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Preparation of pingyangmycin PLGA microspheres and related in vitro/in vivo studies

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ABSTRACT

Using a multiple emulsion solvent evaporation method, pingyangmycin was entrapped in poly(lactic-co-glycolic acid) (PLGA) to prepare a long-acting pingyangmycin PLGA microsphere formulation that can be sustainably released with high entrapment efficiency. Meanwhile, the effects of stirring speed during the multiple emulsion solvent evaporation process were also taken into consideration. Investigation of the in vitro release properties showed that the microsphere formulations could sustainably release the drug over nearly 28 d, and, moreover, it could stably control pingyangmycin release over nearly 24 d when intramuscularly injected into dogs. No serious toxic effect was observed in an acute toxicity test in mice. A subcutaneous xenotransplant model of hepatoma H_{22} in mice was established for pharmacodynamic studies and the results showed that the process of preparing pingyangmycin PLGA microsphere formulations was feasible and that intramuscular injection of this microsphere formulation resulted in anti-tumor activity in vivo.

1. Introduction

Pingyangmycin (PYM) is a streptomycete-produced cytotoxic glycopeptide anti-tumor antibiotic, which was isolated from the soil in the Pingyang, Zhejiang Province in China. When discovered, the bacteria represented a new type of mutation and were called Pingyang Streptomycete (Xu and Zhang, 1980). By virtue of its hydrophobic effect and hydrogen bonding capability, PYM can bind and break 5–6 poly G–C base pairs in the double stranded DNA of tumor cells (Li and Zou, 1996), inducing a chromosome aberration as well as sister-strand crossover, which could affect the metabolic function of tumor cells and lead to degeneration and necrosis (Wang, 1992). The ability of PYM to induce chromosomal aberrations is stronger than that of anti-tumor drugs like bleomycin A2, mitomycin and 5-fluorouracil (Zhen and Li, 1992). PYM is a cell cycle nonspecific agent with little effect on immune response and no obvious harm to hematopoiesis. Studies murine species demonstrated that PYM has a clear inhibitory effect on Walker 256 carcinosarcoma in the rat; sarcoma and melanoma in mice (Han et al., 2009), with inhibition rates close to or exceeding 90%, and it had smaller toxic and side effects on mice than did bleomycin A2 (Zhang et al., 1997).

Currently, PYM is widely used in the clinical treatment of malignant neoplasm. Its significant therapeutic effect has also been shown in lip cancer (Song et al., 1999), tongue cancer (Hua et al., 2002), ocular cancer (Zhao et al., 1998), nasopharyngeal cancer (Zhen et al., 1986), esophageal cancer (Zhang, 2001), breast cancer (Wang et al., 1995), liver cancer (Su, 2008), cervical cancer (Zhang et al., 1998) and penile cancer (Qiao et al., 2006). There are some reports concerning the use of PYM with a satisfactory effect on the treatment of benign tumors and benign lesions, e.g., pediatric lymphangioma (Meng et al., 1999), pediatric hemangioma (Dai et al., 2007), ocular pterygium (Fan, 2004), condyloma acuminate (Lu and Wu, 2008), vitiligo, rhinopolypus (Wang, 1992), etc. On the other hand, a serious disadvantage of PYM is its short half life (Li et al., 1995). The administration of continual i.v., i.a., i.m. and intratumor injections at 4–20 mg each q.d. or q.o.d. is used in practice for at least 20 d or even longer, which adds to the suffering and trouble of patients.

At present, the study of a sustained and controlled release formulation of PYM has mainly been applied in sclerosing and embolization therapy in stomatology. Some investigators used gelatin (Qiu et al., 2007), albumin (Wang et al., 2007) or fibrin (Yang et al., 2008) as the entrapment material for PYM application. The advantages of materials like gelatin and albumin are their biocompatibility with swelling to some degree, which makes them an ideal drug-loading microsphere matrix material for embolization. The ideal particle size of microspheres for embolization is generally

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around 50–200 μm. Microspheres smaller than 40 μm could lead to embolization in non-target organs or uptake by macrophages, while microspheres larger than 200 μm could lead to collateral circulation and a diminished effect of embolization in the long run (Wang et al., 2008). Different target organs in the body have different requirements for particle size. In addition, most of the target organs in which PYM microspheres are applied are not exposed and are therefore not accessible for treatment with microsphere embolization therapy, thus PYM microspheres are only applied in the treatment for hemangioma. Our study is the first in which PLGA was used as the carrier of PYM to prepare microspheres for i.m. injection in the treatment of tumors and other kinds of cancers in areas that are not easily accessible for embolization therapy, resulting in enhanced patient compliance and tolerance, an increased biological stability of the drug and an extended application of the long-acting formulation of PYM.

2. Materials and methods

2.1. Material

PYM was purchased from Bolai Pharmaceuticals, Co. Ltd. Harbin, China (Harbin, China). PLGA was supplied by Lakeshore (USA) and PVA was from Sigma (USA). Sodium hexanesulfonate, disodium ethylenediamine tetraacetic acid, acetic acid, ammonia water, methanol, acetonitrile, formic acid and dichloromethane were purchased from Tianjin Chemicals Reagent Co. Ltd. (Tianjin, China).

Hepatoma H_{22} cells were provided by the School of Public health, Jilin University.

China KM mice were provided by the Experimental Animal Center of Jilin University.

