HPLC, high pressure liquid chromatography; I.V., intravenous; Na-HMP, sodium hexametaphosphate; PBS, phosphate buffered saline; PLGA, poly(D,L-lactic-co-glycolic acid); PNLV, poly (D,L-lactic-co-glycolic acid) nanoparticles loaded with voriconazole; PVA, polyvinyl alcohol; SEM, scanning electron microscope; VRC, voriconazole.

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The present study aims to investigate the feasibility of PLGA nanoparticles loaded with voriconazole, improve their burst release and agglomeration, and also approach the efficacy against fungi compared with voriconazole.

2. Materials and methods

2.1. Chemicals

VRC was a gift from Jiandi Pharmaceutics Technological and Development Co., Ltd. (Harbin, China). Poly(D,L-lactic-co-glycolic acid) (PLGA, Av. Mw 29,000 Data) with a lactide/glycolide ratio of 50:50, both end-capped by a suitable ester group, was synthesized and kindly provided by Shandong Research Institute of Medical Equipment (Ji'nan, China). Polyvinyl alcohol (PVA) 88% hydrolyzed (Av. Mw 30,000) was purchased from Kuraray Co., Ltd. (Korea). All other agents were of analytical grade or higher purity. Water used in the investigation was purified through pure water systems (Pall, USA).

2.2. Animals

KunMing (KM) mice purchased from the Third Affiliated Hospital of Harbin Medical University (Harbin, China) weighing 20–22 g were used in the study. They had access to food and water freely. The Institute's Animal Ethics Committee has approved all of the animal studies. The investigations conform to the *Guide for the Care and Use of Laboratory Animals* published by the US National Institute of Health (NIH Published No. 85–23, revised 1996).

2.3. Fungal inocula

Three days before the challenge, *Candida albican* (*C. albicans*) *ATCC* 10231 was subcultured daily in Sabouraud's dextrose broth. On the day of the challenge, the subculture was pelleted and rinsed twice with 0.01 M phosphate buffered saline (PBS), pH 7.2. The final pellet was resuspended in PBS. The concentration of blastospores was counted with a hemacytometer and prepared for the following experiments.

2.4. Preparation of PLGA nanoparticles loaded with voriconazole

VRC nanoparticles were prepared as previously reported by the emulsion evaporation technique (Jalil and Nixon, 1989; Jaiswal et al., 2004). Briefly, the oil phase was poured into the aqueous phase with or without sodium hexametaphosphate (Na-HMP) (pH 7.0) and dispersed by another high-speed homogenizer (IKA, T18, German) under ice cooling temperature for 1 min at 6000 rpm. The resultant emulsion was then passed through high-pressure homogenizer (Niro Soavi S.P.A., NS1001, Italy) for three times at an operating pressure of 800 bars. The nanodispersion was collected in a glass beaker and kept for 20 h on magnetic stirrer at 500 rpm (IKA, ETS-D4, German) in fume hood in order to remove CH_2CI_2. The hardened nanoparticles were centrifuged (28,300 × *g*) for 30 min and rinsed twice with pure water, freeze dried to complete the removal of organic solvent and water, and finally stored at 4 °C. The compositions of the oil and water phase in the preparation process were described in Table 1.

Table 1
The compositions of oil phase and water phase in the preparation process

Phase	Materials	Amount
Oil phase	CH_2CI_2 (ml)	5
	PLGA (mg)	250
	Span-80 (g)	1.0
	VRC (mg)	20
Water phase	Water (ml)	100
	PVA (g)	2.5
	Tween-80 (g)	0.5
	Na-HMP (%)	0 or 3

