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Original Article

Probucol Nanostructured Lipid Carrier Ameliorates Elastase-induced Abdominal Aortic Aneurysm in Mice

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Abstract

Probucol (PB) is a drug commonly used for the treatment of hyperlipidemia and atherosclerosis. In our previous study, we have proved that PB can ameliorate abdominal aortic aneurysm (AAA) induced by elastase in mice through its anti-inflammatory and anti-oxidative effect. In this study, the water solubility and oral absorption of PB were improved by encapsulating PB into nanostructured lipid carriers (PB-NLC), and the effects of NLC on the anti-AAA effects of PB were evaluated and compared to the PB free drug. AAA mouse model was constructed by incubating the mouse aorta with elastase. Mice in the drug-treated groups were given PB free drug or PB-NLC at a PB dose of either 10 mg/kg or 20 mg/kg daily, and mice in the model group were treated with saline. The arterial wall and the degradation grade of the elastic lamina were investigated by HE staining and aldehyde fuchsine staining of the aortic sections. Infiltration of macrophages and degree of inflammation were evaluated by immunohistochemical staining of CD68 and TNFα, respectively, in the aortic sections. In addition, the absorption improvement of PB by the NLC nano-formulation was evaluated through determining and comparing the steady-state plasma concentration of PB in the PB free drug or PB-NLC treated mice group. When treating the mice with AAA with an equal dose of PB, PB-NLC more significantly inhibited expansion of the abdominal aortas and maintained the integrity of the aortic walls, compared with the PB free

drug. The NLC nano-formulation significantly increased the steady-state plasma concentration of PB. PB-NLC significantly increased the absorption and protective effects of PB against elastase-induced AAA in mice.

Keywords: abdominal aortic aneurysm, elastase, inflammation, nanostructured lipid carrier, probucol, oral absorption.

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1. Introduction

Abdominal aortic aneurysm (AAA) is the segmental and full-thickness dilatation of the abdominal aorta, and such expansion is progressive and irreversible [1]. So far, there is no safe and effective drug that can inhibit the progress of AAA except for surgical treatment, so it is necessary to find an effective medication for AAA treatment.

Although the detailed molecular mechanism of AAA development has not been fully understood, it is known that AAA is closely associated with weakening of the vascular wall caused by inflammation [2]. Inflammation occurs in the early stages of AAA and runs through the whole process of AAA development. In the development of AAA, macrophages, among all inflammatory cells (neutrophils, monocytes and macrophages), are the dominant cells that infiltrate into aorta. A large number of cytokines [3], such as tumor necrosis factor-α (TNFα)，monocyte chemotactic protein 1 (MCP-1), interleukin 6 (IL-6) and matrix metalloproteinases (MMPs) released by the inflammatory cells, will further exacerbate the inflammatory response, leading to the degradation of elastin and collagen, as well as the apoptosis of vascular smooth muscle cells (VSMCs) in the media and adventitia, finally resulting in destruction of the arterial wall and expansion of the aortic lumen [4][5].

Probucol (PB) is a drug with strong anti-oxidative activity, and is broadly used in clinic for treatment of atherosclerosis and hyperlipidemia. It has been reported that PB can inhibit oxidation, inflammation and production

of MMPs, and maintain the function of vascular endothelial cells, which all contribute to the protective effects of PB against aneurysms [1]. However, the poor water solubility of PB results in its weak and variable absorption in the gastrointestinal tract, and hence the drug shows an undesirable oral bioavailability of less than 10%, which limits its clinical applications [6]. At present, various nano-formulations of PB have been developed and reported to increase the solubility of PB, including PB microemulsion [7], PB nanoparticles [8], and PB micelles [9]. However, low PB loading capacity and potential toxicity of the residual organic solvent limit the application of these formulations.

Nanostructured lipid carriers (NLC), a novel nano-scaled drug delivery system developed from solid lipid nanoparticles (SLN), has a matrix structure composed of physiologically compatible and biodegradable solid and liquid lipids. The incorporation of liquid lipid significantly increases the encapsulation as well as the efficacy of drugs with poor water-solubility. The high loading capacity of hydrophobic drugs is a unique advantage of NLC compared to other nano-scaled drug delivery systems. In addition, lipids used in NLC are either natural or semisynthetic, which assures good biocompatibility of NLC. So far, NLC has been broadly used in the development of oral drug delivery systems for treating chronic diseases [10]. Therefore, in this study, in order to improve oral bioavailability of PB and increase its anti-AAA effects, PB was encapsulated into NLC to construct PB-NLC, and its anti-AAA effects were evaluated in an elastase-induced AAA mouse model and the results were compared to those of the PB free drug.