2.2. Preparation of PYM-PLGA microspheres

PYM-PLGA microspheres were prepared by a multiple emulsion solvent evaporation method. Briefly, a pre-treated, highly concentrated solution of PYM in water was added to dichloromethane containing dissolved PLGA, which was introduced into the initial emulsion with water in oil by high-speed stirring. The initial emulsion was then slowly poured into a PVA water solution. In a self-made multiple emulsion stirring reactor, the w/o/w multiple emulsion was formed by low-speed stirring followed by a slower stirring speed. Meanwhile, the vacuum pump pumped gas into the reactor, accompanied by the exhaust fan drawing gas outward to ensure that the reaction was taking place under a pressure balance and that the surplus dichloromethane gas was removed after the microspheres were cured. The cured PYM-PLGA microspheres were thus formed after 4 h of stirring at low speed. The microspheres were washed repeatedly with distilled water to remove PVA and were subsequently freeze-dried to obtain the final PYM-PLGA microsphere formulation. By changing the multiple emulsion stirring speed to 250 rpm, 300 rpm and 350 rpm with other preparation conditions intact, 3 groups of microspheres, called PYM-PLGA microspheres A, B and C, each with different particle sizes and release behaviors, were prepared (Yin et al., 2007).

2.3. Entrapment efficiency (EE%) and morphology of PYM-PLGA microspheres

One hundred milligrams of PYM-PLGA microspheres were transferred into a 50-ml volumetric flask, followed by the addition of 10 ml acetonitrile to dissolve all of the microspheres. Water was added to bring the volume to 50 ml, and the solution was sonicated in a water bath for 2 min (KQ-300DV Digital Ultrasonic Instrument, Shanghai Precision Instruments and Equipments Company, Shanghai, China). After centrifugation, the supernatant was collected for the determination of the PYM concentration by HPLC (Agilent 1100 High Performance Liquid Chromatograph, Agilent, USA) and calculation of the EE% (Han et al., 2009). Particle size evaluation was performed for the freeze-dried PYM-PLGA microspheres, and the morphology was observed by electron microscopy.

Table 1
Gradient profile of liquid phase detection of PYM released in vitro.

Time (min)	Mobile phase A (%)	Mobile phase B (%)
0	70	30
15	67	33
35	60	40
36	70	30
40	70	30

2.4. In vitro release of PYM-PLGA microspheres

Three portions of 40 mg of PYM microspheres each were precisely weighed for Groups A, B, and C, respectively, placed in 25 ml of in vitro release solution in labeled tubes and sealed with parafilm. The tubes were placed in a 37 °C shaker at 75 rpm for incubation (Han et al., 2008). From each tube, 20 ml of supernatant was withdrawn at 4 h, 1 d, 2 d, 4 d, 6 d, 8 d, 10 d, 12 d, 14 d, 16 d, 18 d, 20 d, 24 d, and 28 d and centrifuged at 500 rpm for 4 min while another 20 ml in vitro release solution was added for compensation. The content of PYM in each supernatant at the corresponding time point was determined by HPLC, and the cumulative release percentage at 28 d was calculated. The in vitro liquid phase conditions were: column: C18 (FLM, Guangzhou FLM Scientific Instrument Company, Guangzhou, China) (150 mm × 4.6 mm, 5 μm); flow rate: 1 ml/min; mobile phase A: dissolve in 0.08 mol/l acetic acid to prepare the solution containing 0.753% sodium hexanesulfonate and 0.1% disodium ethylenediamine tetraacetic acid. Adjust the pH to 4.2 with ammonia water. Mobile phase B: methanol:acetonitrile (70:30); detection wave length 254 nm; column temperature 32 °C; injection volume 20 μl. See Table 1 for the gradient profile. The experiment was repeated 6 times in accordance with the above steps. In vitro release curves were compared among Groups A, B, and C PYM microspheres.

2.5. In vivo release of PYM-PLGA microspheres

PYM microspheres of Group B were injected i.m. into the gluteal area in 6 Beagle dogs, half male and half female. Three-milliliter blood samples were collected at 4 h, 1 d, 2 d, 4 d, 6 d, 8 d, 10 d, 12 d, 14 d, 16 d, 18 d, 20 d, 24 d, and 28 d from the forelimb veins of dogs. After pre-treatment, samples were put on LC–MS (API 4000 LC/MS/MS System, Applied Biosystems, USA) (Tang et al., 2000; Shi et al., 2007) for the detection of plasma concentrations, and an in vivo release percentage graph was produced for the analysis of the in vivo release profile of Pingyangmycin PLGA microspheres. The LC–MS detection conditions were as follows: column: C18 (150 mm × 4.6 mm, 5 μm); flow rate 1 ml/min; mobile phase A: 5% acetonitrile, 0.1% formic acid, 5 nmol/l ammonium acetate solution, dissolved with water for injection, pH 3.2 after dissolution: B: 95% acetonitrile, 0.1% formic acid, ammonium acetate. See Table 2 for the gradient profile. The AUC of PYM released in a 24-d period was

Table 2
Gradient profile of LC–MS detection of PYM released in vivo.

Time (min)	Mobile phase A (%)	Mobile phase B (%)
0	80	20
40	67	33
41	80	20
50	80	20

set at 100% since PYM-PLGA microspheres are barely detectable 24 d after being injected into the body. The ratio between AUCs of PYM at each interval and the 24-d total AUC was regarded as the percentage of PYM that was released through PYM-PLGA during each period. The in vivo release of PYM-PLGA microspheres was analyzed.