2.5. Characterization of PLGA nanoparticles loaded with voriconazole

2.5.1. Determination of voriconazole loaded into PLGA nanoparticles

5 mg of PLGA nanoparticles were dissolved in 2 ml of acetone for 30 min. The mixture was centrifuged (9167 × *g*) for 10 min. VRC concentration in the supernatant was measured at 255 nm by reverse phase HPLC using a standard curve. The HPLC was provided with Waters 600 system composed by 2487 UV detector, and Rheodyne 7725i injector (20 μl sample loop). The analytical column purchased from Phenomenex (Jupiter, C18, 5μ, 250 mm × 4.6 mm, 300 E) was eluted with mobile phase containing 42% of acetonitrile and 58% of ammonium phosphate solution (0.04 M, pH 6.0). The flow rate was 1 ml/min. Calibration curves were constructed and the volume injected into the HPLC system was 10 μl. There was a linear relationship between 0.2 and 20 μg/ml ($y = 3{,}041{,}244x - 269{,}675$, $R^2 = 0.9991$).

2.5.2. PLGA nanoparticles size distribution and morphology

The size distribution of particles was analyzed by laser particle size analyzer (Malvern, Mastersizer 2000, UK). The colloid containing PNLV was pumped into detector pool by peristaltic pump. The volumes of the particles were expressed as the mean diameter ± S.D. Data was collected from five different batches.

Freeze dried samples were dialyzed and salts were removed followed by drying onto an aluminum lamella at room temperature. The surface morphology and size of the samples were imaged by scanning electron microscope (SEM) (Hitachi, S-4700, JP).

2.5.3. Decrease of initial burst release of voriconazole from PLGA nanoparticles

VRC samples of 2 mg for the two preparations were suspended in 1 ml PBS (pH 7.4, 0.5% SDS) or 20% ethanol and violently mixed for 5 min. Then, the supernatant was removed and the VRC content was determined after centrifu-

gation (9167 × *g*) for 30 min. The precipitate was oven dried at 110 °C for 1 h and the weight loss was determined.

2.5.4. In vitro release of voriconazole from PLGA nanoparticles

Two of four groups contained 20 mg samples of different preparations (contained or not contained sodium hexametaphosphate in the water phase) in each tube, and were dealt by PBS or 20% ethanol cleanse. Then, the precipitates and the other two groups without cleanse were suspended in 5 ml PBS and cultured at 37 °C with 50 rpm shaking in water bathing constant temperature vibrator (JinTan, SHA-B, China). At appropriate intervals, 200 μl of supernatant was collected after centrifugation (9167 × *g*) for 30 min, and 200 μl of the buffer was added and incubated. The VRC content in the supernatant was determined as described in Section 2.5.1. Experiments were carried out in triplicate for each point of release kinetics. In order to investigate the degradation of VRC during the release period, free drug was cultured under the same conditions as in the other tubes.

2.6. Antifungal effects in vitro and in vivo

2.6.1. In vitro

The yeast cells were prepared as described above in Section 2.3. 10 μl of the suspension (i.e., 5×10^4 blastospores) was dispensed into each test tube containing 5 ml of Sabouraud's dextrose broth. The groups were the PNLV group (1.75 and 3.5 mg/tube, equal amount calculated according to VRC), the VRC group (35 and 70 μg/tube), and the PBS group. The numbers of fungi in each test tube were diluted properly and counted every 24 h for 7 consecutive days by light microscope (Olympus, BX-51, JP).

2.6.2. In vivo

In vivo; the suspension was diluted with PBS to 5×10^6 blastospores/ml. 40 KM mice were used to carry out *in vivo* experiments, which included 10 mice in each group. One group was used as a negative control, and the rest were immunocompromised with 50 mg/kg hydrocortisone (Zizhu pharmaceutical Co., Ltd., Beijing, China) by intraperitoneal administration 3 days prior to the challenge. Mice were challenged intravenously via the tail vein with 5×10^5 *C. albicans*.

The model mice were treated with 200 μl VRC (5 mg/kg), PNLV (5 mg/kg, calculated by VRC), or PBS intragastric administration 24 h after challenge, respectively. Mice were sacrificed with ether anaesthesia 24 h after the final administration, right kidney tissue was then removed and ground through wire mesh. The culture suspension containing fungi was diluted to 100 multiples, and was cultured by plates. The CFU/g ($\log_{10}$ clonal forming units per g of kidney tissue) was calculated according to the CFU results in a specific plate on day 3 in culture. The left kidney tissue was embedded with paraffin and sectioned. H&E staining was performed to detect pathology changes.