2. Materials and Methods

2.1. Materials

Probucol was purchased from Japan Otsuka Pharmaceutical (Tokyo, Japan). Elastase and chloral hydrate were purchased from Sigma Aldrich (Beijing, China). Glyceryl monostearate, stearic acid, and oleic acid were purchased from Shanghai Changwei Pharmaceutical raw materials technology (Shanghai, China). Soybean lecithin was purchased from Shanghai Taiwei Pharmaceutical (Shanghai, China). Pluronic F68 was purchased from AMRESCO (Houston, USA). Tissue-Tek O.C.T. Compound was obtained from Sakura Finetek Japan Co., Ltd. (Tokyo, Japan). Anhydrous ethanol,

methanol, sodium chloride, sucrose, and paraformaldehyde were purchased from local suppliers in China. Methanol and acetonitrile used for HPLC analysis (chromatographic grade) were purchased from Merck (Germany).

2.2. Animals

All study protocols conformed to the Animal Management Rules of China (Documentation No. 55, 2001, Ministry of Health, China). All experiments were approved by the Committee for Ethics of Animal Experiments and were conducted in accordance with the Guidelines for Animal Experiments, Peking University Health Science Center. All mice were raised under a 12-hour light/dark cycle with free access to food and water.

2.3. Preparation and Characterization of NLC

NLC was prepared by emulsion evaporation and low temperature-solidification techniques. We mixed 20 mg glyceryl monostearate and 20 mg stearic acid (1: 1) as the solid lipid, and oleic acid (20 mg) was used as the liquid lipid. The proportion of the auxiliary emulsifier lecithin (40 mg) and solid lipid was 1:1. The lipids, lecithin and PB (60 mg) were dissolved in 5 mL ethanol as the oil phase. Aqueous solution of Pluronic F68 (3%, w/v) was the aqueous phase. The two phases were heated in water bath to 75 °C, and the oil phase was added dropwise to 40 mL aqueous phase at a rate of 15 $mL \cdot h^{-1}$ using a microinjection pump (Longer Precision Pump Company, Baoding, China), and the mixture of the two phases was stirred at 1000 rpm. After the addition of the oil phase, the mixture was stirred and emulsified until the drug concentration was 2 mg/mL. Then the mixture was quickly transferred to an ice-water bath and stirred for 1 h at a low temperature to obtain the suspension of PB-NLC. In the preparation of blank NLC, PB was not added in the oil phase, and other steps were the same as the preparation of PB-NLC.

Particle size and zeta potential of the NLC were measured by Malvern Zetasizer Nano-ZS (UK). In order to determine drug loading capacity of PB-NLC, free drugs in PB-NLC were removed by filtration, and the filtrate was dissolved in methanol and then diluted with the HPLC mobile phase. PB concentration in the NLC was determined by HPLC (Shimadzu Company, Japan) and recorded as C_{encap}. The whole suspension of PB-NLC without filtration was dissolved in methanol and diluted with the mobile phase. PB concentration determined by

HPLC was recorded as C_0. The encapsulation efficiency and drug loading of PB-NLC were calculated according to the following formulas:

Encapsulation efficacy (EE, %) = $C_{encap}/C_0 \times 100\%$;

Drug loading (DL, %) = $C_{encap} \times V/W_{lipid} \times 100\%$.

C_{encap} was the concentration of PB contained within the NLC nanoparticles per unit volume; C_0 was the total PB concentration in the NLC formulation suspension per unit volume; V was the volume of the PB-NLC suspension. W_{lipid} was the total mass of lipids (stearic acid, monostearate, lecithin, oleic acid) in the PB-NLC.

2.4. Elastase-Induced Mice Abdominal Aortic Aneurysm and Drug Treatment

C57BL/6 male wild type mice (22 ± 2 g) were randomly divided into 5 groups, with 4 to 7 animals in each group: Model group, 10 mg/kg PB (PB10), 10 mg/kg PB-NLC group (PB-NLC10), 20 mg/kg PB (PB20) and 20 mg/kg PB-NLC group (PB-NLC20).