2.6. Acute toxicity test in mice

Forty China KM mice (20 ± 2 g, 3–4 weeks old), half male and half female, were used for the acute toxicity test. The mice were fasted for 12 h before medication. According to the maximum dosage method and considering that the available maximum drug concentration of PYM-PLGA microspheres was 600 mg/ml and the highest volume per injection was 0.2 ml, the highest dose (equivalent to about 40 times the normal clinical dose for humans in terms of body surface area), the medium dose and the lowest dose were 360 mg/kg, 120 mg/kg and 36 mg/kg, respectively. After intramuscular injection once into the hind limbs, the mice were observed for 1 month, and detailed weights, behaviors, conditions, diets, hair, secretions, excretions and deaths were recorded. At the end of 1 month of observation, all mice were sacrificed and their hearts, lungs, livers and kidneys were examined to determine any pathological changes.

2.7. Establishment of a xenotransplant model of hepatoma H_{22} in mice

China KM mice were used as the animal model for the pharmacodynamic study. Mice hepatoma H_{22} cells were cultured in vitro and collected when they were in the logarithmic growth phase. After centrifugation, serum free medium was added to adjust the cell concentration to about 5×10^7 cells/ml in the suspension (Zhang, 2000). Using a 1-ml syringe, about 0.2 ml of the cell suspension was withdrawn and injected into the abdominal cavity of mice where the skin had been disinfected by alcohol. Peritoneal fluid was available 6–7 d after the first transplantation into KM mice. Under aseptic conditions, all mouse hair was disinfected with alcohol. Hepatoma H_{22} cells in peritoneal fluid were withdrawn using a syringe, diluted with sterile saline to a final tumor cell concentration of 1×10^7 cells/ml (Zhang et al., 2004), stored at 37 °C and used within 1 h for inoculation into the underarms of the mice for the pharmacodynamic study. Forty KM mice (20 ± 2 g, 3–4 weeks old), half male and half female, were used for the experiment, 0.2 ml of the hepatoma H_{22} cell suspension was inoculated in the left underarm of each KM mouse.

2.8. Mouse grouping and treatment

Twenty-four hours after the inoculation of H_{22} hepatoma cells, all KM mice were randomly divided into 4 groups of 10 mice each, consisting of a high, medium, and low dose of PYM-PLGA microspheres group and a blank control group. On the 4th day after inoculation, a small tumor nodule was found in the left underarm where the tumor cells had been transplanted into every mouse, and the treatment was started. According to the results of the acute toxicity test and the clinical application of the PYM common formulation (Wu and Lu, 2001), the doses in the pharmacodynamic studies of PYM-PLGA microspheres were determined as 36 mg/kg for the high dose group, 18 mg/kg for the medium dose group and 9 mg/kg for the low dose group. All of the drugs were administered by injection into the mouse hind limbs as 0.2 ml mixed water solution of sodium carboxymethylcellulose and mannitol. Mice in the blank control group were injected with 0.2 ml mixed water solution of sodium carboxymethylcellulose and mannitol. Treatment was administered only once to the mice in each group.

2.9. Inhibition study of the PYM-PLGA microsphere formulation on xenotransplanted hepatoma H_{22} tumors in mice

Tumor size changes were observed by measuring the tumor size of live mice using a vernier caliper. On the 20th day after medication, all of the mice were sacrificed. Before being sacrificed, the longest diameter (a) and the corresponding longest diameter (b), which is vertical to (a), were measured to calculate the tumor volume and the inhibition rate A according to the tumor volume change. The tumor volume was calculated as $v = \pi \times ab^2/2$, and the tumor inhibition rate A (%) was calculated as A (%) = (1 − average volume change in treatment group/average volume change in control group) × 100 (Hu and Li, 1997). After being sacrificed, the hearts, livers, kidneys and lungs of the mice in each group were dissected, and the whole subcutaneous tumor was obtained and weighed. The changes in each organ were observed, and tumor inhibition rate B was calculated according to the tumor weight after the PYM-PLGA microsphere formulation treatment As the tumor inhibition rate B (%) = (tumor weight in blank control group − tumor weight in treatment group)/tumor weight in blank control group × 100 (Han et al., 2009).

3. Results

3.1. Entrapment rate and morphology of PYM-PLGA microspheres

PYM-PLGA microsphere formulations A, B and C were a white powder with a dense spherical roundness but without any collapsing or shrinking (Fig. 1). The average size of the PYM-PLGA microsphere formulation A was 3.9 μm, with a size range of 300–15 μm and an entrapment rate of greater than 64%. The average size of PYM-PLGA microsphere formulation B was 3.7 μm, with a size range of 300–10 μm and an entrapment rate of greater than 65%. The average size of PYM-PLGA microsphere formulation C was 3.4 μm, with size range of 200–10 μm and an entrapment rate of greater than 60%. The entrapment rates of microsphere formulations A, B and C were all high, and the particle sizes were suitable for intramuscular injection. The formulations were studied together to compare the differences in the in vitro release behavior.

