




## Preparation of long-acting microspheres loaded with octreotide for the treatment of portal hypertensive

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### ABSTRACT

The purpose of this study was to optimize the preparation method of injectable Octreotide microspheres. To explore the correlation between the solvent system and the general properties of microspheres to reduce burst release and enable them to be used for portal hypertension. Octreotide microspheres were prepared by modified double emulsion solution evaporation method after optimizing preparation conditions. The results showed that Octreotide microspheres had a particle size of  $57.48 \pm 15.24 \mu\text{m}$ , and the initial release was significantly reduced. In vitro release and in vivo pharmacokinetic data indicated that Octreotide was released stably within 1200 h. The effects on portal vein pressure, liver tissue morphology and other related indexes were observed after administration. As obvious results, injection of Octreotide microspheres could significantly reduce portal vein pressure and reduce the portal vein lumen area in experimental cirrhotic portal hypertensive rats. The optimized Octreotide PLGA microsphere preparation has been proved to have a good effect on PHT in vivo after detecting aminotransferase (AST) and alanine aminotransferase (ALT) activity, liver tissue hydroxyproline (Hyp) content, serum and liver tissue malondialdehyde (MDA) levels, plasma prostacyclin ( $\text{PGI}_2$ ) levels, and liver tissue tumor necrosis factor ( $\text{TNF}\alpha$ ) content. In addition, serum and liver tissue superoxide dismutase (SOD) activity and liver tissue glutathione (GSH) content, plasma thromboxane ( $\text{TXA}_2$ ), serum nitric oxide (NO), liver tissue nitric oxide synthase (NOS), and plasma and liver tissue endothelin (ET) were significantly increased.

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### KEYWORDS

Octreotide; microspheres; PLGA; controlled release; portal hypertension

## 1. Introduction

Somatostatin and its derivatives act selectively and directly on visceral vascular smooth muscle to induce contractions, inhibition of vasoactive substances, such as glucagon, vasoactive peptide, substance P, and calcitonin gene-related peptide indirectly block visceral vasodilation, reduce hepatic arterial blood flow and intrahepatic vascular resistance, so most of the portal vein blood flow through the intrahepatic blood vessels has low resistance to reduce portal vein pressure (Mejias et al., 2008). For somatostatin, they have a very short half-life, approximately 2–3 min. In clinical applications, the first intravenous infusion must be followed by continuous infusions to maintain an effective blood concentration. Octreotide, a long-term somatostatin analog, has a cyclic peptide molecular structure with the same sequence arrangement of the four active amino acids as somatostatin, but an extended half-life of 80–160 min (Currow et al., 2015; Ismail et al., 2015; Loyaga-Rendon et al., 2015). Octreotide acts by inhibiting glucagon and delaying the increase in post-prandial hepatic venous pressure gradient (HVPG) and portal blood flow (Wells et al., 2012; Tripathi et al., 2015). Currently, Octreotide is mainly used to treat gastrointestinal diseases and various endocrine tumors. To reduce the number of medications for patients and greatly improve patient

compliance, Octreotide microspheres for injection were developed. However, the main problem to limit the safety of these current sustained-release preparations is the sudden release of microspheres, which causes the blood concentration in the body to exceed a safe range (Xu et al., 2013). Poly(D,L-lactide-co-glycolic) PLGA is widely used in biomedical engineering because of its great biocompatibility and biodegradability and controllable rate of degradation (Pean et al., 1998; Wu et al., 2017). It has been prepared as various tissue engineering scaffold materials, as well as drug controlled release carriers (Han et al., 2010; Zhang et al., 2011). The release characteristics of most PLGA microspheres are burst release, followed by plateaus and rapid drug release. The initial release and plateau are the main reasons for fluctuations in blood concentrations, and may cause various adverse reactions (Hu et al., 2011). As the most commonly used method for preparing drug-loaded sustained-release microspheres, the emulsification-solvent volatilization affects microsphere morphology, particle size, and drug release rate by adjusting the physicochemical properties and removal rate of the solvent used (Ito et al., 2009). A key factor in the preparation of microspheres is the successful selection of organic solvents. In addition to the physical properties of organic solvents, the properties of dissolved carrier materials

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and drugs will also affect the particle size and encapsulation efficiency of the microspheres.

Portal hypertension (PHT) is the most important complication of cirrhosis and the leading cause of mortality and liver transplantation worldwide (Gracia-Sancho et al., 2015; Mercado, 2015). In many cases, clinical treatment, such as conventional medical treatment or surgery, cannot prevent the emergency or release pain (Verbeke et al., 2014; Schouten et al., 2015). Long-acting medicines, as an effective treatment, offer opportunities to improve the quality of life of patients. In this study, the correlation between the solvent system and the conventional properties of microspheres such as drug loading and drug burst release was investigated. It was found that there were significant difference in drug release behavior and distribution of microspheres with different mixed solvents. PLGA microspheres supported by Octreotide were prepared to increase the concentration of Octreotide in internal aqueous phase (Chen et al., 2014). At the same time, cracking was further reduced by changing the proportion of organic solvents. Microspheres with low initial explosive release and high drug loading were prepared by the optimized protocol. The drug release behavior of the prepared microspheres in vitro showed that they had obvious sustained release effect and lower burst release rates. In vivo blood concentration was more stable than somatostatin. Pharmacodynamics of PHT animal models showed good biological activity.

## 2. Materials and methods

### 2.1. Materials

The following reagents, drugs, and chemicals were used in this study: PVA (Mw 20,000–30,000, ACROS ORGANICS); Octreotide acetate (Shanghai Tash Biotechnology Co., Ltd); PLGA (L/G 50/50, Mw 35,000, Birmingham, Germany). Polyvinyl alcohol (PVA, Mw 30,000–70,000, Acros, NJ); All other chemicals (Sinopharm chemical reagent Co., Ltd, Shanghai, China); Sandostatin (Novartis, Ltd.); ALT, AST, NO, MDA, SOD, NOS, GSH and Hyp assay kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China); ET, TXB<sub>2</sub> and TNF $\alpha$  (Liberation and Exemption Institute of Science and Technology Development Center of PLA General Hospita, China).

### 2.2. Animals

Male Wistar rats (weighing 180–220 g) were purchased from the Experimental Animal Center of the Basic Medical College of Jilin University (animal license number: SCXK Jilin 2003-0001 (Jilin, China)). Beagle dogs (weighing 10–12 kg), were purchased from Natural Pharmatech (Jilin, China). The animals had free access to a standard rodent pelleted diet and water ad libitum. The study followed the principles of the Helsinki declaration. The Jilin University Animal Ethics Committee approved all the animal experiments.

### 2.3. Preparation of octreotide-loaded PLGA microspheres

The preparation of Octreotide microspheres was optimized by double emulsion solvent evaporation method. First, dichloromethane was added to a distillation solution containing 2.5 g powder of Octreotide and soaked in Octreotide to obtain a cloudy liquid. A fraction of water (1 mL) was then added to the turbid solution. After evaporation of dichloromethane, a highly viscous internal aqueous phase was produced. PLGA (29% w/v in dichloromethane, dichloromethane–acetone, dichloromethane–ethyl acetate, or dichloromethane–dimethyl sulfoxide (DMSO)) was added to the vortex mixture. A high-speed agitator (T18; IKA) stirred the mixture for 10 min at 10,000 rpm at 15 °C to initiate emulsion formation. After initial incubation at 4 °C for 30 min, the initial emulsion was dripped into 0.5% PVA aqueous solution to form a double emulsion. The mixture was stirred at 800 rpm for 10 min using a high-speed mixer at 15 °C. The solution was decompressed to remove the organic solvent (1 h) to form solidified microspheres. The microspheres were centrifuged at 3,000 rpm for 5 min to collect the microspheres, which were washed three times with acetate buffer (pH 4.0). After thorough washing and freeze-drying, purified microspheres were obtained.

### 2.4. Determination of the drug loading and morphology of the PLGA microspheres loaded with octreotide

The encapsulation efficiency was determined by high performance liquid chromatography (HPLC, Agilent Technologies, Beijing, China). Briefly, 0.2 g microspheres were accurately weighed, placed in a 250 ml volumetric flask, 50 ml of tetrahydrofuran and a stopper was added followed by shaking to dissolve. The diluted solution, 2.0 g glacial acetic acid, 2.0 g sodium acetate (NaC<sub>2</sub>H<sub>3</sub>O<sub>2</sub>·3H<sub>2</sub>O), and 7.0 g sodium chloride were dissolved in water and diluted to 1,000 ml, diluted to the mark, shaken well for 20 min to precipitate the macromolecules. The solution was filtered (Millex HV 0.45  $\mu$ m), and an accurate determination of the filtrate was obtained by injecting 10  $\mu$ l into the liquid chromatograph, and recording the chromatograms. In addition, the appropriate amount of Octreotide reference substance was determined accurately, and the diluted solution was added in the same way to prepare a 50  $\mu$ g/ml solution. The content of Octreotide acetate in the sample was calculated from the peak area according to the external standard method (Zhang et al., 2010; Chen et al., 2014). Microspheres were dispersed in 0.1% (w/w) Tween 20 aqueous solution, and the particle size was measured by a Mastersizer 2000 particle analyzer (Malvern, England).

### 2.5. Circular Dichroism

The octreotide particles were immersed in an appropriate amount of dichloromethane to fully dissolve, and then extracted with water for injection, and the extracts were combined. Prepared a 50  $\mu$ g/ml octreotide solution for later

use. CD spectra of the octreotide solution were recorded. The CD spectra of natural octreotide was recorded for reference.

