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Biocompatibility study of theophylline/chitosan/β-cyclodextrin microspheres as pulmonary delivery carriers

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Abstract To evaluate the biocompatibility of the theophylline/chitosan/β-cyclodextrin microspheres, which has a potential application in pulmonary delivery system. The detection of LDH and protein in BALF was examined acute cell toxicity, hemolysis test was carried out to estimate blood toxicity; Micronucleus Test was reckoned to identify genotoxicity, MTT assay was used to evaluate in vitro cytotoxicity, and muscle implantation investigated the tissue biocompatibility. The results demonstrated that the total contents of protein and LDH in BALF were not significantly different from that of normal group. The experiments showed that the cytotoxicity was depended on the concentration and had no cytoxicity at low concentration and no hemolysis activity. The micronucleus frequency of MS B was 0.99‰, which showed no genotoxic effects either. The results of implantation showed that the microspheres had no effect on hemoglobin and no toxicity in the liver and kidney. The inflammations of muscle tissue were not significantly different from that of operative suture, therefore, the MS B possess high good biocompatibility and can be applied in pulmonary sustained release systems.

Keywords Chitosan · Theophylline · Microspheres · Biocompatibility · Pulmonary delivery

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1 Introduction

Increasing attention has been paid to pulmonary drug delivery system due to its effective ways of delivering drugs for the treatment of asthma, chronic obstructive pulmonary disease and other respiratory diseases. Recently, sustained release microspheres have been proposed for pulmonary delivery, which major advantage is the formulation parameters targeted to the deep lung, therefore can be used as targeted and sustained drug release carriers [1, 2]. Being small in size, microspheres have large surface to volume ratios and can be used for controlled release of insoluble drugs [3]. Microsphere-based systems may increase the life span of active constituents and control the release of bioactive agents. Furthermore, a number of biodegradable microspheres have proved to be non-toxic, and non-immunogenic. In recent years, biodegradable microspheres are becoming increasingly popular in the design of pulmonary drug delivery systems. Drugs encapsulated into a polymeric particulate system possess sustained release characteristics as well as targeted effect, however, as inhaled microspheres, biocompatibility is an important index to evaluate whether the microspheres can be applied in the pulmonary drug delivery systems because the inferior biocompatibility which are known to be an obstacle especially for pulmonary delivery systems. Therefore, speaking about the pulmonary delivery,

especially for disease of the lung, are plagued by safety concerns.

Chitosan (CTS) is a cationic natural copolymer of glucosamine, obtained from the deacetylation of chitin, which is considered to be a good candidate for use in pulmonary delivery of biotherapeutic compounds because of its biodegradable, mucoadhesive and biocompatible properties [4]. Furthermore, its degraded products are also considered to be non-toxic and non-immunogenic [5]. Previous study reported that it can bind with mucosal surfaces because of its cationic nature, which leads to bioadhesion, reduces mucociliary clearance thereby provides a longer contact time for drugs [6]. In addition, it also has another dramatic effect in terms of improving the drug absorption by opening the intercellular tight junctions of the lung epithelium [7]. CTS has been used as a drug carrier to attain the desirable drug release profile and enhance the dissolution rate of low water soluble drugs [8–10]. For most of the drug delivery applications, the chitosan microspheres were entrapped some additives such as absorption enhancers, crosslinking reagent and propellant et al. In vitro and in vivo evaluations of the biocompatibility of CTS have been reported in previous studies [11, 12]. Mi et al. [11] investigated the in vivo biocompatibility of the genipin-crosslinked injectable CTS microspheres and reported the microspheres had a superior biocompatibility. Williams III et al. [13] also evaluated the compatibility of CTS microspheres and the propellant. Although CTS has been considered to be a biocompatible polymer, previous study reported that the high concentration of CTS has cell toxictic effect [14]. Dekie et al. [15] have reported the primary amino groups are toxic to red blood cells. Therefore, the biocompatibility of the microspheres was to evaluate which are known to be an obstacle especially for pulmonary delivery systems.

Our previous study reported the characteristics of the theophylline(TH)/CTS/β-cyclodextrin(β-CD) microspheres made by spray drying [16, 17]. The aim of this study was to evaluate the biocompatibility of TH/CTS/β-CD microspheres (that is MS B) such as cell toxicity, hemolytic test, acute cell toxicity and in vivo implantation et al., which may help us to examine the feasibility of applying TH/CTS/β-CD microspheres as a sustained release pulmonary drug delivery carrier.

2 Materials and methods

2.1 Materials

Microspheres were prepared in our laboratory by spray drying method [16], which the ratio of MS B was TH/CTS/β-CD 1/3/1 (w/w/w), while the blank microspheres was MS K (CTS/β-CD 3/1). In brief, a predetermined amounts of TH and β-CD were dissolved in 200 ml of 1% CTS in acetic acid solution according to the above formulations. The solutions were spray-dried, using a spray-dryer (Büchi® Mini Spray Dryer, B-191, Switzerland), co-current flow type with a two fluid nozzle (diameter 0.7 mm). The operating parameters were as follows: 600 l/h airflow rate, aspiration 90%, 6 ml/min feed rate and inlet temperatures 150 ± 2°C, resulting in outlet temperatures 81 ± 2°C. The spray-dried microspheres were collected and stored in a desiccator (with anhydrous $CaCl_2$) at room temperature.