The AAA model was induced by elastase. The mice were intraperitoneally injected with 5% chloral hydrate for general anesthesia. The mice were then fixed supinely and their abdominal cavity was exposed layer by layer in the lower abdomen median incision. Then the abdominal aorta between the renal artery and iliac artery (about 1 cm) was bluntly separated. The polyethylene sponge cloth, which was immersed in a 1.3 U elastase solution, was used to completely wrap the separated abdominal aorta. The mice were placed on a 37 ℃ thermostat plate. After incubation with elastase for 40 min, the sponge cloth was removed and the abdominal incision was sutured. The control group with sham operation was treated with the polyethylene sponge immersed in physiological saline, and other operations were the same as the model group.

Daily oral drug administration began on the first day of the AAA surgery. Mice in the Model group were fed with saline. Mice in the PB10 group and PB20 group were fed with 10 mg/kg and 20 mg/kg of PB, respectively. Mice in the PB-NLC10 group and PB-NLC20 group were fed with PB-NLC (equivalent to 10 mg/kg and 20 mg/kg PB, respectively). Each group was given continuous drug administration for 14 days while fed with chow diet.

2.5. Pharmacodynamic Analysis

After removal from the mice, the infrarenal abdominal aorta was fixed in 4% paraformaldehyde solution and then transferred to 20% sucrose solution for dehydration. The maximal diameter of the aorta and the area of arterial dilatation were measured with Image J. AAA was defined as the largest diameter of the abdominal aorta exceeding 50% of the maximum diameter of the normal control group. After the measurement, the infrarenal part of the abdominal aorta was cut and embedded in Tissue-Tek O.C.T. Compound, then frozen in liquid nitrogen. The aortic tissues were cut into 7 μm cryo-sections for staining. The efficacy of PB-NLC and PB in inhibiting AAA was compared by evaluating the change of AAA diameter as well as histology and morphology of the arterial wall between the Model group and the PB-NLC or PB treated groups.

2.6. Histological Analysis

Hematoxylin-eosin (HE) staining was used to observe the basic morphological structure of the aortic wall, and aldehyde fuchsin staining was used to visualize elastin of the aortic wall. The degradation grade of the elastic lamina was rated as four grades according to the following scoring criteria: level 1, less than 25% degradation; level 2, 25%–50% degradation; level 3, 50%–75% degradation; level 4, greater than 75% degradation.

2.7. Immunohistochemistry

Immunohistochemical staining of CD68 and TNF-α was used to evaluate macrophage infiltration and inflammation in the aortic wall. The slices of the aortas were incubated in H_2O_2 for 10 min to eliminate endogenous peroxidase activity and blocked with goat serum for 1 h at room temperature, and then incubated with primary antibodies overnight at 4 ℃. Subsequently, the slices were washed with phosphate buffer saline (PBS) and incubated (1 h, 37 ℃) with second antibodies. Finally, the slices were incubated with diaminobenzidine (DAB) and observed under light microscopy.

2.8. Determination of Steady-State Plasma PB Concentration

In pharmacodynamic experiments, mice were sacrificed after 14 days of drug administration, and their aortas and plasma were taken. The plasma was treated by direct precipitation. 150 μL methanol was added to 50 μL plasma and the mixture was vortexed for 3 min, followed by

centrifugation at 12000 rpm at 4 °C for 10 min. PB concentrations in the supernatant were determined by HPLC to analyze the plasma concentrations of PB in different administration groups. The detection conditions of HPLC were as follows: mobile phase: acetonitrile: water = 9:1 (v/v); detection wavelength: 290 nm; velocity: 1.0 mL/min; injection volume: 20 μL.

2.9. Statistical Analysis

The software SPSS 20.0 was used for statistical analysis, and the data were presented as mean ± SEM. The non-pairing t test was used to compare the data between the two groups, and data between multiple groups were compared using one-way ANOVA with Bonferroni post-hoc analysis. Pathological scores were analyzed by two-sample rank test (Mann-Whitney U). AAA incidence was analyzed by Chi-square test. Differences with $P<0.05$ were considered statistically significant.