3.2. Results of the in vitro release study of PYM-PLGA microspheres

PYM-PLGA microsphere formulations A, B and C were selected to be released in our in vitro environment, and the results showed that in all three formulations PYM could be completely released from the microspheres in 28 d (Fig. 2). The in vitro release curves of PYM-PLGA microsphere formulations A, B and C showed that average cumulative release rates were all above 45% in 10 d, above 80% in 20 d and were almost 100% in 28 d. According to the administration and dosage of the PYM common formulation, one shot of the PYM-PLGA microsphere formulation every 20 d would be appropriate. Comparing the in vitro release results of formulations A, B and C, formulations A and C had an obvious burst release effect while formulation B had the highest cumulative release rate in 20 d with a release amount higher than 87% of the total drug amount, and it also had a smoother in vitro release curve compared to formulations A and C. In addition, formulation B had the highest entrapment rate, and the particle size was more evenly distributed. Therefore, PYM-PLGA microsphere formulation B was selected for further in vivo release, acute toxicity and pharmacodynamic studies.

3.3. Results of the in vivo release studies of PYM-PLGA microspheres

According to the in vivo release curve (Fig. 3), the PYM-PLGA microsphere formulation B could be stably released in beagles. PYM

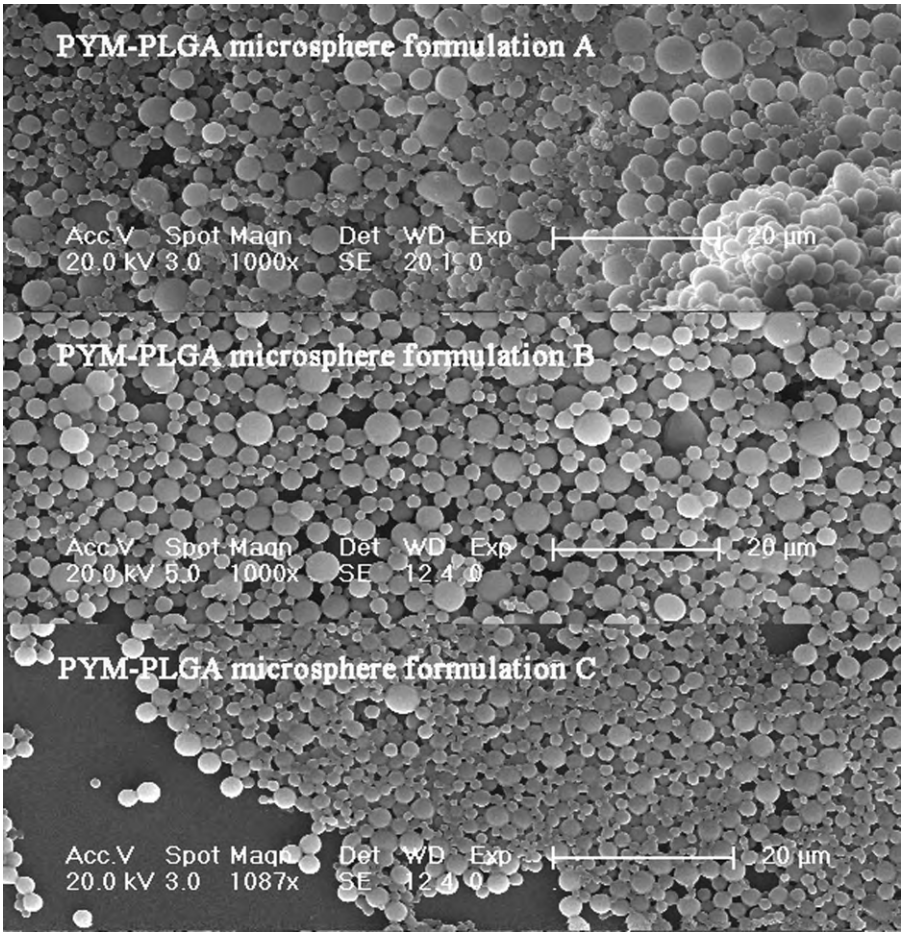

Fig. 1. Morphology of PYM-PLGA microspheres by electron microscopy.

could be completely released from the microspheres in 24 d. The in vivo release curve of PYM-PLGA microspheres showed that the average cumulative release rate was above 60% in 10 d and above 97% in 20 d. This PYM-PLGA microsphere formulation B met the drug administration requirement of one shot every 20 d as one course of treatment.

3.4. Acute toxicity test results

The mice were generally in good condition with bright and smooth hair, normal behaviors and diets, and normally shaped feces. No animals died. On the day after the test was finished, all of the animals were sacrificed for anatomical studies. No changes in the volume, color or texture of the organs, including essential organs such as the heart, liver, lung and kidney, were observed by the naked eye. No bleeding, hyperemia, exudation, ulceration, perforation, inflammation, or effusion in the thorax cavity, abdominal cavity and pericardial cavity was observed by the naked eye. For the mice in the high dose group, the heart cells appeared in order without edema, necrosis, or inflammatory cell infiltration; the alveolar wall had thickened and edema in the interstitium and hyperemia

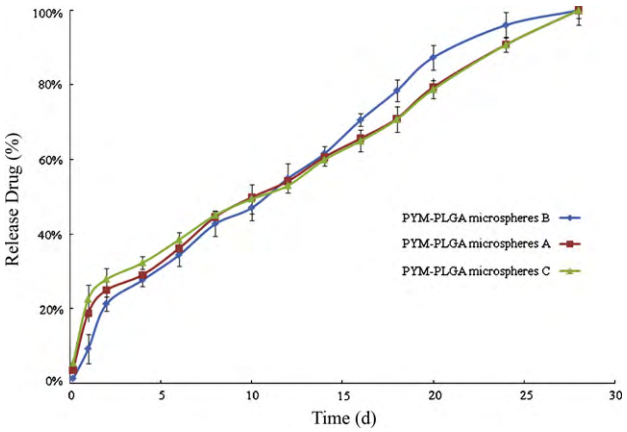

Fig. 2. In vitro drug release curves of PYM-PLGA microsphere formulations A, B and C in 28 d.