Coomassia Briliant Blue, Triton-X 100, Dulbecco's modification of eagle's medium (DMEM) and Newborn calf serum (NBCS) were obtained from Hyclone. Co., New Zealand. 3-(4, 5-Dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) and Tween-20 (Polyoxyethene-20-Sorbitan Monolaurate, MW1227.54) were obtained from Amresco. All other chemicals and reagents used were of analytical grade and provided by Shanghai Chemical Reagent Company (Sigma Co. ST. Louis, USA).

Male Sprague–Dawley rats and other animals were all purchased from Qingdao Municipal Institute for evaluation biocompatibility in vivo. The animal protocol was approved by Shandong Medical Laboratorial Animal Administration Committee. They were housed in a room with controlled temperature and humidity. All animal experiments were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH publication No. 80-23) revised in 1996. All efforts were made to minimize the number of animals used and their suffering.

2.2 Assay for lactate dehydrogenase (LDH) and protein in Bronchoalaeolar Lavage Fluid (BALF)

Male Sprague–Dawley rats weighing 160–180 g were divided into four groups (4 rats in each group): Normal group, PBS group, Triton-X 100 group and MS B group, anaesthetised with pentobarbital (40 mg/kg, i.p.) and secured on their backs on a board during the experiments. The trachea was exposed and the needle of a syringe containing solutions of 100 µl of PBS (pH 7.4) or 100 µl of Triton-X 100 was inserted to a depth of 0.6 cm between the fifth and sixth tracheal rings caudal to thyroid cartilage. Dry powder of microspheres was administrated through the rat trachea according to the method of Todo et al. [18]. Normal group was not given any treatment. The rats were sacrificed 24 h after obtaining blood sampling from the heart and the lungs were washed three times with 10 ml of PBS to obtain BALF. The activity of LDH in the supernate in the lavage centrifuged at 1,500 rpm at 4°C for 5 min was determined using Full Automatic Biochemical Analysor 7080 (Hitachi Instrument Ltd., Tokyo, Japan). The

protein content was determined using an assay kit of Coomassia Briliant Blue protein (Nanjing Jiancheng bioengineering institute, Nanjing, China). An SDS-PAGE was also run to testify the protein content. Conditions: 8% stack, Protein samples 25 μl, Gels are run at 200 V (constant voltage).

2.3 Evaluation of cytotoxicity

The cytotoxicity was determined using Fibroblasts by the MTT method [19]. The microspheres sample (MS B and MS K) diluted with 75% alcohol for 30 min, then washed two times with D-Hank's was sterilized through a 0.22 μm filter and the diluted solution was sterilized using autoclave sterilization method under 121°C for 30 min. Mouse Embryonic Fibroblasts (MEF) were used to evaluate cytotoxicity. Cells at a density of 3–7 × 10^4/ml were seeded into 96-well microtitre plates. Samples of microspheres were added to the plates at various concentrations (0.1172, 0.1563, 0.2344, 0.3125, 0.4688, 1.2500 mg/ml) in a final volume of 220 μl $well^{-1}$. After incubation, supernatants were removed, and 100 μl DMSO was added. Plates were placed on a shaking water bath at 37°C for 10 min to solubilize the formazan products and the absorbance was recorded at 570 nm. Cells treated with blank medium without any sample and with medium containing 500 μg ml^{-1} phenol were used as the negative control and positive control, respectively. They were treated the same way as above. Every group had 4 replications. The percent of viability was expressed as the relative growth rate (RGR) as follows:

$$RGR\% = \frac{D_t}{D_{nc}} \times 100\% \quad (1)$$

where D_t and D_{nc} are the absorbances of the tested sample and the negative control.

2.4 Blood compatibility

The hemolytic activities of the microspheres were investigated according to Singhal and Ray [20]. Blood was obtained from male rabbit after death by cardiac puncture. About 8 ml rabbit blood was diluted with 10 ml physiological saline. All TH microspheres 5 mg were placed in physiological saline (5 ml) were incubated for 30 min at 37°C. At the same time, physiological saline solution and distilled water were used as negative control and positive control respectively. After incubation, sample tubes were added 0.2 ml pooled rabbit blood dilution, continue incubated for 60 min, centrifuged at 3,000 rpm for 10 min at room temperature. The supernatant was transferred into the cuvette and the absorbance (D) was measured for haemoglobin at 545 nm using UV spectrophotometer. The hemolysis rate (HR) was calculated according to the equation:

$$HR = \frac{D_t - D_{nc}}{D_{pc} - D_{nc}} \times 100\% \quad (2)$$

where D_t, D_{nc} and D_{pc} are the absorbance of the sample, the negative control and the positive control, respectively.