2.7. Statistics analysis

Qualitative data such as VRC content loaded in PNLV, median volume diameter of PNLV, the fungal numbers in tubes, and fungal burden in the mice kidney tissue (CFU per gram of kidney tissue) were expressed as means ± S.D. The numbers of fungi in culture medium and in the animal body were analyzed by Student's unpaired *t*-test. Probability values of $P < 0.05$ were considered to be statistically significant.

3. Results and discussion

3.1. Characterization of PLGA nanoparticles loaded with voriconazole

3.1.1. Determination of voriconazole loaded into nanoparticles

According to the results of HPLC, the amount of VRC in PLGA nanoparticles with sodium hexametaphosphate and that in particles without salt were 2.01 ± 0.27% and 1.73 ± 0.24%, respectively ($n = 3$). This result may be due to several reasons such as the salt in the water phase increased the ζ potential of nanoparticles, which improved the stability of o/w emulsion (Yang et al., 2001). Also, ionic strength increase reduced free drug to diffuse from oil phase into aqueous phase. Traditionally, drug-loaded content in particles is measured by a direct or an indirect method (Li et al., 2001; Pandey et al., 2005). Data from the indirect method equaled the general drug amount used in the experiments minus the drug amount in supernatant, which neglected the weight loss in the preparation process. Direct result was nearer to actual value than the indirect one because the latter was greater than the actual one. The weight loss of freeze dried samples dispersed by 20% ethanol solution (about 8 mg/ml) dealt by ethanol solution nearly equaled that by PBS in this study. However, the ratio of active agent between ethanol and PBS supernatant was 152.8 ± 3.09% ($n = 3$). The direct result was also greater than the actual value because of the ignorance of surfactant solubilization, especially for chemicals with low solubility. Venier-Julienne and Benoit (1996) found an aggregate form of amphotericin B mixed in PLGA nanoparticles, and the resin bead was used to remove it. We speculated that part of VRC was not encapsulated in the preparation process and formed aggregates like amphotericin B. It was reported that $PVA_{05–88}$ was easy to dissolve in 10–60% ethanol solution (Zheng, 1993). The ethanol solution may dissolve VRC aggregates and drug on the surface of particles more easily than PBS does. Therefore, the VRC concentration in supernatant liquid cleansed by ethanol solution was higher than that by PBS, whereas the loss of particles dealt by ethanol solution was lower than that by PBS.

3.1.2. PLGA nanoparticles size distribution and morphology

The morphology of PNLV was observed by SEM (Fig. 1A and B), and the image showed that PNLV with Na-HMP was regularly spherical in shape (Fig. 1A) and PNLV without sodium salts was irregularly spherical in shape and agglomerated (Fig. 1B). The particle size and distribution were measured