## 2.6. In vitro release of octreotide-loaded PLGA microspheres

The in vitro release of Octreotide-loaded PLGA microspheres was performed in PBS. Briefly, 5 mg Octreotide PLGA microspheres were dissolved in PBS (pH = 7.4, with 0.02% sodium azide) (Habraken et al., 2006; Kempen et al., 2006; Bhardwaj et al., 2007). A series of dilutions was conducted to establish a standard curve for Octreotide. Then, 20 mg of Octreotide-loaded microspheres were placed in 30 ml of PBS at 37 °C with shaking at 75 rpm. 10 mL of supernatant was taken at 4 h, 24 h, 48 h, 96 h, 144 h, 192 h, 240 h, 336 h, 432 h, 528 h, 624 h, 720 h, 816 h, 912 h, 1008 h, 1104 h and 1200 h, centrifuged at 700 r/min for 3 min, and the obtained supernatant for liquid chromatography analysis. The precipitate at the bottom of the centrifuge tube was placed back into the release tubes, and approximately 10 mL of in vitro release solution was added to the concave label. The Octreotide content in the supernatant at each time point was determined by HPLC, and the cumulative release percent over 50 d was determined.

## 2.7. Pharmacokinetics of octreotide-loaded PLGA microspheres

Each beagle was intramuscular injected with 1.4 mg/kg Octreotide-loaded PLGA microspheres and Sandostatin separately ( $n=6$ ). Blood samples (3.5 ml) were collected at 4 h, 24 h, 48 h, 96 h, 144 h, 192 h, 240 h, 336 h, 432 h, 528 h, 624 h, 720 h, 816 h, 912 h, 1008 h, 1104 h and 1200 h for the determination of plasma concentration of Octreotide by LC-MS (Habraken et al., 2006). Blood concentration curve was established to analyze the release rate.

## 2.8. Pharmacodynamics

Fifteen rats were randomly selected from 180 male Wistar rats by weight as the blank control group, 15 rats were subjected to sham operation, and the other 150 rats were treated as the model group to replicate the experimental portal hypertension model of liver cirrhosis (Zhou et al., 2000). All rats in the sham group underwent surgery no ligation of the superior renal vein. In this experiment, the left superior renal vein and carbon tetrachloride were disconnected for 15 weeks to model portal hypertension in rats with cirrhosis. The model conformed to the clinical practice of cirrhosis portal hypertension. Rats were fed a normal diet and injected with penicillin sodium daily at a dose of 100,000 units/100 g to avoid infection 1 week after operation. Two weeks after operation, the rats were randomly divided into model group, high-, medium-, and low-dose Octreotide microsphere injection groups and positive drug group, with 26 rats in each group.

The typical hepatotoxic drug  $\text{CCl}_4$  was administered in the sham operation group, model group, high-, medium-, and low-dose Octreotide microsphere injection groups and positive drug group. To ensure that the drug concentration in vivo was high enough when the rat liver cirrhosis portal hypertension model was established, the drug was administered once 2 weeks before formation of the model (the plasma concentration rises 1 week after a single intramuscular injection and reaches a plateau after approximately 2 weeks, according to the pharmacokinetics characteristics). Then, 8, 4, or 2 mg/kg (4 times, 2 times, or equal to that of the clinical common dose) of Octreotide microspheres were injected intramuscularly in the high-, medium- and low-dose groups, respectively. Sandostatin (8 mg/kg) was injected intramuscularly in the positive drug group. Blank microspheres were administered to the blank control group, sham operation group and model group. Rats were fasted for 12 h after 4 weeks of administration and then anesthetized by intraperitoneal injection of 10% chloral hydrate at a dose of 300 mg/kg.

After that, the rats were anesthetized, fixed in supine position and incised in the middle abdomen. The mesenteric vein and portal vein were exposed. The PC intubation filled with heparin saline and connected with a pressure transducer was inserted conversely into the mesenteric vein. After the pressure curve was stabilized, the pressure of the mesenteric vein was determined, considering that the pressure of the right atrium was zero. The intubation followed the mesenteric vein to the main portal vein. The pressure of portal vein was determined by the above method. The anterior mesenteric vein at the punctured site was then ligated. The main left gastric vein was exposed and punctured in the direction of reverse blood flow to measure the pressure of the left gastric vein. Finally, the puncture site of the blood vessel was ligated.

Blood and tissue samples were collected and AST, ALT, Hyp, MDA,  $\text{PGI}_2$ ,  $\text{TNF}\alpha$ , SOD, GSH,  $\text{TXA}_2$ , NO, NOS, ET were detected according to the manufacturer's instructions. The portal vein was about 1.0 cm, fixed in 10% formaldehyde, and stained with H&E. The lumen area of the portal vein was determined by a computer image analysis. Portal veins (1 ~ 1.5 cm) were collected and fixed in 2.5% glutaraldehyde. The ultrastructure of the portal vein was observed by electron microscopy. The thymus, spleen, kidney and liver of the rats were collected and weighed. The organ coefficient (organ weight/weight  $\times$  100%) was calculated according to the weight of each rat. A partial sample of the right lobe of the liver was collected and fixed in 10% formaldehyde and stained with H&E for pathological morphological detection.

## 2.9. Statistical analysis

Statistical analysis was performed using GraphPad Prism 5.0 software. Intergroup  $t$ -test comparisons were performed using the mean  $\pm$  standard deviation ( $\bar{x} \pm s$ ), and  $p < .05$  was considered statistically significant.

### 3. Results

#### 3.1. Optimization of microsphere preparation

The physical properties of the organic solvent and the ability to dissolve the carrier material and drug affect the particle size and encapsulation efficiency of the microspheres. Therefore, Octreotide microspheres were prepared by using different proportions of solvents ( $\text{CH}_2\text{Cl}_2$ - $\text{CH}_3\text{COCH}_3$ (9:1)(M1),  $\text{CH}_2\text{Cl}_2$ (M2),  $\text{CH}_2\text{Cl}_2$ - $\text{CH}_3\text{COOC}_2\text{H}_5$ (9:1)(M3),  $\text{CH}_2\text{Cl}_2$ -DMSO(9:1)(M4),  $\text{CH}_2\text{Cl}_2$ - $\text{CH}_3\text{COCH}_3$ (4:1)(M5),  $\text{CH}_2\text{Cl}_2$ - $\text{CH}_3\text{COOC}_2\text{H}_5$ (4:1)(M6),  $\text{CH}_2\text{Cl}_2$ -DMSO(4:1)(M7)).

#### 3.2. In vitro and in vivo drug release of octreotide microspheres

The samples prepared by optimized and conventional methods were characterized. Characterization was based on particle size distribution, loading (LC), and initial burst release (Figure 1, Table 1). Scanning electron microscopy (SEM) study on the surface morphology of Octreotide microspheres showed that the prepared microspheres were all round with uniform pores on the surface and inside, which was the main reason for their sudden release. The surface of M7 microsphere was smoother and more uniform than that of M4. These results could be attributed to co-solvents and their removal process that created desired polymer matrix structure to reduced burst. The initial burst release of the sample was determined during the first 24 h of the double emulsion process. At each time point, Octreotide microspheres were collected by centrifugation at 3000 rpm for 5 min at 4°C. The effect of organic solvent on initial burst release was determined. The results are shown in Figure 2. M1, M2, M3, M4, M5, M6 and M7 were similarly loaded, and the initial burst release (6.47%) of M7 was smaller than that of M3 (8.33%). The initial burst release results showed that initial burst release decreased with the increase of DMSO ratio. These results indicated that the difference in particle size of PLGA microspheres was related to the interfacial tension and viscosity of the organic phase. The greater the interfacial tension and viscosity in the formation of the emulsion, the larger the particle size and the lower the burst

release. The microspheres prepared by M7 were used for verification in subsequent experiments.

#### 3.3. Structural study by circular dichroism

For octreotide microsphere delivery systems, it is very important to evaluate the structural integrity of octreotide released from the microparticles. Figure 3 showed the CD spectra of released octreotide and native octreotide. The CD spectrum of the released octreotide showed negative ellipticity at 202 and 217 nm, which was the same as that of natural octreotide. These results showed that no conformational changes were induced in the released polypeptide chain of octreotide, indicating that the biological activity of octreotide was retained.

#### 3.4. In vitro and in vivo release and pharmacokinetics of octreotide microspheres

In vitro release evaluation, 10 ml of release solution was taken separately at 4 h, 24 h, 48 h, 96 h, 144 h, 192 h, 240 h, 336 h, 432 h, 528 h, 624 h, 720 h, 816 h, 912 h, 1008 h, 1104 h and 1200 h, and the in vitro release was detected by HPLC. Figure 4 indicated that the Octreotide-loaded PLGA microspheres and Sandostatin had sustained release in PBS for 50d. Compared with Sandostatin, the initial release of Octreotide microspheres was lower, and the burst release effect was not obvious. The blood Octreotide concentration in beagle was detected by LC-MS at 4 h, 24 h, 48 h, 96 h, 144 h, 192 h, 240 h, 336 h, 432 h, 528 h, 624 h, 720 h, 816 h, 912 h, 1008 h, 1104 h and 1200 h (Figure 5). Compared with Sandostatin, Octreotide microspheres showed a significant

Table 1. Microsphere performance inspection results.