2.5 Micronucleus test

Twelve mice were averagely divided into four groups: normal group, negative group (0.9% physiological saline group), positive group (Cyclophosphamide group) and MS B group (The microspheres 40 mg were diluted with 10 ml 0.9% physiological saline for 72 h at 37°C, and the diluted solution was sterilized using autoclave sterilization method under 121°C for 30 min). As negative control, 3 animals was injected s.c. with 50 ml/kg of 0.9% physiological saline. Cyclophosphamide was used as positive control, therefore 3 mice received injections of 100 mg/kg of cyclophosphamide. MS B group of 3 mice received injections of 50 ml/kg of diluted solution of microspheres. Each animal received 2 injections under the abdominal skin, separated by a 24-h interval. The animals were sacrificed by neck extension, 6 h after the last injection according to 2 alternative protocols. With the help of a syringe, the bone marrow from both femurs of the sacrificed animals was processed according to Schmid [21], with the only modification being the suspension of the cells in 1 ml of bovine calf serum and used for smear preparation. One drop of this suspension was applied to a slide, lying on a chilled glass top. Slides were prepared in triplicate for each animal. The slides were air dried for 12 h, fixed with Methanol, stained in Giemsa solution for 10–15 min and washed once again. After air drying for 30 min, the smears were in xylene for 10 min, two drops of Entellan were added and the slides were dried again. Micronucleated polychromatic erythrocytes were scored from 1,000 polychromatic erythrocytes counted for each animal using a Nikon microscope, cell counts were made on coded slides.

2.6 In vivo implant experiment

The in vivo biocompatibility of the CTS microspheres were examined by implanting the microspheres in the skeletal muscle of SD rats. The test microspheres (MS B and MS K) were sterilized using UV method. Subsequently, the sterilized microspheres, 60 mg, were implanted into the skeletal muscle after the rat being carried out anesthesia. Each animal received two implantation in the back of the rat just lateral to the midline. Rats were killed after defined time intervals, obtained blood sampling from the heart and the Haemoglobin (Hb), Alaninetransaminase (ALT) and Creatinine (Cr) were determined using a Full-Automated Blood Analyzer (XT-1800i, Sysmex. Corporation, Kobe,

Japan) and Full Automatic Biochemical Analyzer 7080 (Hitachi Instrument Ltd., Tokyo, Japan). The excised surrounding tissues were fixed with formalin and prepared histological sections which were stained with hematoxylin and eosin, observed by light microscope (Olympus, Japan). Similarly, sections of operative suture group (Group X) were obtained to serve as control.

2.7 Statistical evaluation

All the data were the arithmetic mean ± SD, One-way analysis of variance (ANOVA) was performed to compare different independent groups at 5% significance level.

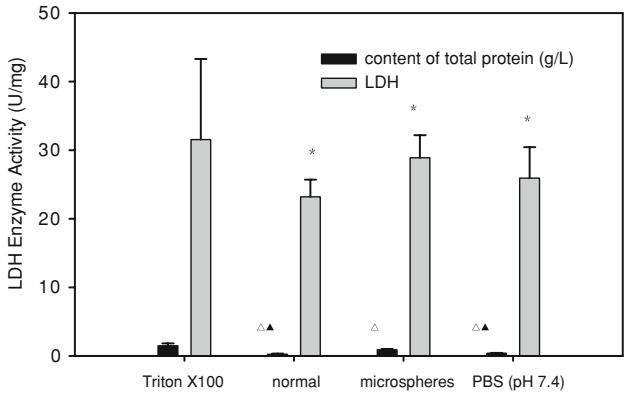

Fig. 1 LDH activity in BALF recovered 24 h after administration of Triton-X 100, Microspheres B and PBS solution. Statistical significance (*compared with Triton-X 100 $P < 0.05$) was observed for (Triton-X 100 versus microspheres B), (Triton-X 100 versus PBS), (Triton-X 100 versus normal); △ compared with Triton-X 100 $P < 0.05$; ▲compared with microspheres B $P < 0.05$

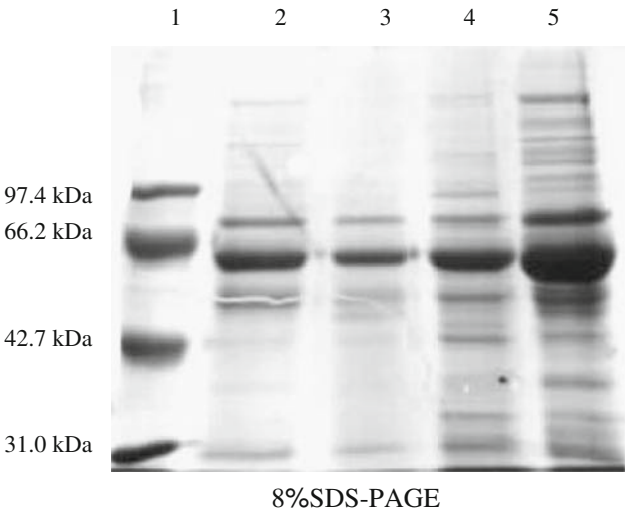

Fig. 2 SDS-PAGE electrophoresis gel stained by Comessie Blue: Lane 1: marker; lane 2: normal group; lane 3: PBS group; lane 4: microspheres B; lane 5: Triton-X 100