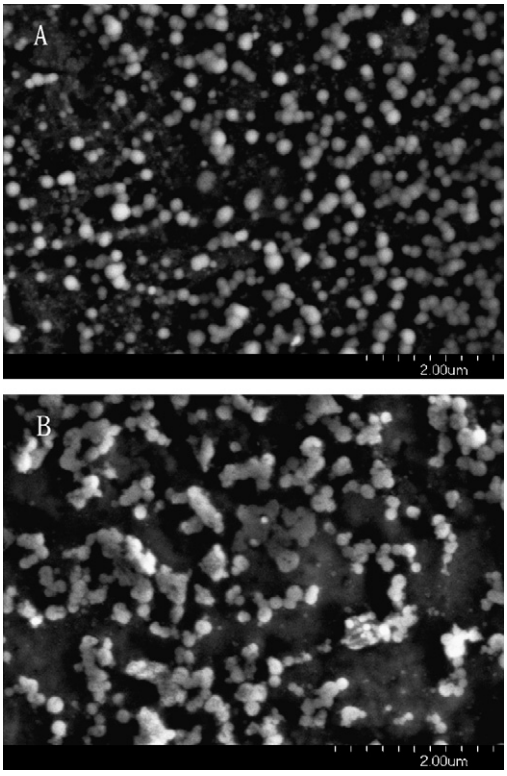

Fig. 1. (A) Scanning electron micrographs of VRC-loaded PLGA with Na-HMP nanoparticles. Accelerated volts were 5.0 kV, magnitudes were 20,000 (Hitachi S-4700, JP). (B) Scanning electron micrographs of VRC-loaded PLGA without Na-HMP nanoparticles. Accelerated volts were 5.0 kV, magnitudes were 20,000 (Hitachi, S-4700, JP).

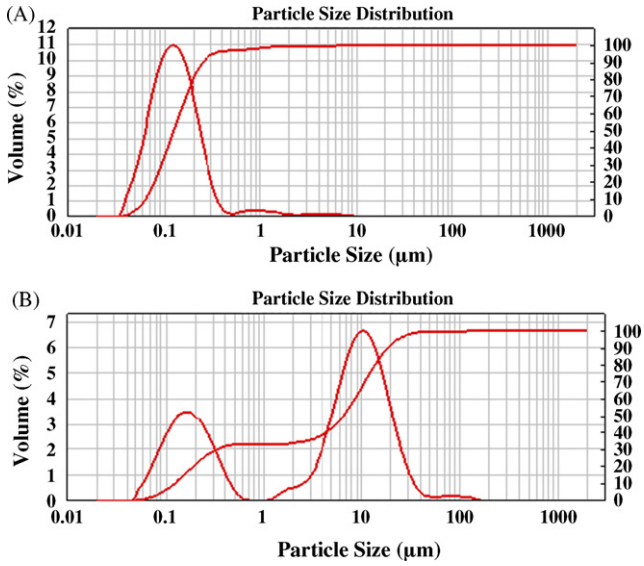

Fig. 2. (A) Distribution of VRC PLGA nanoparticles with Na-HMP by laser particles size analyzer. Span was 1.5. D_{50} and D_{90} were 0.127 μm, and 0.261 μm, respectively. Specific Surface Area was 54.7 m^2/g calculated by the density of glass beads (RI: 1.520). Particle diameter is presented by volume diameter and the same experiment was carried out five times (Malvern, Mastersizer 2000, UK). (B) Distribution of VRC PLGA nanoparticles without Na-HMP by laser particles size analyzer. Span was 2.8. D_{50} and D_{90} were 6.739 μm, and 19.020 μm, respectively. Specific Surface Area was 5.71 m^2/g calculated by the density of glass beads (RI: 1.520). Particle diameter is presented by volume diameter and the same experiment was carried out five times (Malvern, Mastersizer 2000, UK).

using Malvern mastersizer 2000, the results of which were in agreement with the observation of SEM. The graph showed that Na-HMP improved the agglomeration of PNLV. The nanoparticles containing sodium salts showed a good dispersity, and the median volume diameter of particles was 132.8 ± 8.4 nm (n = 5) (Fig. 2A), whereas that of PNLV without sodium salts was 6.3 ± 1.9 μm (n = 5) due to agglomeration (Fig. 2B). These results indicated that Na-HMP played a role in improving agglomeration by changing the ζ potential of PNLV (Kumar and Jain, 2007). In addition, the specific surface area of PNLV with and without the salt was increased by about 10 times. Previously, the specific surface area had positive correlation with the release speed and amount of the drug. Smaller particles had more opportunity to be absorbed by digestive tube as well as phagocytized by fungi cells (Desai et al., 1996; He et al., 2005). It is previously reported that a particle size below 500 nm can pass through intestine Peyer's patches into the body (Pandey et al., 2005). In our study, D_{90} was less than 336.2 ± 71.8 nm (n = 5) and the specific surface area was 52.3 ± 2.9 m^2/g (n = 5), which would benefit for absorption. The size results from SEM