Method	Solvent	Particle size ( $\mu\text{m}$ )	Drug loading (%)	Release (%)
M1	$\text{CH}_2\text{Cl}_2$ - $\text{CH}_3\text{COCH}_3$ (9:1)	$48.71 \pm 14.82$	$10.06 \pm 1.32$	$9.31 \pm 1.54$
M2	$\text{CH}_2\text{Cl}_2$	$53.13 \pm 16.56$	$12.28 \pm 1.28$	$8.94 \pm 1.88$
M3	$\text{CH}_2\text{Cl}_2$ - $\text{CH}_3\text{COOC}_2\text{H}_5$ (9:1)	$51.47 \pm 17.91$	$11.07 \pm 1.33$	$8.33 \pm 1.26$
M4	$\text{CH}_2\text{Cl}_2$ -DMSO(9:1)	$55.17 \pm 15.85$	$14.38 \pm 2.31$	$7.61 \pm 1.53$
M5	$\text{CH}_2\text{Cl}_2$ - $\text{CH}_3\text{COCH}_3$ (4:1)	$43.24 \pm 13.89$	$8.51 \pm 1.79$	$10.73 \pm 1.67$
M6	$\text{CH}_2\text{Cl}_2$ - $\text{CH}_3\text{COOC}_2\text{H}_5$ (4:1)	$49.12 \pm 14.62$	$10.87 \pm 1.26$	$8.05 \pm 1.11$
M7	$\text{CH}_2\text{Cl}_2$ -DMSO(4:1)	$57.48 \pm 15.24$	$16.43 \pm 2.45$	$6.47 \pm 1.09$

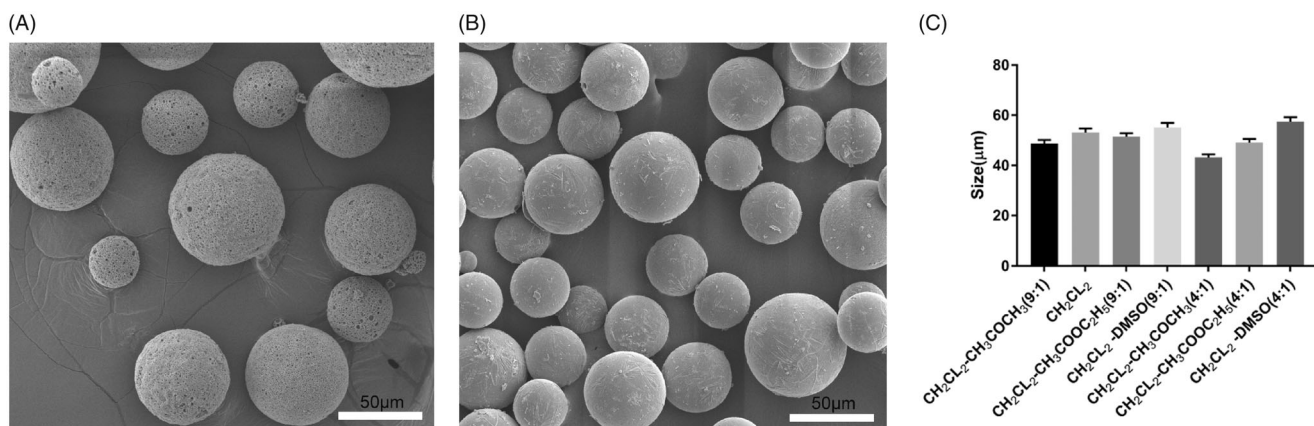


Figure 1. Morphological and physicochemical characterization of Octreotide Microsphere. (A) Transmission electron micrographs of Octreotide Microsphere ( $\text{CH}_2\text{Cl}_2$ - $\text{CH}_3\text{COCH}_3$ (9:1)), (B) The Octreotide Microsphere were characterized by dynamic light scattering (DLS) for their size.

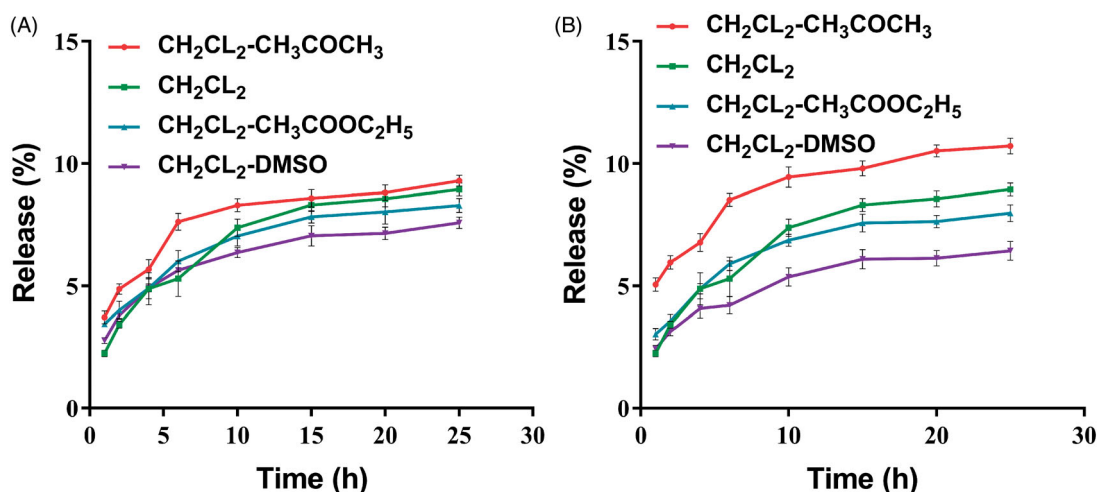


Figure 2. The release curve over the first 24 h. (A) Dichloromethane and different solvent ratios of 9:1, (B) dichloromethane and different solvent ratios of 4:1.

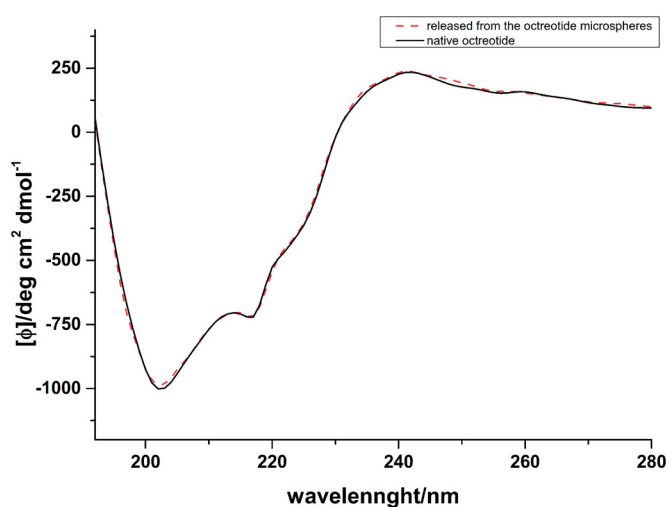


Figure 3. CD spectra of the octreotide released from the octreotide microspheres (red line) and native octreotide (black line).

decrease in plasma concentration during the initial stage after administration. Furthermore, Octreotide can be stably released during the period from 48 h to 624 h.

### 3.5. Pharmacodynamics of the inhibition of PHT

The effects of Octreotide microsphere injection on portal vein pressure were studied. In contrast to the blank control group, there was no significant difference in the rat portal vein pressure (PP), mesenteric vein pressure (PM) or left gastric vein pressure (PG) of the sham operation group. Compared with the sham operation group, the PP, PM and PG of the model group were significantly increased, indicating that the model of portal hypertension in rats with cirrhosis was successfully developed. Compared with the model group, the injection of 4 or 8 mg/kg Octreotide microspheres and 8 mg/kg Sandostatin significantly reduced the PP, PM and PG in rats, and the injection of 2 mg/kg Octreotide acetate microspheres exerted no obvious influence on the PP, PM and PM of rats. The effects above were not significantly different between 4 and 8 mg/kg Octreotide acetate microspheres and 8 mg/kg Sandostatin administration (Figure 6).

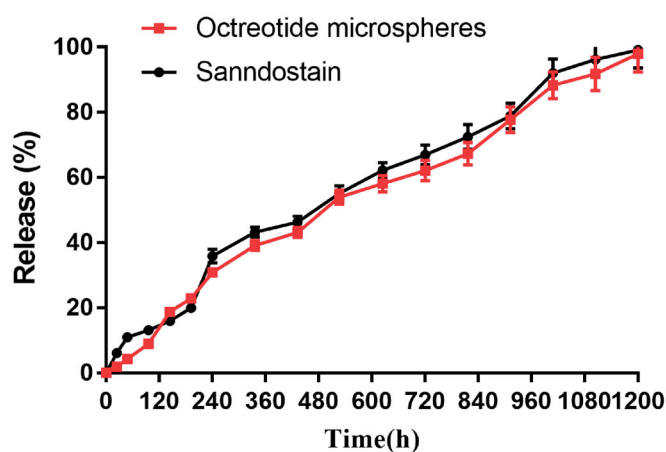


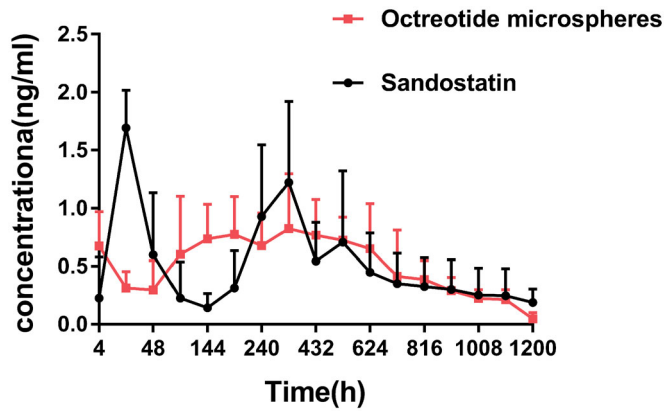
Figure 4. In vitro release profile of Octreotide microspheres or Sandostatin in PBS solution for 50 d.

#### 3.5.1. Visual inspection

Rats in the blank control group and sham group had red and brown, soft and brittle livers with thin margins. In the model group, the brownish, yellow livers were significantly stiffened, blunt and thick without their normal luster, and the surfaces were uneven. Livers in the Octreotide microsphere and Sandostatin injection groups hardened without normal gloss. The surface was uneven with nodules. However, the level of cirrhosis in the model group rats was not as high as that in the control group (Figure 7).