3 Result and discussion

3.1 Assay for LDH and protein in BALF

Since LDH is an intracellular enzyme, its leakage into the BALF is an indicator of membrane irritation and often regarded as a sensitive indicator of an inflammatory response [22]. In order to accurately appraise the biocompatibility of the microspheres, the LDH activities and the total proteins were employed in this study. Previous investigation reported that the LDH of the lavage fluid increased after administration Triton-X 100 [23], which was used as positive control. The LDH activities in BALF after administration of the dry powders was compared with those observed after administration of PBS group, Triton-X

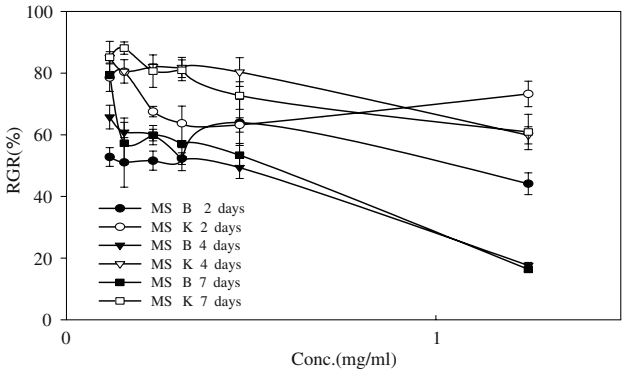

Fig. 3 Cell cytotoxicity results of different microspheres at different concentration and time ($n = 4$)

Table 1 Hemolysis of blood by the microspheres ($n = 3$, mean ± SD)

Sample	Optical density at 545 nm	Hemolysis (%)
Distilled water	0.557 ± 0.021	+ Control
Normal saline	0.012 ± 0.003	− Control
MS B	0.036 ± 0.006	4.77
MS K	0.022 ± 0.005	1.84

Table 2 The effect of microspheres on the rate of micronucleus and PCE/RBC in mice marrow cells (‰) ($n = 3$, mean ± SD)

Group	No. of animals	No. of cells	Frequencies of MNs (‰)
Normal	3	1000	0.91 ± 0.26△▲
Negative control	3	1000	1.10 ± 0.14△
Positive control	3	1000	21.20 ± 2.35
MS B	3	1000	0.99 ± 0.17△▲

△$P < 0.05$: Compared with Positive control; ▲$P > 0.05$: compared with Negative control

100 group and normal group (see in Fig. 1). The LDH activity of Triton-X 100 administrated after 24 h was significantly increased compared with MS B group, PBS group and normal group ($P < 0.05$), as for the MS B group, the LDH activity was increased but no statistical difference compared with PBS group and normal group ($P > 0.05$). The results of the protein content are seen in Fig. 1. The same tendency with the LDH activity was found. The

Table 3 The results of Hb in different time (g/l), ALT (μ/l) and Cr (μmol/l) of different microspheres and operative suture at different time ($n = 3$, mean ± SD)

Time (w)	Hb			ALT			Cr		
	MS B	MS K	Group X	MS B	MS K	Group X	MS B	MS K	Group X
0.5	114 ± 1.98	115 ± 2.34	122 ± 0.56	35.75 ± 1.09	35.96 ± 0.67	50.07 ± 0.34	68.89 ± 0.21	61.11 ± 2.21	84.31 ± 6.54
1	115 ± 2.34	83 ± 1.94	121 ± 2.46	53.3 ± 2.07	45.25 ± 1.89	43.59 ± 2.78	51.62 ± 2.56	45.96 ± 5.43	51.32 ± 0.98
4	127 ± 0.90	127 ± 0.45	120 ± 3.02	67.82 ± 4.56	73.63 ± 3.45	36.77 ± 4.67	84.84 ± 5.02	57.98 ± 1.34	89.12 ± 6.87
6	108 ± 0.45	115 ± 3.21	128 ± 5.02	29.48 ± 0.46	33.27 ± 0.97	33.31 ± 0.09	48.22 ± 3.12	44.89 ± 3.45	45.99 ± 2.19
12	123 ± 1.56	132 ± 0.70	94 ± 0.68	25.84 ± 3.09	26.68 ± 3.45	45.55 ± 0.67	34.36 ± 2.90	58.67 ± 3.58	31.22 ± 6.32