Table 2
Activity of PNLV and VRC against *Candida albicans in vitro* (log_{10} CFU)

		C_{VRC}		C_{PNLV}	
		70 μg/ml	35 μg/ml	3.5 mg/ml	1.75 mg/ml
Day 1	5.11 ± 0.20	3.73 ± 0.16[a]	4.23 ± 0.23[a,b]	4.51 ± 0.18[a,e]	4.73 ± 0.11[a,c,f]
Day 2	6.09 ± 0.22	4.41 ± 0.12[a]	4.99 ± 0.22[a,b]	5.08 ± 0.14[a,d]	5.31 ± 0.14[a,c]
Day 3	7.07 ± 0.46	5.37 ± 0.19[a]	5.77 ± 0.20[a,b]	5.59 ± 0.06[a]	5.96 ± 0.04[a,c]
Day 5	7.06 ± 0.18	6.05 ± 0.15[a]	6.11 ± 0.24[a]	5.70 ± 0.09[a]	6.09 ± 0.06[a,c]
Day 7	7.15 ± 0.10	6.72 ± 0.24[a]	6.83 ± 0.18[a]	5.74 ± 0.10[a,d]	6.29 ± 0.08[a,c]

Note: Initial blastospores density in each test tube was 1 × 10^4 /ml at day 0. Values are means ± S.D. of the results obtained from 3 separated experiments with 10 animals.

[a] P < 0.001, vs. control.
[b] P < 0.001, vs. 70 μg/ml group.
[c] P < 0.001, vs. 3.5 mg/ml group.
[d] P < 0.05, vs. 70 μg/ml group.
[e] P < 0.001, vs. 70 μg/ml group.
[f] P < 0.05, vs. 35 μg/ml group.