#### 3.5.2. Pathomorphological observation

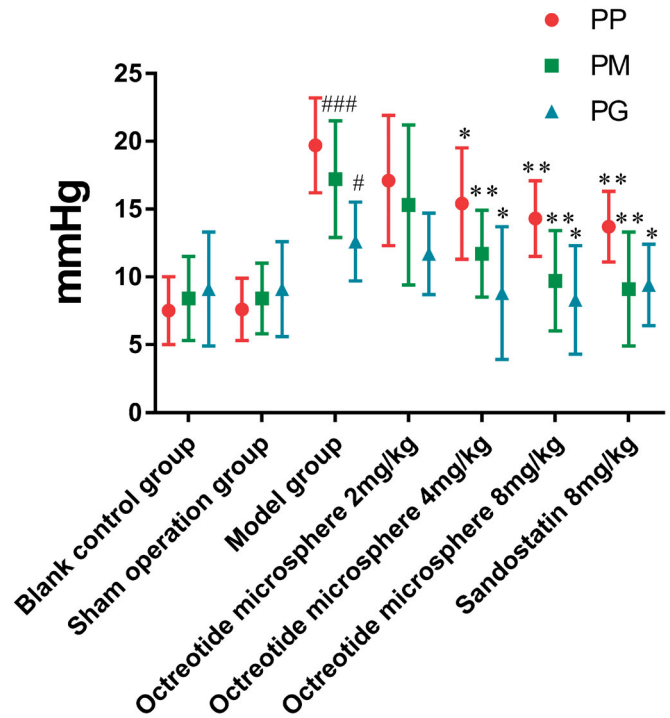
Livers of blank control group were normal in structure. Hepatocytes were polygonal in shape, surrounding the lobules and radiating out from the central vein. The cytoplasm was stained pink and the nuclei were round or oval. The hepatic sinusoids were clear, and endothelial cells could be seen inside. Blood vessels and bile ducts could be seen in the portal area (Figure 8(A)). The liver tissues of the sham operation group had integrated structure, and the hepatic lobules were clear. Hepatocytes surrounded the lobules, radiating from the central vein. Most cells were morphologically normal, and only a handful of cells in the central lobule area



**Figure 5.** Octreotide blood concentration–time curve after a single intramuscular administration of Octreotide microspheres or Sandostatin in beagles (mean  $\pm$  SD,  $n = 6$ ).

showed steatosis. Inflammatory cell infiltration could be seen in parts of the portal area. The other groups were the same as the blank control group (Figure 8(B)). The lobular structure of the model group was damaged with disordered hepatocyte arrangement. The liver interstitium had fibrous tissue proliferation, which could divide the hepatic lobules into different sizes of oval or irregularly shaped pseudolobules in some areas. Hepatocytes were eosinophilic and diffuse. Steatosis, focal necrosis, hyaline degeneration, cellular swelling and hepatocyte regeneration were observed. Lymphocyte infiltration and bile duct hyperplasia were observed in the fibrous septum of the portal area (Figure 8(C)). In the low-dose and medium-dose Octreotide acetate microsphere injection groups, pseudolobules could be seen clearly. Active proliferation of fibrous tissue in the interstitial area is shown. A large number of lymphocytes infiltrated. Steatosis and focal necrosis of hepatocytes can be observed. The degree of pathological changes was less than that in the model group (Figure 8(D,E)). In the group injected with high-dose Octreotide acetate microspheres and Sandostatin, fibrous tissue hyperplasia was observed in the liver tissue. A few pseudolobules could be spotted, and the degree of steatosis and focal necrosis was less than that in the model group. Lymphocyte infiltration could also be seen in some areas (Figure 8(F,G)).

In the blank control group, cells in the intima, media and adventitia of the vein were normal, and the cytoplasm and nucleus were also normal (Figure 9(A,B)). In the sham operation group, cells in media and adventitia of vein were normal, as well as the cytoplasm and nucleus inside (Figure 9(C,D)). In the model group, platelets could be seen inside the vessel. The integrity of the endothelial basement membrane was damaged, and the connection was loose. Endothelial cells, in folded shapes, were removed and were in different degrees of degeneration. The intima of the veins was exposed, and platelets adhered to the endothelium. The smooth muscle, elastic membrane and elastic fiber of the media were in a disordered arrangement. Some of the elastic plates ruptured, necrotic tissues and scattered smooth muscle cells could be seen, and the smooth muscle layer



**Figure 6.** Effects of Octreotide microspheres on the pressure of the portal vein, vena mesenteric anterior and gastric vein in portal hypertensive rats. Compared with the sham operation group,  $^{\#}p < .05$ ,  $^{\#\#\#}p < .001$ ; compared with the model group,  $^*p < .05$ ,  $^{**}p < .01$ .

was significantly thickened. Smooth muscle cells increased in size with degeneration, the mitochondria in the cells increased, and residual body and lipofuscin could be seen. Collagen fibers and extracellular matrix increased, and fibroblasts also increased with degeneration (Figure 9(E,F)). In the low-dose Octreotide group, the endothelium dropped and platelets and necrotic tissues could be seen on the surface of the intima. The elastic membrane and smooth muscle were visible in the media, and the smooth muscle was pyknotic. The mitochondria in the smooth muscle cells of the outer membrane increased in number, and exudate was visible. Some smooth muscle cells were stained deeply and homogeneously (Figure 9(G,H)). In the middle-dose Octreotide group, endothelial cells dropped, with few remaining. Platelets adhered to the intima surface. The mitochondria in the smooth muscle cells of the media were swollen, with collagen fibers and elastic around; a few necrotic tissues could also be seen. Smooth muscle cells in the adventitia increased in number, with more vacuoles inside the mitochondria (Figure 9(I,J)). In the high-dose Octreotide group, most of the endothelium was exfoliated, but the endothelial residue was visible, with platelets adhered to the surface. The endothelial cells and nucleus were larger. An elastic membrane and smooth muscle cells were observed in the media. Platelet infiltration and aggregation were observed in the intercellular space of the smooth muscle cells. The smooth muscle cell nuclei in the adventitia were irregularly shaped, with less myofibril inside the cytoplasm (Figure 9(K,L)). In the Sandostatin group, most of endothelial cells were exfoliated, but endothelial residue could be observed. The intima was thin, with more platelets adhered to the surface. An elastic

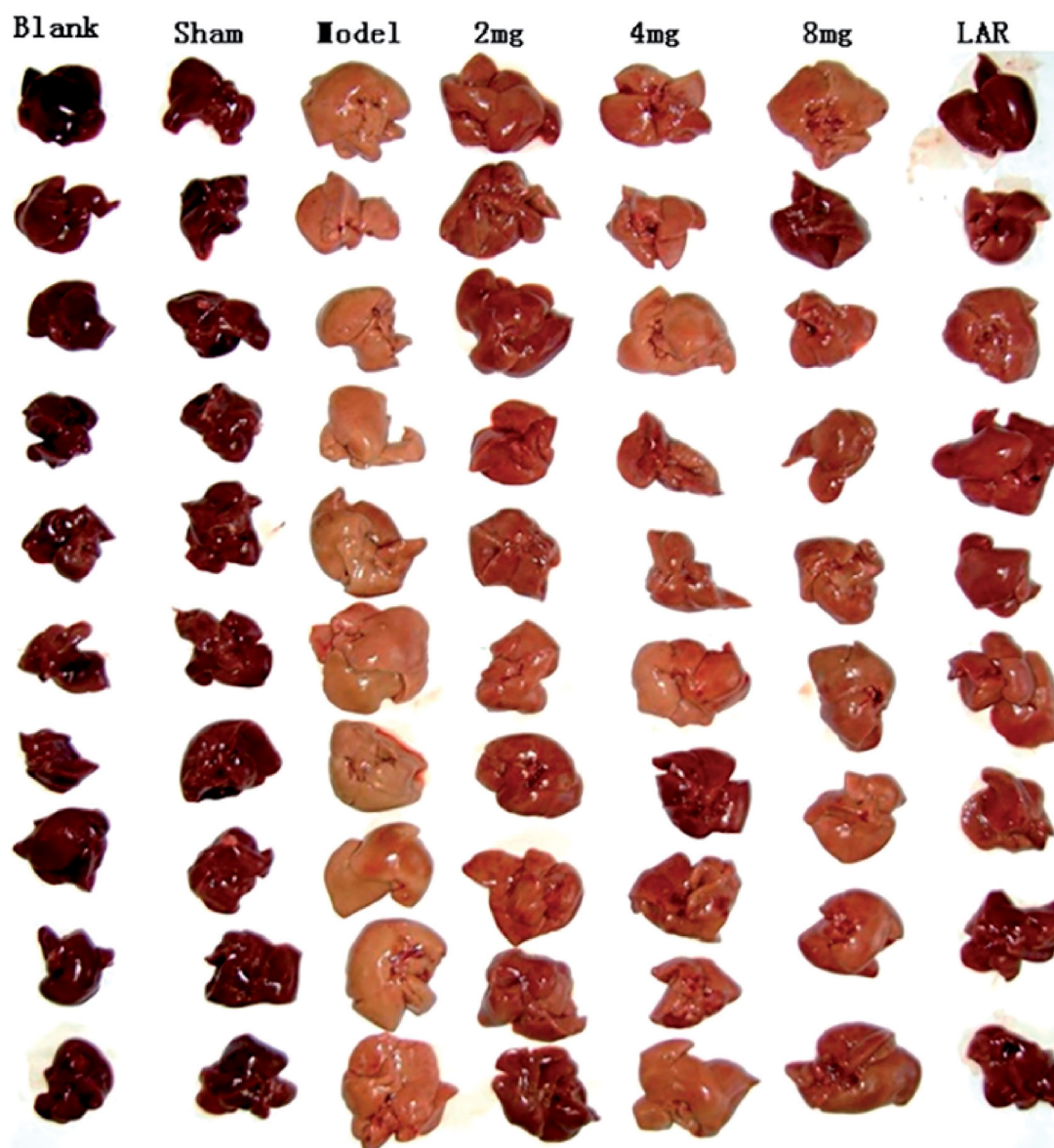


Figure 7. Liver observation photo.