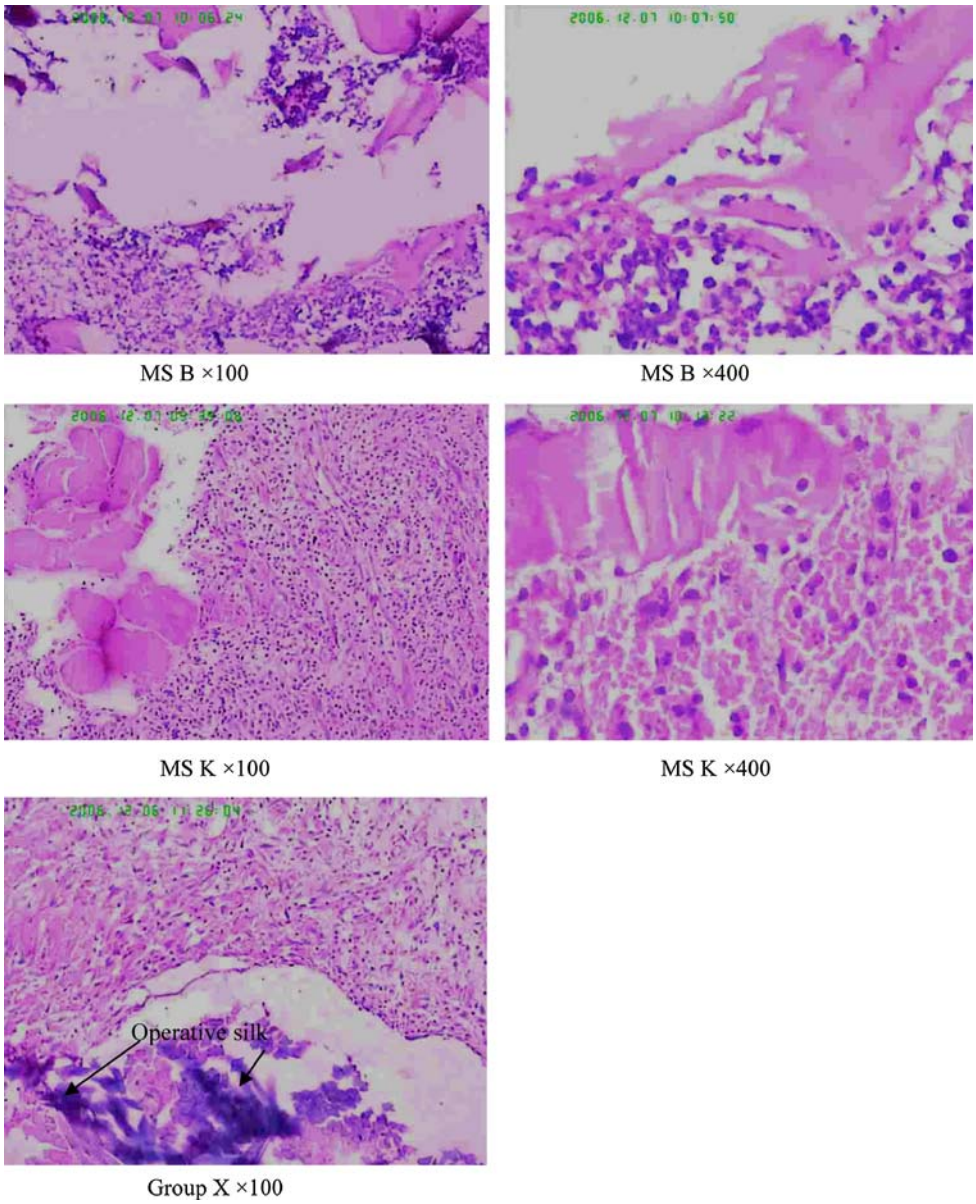

Fig. 4 Pathological photograph of muscle tissue at the implantation sites from SD rat receiving different microspheres and operative suture at 3.5 d

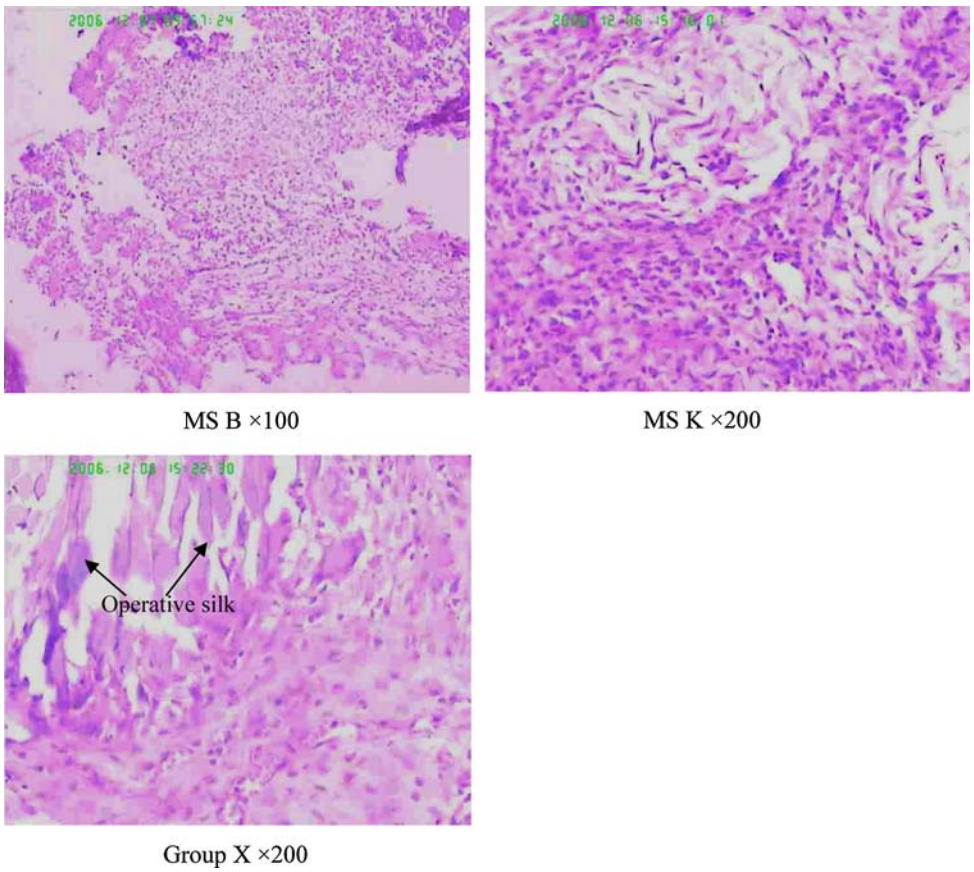

Fig. 5 Pathological photograph of muscle tissue at the implantation sites from SD rat receiving different microspheres and operative suture at 7 d