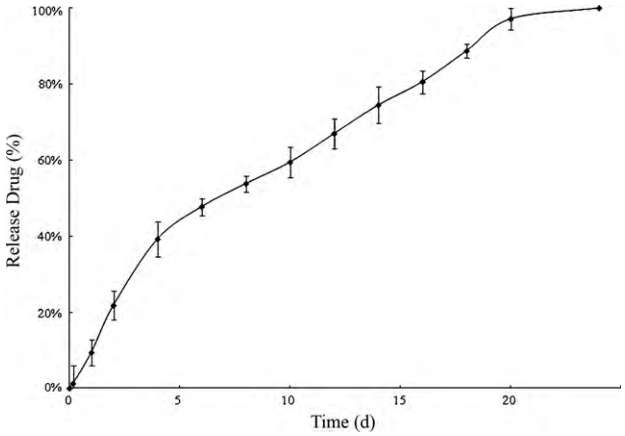

Fig. 3. Average in vivo drug release curves of PYM-PLGA microsphere formulation B in 28 d.

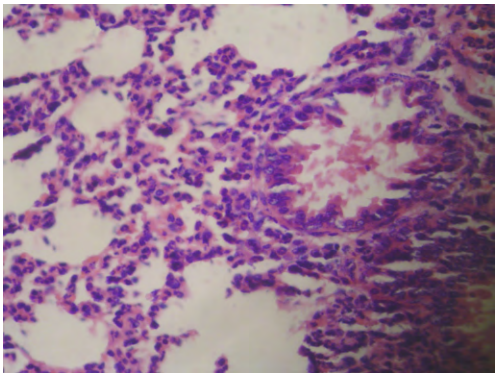

Fig. 4. Mouse lung tissue section in the PYM microsphere high dose group in mice acute toxicity test.

and dissolution in some alveolar cavities were observed (Fig. 4); in the liver cells, edema and an increased volume were observed, and an infiltration of chronic inflammatory cells in the interstitium, observable focal necrosis and dilatation and hyperemia in the central vein were present (Fig. 5); in the kidneys, the structures of the renal glomerulus and renal tubules were clear with no obvious cellular swelling and chronic inflammatory cell infiltration. In the medium and low dose groups, no abnormalities were observed for the organs observed under the microscope. In the group receiving treatment as well as the control group, tiny white cones at the administration area were observed. Under microscopic observation, these white cones were determined to be tiny foreign body granulomas coated with fiber tissue. In the granuloma, there was floccus pink material.

3.5. Pharmacodynamic results

These experiments showed that the PYM long-acting formulations could be administered intramuscularly in mice with transplanted hepatocarcinoma H_{22} tumors. During the treatment, the tumor body was observed to resolve and the body weight declined in the high dose treatment group, but this change was found to be smaller than the effects in its early treatment. During the treatment, mouse activity and diet did not display any significant change compared to during the pre-treatment, nor did the skin color. The medium dose treatment group was observed to have a slightly poorer diet and activity as well as poor skin color. The blank control group had a delayed reaction, reduced diet and activity, and a poor skin color. The microscopic observation of the slices of tumor, heart, liver and lung tissues of all experimental animals revealed no apparent tissue necrosis in the blank control group; the cells grew actively. The low dose treatment group was observed to have a smaller tumor size and evident nuclear mitotic figures; the medium dose treatment group displayed scarce necrosis, with some nuclei showing pycnosis and caryoclasis (Fig. 6), while the high dose treatment group showed extensive necrosis, evident pycnosis and caryoclasis, and some translucent cytoplasm with vacuole-like denaturization (Fig. 7). There were no apparent abnormal changes observed in the heart, liver, lung and kidney tissues of all experimental groups.

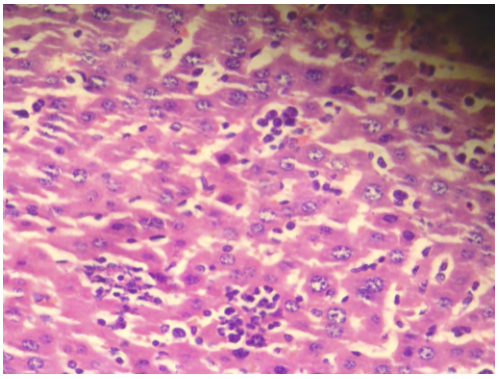

Fig. 5. Mouse liver tissue section in the PYM microsphere high dose group in mice acute toxicity test.

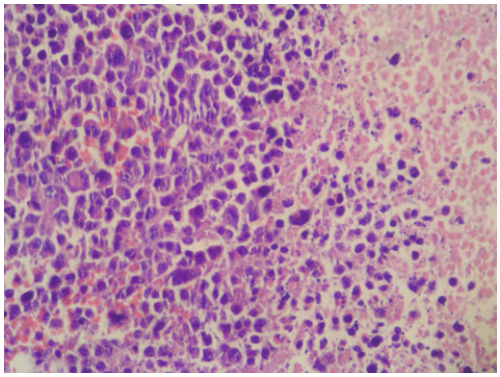

Fig. 6. Mouse tumor tissue section in the PYM microsphere medium dose group in mice in vivo pharmacodynamic study.