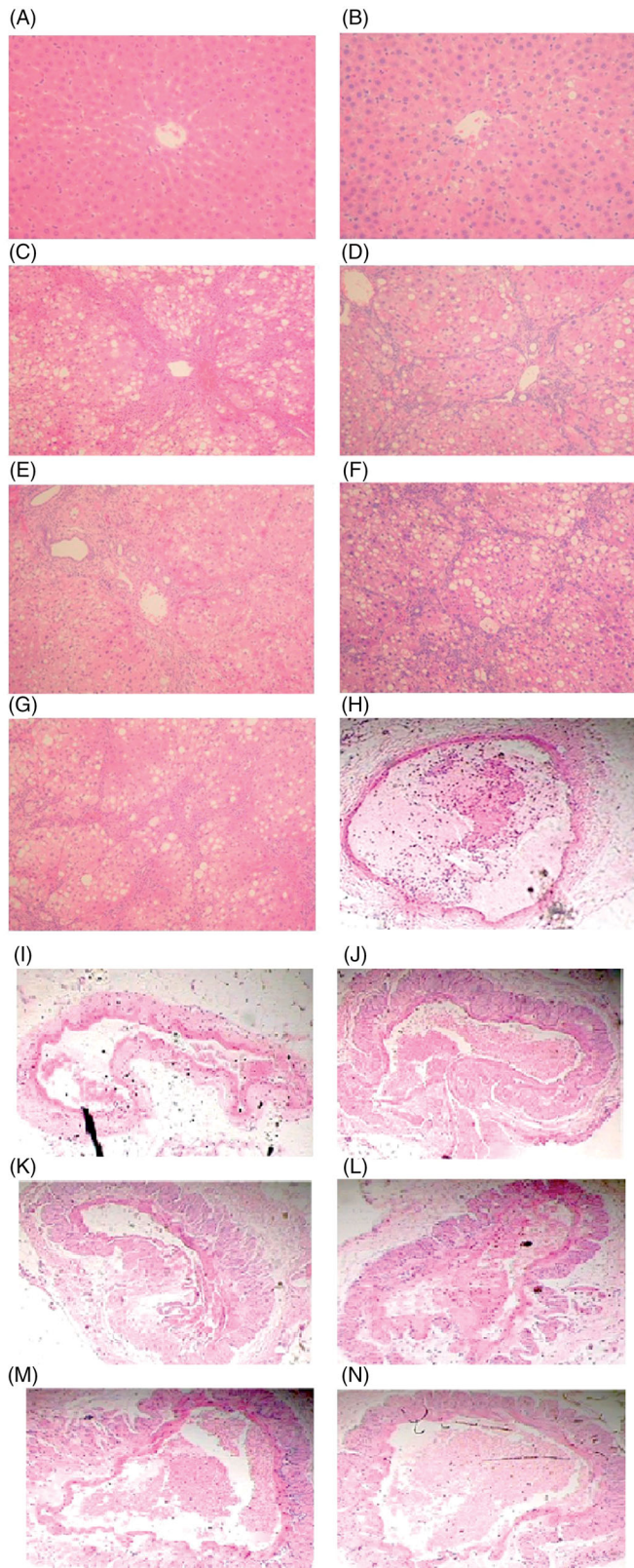
membrane and smooth muscle cells were visible, the mitochondria in the smooth muscle cells increased and had vacuoles inside. Many smooth muscle cells existed in the adventitia, with more mitochondria inside. Cells were stained lightly with fewer myofilaments, dense bodies and dense patches (Figure 9(M,N)).

The portal veins of the rats in each group were fixed, and the portal vein area was measured by computer image analysis, which indirectly reflected the portal vein size. These results showed that there were no significant differences in the portal vein between sham operation group and blank control group. The portal vein area in the model group was significantly increased than that of the sham operation group, indicating that the hypertension model in rats with hepatic cirrhosis was accompanied by enlargement of the portal vein diameter. Compared with the model group, injection of 8 mg/kg Octreotide microspheres and Sandostatin significantly reduced the portal vein area, and no significant difference between the 8 mg/kg Octreotide microsphere and

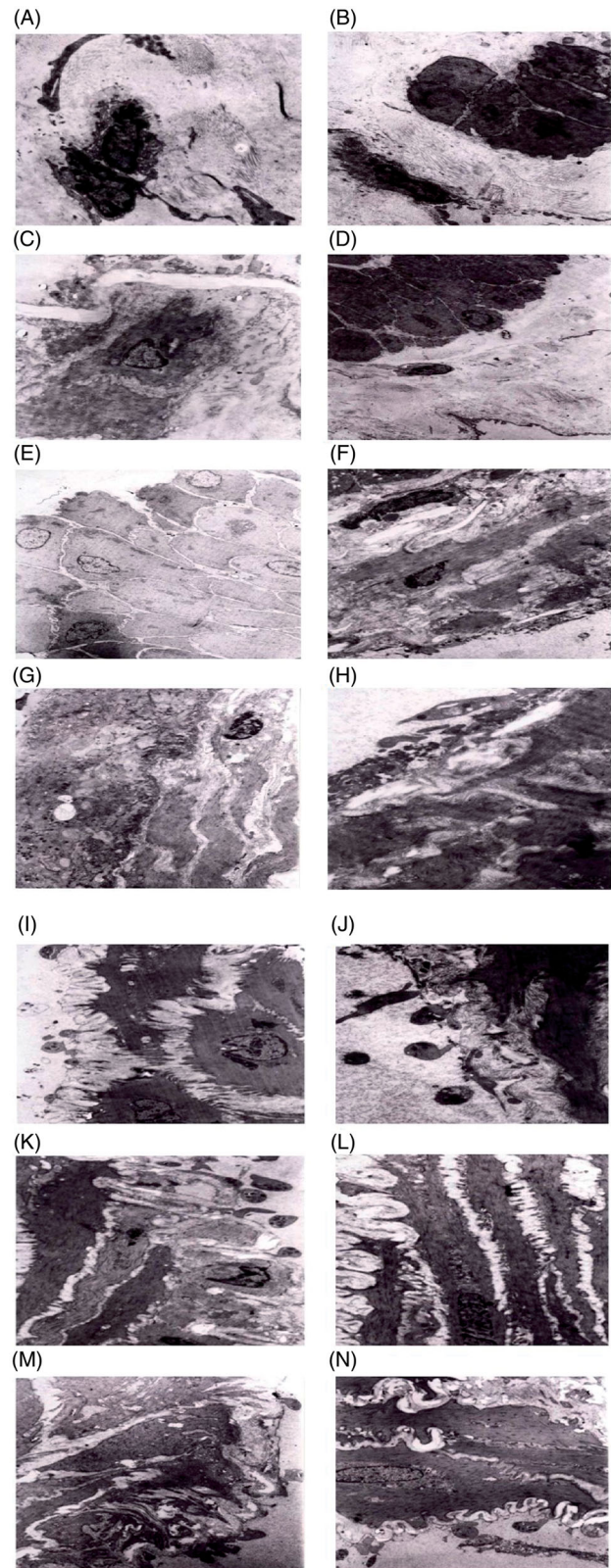
Sandostatin groups. Injection of 2 or 4 mg/kg Octreotide acetate microspheres had no significant effect on the portal vein area (Figures 8(H-N) and 10).

There were no significant differences in liver coefficient, thymus coefficient, spleen coefficient and renal coefficient between sham operation group and blank control group. Compared to the sham group, the thymus index decreased slightly without a significant difference in the model group, but all the above indexes increased significantly. Compared with the model group, injection with 8 mg/kg Octreotide microspheres and Sandostatin significantly reduced the liver index, and injection with 4 or 8 mg/kg Octreotide microspheres and Sandostatin also reduced the liver index, with no significant difference among each of the groups. There was no significant effect on the thymus index or renal index of rats injected with Octreotide acetate microspheres or Sandostatin (Figure 11).

The serum AST and ALT activity in the blank control group was not clearly different from that in the sham group.

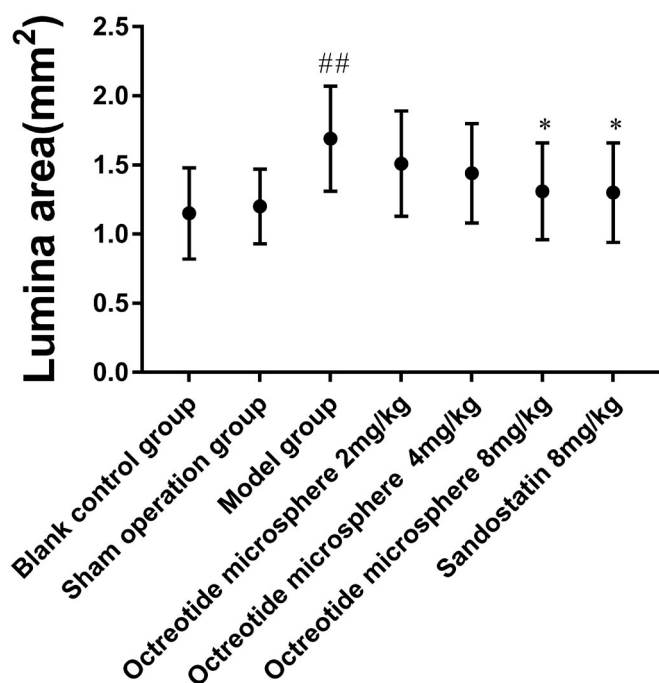


**Figure 8.** Histopathological observations of the liver tissues: (A) Liver tissues of the blank control group; (B) Liver tissues of the sham operation group; (C) Liver tissues of the model group; (D) Liver tissues of low-dose Octreotide microsphere injection group; (E) Liver tissues of medium-dose Octreotide microsphere injection group; (F) Liver tissues of high-dose Octreotide microsphere injection group; (G) Liver tissues of the Sandostatin injection group. Effects of Octreotide microsphere injection on the vascular lumen area of the portal vein in the (H) Blank control group; (I) Sham operation group; (J) Model group; (K) Low-dose Octreotide microsphere injection group; (L) Medium-dose Octreotide microsphere injection group; (M) Medium-dose Octreotide microsphere injection group; and (N) Sandostatin injection group.