results of SDS-PAGE electrophores are seen in Fig. 2. There was a distinct increase in the protein banding of the Triton-X 100 positive control (banding at around 66 kDa). The microsphere treated samples also increased significantly compared with the normal group and the PBS group but was lower than that of the Triton-X 100 group. This results verified the above results (Fig. 1). Therefore, these results indicate that MS B could be regarded as a potential safe sustain-release pulmonary drug carrier.

3.2 Evaluation of cytotoxicity

MTT assay, a quick effective method for testing mitochondrial impairment and correlating quite well with cell proliferation [24], therefore often were performed to evaluate the cytotoxicity of polymers [19]. In this paper, MTT assay was used to evaluate the cytotoxicity of the microspheres. The cell cytotoxicity results of MS B and MS K are shown in Fig. 3. The MS B at low concentration (0.1172 mg/ml) showed no significant toxicity on the cells, with concentration increased (from 0.4688 mg/ml to 1.2500 mg/ml), the cell cytotoxicity increased because the cell viabilities significantly decreased from 80% to 20%. The cell cytotoxicity had no significant increase with time prolonged. The MS K showed relatively less significant toxicity on the cells compared with MS B. An explanation for the cell toxicity of MS B could be the strong alkalic properties of TH consequently suppress the interaction with cell membrane. From cytotoxicity results, it was shown that the MS B and MS K suspension was less toxic to the cell at low concentration, while at the higher concentration, the cytotoxicity increased significantly. Therefore, the cytotoxicity is concentration dependent (see Fig. 3). This observation is in agreement with the previous studies [11], and MS B must choose proper concentrations when used as pulmonary drug carrier.

3.3 Hemolysis test

In vitro hemolysis test is often used as an indicator of the injuries of the red blood cell membrane and consider to be a simple and reliable measure for estimating blood compatibility of materials. Hemolysis index is regarded as safe when it is less than 5% [25, 26]. CTS microspheres

interaction with blood components has been reported elsewhere [11, 27]. Wang et al. [12] evaluated the hemolysis of CTS films and considered the films are a safe carrier. Li et al. [28] reported that oleoylchitosan nanoparticles was suitable for intravenous administration because lower than 5% of hemolytic activity was a wide safe margin. Table 1 showed the result of hemolysis test. Hemolysis indexes of MS B and MS K were 4.77% and 1.84% at the concentration of 1 mg/ml. Base on the above consideration, the microspheres of MS B and MS K had no hemolysis and could be used as pulmonary delivery carrier.

3.4 Micronucleus test

Micronucleus test is an important measure to evaluate genetic toxicology, and the quantitative assessment of micronucleus containing cells is considered to be an indicator for the induction of structural and/or numerical chromosomal aberrations. The mouse bone-marrow micronucleus test (MNT) is accepted and widely used as a rapid test in science and industry [29]. The genotoxic potency of MS B in this test is assessed by scoring micronuclei in polychromatic erythrocytes. As can be seen from the Table 2, the micronuclei frequencies of normal group and negative control were 0.91 ‰ and 1.10 ‰, respectively, for the positive control, the micronuclei frequency was 21.20 ‰, which had significant differences compared with the normal group and negative control ($P < 0.05$). The micronuclei frequency of the MS B was 0.99‰, ranged from the normal group to the negative group and had no significant difference ($P > 0.05$), while compared with positive group, there was significant difference ($P < 0.05$). This result implies that there is no significant contribution to micronuclei scores after MS B treatment.

3.5 Histological evaluation

Table 3 shows the results of Hb in different time (g/l), ALT (μ/l) and Cr (μmol/l) of different microspheres and operative suture at different time. As shown in Table 3, the Hbs of MS B, MS K and Group X were all in the normal range, no significant change in the levels of Hbs was observed, whenever the time, as compared to each other. The levels of serum ALT and Cr were no significant changes observed in microsphere group (MS B, MS K) as compared to Group