3. Results and Discussion

3.1. Characterizations of PB-NLC

Particle size and zeta potential of the PB-NLC were measured by dynamic light scattering. Figure 1 shows that polydispersity indexes of the Blank-NLC and PB-NLC were 0.28 ± 0.03 and 0.23 ± 0.03, respectively, which were less than 0.3, indicating that the prepared NLC was dispersed evenly.

The particle size of the Blank-NLC was 189.46 ± 20.74 nm, and the PB-NLC was about 246.67 ± 10.60 nm. Loading PB into the NLC increased the particle size of the NLC to a certain extent, and the zeta potential increased from 0.02 ± 0.01 mV of the Blank-NLC to 0.23 ± 0.06 mV of the PB-NLC (Figure 1 and Table 1). Table 1 shows that the encapsulation efficiency of PB-NLC was 89.28 ± 1.32%, and the drug loading percentage was 23.76 ± 3.88%.

High hydrophobicity of the drugs can cause their poor gastrointestinal absorption and the subsequent low bioavailability, which limit the development and clinical application of the drugs. Therefore, it is one of the research priorities of pharmaceutical science to improve dissolution and release of the drugs with poor water solubility. In this study, NLC was used to improve water solubility and oral absorption of the hydrophobic drug, PB, and to increase its anti-AAA effects. The emulsion evaporation and low temperature-solidification techniques were used to prepare the PB-NLC. The particle size of the PB-NLC was about 246 nm and the NLC were dispersed evenly. Our previous study

Table 1. Characterization of the Blank-NLC and the PB-NLC. (Mean ± SEM, n=3).

Sample	Particle size(nm)	Polydispersity Coefficient	Zeta potential (mV)	Entrapment efficiency (%)	Drug loading percentage (g/g, %)
Blank-NLC	189.46± 20.74	0.28±0.03	0.02±0.01	-	-
PB-NLC	246.67± 10.60	0.23±0.03	0.23±0.06	89.28.±1.32	23.76±3.88

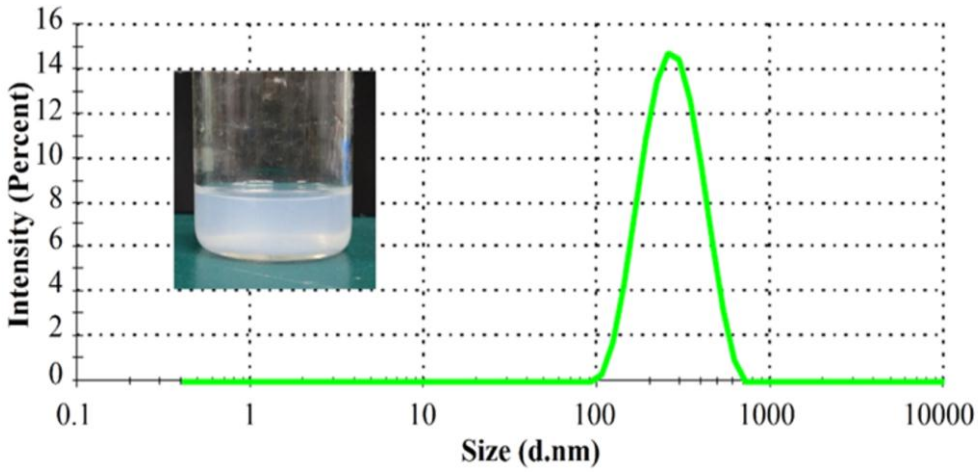

Figure 1. 1 Gross appearance and particle size distribution of the prepared PB-NLC.

showed that the nano-scaled particle size of the PB-NLC increased the dispersion and absorption efficiency of PB in the gastrointestinal tract. The better preventive effects of PB-NLC against AAA than the free PB when administrated at the equal dose would be attributed to various advantages of the NLC formulation, including the high encapsulation efficiency and drug loading percentage of the PB-NLC, as well as the controlled release and the increased stability of PB in the gastro-intestine and circulation [10].