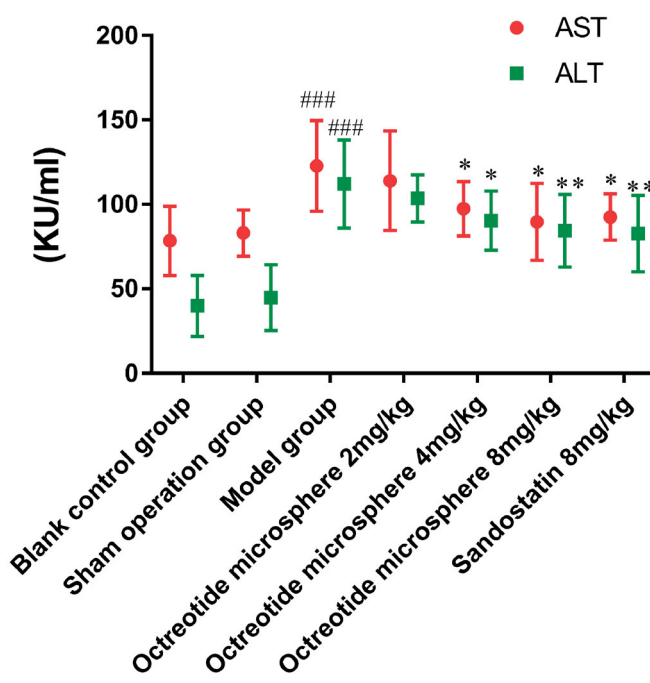


**Figure 9.** Morphological electron microscopy images of the portal vein. (A, B) Blank control group; (C, D) Sham operation group; (E, F) Model group; (G, H) Low-dose Octreotide microsphere injection group; (I, J) Medium-dose Octreotide microsphere injection group; (K, L) High-dose Octreotide microsphere injection group; and (M, N) Sandostatin injection group.

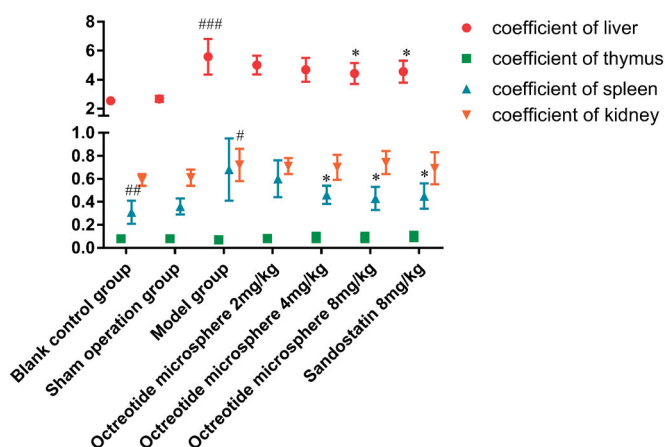




**Figure 10.** Effect of Octreotide microspheres on the luminal area of the portal vein in portal hypertensive rats. Compared with the sham group,  $^{\#}p < .05$ ,  $^{##}p < .01$ ; compared with model group,  $^{*}p < .05$ .



**Figure 12.** Effect of Octreotide microspheres on the AST and ALT activities in the serum in portal hypertensive rats. Compared with the sham group,  $^{###}p < .01$ ; compared to the model group,  $^{*}p < .05$ ,  $^{**}p < .01$ .



**Figure 11.** Effects of Octreotide microsphere acetate injection on the organ coefficient. Compared with the false operation group,  $^{###}p < .001$ ,  $^{##}p < .01$ ,  $^{\#}p < .05$ ; compared with the model group,  $^{*}p < .05$ .

Compared with the sham operation group, the serum AST and ALT activities in the model group were significantly increased. Compared with the model group, injection of 4 and 8 mg/kg Octreotide acetate microspheres and Sandostatin significantly reduced serum AST and ALT activity, and there was no significant difference between the Octreotide acetate microsphere and Sandostatin groups (Figure 12).

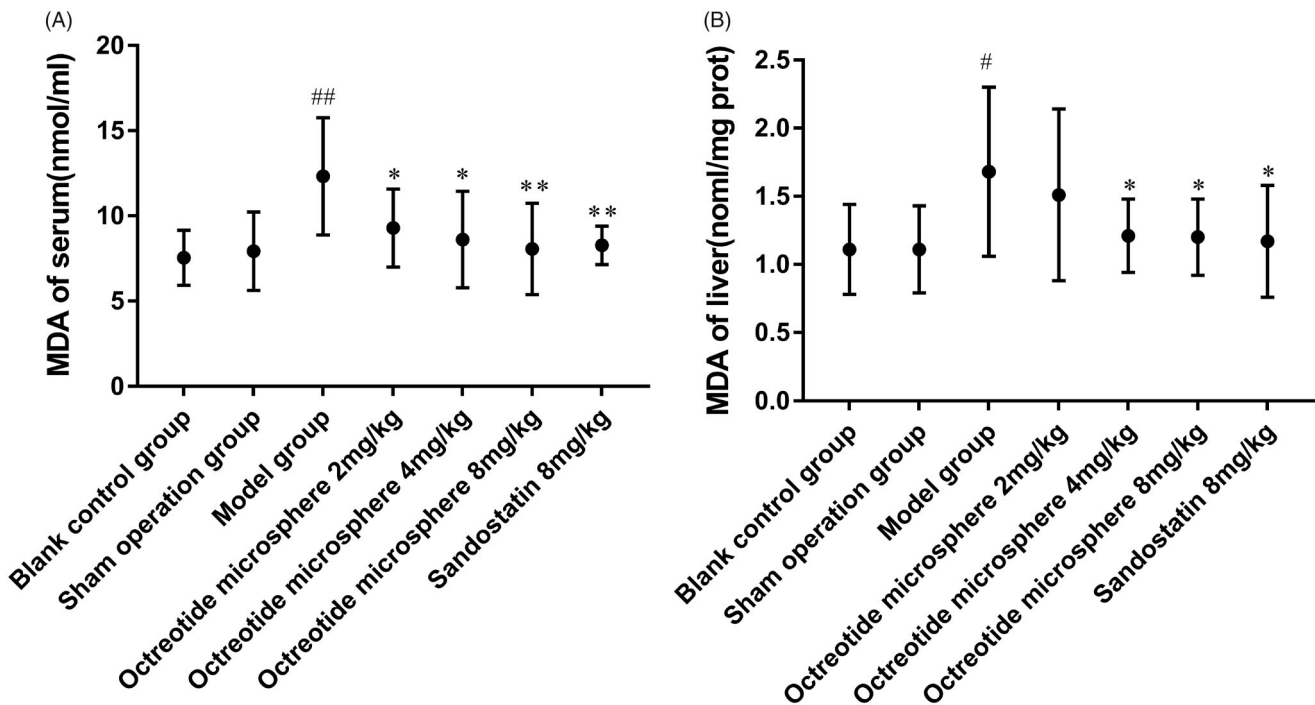
There were no significant differences in MDA levels in serum and liver tissues between the sham group and blank control group ( $p > .05$ ). Compared with the sham group, MDA levels in serum and liver tissues of the former group were significantly increased ( $p < .05$  or  $p < .01$ ). Compared with the model group, injection of 2, 4, or 8 mg/kg Octreotide acetate microspheres and Sandostatin significantly

decreased MDA levels in serum ( $p < .05$  or  $p < .01$ ), injection of 4 or 8 mg/kg Octreotide acetate microspheres and Sandostatin significantly reduced MDA levels in liver tissues ( $p < .05$ ), and no significant difference between the Octreotide acetate microsphere and Sandostatin groups ( $p > .05$ ) (Figure 13).