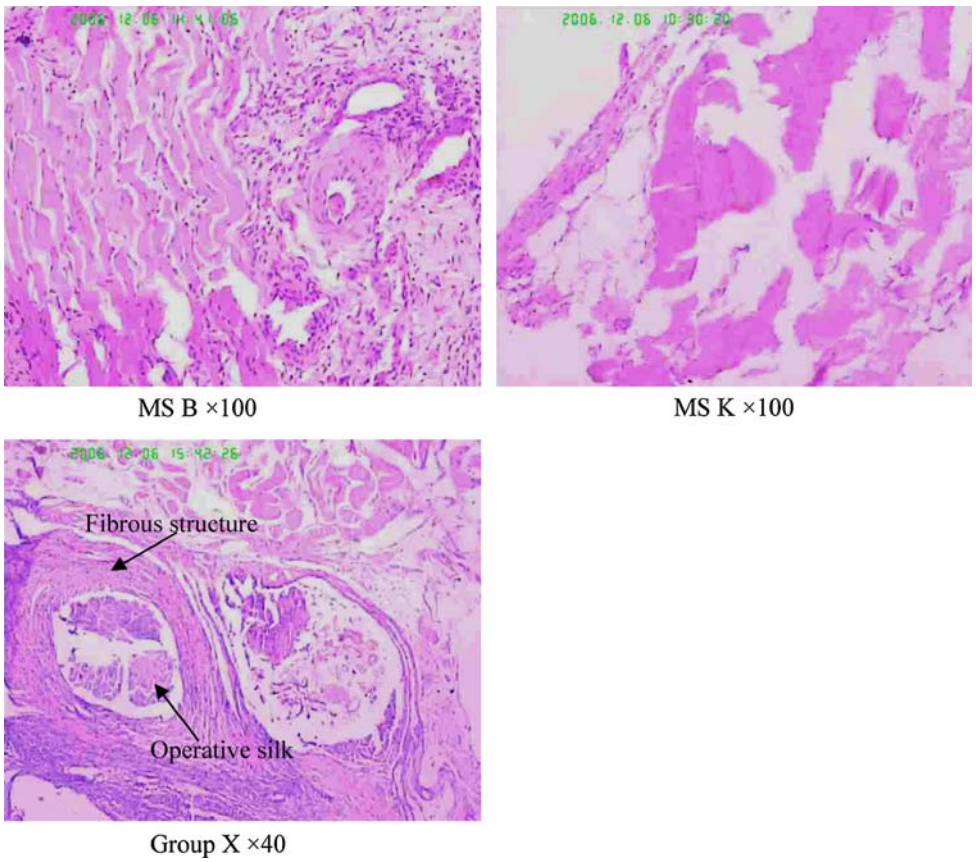

Fig. 6 Pathological photograph of muscle tissue at the implantation sites from SD rat receiving different microspheres and operative suture at 4 w

X, which is in line with the good biocompatibility of CTS reported previously [30, 31], so it can be considered that there is no hepatotoxicity, renal toxicity and hemoglobin synthesis was toxicity. Figures 4, 5, 6, 7 and 8 show the photomicrographs of the tissues implanted with MS B, MS K and operative suture in rat at different intervals. The wounds were free from suppuration and necrosis after muscle implantation in all periods. As can be seen from the Fig. 4, there was a notable acute inflammatory reaction with many neutrophils infiltration, a few lymphocytes and macrophages observed on the tissues among all studies groups. Further, operative suture inside the implanted tissues was observed. At one week after implantation, the degree of inflammatory reaction was much milder than those in Fig. 4, and neutrophils infiltration were significant alleviated (see in Fig. 5). Figure 6 shows the results of implantation in rat for 4 weeks. The degree in inflammatory reaction for the tissues implanted with MS K and MS B was less than those retrieved at 3.5-day and 7-day postoperatively, especially for MS K. For the Group X, the degree in inflammatory reaction was also markedly lightened, however, fibrous structure which was located at the interface surrounding operative silk was clearly observed, and even became a thicker wall (Fig. 5). At 6 and 12-week postoperatively, the inflammatory reaction of the three tested group had almost disappeared. From the above comparison, it can be seen that with time prolonged, the inflammatory reaction was gradually lighted and disappeared in the end. The tissue compatibility evaluations of CTS were studied elsewhere [32]. Similar result has been reported by Carreño-Gómez and Duncan [33]. This observation reflects good tissue compatibility between the MS B, MS K and the surrounding tissues, moreover, which had no significant difference compared with Group X.

4 Conclusion

The present work shows that microspheres (MS B) can be delivered into the lung as a high biocompatible pulmonary drug system, taking into account that these microspheres have promising potential for use as safe carrier because of neither hemolysis nor toxicity to rat implantation, even no genetic toxicity. The test of muscle implantation test also