3.2. NLC Promotes Inhibitory Effects of PB on AAA

3.2.1. PB-NLC Enhanced the Inhibitory Effect of PB on Expansion of the Abdominal Aorta of the Mice with AAA

Figure 2 shows that after 14 days of AAA inducement, the abdominal aortas of the model group expanded obviously. Administration of free PB at a dose of 20 mg/kg (PB20) for 14 days inhibited the expansion of abdominal aortic diameter (P<0.05 *vs* Model group), but 10 mg/kg PB (PB10) was not effective. However, PB-NLC at the equal PB dose of 10 mg/kg (PB-NLC10) significantly inhibited the expansion of the abdominal aortas in the mice (P<0.05 *vs* Model group), and effect of PB-NLC at 20 mg/kg (PB-NLC20) was better than PB-NLC10 and PB20 (P<0.01 *vs* Model group, P<0.05 *vs* PB20 or PB-NLC10 group).

In this AAA mouse model induced by elastase in C57BL/6 mice, after treating the mice with different doses of PB for 14 days, we found that PB could inhibit the development of AAA in a dose-dependent manner, as 20 mg/kg was better than 10 mg/kg, but PB-NLC could inhibit the formation and expansion of AAA at both doses.

3.2.2. PB-NLC Enhanced the Protective Effects of PB on Structural Integrity of the Abdominal Aorta of the Mice with AAA

The results of HE staining in Figure 3 show that the abdominal aorta lumen significantly enlarged and the structural integrity of the

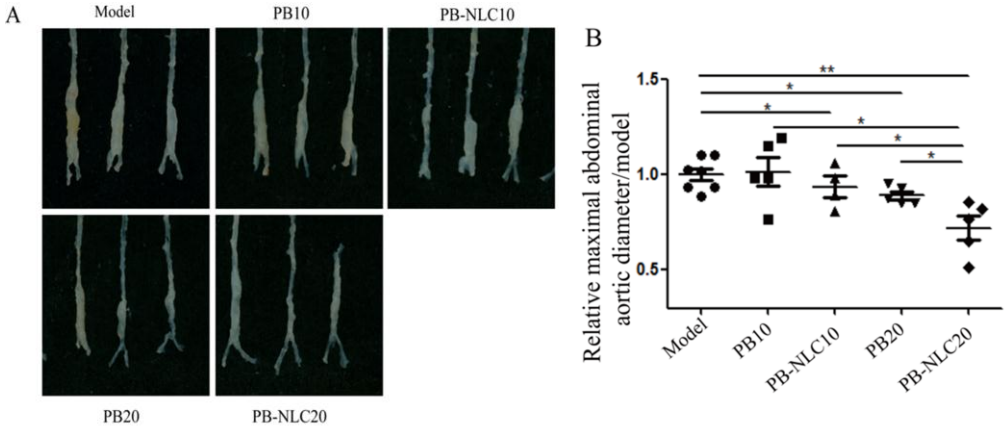

Figure 2. PB-NLC enhanced the inhibitory effects of PB on the dilation of the abdominal aorta of the AAA mice. A: Gross appearance of the abdominal aortas from each group; B: Ratios of the maximal abdominal aortic diameter of the PB-treated mice groups with the Model group. *P <0.05, **P < 0.01. n=4-7.

arterial wall was severely damaged in the Model group. Figure 3A and 3B also show the fractured elastic lamina in the tunica media as well as the compensatorily remodeled and thickened tunica adventitia in the Model group. In addition, the most severe infiltration of