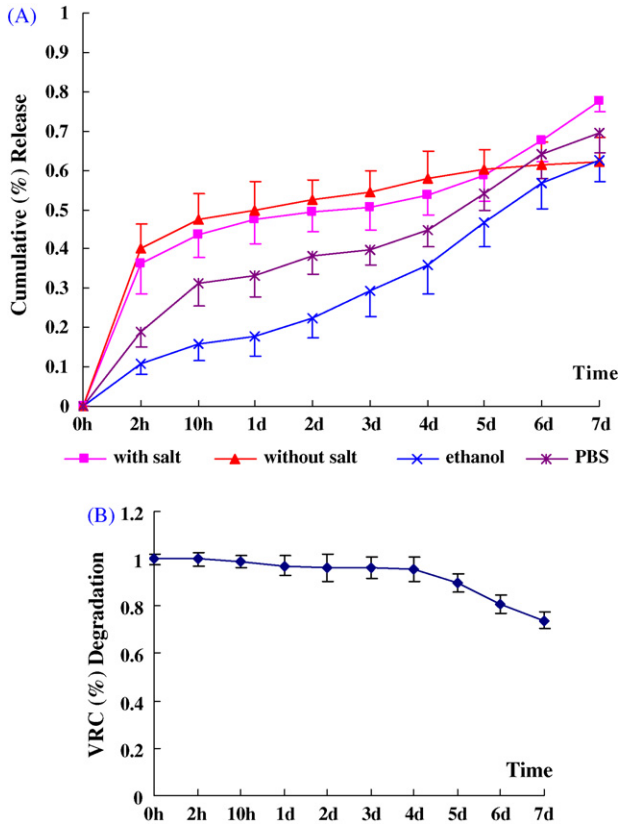

Fig. 3. (A) Release profiles of VRC-loaded PLGA nanoparticles in PBS ($n = 3$). The release profiles of two samples from different preparation processes (contained or not contained sodium hexametaphosphate in water phase) and the samples containing this salt in the water phase dealt by PBS or 20% ethanol solution were investigated. *Y*-axis means the cumulative (%) release of VRC by HPLC and *X*-axis represents the time points of drug release in this figure. The HPLC (Waters, 600, US) was provided with 2487 UV detector. The analytical column (Jupiter, C18, 5μ, 250 mm × 4.6 mm, 300 E) was eluted with mobile phase containing 42% of acetonitrile and 58% of ammonium phosphate solution (0.04 M, pH 6.0). The flow rate was 1 min/ml. Calibration curves were constructed. There was a linear relationship between 0.2 and 20 μg/ml ($y = 3{,}041{,}244x - 269{,}675$, $R^2 = 0.9991$). (B) VRC degradation ratio was 25.2% at 37 °C during 7 days. *Y*-axis means the VRC percent of degradation by HPLC and *X*-axis represents the time points of drug analysis in this figure. The HPLC (Waters, 600, US) was provided with 2487 UV detector. The analytical column (Jupiter, C18, 5μ, 250 mm × 4.6 mm, 300 E) was eluted with mobile phase containing 42% of acetonitrile and 58% of ammonium phosphate solution (0.04 M, pH 6.0). The flow rate was 1 min/ml. Calibration curves were constructed. There was a linear relationship between 0.2 and 20 μg/ml ($y = 3{,}041{,}244x - 269{,}675$, $R^2 = 0.9991$).

analysis were in agreement with those by laser size analyzer in the present study.

In the present experiments, the salt concentration in the external water phase improved the morphology of particles, and 3.0% Na-HMP in this phase improved the smoothness of particles as well as the dispersity. Improving agglomeration of PNLV by Na-HMP increased uptake of particles as well as changed release kinetics.

3.1.3. *In vitro release of voriconazole from PLGA nanoparticles*

Freeze dried sample release cleansed with PBS *in vitro*, indicated that there were two stages in an over 7 day release period. The first stage lasted 24 h, which was called the initial "burst release", which mainly occurred within 2 h according to HPLC analysis. The samples from the composition contained Na-HMP in the water phase had lower initial burst effect than those without the salt in this phase and the release speed of the former was quicker than that of the latter except for the initial release period. However, the burst release within first 2 h was weakened significantly by rinsing with 20% ethanol solution in our *in vitro* study.

Initial burst release of protein from microparticles can happen in two ways. Firstly, the drug associated with the surface and/or embedded in the surface layer dissolves in release medium. Secondly, the drug escaped from the polymeric matrices through the pores and cracks of particles goes into the release medium (Yeo and Park, 2004). As for chemical molecules, they mainly depend on the diffusion mechanism after initial burst release (Mu and Feng, 2003; Polakovic et al., 1999). Generally, researchers hold that the second release stage following the initial release is due to uniform degradation and erosion of the wall of nanoparticles. But we could not explain why the residue of VRC on the surface of particles was not removed in the solidification and rinse process. On the contrary, burst release did happen in PBS within 2 h. In this study, we found that cleaning with 20% ethanol can reduce burst release, which was more effective than PBS did in a sink condition. We also found that the drug amount in supernatant decreased gradually with solidification elongation. Drug in supernatant in the solidification process may adhere on the particles surface with increased viscosity of the organic phase due to Brownian movement during the ongoing solidification. It may partly also contribute to the initial burst release. We inferred that freeze dried sample was a mixture of PNLV and VRC aggregates after centrifugation because of the high bulk density of the drug (Venier-Julienne and Benoit, 1996). Ethanol solution rinse dissolved drug aggregates and part of drug on the particles surface and reduced the resource of drug burst release. The drug release curve is shown in Fig. 3A. Calculated by the degradation of VRC, samples with salt dealt by ethanol solution conform to quasi-zero order kinetics. Drug degradation curve is shown in Fig. 3B.

3.2. *Antifungal effects in vitro and in vivo*

Generally, Candida continues to be the predominant fungal pathogen (Wingard and Leather, 2004). *C. albicans* were the most common causative species, being responsible for about 50% of systemic infections in specific surveillance program (Pfaller et al., 2000).