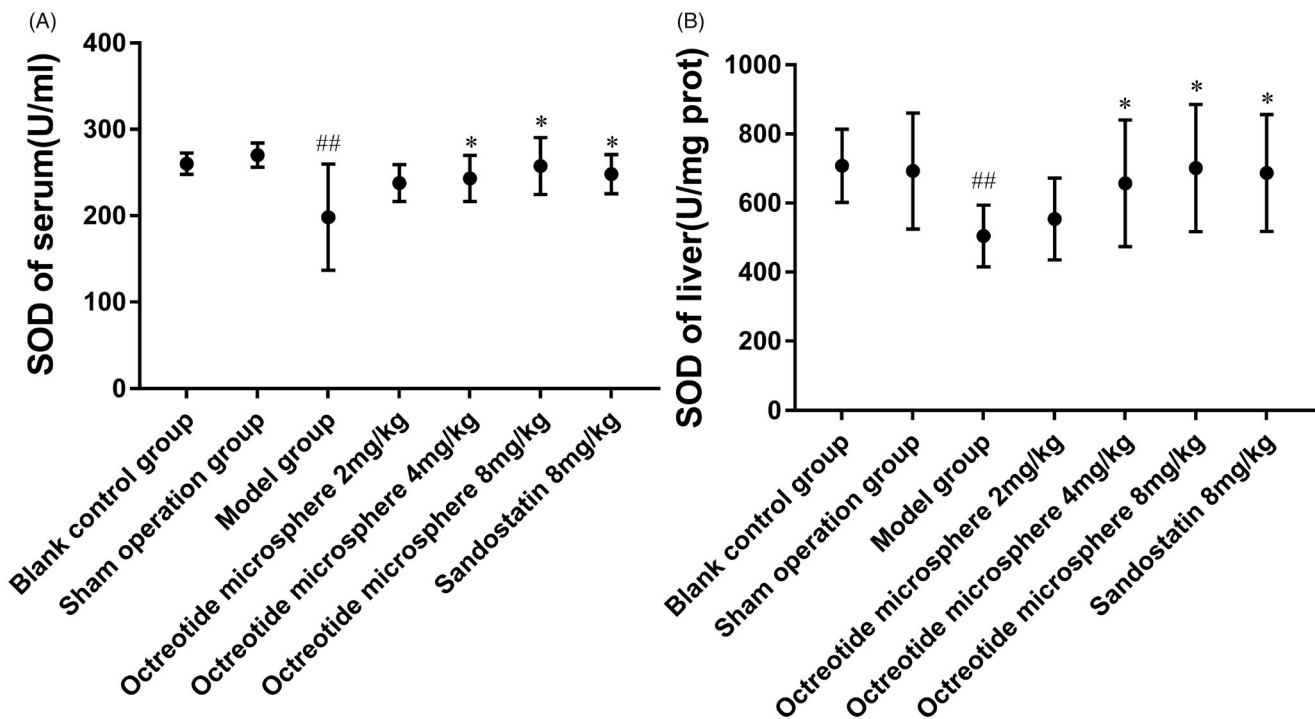
There were no significant differences in SOD activity in the serum and liver tissues between the sham operation group and blank control group. Compared with the sham operation group, the activity of SOD in the serum and liver tissue was significantly decreased in the model group. Injection of 4 or 8 mg/kg Octreotide microspheres and Sandostatin significantly increased the activity of SOD in the serum and liver tissue compared with the model group. No obvious differences on the activity of SOD in the serum and liver tissue after injection of 2 mg/kg Octreotide acetate microspheres compared with the model group. (Figure 14).

There were no significant differences in the levels of Hyp, GSH and  $TNF\alpha$  in the liver tissue between the sham operation group and blank control group. The levels of Hyp and  $TNF\alpha$  in the liver tissue of the model group rats increased significantly compared with the sham operation group, and the GSH level decreased significantly. Compared with the model group, injection of 4 and 8 mg/kg Octreotide acetate microspheres and Sandostatin significantly reduced Hyp and  $TNF\alpha$  levels, while injection of 2, 4, and 8 mg/kg Octreotide acetate microspheres and Sandostatin significantly increased GSH levels in the liver; no significant difference between the Octreotide acetate microspheres and Sandostatin groups (Figure 15).

There were no significant differences in the levels of plasma  $PGI_2$  and  $TXA_2$  between the sham operation group and blank control group. Compared with the sham operation group, the levels of plasma  $TXA_2$  and  $PGI_2$  in the model



**Figure 13.** Effect of Octreotide microspheres on MDA in the (A) serum and (B) liver of portal hypertensive rats. Compared to sham group, <sup>#</sup> $p < .05$ , <sup>##</sup> $p < .01$ ; compared with model group, <sup>\*</sup> $p < .05$ , <sup>\*\*</sup> $p < .01$ .

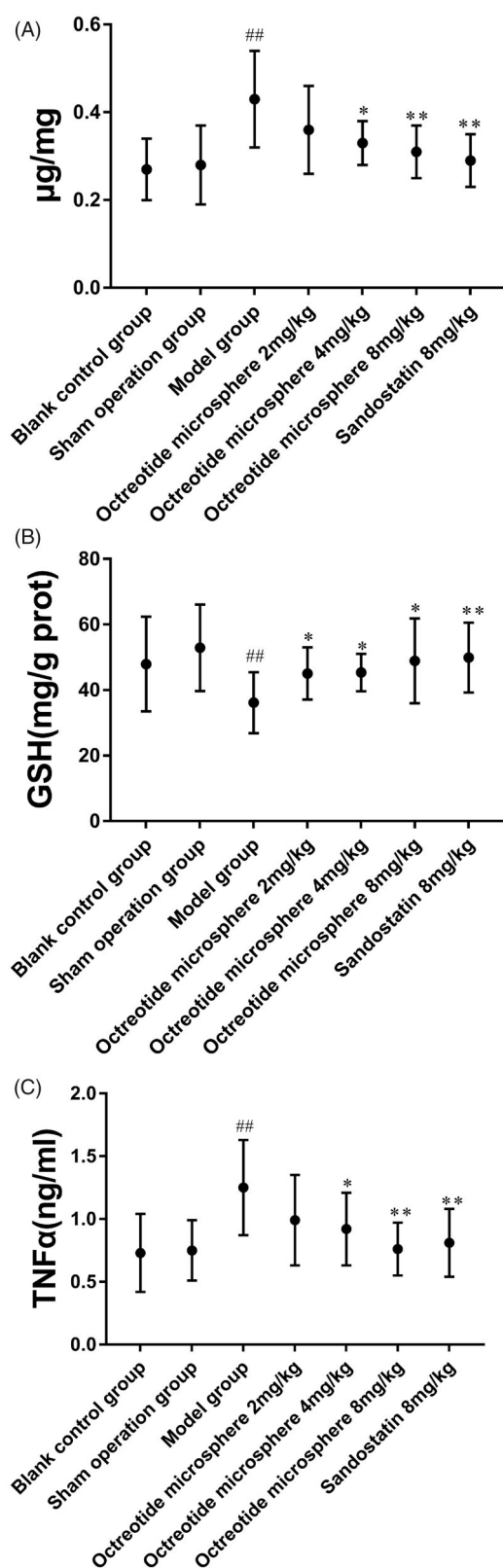


**Figure 14.** Effects of Octreotide microspheres on SOD in the (A) serum and (B) liver of portal hypertensive rats. Compared with the sham operation group, <sup>##</sup> $p < .01$ ; compared with the model group, <sup>\*</sup> $p < .05$ , <sup>\*\*</sup> $p < .01$ .

group increased significantly. Compared with the model group, injection of 4 and 8 mg/kg Octreotide acetate microspheres and Sandostatin significantly reduced the plasma PGI<sub>2</sub> level, while the plasma TXA<sub>2</sub> level had a decreasing tendency, but no significant difference. There was no significant difference between the levels of plasma PGI<sub>2</sub> and TXA<sub>2</sub> in

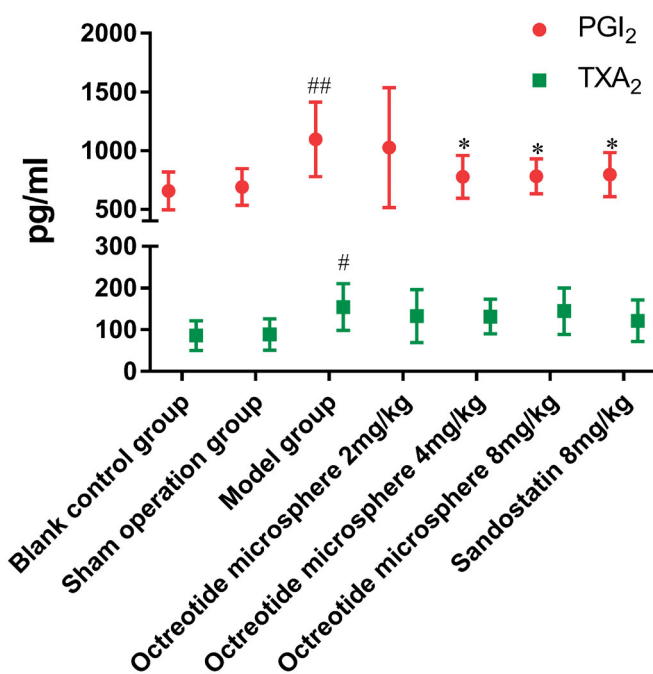
rats injected with Octreotide acetate microspheres and Sandostatin (Figure 16).

There was no significant difference in NOS activity or serum NO levels in the liver tissues between the sham operation group and blank control group. Compared with the sham operation group, the serum NO level of the model



**Figure 15.** Effects of Octreotide microsphere acetate microsphere injection on liver (A) Hyp, (B) GSH and (C) TNF $\alpha$  levels. Compared with the sham operation group, ##  $p < .01$ ; compared with the model group, \*  $p < .05$ , \*\*  $p < .01$ .

group rats significantly increased, while the activity of NOS in liver tissue slightly increased, but the difference was not significant. Compared with the model group, injection of 2, 4, and 8 mg/kg Octreotide acetate microspheres and



**Figure 16.** Effects of Octreotide microsphere acetate microsphere injection on plasma (A) PGI $_2$  and (B) TXA $_2$  levels. Compared with the sham operation group, #  $p < .05$ , ##  $p < .01$ ; compared with the model group, \*  $p < .05$ .

Sandostatin had different degrees of reducing effects on serum NO levels and NOS activity in liver tissues, but there was no significant difference between the test group and the model group or between the test groups (Figure 17).

There was no obvious difference in the level of ET in the plasma or liver between the sham operation group and the blank control group. In the model group, the plasma ET level decreased significantly, while the ET level in liver tissue increased significantly compared with the sham group. Compared with the model group, injection of 2, 4, and 8 mg/kg Octreotide acetate microsphere and Sandostatin increased plasma ET levels while decreasing ET levels in liver tissues to a certain extent, but there was no obvious difference. The difference in the change in ET levels in the plasma and liver tissue in each test group was not obvious (Figure 18).