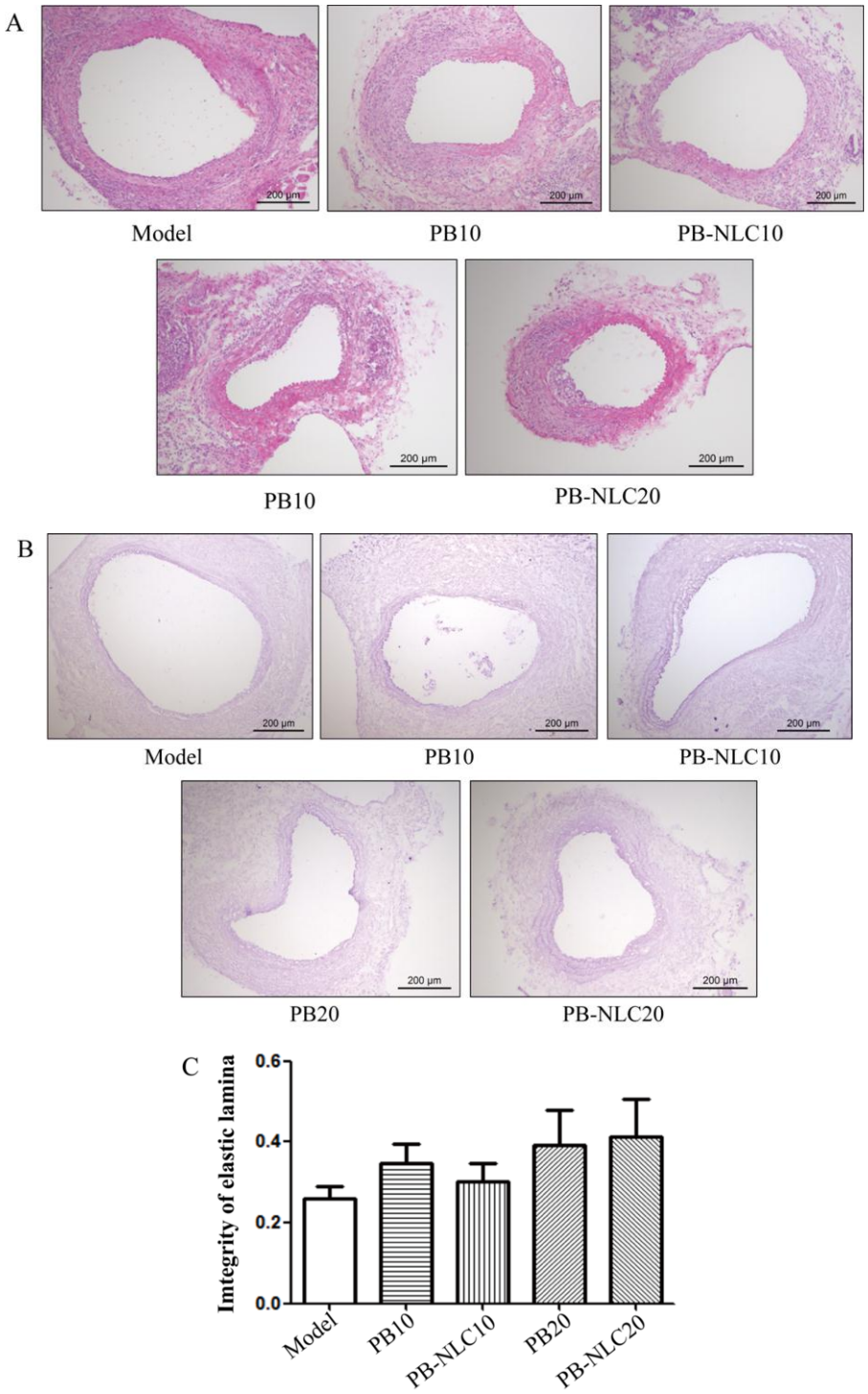

Figure 3. Protective effects of PB-NLC on the structural integrity of the abdominal aorta wall of the AAA mice. A: HE staining of the mice abdominal aortas from each group (×100); B: Aldehyde fuchsine staining

inflammatory cells and the highest degradation degree of elastic lamina were observed in the Model group. Compared to the Model group, PB20, PB-NLC10 and PB-NLC20 all inhibited the expansion of the abdominal aorta lumen and protected the integrity of the arterial wall. PB and PB-NLC at the above doses also alleviated the thickening of the intima. Figure 3 shows that PB-NLC20 showed certain extent of protective effect on the degradation of elastic lamina in the tunica media but there was no statistical difference compared to the Model group.

The inhibitory effects of PB and PB-NLC on AAA at the equal dose of 20 mg/kg were compared. The free PB had certain inhibitory effects on AAA, while the PB-NLC more significantly decreased the diameter of the aneurysm and reduced the damage of the arterial wall. Further histomorphological evaluation of the arterial wall shows that the PB-NLC enhanced the protective effects of PB on the structure integrity of the arterial wall, including reducing the disorder and degradation of elastic lamina in the tunica media, as well as decreasing the thickening of the tunica adventitia. These results indicate that NLC significantly increased the anti-AAA effects of PB.