3.2.1. *In vitro*

In order to compare antifungal efficacy between the PNLV and VRC, we carried out drug antifungal experiments *in vitro* and *in vivo*. *In vitro* results are shown in Table 2. The fungal numbers in tubes containing drug were significantly lower than those in control tubes ($P < 0.001$) at any point in time. Compared with the control group, the variation of fungal numbers in tubes at doses of 70 μg/ml VRC and 3.5 mg/ml PNLV were changed from 1.4 and 0.6 $\log_{10}$ on day 1 to 0.4 and

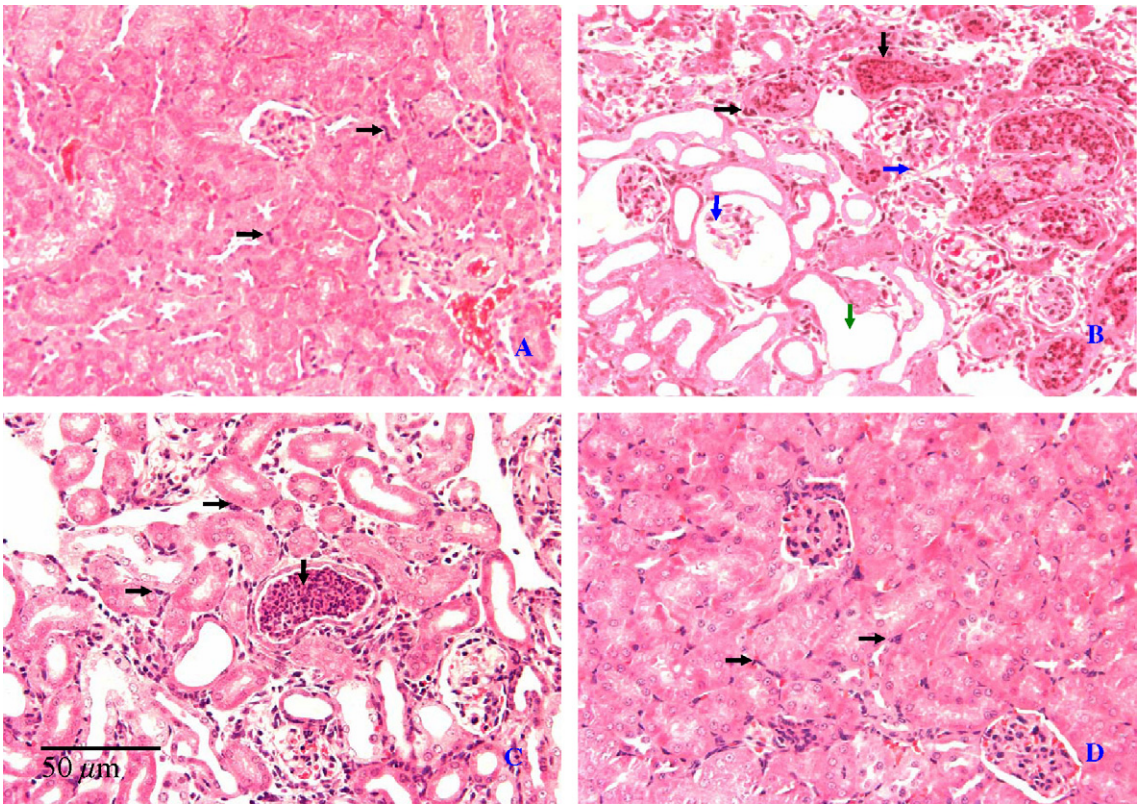

Fig. 4. H&E staining micrographs of fungi infected mice kidney. Inflammatory cell infiltration was observed in model mice kidney, with the severity of infiltration associated with fungi infected conditions (B–D). In the positive control group multifocal fibrosis, sporadic renal glomerulus atrophy and renal tubular vacuolation was observed (B). There were casts in renal tubule in mice kidney administrated by VRC (C). Magnitudes were 200 (OLYPUS, BX-51, JP) The arrows (→, ↓, →, ↓, and ↓) represent infiltration, cast, fibrosis, renal glomerulus atrophy and renal tubular vacuolation.