#### 4. Discussion

Octreotide is soluble in water, double emulsion solvent evaporation method is the most common method for preparing peptide-loaded PLGA microspheres (Iqbal et al., 2015). The present study used w/o/w method to prepare Octreotide-loaded PLGA microspheres. It is simple and easy to perform, although there are many factors that can affect the process, including the concentration of PLGA, the volume of the aqueous phase, the formation speed of the initial and double emulsion, the mixing equipment, and the ratio of oil and water, and so on (Yang et al., 2001). This method is suitable for mass production because it allows for better homogeneity in one step without the need for postprocessing. Octreotide microspheres prepared by using the double emulsion method have advantages in the release process. As long as the processing parameters are well controlled and a sterile

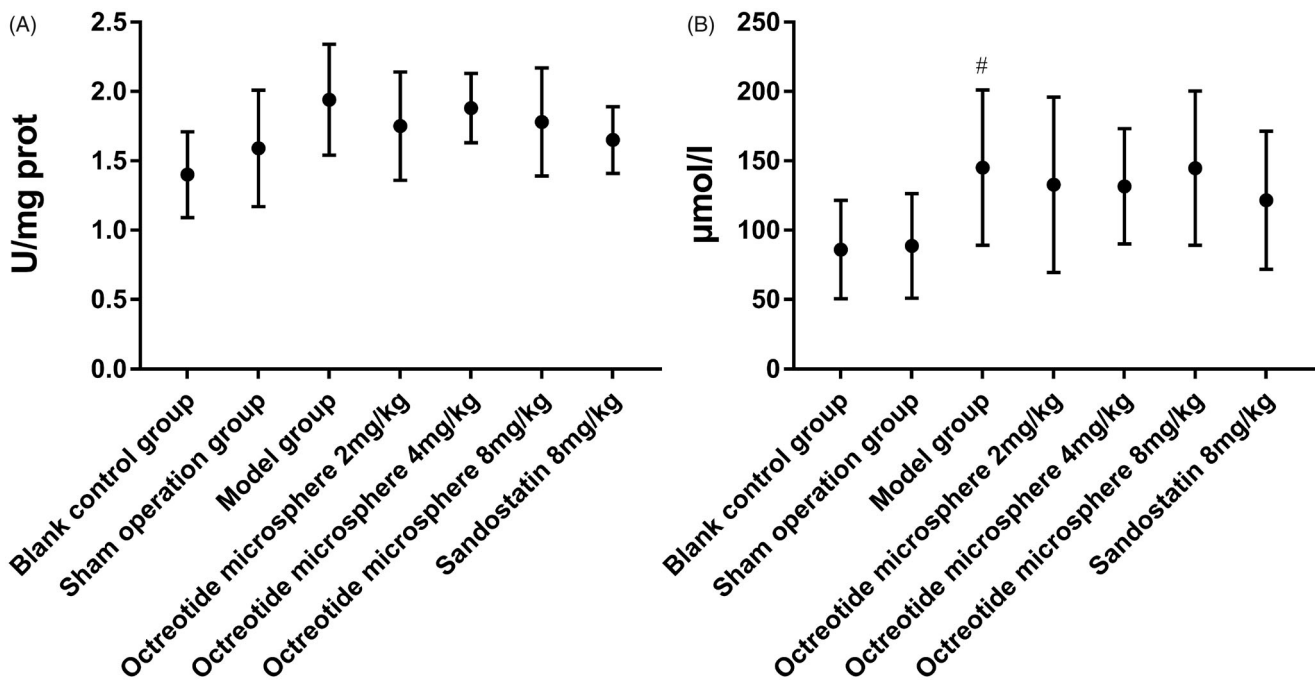


Figure 17. Effects of Octreotide acetate microsphere injection on liver (A) NOS activity and (B) serum NO levels. Compared with the model group,  $^{\#}p < .05$ .

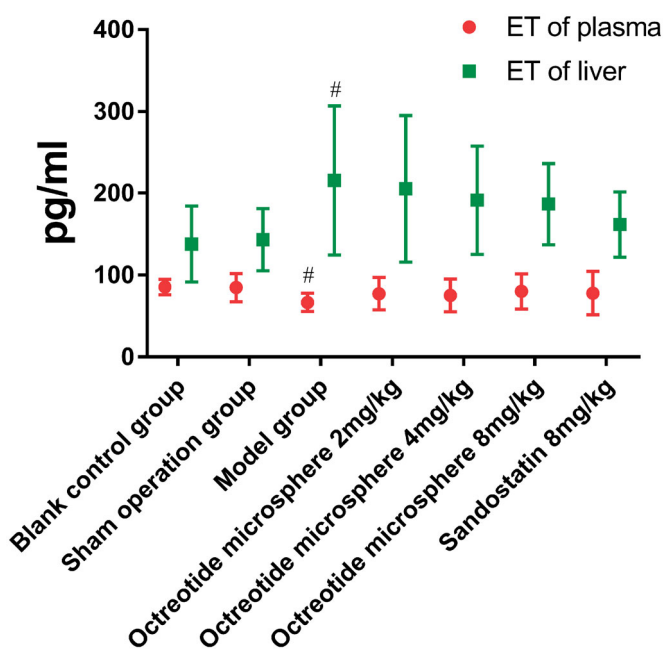


Figure 18. Effects of Octreotide acetate microsphere injection on the ET levels in (A) plasma and (B) liver. Compared with the sham operation group,  $^{\#}p < .05$ .

operation is ensured, the double emulsion method can be applied to medium-scale and large-scale production.

To accurately reflect the *in vivo* release process of the medicated microspheres, many researchers have designed a number of *in vitro* release methods, including designing *in vitro* release vessels of various shapes and different stirring and temperatures to simulate the conditions inside the organism's body (Yang et al., 2001; Wei et al., 2004). We believe that the main purpose of *in vitro* release is to guide the preparation conditions; thus, we chose an *in vitro* release

model that is easy to manipulate and easy to control in the present *in vitro* release experiments. For the choice of *in vitro* release solutions, we selected PBS as the buffer for the *in vitro* release system to simulate *in vivo* release conditions. Generally, the *in vitro* release curve of PBS is closest to that of *in vivo* release. The Octreotide microspheres were immersed in a system of a fixed volume (30 ml) and in a closed and relatively static environment. Therefore, *in vitro* release tests could provide momentous parameters for *in vivo* research to a certain extent, but they could never replace *in vivo* tests. It was not difficult to find that the plasma concentration of Octreotide microspheres we prepared was more stable than that of Sandostatin. The particle size range of microspheres is generally 1 ~ 500µm. Controlling the particle size is an important process for preparing microspheres. The stirring speed and polyvinyl alcohol (PVA) concentration during the solvent evaporation process will affect the particle size (Samati et al., 2006; Szłęk et al., 2016). Intramuscular injection is a common injection method with the particle size can up to several hundred microns in size. Microspheres with a diameter in this specific range are retained in the interstitial tissue acting as sustained release depots (Zhang et al., 2018). The drugs embedded or adsorbed in the microspheres are gradually released into the blood along with the degradation of the microspheres (Yamaguchi et al., 2002). However, the initial burst release of microspheres would be affected by the particle size, according to the smaller the average particle size is, the more uniform distribution of drugs in the microspheres, the larger the specific surface area is, the larger the distribution of drugs on the microsphere surface is, and the greater burst release is. Moreover, microspheres with bigger size could result in the blockage of the syringe needle. Therefore, the particle size of the microspheres was controlled at about 50 µm.

Based on the results of the Octreotide PLGA microsphere formulation in vivo release study in beagles, Octreotide had sustained release over 50 d. According to the in vivo release of plasma Octreotide, the percent of the  $AUC_{0-4h}$  in total  $AUC_{0-50d}$  after a single administration of the microsphere formulations was less than 5%. The results indicated that the release amounts of Octreotide 0–4 h after administration of the sustained-release drugs were lower than those reported before (Rhee et al., 2011; Ghassemi et al., 2012), which means that the formulation was sufficiently safe.

PHT is associated with the most serious complications of cirrhosis, including liver cancer (Lee & Kim, 2007; Ikenaga et al., 2015). The results showed that Octreotide microspheres for injection could significantly reduce the portal vein pressure and the area of portal vein lumen, the activity of AST and ALT in serum, the content of Hyp in liver tissue, the content of MDA in serum and liver tissue, the level of PGI<sub>2</sub> in plasma and the content of TNF $\alpha$  in liver tissue, the activity of SOD in serum and liver tissue, and the content of GSH in plasma and liver tissue. The TXA<sub>2</sub>, NO level in serum and NOS activity in liver tissue had no significant effects. It is therefore suggested that injected Octreotide microspheres may be a promising drug for the treatment of cirrhosis and portal hypertension by reducing transaminase activity and collagen content of liver tissue, antagonizing oxidative damage and regulating the levels of vasoactive substances and cytokines.

## 5. Conclusion

This research examined the correlation between the solvent system of preparations and the conventional properties of microspheres, such as drug loading and drug burst release, and found that there were significant differences in the in vitro release behavior and drug distribution of microspheres from different mixed solvents. The Octreotide microsphere preparation conditions were optimized, and the new long-acting Octreotide can significantly reduce portal pressure, which may be attributed to reduced liver fibrosis. The long-acting formulation of Octreotide microspheres prepared in this study had good bioavailability and a more stable release curve. The Octreotide microspheres inhibited portal hypertension, and the results demonstrated that all Octreotide PLGA microsphere formulations in the high-, mid- and low-dose groups could reduce PHT. The new Octreotide PLGA microsphere preparation was proven to have a good effect on PHT in vivo and no additional toxicity after detecting ALT, AST, Hyp, PGI<sub>2</sub>, TNF $\alpha$ , SOD, GSH, TXA<sub>2</sub>, NO, NOS and ET. This will positively impact the extension of indications for incoming Octreotide prolonged formulations.

## Disclosure statement

The authors declared that they have no conflicts of interest to this work.

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