3.2.3. PB-NLC Enhanced the Anti-Inflammatory Effects of PB

HE staining of the abdominal aorta sections shows that in the Model group, infiltration of inflammatory cells in the tunica adventitia increased significantly. Immunohistochemical staining in Figure 4A and Figure 4B further reveals that the inflammatory cells in the tunica adventitia were mainly macrophages and the inflammatory factor TNFα was significantly increased in the tunica adventitia. Compared to the Model group, PB10 and PB20 inhibited the infiltration of macrophages and the expression of TNFα in the arterial wall, and such anti-inflammatory effects of PB20 were more significant than PB10. Compared to the PB10 group, PB-NLC10 more significantly inhibited macrophage infiltration and the expression of TNFα in the tunica intima and media.

The molecular pathogenesis of aneurysms is complicated. At present, studies have shown that inflammatory cells and inflammatory factors play an important role in the development of aneurysms. Macrophages are widely distributed in the medial and adventitia of AAA. During their migration and infiltration into the aortic intima, various inflammatory factors, such as TNFα and IL-1β, are released by

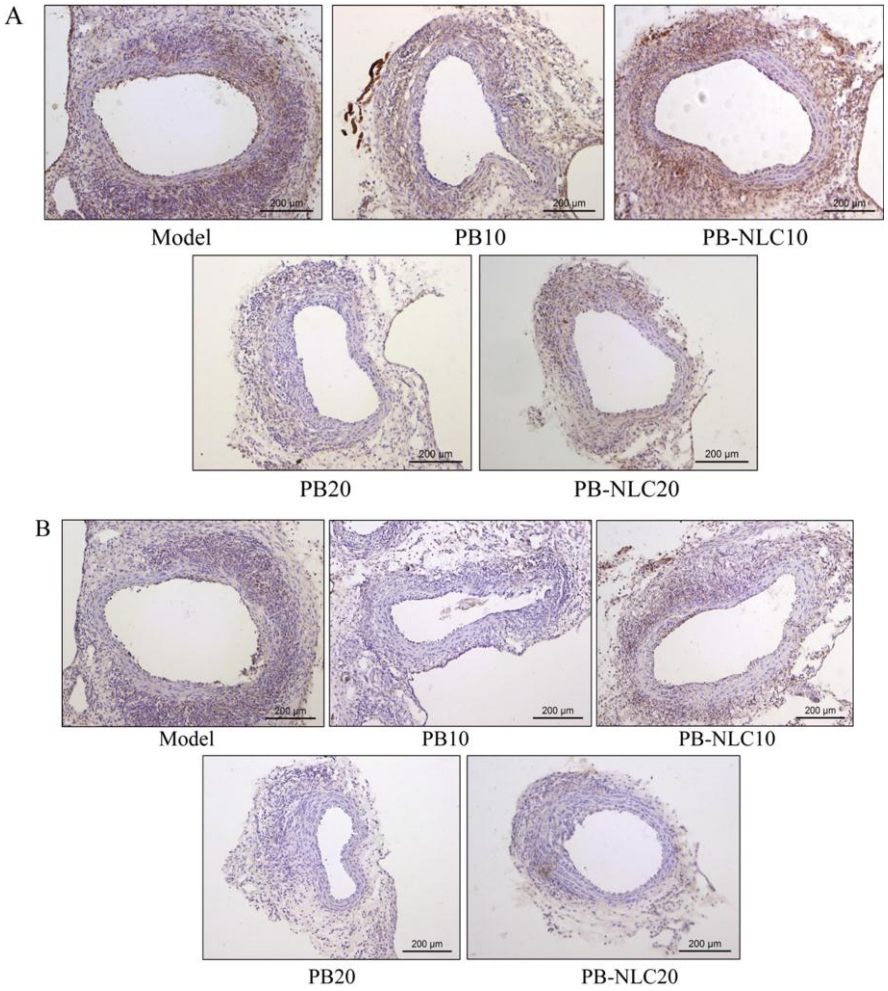

Figure 4. PB-NLC significantly enhanced the effect of PB on decreasing macrophage infiltration and the expression of TNFα in the abdominal aorta wall of the AAA mice. A: Representative images of immunostaining for $CD68^{+}$ macrophages (×100); B: Representative images of immunostaining for TNFα (×100).

macrophages. TNFα can activate nuclear factor-κB (NF-κB), which further promotes the expression of pro-inflammatory factors. And a large number of inflammatory factors can stimulate vascular smooth muscle cells (VSMCs) and induce their conversion from the contractile type into the secretory type. The VSMCs activated by inflammation then secrete pro-inflammatory factors and MMPs. MMPs accelerate the degradation of elastin. The decreased integrity of elastic fiber not only affects cell biology of VSMCs but also reduces aortic resilience, which allows the aortic wall to dilate into AAA. In addition, loss in the integrity of collagen fiber decreases aortic strength,

which can render the aortic wall vulnerable to rupture [11].