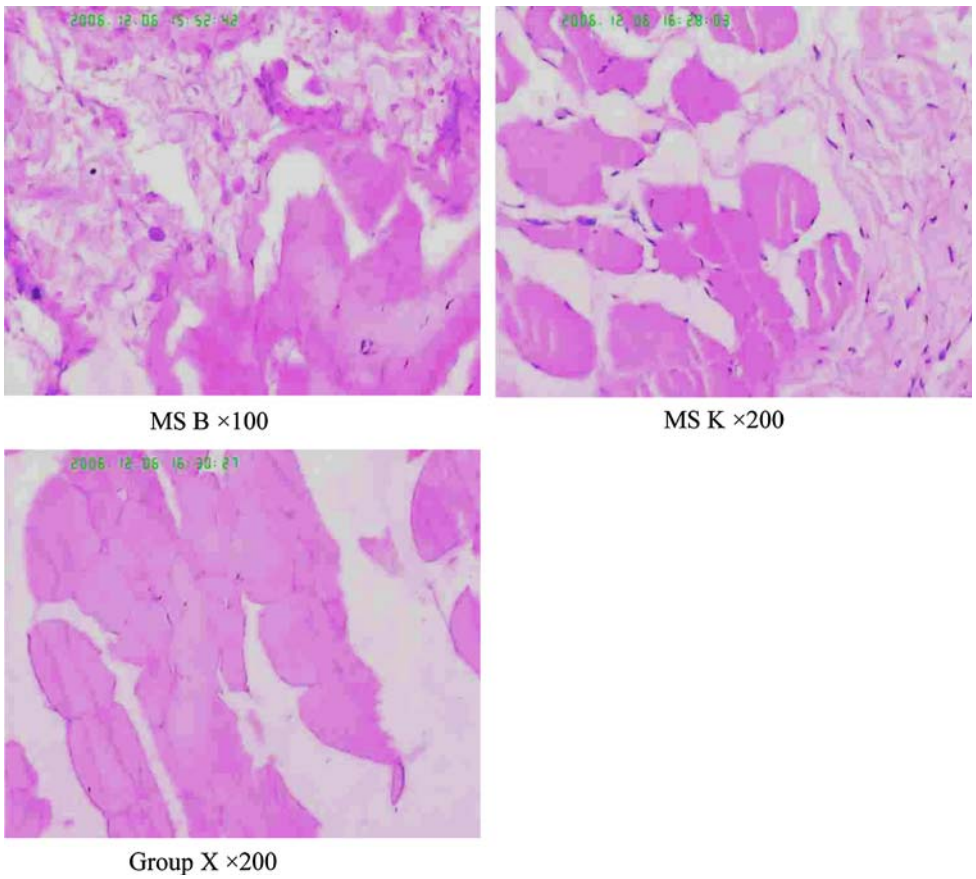

Fig. 7 Pathological photograph of muscle tissue at the implantation sites from SD rat receiving different microspheres and operative suture at 6 w

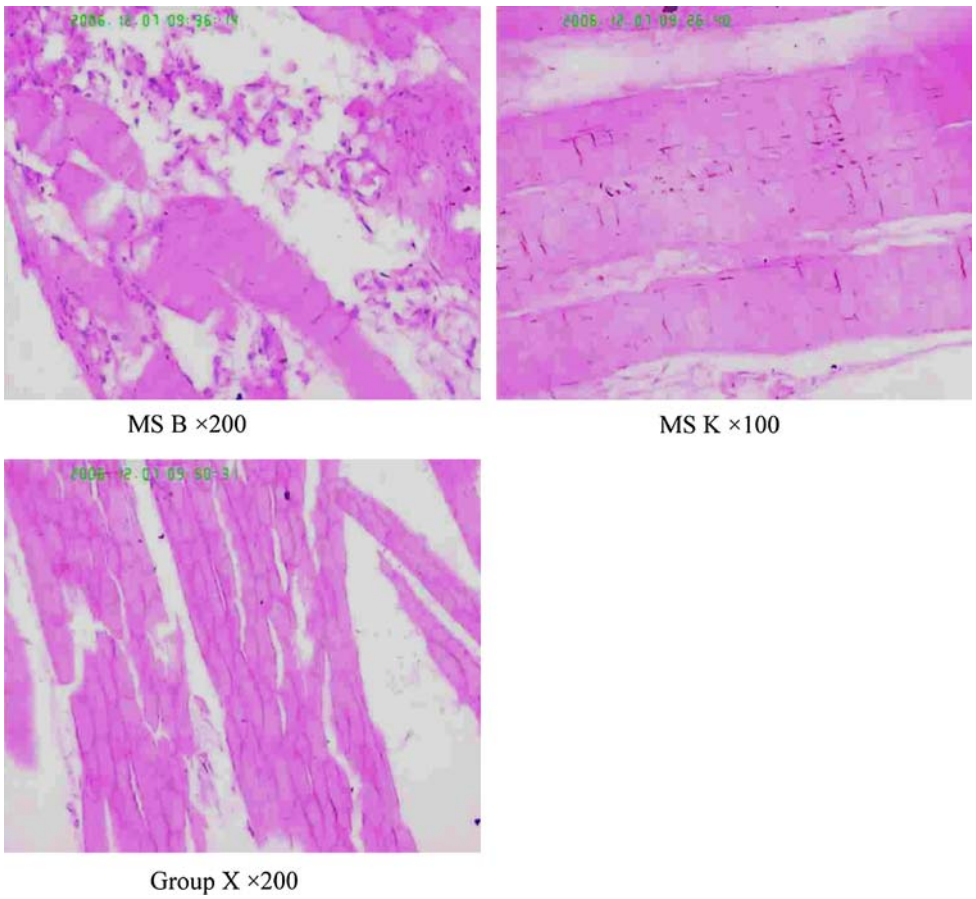

Fig. 8 Pathological photograph of muscle tissue at the implantation sites from SD rat receiving different microspheres and operative suture at 12 w

indicates that the wounds were free from suppuration and necrosis after muscle implantation in all periods, even did not cause any adverse effect such as hepatotoxicity, renal toxicity and hemoglobin synthesis toxicity. With time prolonged, the inflammatory reaction is gradually lighted and disappeared in the end.

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