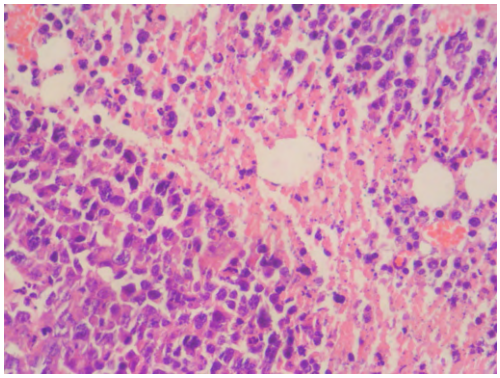

Fig. 7. Mouse tumor tissue section in the PYM microsphere high dose group in mice in vivo pharmacodynamic study.

3.6. Tumor inhibition rate of the PYM-PLGA microsphere formulation on xenotransplanted hepatoma H_{22} tumors in mice

Forty mice were successfully inoculated, and subcutaneous hepatoma H_{22} xenotransplant tumors were observable by the 4th day of inoculation. After 20 d of treatment, the tumor size under the left arm was measured in each mouse group. The tumor inhibition rate *A*, calculated by the tumor volume change in mice from the high, medium and low dose treatment groups, is presented in Table 3. The high, medium and low dose groups were significantly

Table 3
Comparison of tumor volume and inhibition rate *A* in different groups after administration of the PYM-PLGA microsphere formulation. $P < 0.05$ between each group.

Groups	Tumor volume (cm^3)	Tumor inhibition rate *A*
PYM long-acting formulation, high dose	3.5112 ± 0.3005	56.18%
PYM long-acting formulation, medium dose	4.8432 ± 0.4612	39.56%
PYM long-acting formulation, low dose	6.1239 ± 0.9496	23.58%
Blank	8.0135 ± 1.3241	

Table 4
Comparison of tumor weight and inhibition rate *B* in different groups after administration of the PYM-PLGA microsphere formulation. $P < 0.05$ between each group.

Groups	Tumor weight (g)	Tumor inhibition rate *B*
PYM long-acting formulation, high dose	0.8934 ± 0.1005	89.55%
PYM long-acting formulation, medium dose	2.8172 ± 0.8383	67.04%
PYM long-acting formulation, low dose	5.1239 ± 0.8993	40.05%
Blank	8.5474 ± 0.9726	

different from the blank control group ($P < 0.05$). The inhibition rate *A* of the PYM long-acting formulation among the high, medium and low dose groups was also significantly different ($P < 0.05$). The tumor inhibition rate *B*, calculated by the tumor weight in high, medium and low dose groups after treatment, is shown in Table 4. The difference of the high, medium and low dose groups compared to the blank control group was significant ($P < 0.05$), and among the high, medium, and low PYM long-acting formulation groups, the tumor inhibition rate *B* was significantly different ($P < 0.05$). Different doses of PYM-PLGA microsphere formulations all had an inhibitory effect on the H_{22} solid tumor in KM mice.

4. Discussion

Studies to design a long-acting PYM formulation began at the beginning of this century. The coating materials have included bovine serum albumin, chitosan, gelatin, etc., and most of them were used for embolization therapy and for the treatment of human oral and maxillofacial vascular tumors, oral squamous cell carcinoma, lymphoma, breast cancer, esophageal cancer and nasopharyngeal cancer. There are not many reports on PYM microspheres in China or abroad, because the size and the drug release profile of PYM microspheres were hard to control.

Researchers found that PYM could induce cell accumulation in the early S phase and had an inhibitory effect on DNA synthesis in cells at early S phase, indicating that cells at this stage were sensitive to PYM and that PYM's mechanism of action may be related to the abundant G–C composition of DNA in cells at early S phase. Early S phase was longer than the Tmax of a single dose. Previous reports showed that the first Tmaxes of the PYM sustained release formulation were all shorter than S phase. In our study, PYM-PLGA microspheres were sustainably released for a long period of time, which would ensure the presence of PYM during S phase in cell proliferation and therefore maximize the effect of PYM.

Some researchers used a radioactive labeling method for the determination of PYM in vivo and found that the distribution in body was concentrated in the head (4.76 ± 1.52%), heart and lung (71.70 ± 3.21%), kidney (4.40 ± 0.32%) and bladder (6.19 ± 1.25%) (Zhang and Wu, 2004), demonstrating that most PYM accumulated in the heart and lung. In our acute toxicity test for the PYM-PLGA microsphere formulation, the results showed that there was no serious adverse pathological reaction in the heart. The main adverse reaction when the PYM-PLGA microsphere formulation was employed in mice was pulmonary toxicity. It was reported that, in clinical application, the incidence rate of pulmonary toxicity when using the PYM common formulation for less than 3 months was about 3%-5% compared with up to 15% in the long-term application, in which case the effect mainly consisted of lung fibrosis leading to pulmonary dysfunction. According to the results of the acute toxicity test and the dosage in the high dose group, pulmonary fibrosis would appear when the dose of the PYM-PLGA microsphere formulation in a single mouse was more than 7.2 mg (calculated by PYM). If PYM-PLGA microspheres were to be used in human treatment combined with radiotherapy of the chest, pulmonary fibrosis would be more likely to occur. Thus, older patients or those with congenital poor pulmonary repair abilities would not be suitable candidates for the long-term administration of the PYM-PLGA microsphere formulation. In general, the pulmonary toxicity in mice resulted from the pharmacological reaction, immunological reaction, cortin-induced susceptible simulating reaction and drug overreaction due to intolerance.