1.5 $\log_{10}$ on day 7, respectively. In our study, high dose nanoparticles were less effective in reducing fungi in tubes than the high dose VRC group at the initial 2-day period after therapy ($P < 0.05$), and there were no significant changes in the following 3 days. However, the numbers of fungi in PNLV tubes (3.5 mg/tube) were significantly smaller than that of equal amount VRC tubes (70 μg/tube) on day 7 ($P < 0.05$), and proliferation of fungi was more strongly inhibited in PNLV tubes. This suggests that PNLV had a more persistent and more potent antifungal effect than the 7-day experiment of VRC *in vitro*.

3.2.2. *In vivo*

On the basis of *in vitro* results, we further observed the antifungal efficacy of PNLV *in vivo*. Equal amount of VRC was single orally administrated to each group except for negative control animals. We found that the CFU/g kidney tissue from mice treated with PNLV, VRC, or PBS were $0.64 \pm 0.19 \log_{10}$, $2.61 \pm 0.27 \log_{10}$, or $3.44 \pm 0.32 \log_{10}$, respectively. The PNLV and VRC were effective in reducing fungal burden in the kidneys about 2.8 $\log_{10}$ and 0.8 $\log_{10}$ on day 7 after being administered ($P < 0.001$, $P < 0.001$, vs. PBS). However, the PNLV group showed more potency and longer kill-fungi time than the VRC group ($P < 0.001$, vs. VRC). Compared with *in vitro* experiments, PNLV also gained an advantage over VRC in long-term antifungal aspects *in vivo*.

3.2.3. *Hematoxylin–eosin stain*

In vivo; inflammatory infiltration, which was possibly related to fungi infection, was observed in the mice kidney, and the severity of infiltration was associated with fungi conditions (Fig. 4B–D). The positive control group showed that multifocal fibrosis, sporadic renal glomerulus atrophy and renal tubular vacuolation were related to fungi infections (Fig. 4B). There were casts and cell debris in renal tubule in the mice kidney treated with VRC (Fig. 4C). However, those lesions were not detected in the mice treated with PLVN (Fig. 4D). It may be related to high dose exposure of active agent.

It was reported that VRC therapy needed loading dose (IC_{50} value 2 μg/L for *C. albicans*) (Purkins et al., 2003; Leveque et al., 2006), the effect of burst release drug played a role of the loading dose in killing fungi to some extent. Ideally, burst release could meet the needs of the loading dose and the follow-up quasi-zero release could balance the amount of metabolism and clearance to maintain the stable minimal plasma concentration for fungi killing. According to previous clinical reports, ID_{50} of VRC was 10 ng/ml for *C. albicans* and 230 ng/ml for *C. krusei* (intrinsically resistant to fluconazole), and the time of drug peak concentration in the blood was 3 h after administration, reaching 1.88–5.27 μg/ml which was far higher than the concentration needed (Leveque et al., 2006). Too many drugs exposed to the body due to the premature degradation can bring waste of drug (Fromtling, 1988), adverse effects as well as induction of fungi

tolerance to the antifungal drug. However, controlled release can avoid this problem effectively. In addition, a controlled release formulation can decrease the frequency of administration and the drug concentration fluctuation of repeated administration. Furthermore, patients' plasma concentration fluctuates 100 times among individuals after administration of VRC formulation available in the market (Purkins et al., 2002). Therefore, it is important to sustain balance between exposure and clearance to reduce adverse effects. The PNLV controlled delivery system has quasi-zero release properties, and could ameliorate this problem.

4. Conclusion

These results suggest that the emulsion–solvent evaporation process is feasible in preparing PNLV. Moreover, ethanol solution could decrease burst release and Na-HMP could improve agglomeration of PNLV. PNLV possesses great potential as an antifungal delivery system, more than VRC both *in vitro* and *in vivo*. But further research is still needed to optimize the preparation process.

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