Further histomorphological evaluation of the arterial wall shows that the PB-NLC enhanced the protective effects of PB on the structural integrity of the arterial wall, including reducing the disorder and degradation of elastic lamina in the tunica media, as well as decreasing the thickening of the tunica adventitia. The results of immunohistochemical staining show that a significant number of macrophages were infiltrated in the thickening tunica adventitia of the Model group, together with an abundant expression of TNFα. However, treatment of the mice with PB or PB-NLC partly inhibited the infiltration of macrophages and decreased expression of the inflammatory factor TNFα.

In our study, we found that when PB-NLC was administrated at 10 mg/kg, the inhibition of inflammation in the aortic wall and the inhibitory effects on aneurysm expansion were much stronger than free PB at the equal dose of 10 mg/kg. But at 20 mg/kg, the protective effect on the degradation of elastic lamina in the tunica media was not significantly enhanced in the PB-NLC group, compared to free PB at the equal dose. This may be explained as such that inflammatory responses play an important role in the initiation of AAA and lead to the following pathological processes by causing the release of MMPs and inflammatory factors; however, the degradation of elastic lamina could be affected by many complex factors in the progression of aneurysm, such as inflammation, oxidative stress and activation of MMPs, etc. Therefore, a higher dose of PB is required to achieve significant protective effect on the degradation of elastic lamina in the tunica media.

3.2.4. PB-NLC Significantly Increased the Steady-State Plasma Concentrations of PB

After 14 days of administration, PB plasma concentration reached its steady state level. Blood samples were taken two hours after drug administration on day 14, and the steady-state plasma concentrations of PB were determined. The results in Figure 5 show that the steady-state plasma concentration of PB in the PB-NLC group was significantly higher than that in the PB group at the equal dose (PB-NLC10, $P < 0.01$ *vs* PB10; PB-NLC20, $P < 0.001$ *vs* PB20).

We found that NLC significantly increased the steady-state plasma concentration of PB. In our previous study, we have reported that

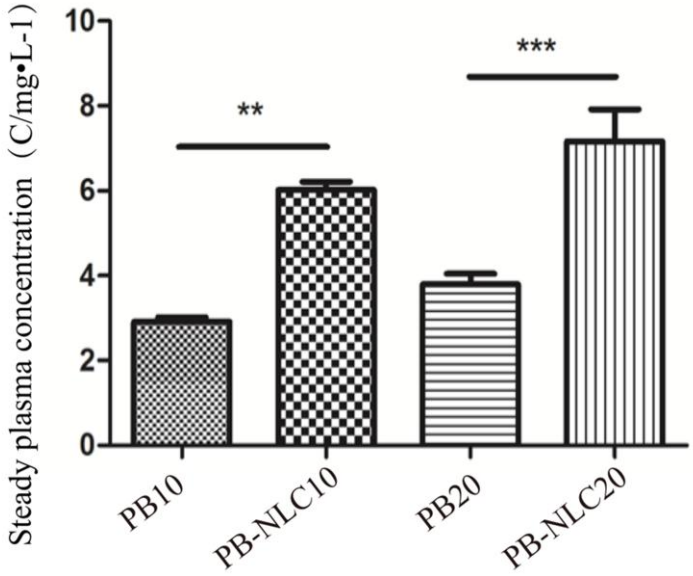

Figure 5. PB-NLC significantly increased the steady state plasma concentrations of PB. ** $P < 0.01$, *** $P < 0.001$. n=3.

PB-NLC significant increase the C_{max}, $AUC_{0\rightarrow12h}$ and bioavailability of PB. Therefore, NLC improved the oral absorption of PB by increasing its solubility and absorption in the intestines, thus enhancing the anti-AAA effects of PB.

4. Conclusion

Based on the data presented here, the PB-NLC prepared in this study significantly enhanced anti-AAA effects of PB by improving its solubility and oral bioavailability. This study is instructive for the development of new nano-formulations of drugs with poor water solubility, and also provides evidence of using PB nano-formulations for the treatment of AAA.

Acknowledgements

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