The combination of PYM with DNA releases about $-28\ kJ\ mol^{-1}$, which is not high, being approximately equivalent to Van der Waals forces or a hydrogen bond. The binding of PYM to DNA mainly depends on the combined effect of hydrophobic and hydrogen bonds, including all of the energy associated with DNA spreading, transformation and drug insertion. Hence, the location of PYM administration related to the distance to the target organ could not guarantee that a huge amount of PYM would bind to the tumor cell DNA. However, the proliferation of tumor cells produces a lot of half-naked DNA, which facilitates the action of PYM when it enters the cells during circulation, approaching and cutting off the DNA. Therefore, an intramuscular injection of PYM would not have worse therapeutic effect than in situ administration.

According to the results of the PYM-PLGA microsphere formulation in vivo release study in beagles, PYM was sustainably released over 20 d, which indicates that one shot could basically sustain the therapeutic effect for 20 d. The difference between intramuscular injection and intratumoral injection of PYM microspheres, as stated by some researchers, lies only in the difference of the local drug concentration. The blockage of local blood circulation could lead to a longer retention time of the drug in local tissues. However, this difference could only be sustained for a short period of time. Without aid from other outside methods, the local drug concentration will not be maintained at a high level. This short period of high local concentration would not assure a fundamentally better therapeutic effect than that of intramuscular injection. Compared with the in vitro drug release study, drug-loaded microspheres had a more obvious sustainable release effect in vivo. In other words, the drug concentration in the circulation was maintained for a long time.

The reason that the xenotransplant tumor-bearing animal model was established for screening PYM-PLGA microsphere formulations was that after inoculation of a certain amount of tumor cells, mice in all groups would have tumors of similar characteristics and growth rates with few differences between individuals. Moreover, the influence of the mice H_{22} xenotransplant tumor on the host was similar to the influence of tumors on the human body. Furthermore, the tumor growth process in mice was much more similar to the growth environment of human solid tumors.

Tumor inhibition rate *A* was smaller than tumor inhibition rate *B*, because during the growth of the xenotransplanted H_{22} tumor, the tumor volume would decrease rather than increase due to PYM's inhibitory effect. The tumor would gradually become hollow in the center, full of effusion. In the treatment, the effect on the decrease of tumor weight was much faster than the shrinkage of the tumor volume. Therefore, although the calculation methods for the tumor inhibition rate had different results, they both showed that the PYM-PLGA microsphere formulation had an inhibitory effect on xenotransplanted H_{22} tumors in vivo.

In the high, medium and low dose groups of PYM-PLGA microsphere formulations, the mouse tumor weight was smaller compared to the blank control group and there was an obvious pathological and histological difference among the mouse tumors. All of the tumor tissues in the high, medium and low dose groups exhibited necrosis to some extent. Some tumor cell nuclei swelled or broke. The tumor cells proliferated while mitosis decreased and stromal cells increased. Using therapeutic doses, all of the hearts, lungs, livers and kidneys of mice in the high, medium, and low dose groups of PYM-PLGA microsphere formulations displayed no obvious pathological changes.

5. Conclusion

This study was the first to apply poly(lactic-co-glycolic acid) in the coating of PYM and to use intramuscular injection as an administration route. The present findings are novel in regards to the previous dosage limitation as only a single administration was used for the PYM long-acting formulation. The results showed that the PYM-PLGA microsphere formulation in high, medium and low doses could all induce tumor tissue necrosis to some extent in mouse hepatoma H_{22} xenotransplanted tumors, which will impact the potential uses of PYM long-acting formulations in the future. If this PYM long-acting formulation was to be successfully applied in human clinical trials, it could greatly reduce patients' suffering and also increase their compliance.

References

Dai, C.J., Ye, Z.P., Niu, J., Hu, B., 2007. Local injection of low concentration pingyangmycin in children with hemangioma. J. Clin. Pediatr. Surg. 6, 36–37.

Fan, Y.F., 2004. Pingyangmycin injection treating 196 eyes with ptertgium. Mod. Diagn. Treat. 15, 10.

Han, B., Gao, Y., Pei, J., 2008. Preparation of aclarubicin loaded PLGA nanoparticles. China Trop. Med. 8, 1115–1116.

Han, B., Zhang, X.H., Liu, J.T., 2009. The anti-tumor effects of pingyangmycin plga microspheres on murine transplanted tumor H_{22}. Chin. J. Lab. Diagn. 13, 1244–1245.

Hu, X.G., Li, D.L., 1997. Inhibitory effects of targeting of pingyangmycin associated magnetic album in microspheres on mucoepidermoid carcinoma in vitro and in nude mice. China J. Oral Maxillofac. Surg. 7, 105–108.

Hua, H., Zeng, Z.Y., Xu, G.P., Chen, F.J., Guo, Z.M., Wu, G.H., Zhang, Q., Yang, A.K., 2002. Role of introduction chemotherapy with pingyangmycin in treating stage T2 tongue squamous cell carcinomas. Chin. J. Cancer 21, 1372–1375.

Li, H.S., Wei, S.L., Lu, W., 1995. Studies on gelatin microspheres-in-oil emulsion of pingyangmycin. Acta Pharmacol. Sin. 30, 390–394.

Li, P., Zou, G.L., 1996. Sequence selective binding of pingyangmycin to DNA. J. Wuhan Univ. 42, 764–768.

Lu, M.R., Wu, J.L., 2008. Clinical observation on condylomata acuminate treated with pingyangmycin in 30 cases. Acta Med. Sin. 21, 680–681.

Meng, X.Y., Yang, X.F., Shen, T.B., 1999. Effect of pingyangmycin in treatment of lymphangiomas in children. Nei Mongol. Med. J. 31, 310.

Qiao, Z.J., Zhao, P.X., Chen, H., Cui, Y., 2006. Initial study of pre-operational pingyangmycin chemotherapy plus improved operation in treating penile cancer. Prog. Mod. Biomed. 6, 76–80.

Qiu, L., Feng, X.H., Wu, H., Ding, Y.Y., 2007. Preparation of a new kind of gelatin-PYM-microspheres for arterial embolization. J. Pract. Stomatol. 23, 345–348.

Shi, J., Gao, Z.B., Wei, J., Ding, P.T., Chen, D.W., 2007. Pharmacokinetics of pingyangmycin hydrochloride in rabbits determined by microdialysis coupled with RP-HPLC. Acta Pharm. Sin. 42, 297–300.

Song, L.H., Liu, Z.G., Cai, S.P., 1999. Effects of pingyangmycin on lower lip cancer. Henan J. Oncol. 12, 141.

Su, W.Y., 2008. Observation of therapeutic effect of pingyangmycin lipodol emulsion embolization combined with hyperthermic perfusion in the treatment of 60 patients with liver cancer and nursing care. J. Qilu Nurs. 14, 8–9.

Tang, G.H., Jiang, G.H., Wang, S.Z., Zheng, L.F., 2000. Determination of perlolyrine in rat plasma by GC–MS. J. Pract. Stomatol. 20, 149–151.

Wang, C.G., Liu, J., Gao, Q.H., Bi, Y.Q., Gan, L.C., Wang, X.C., Hou, S.X., 2007. Reparation and characterization of pingyangmycin loaded bovine serum albumin microspheres for embolization therapy. Int. J. Pharm. 336, 361–366.

Wang, C.G., Liu, J., Pan, W.S., Wang, X.C., Gao, Q.H., Hou, S.X., 2008. Pingyangmycin loaded bovine serum albumin microspheres for chemoembolization therapy—in vitro and in vivo studies. Int. J. Pharm. 351, 219–226.

Wang, W.J., 1992. Mechanisms and clinical application of pingyangmycin. China Cancer Clin. Res. 19, 223–224.

Wang, W.G., Wang, S.H., Xue, Y.C., Zhen, Y.S., 1995. Effect of the conjugate composed of a human monoclonal antibody and pingyangmycin on mammary cancer. Acta Pharm. Sin. 30, 583–587.

Wu, H.B., Lu, G.C., 2001. Adverse effects of pingyangmycin. Chin. J. Clin. Pharm. 10, 53–55.

Xu, H.Z., Zhang, H.Y., 1980. The isolation and identification of pingyangmycin. Acta Pharm. Sin. 15, 609–614.

Yang, Y.W., Li, J.H., Sun, M.Y., Lv, J.H., Yan, Z.W., Lei, D.L., Hu, X.G., Cheng, X.B., Zhang, P., Ma, Q., 2008. Embolism and sclerotherapy of venous malformation in oral and maxillofacial region by using fibrin glue combined with pingyangmycin. J. Pract. Stomatol. 24, 209–212.

Yin, W.G., Xu, W.G., Li, Y.H., Su, C., Han, B., 2007. Preparation and investigation of HBsAg loaded poly(lactic-co-glycolic acid) microspheres. China Trop. Med. 7, 1766–1767.

Zhang, Q., Cheng, J.X., Xu, X.B., 1998. Clinical observation on cervical cancer treated with combination of pingyangmycin and radiotherapy. Mod. Rehabil. 2, 1370.

Zhang, S., Wu, H.J., 2004. Distribution of magnetic pingyangmycin bearing microspheres. J. Chin. Phys. 6, 1076–1078.

Zhang, W.F., 2000. Inhibition of Chinese medicine Xihuangcao on tumor growth of transplanted H_{22} in mice. Acta Chin. Med. Pharmacol. 6, 58.

Zhang, X.W., Zhang, Y.M., Guan, L.P., Quan, Y.C., Sun, Q., 2004. Study on extraction and isolation of active constituents from *Sorbaria sorbifolia* and antitumor effect of the constituents in vivo. J. Chin. Med. Mater. 27, 38–40.

Zhang, X.Y., Liu, W.Q., Zhu, M., Shimura, S., Masuda, T., Saitoh, H., 1997. Different damaging effect of bleomycin and pingyangmycin on lung tissues. Acta Lab. Anim. Sci. Sin. 15, 326–330.

Zhang, 2001. Combination chemotherapy of pingyangmycin and cisplatin in 30 cases with esophageal carcinoma. J. Bengbu Med. Coll. 26, 44.

Zhao, Y.X., Chen, Liu, H.R., Liu, H., Wang, Y.Y., Lang, Y.J., 1998. Local injection of pingyangmycin to treat ocular hemangioma. Recent Adv. Ophthalmol. 18, 151–152.

Zhen, Y.S., Li, D.D., 1992. Anticancer research of pingyangmycin. China Cancer Clin. Res. 19, 58–59.

Zhen, Y.S., Zhang, Z.H., Wu, S.Y., Huang, J., 1986. Inhibitory effect of pingyangmycin on human nanospharyngeal cancer transplanted in nude mice. Acta Acad. Med. Sin. 8, 51–53.