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RESEARCH ARTICLE

Preparation and characterization of lung-targeting ceftiofur-loaded gelatin microspheres

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Abstract

Background: Ceftiofur is an effective antibiotic against respiratory infections in livestock. However, ceftiofur concentration that is found in lungs after intravenous injection is not effective. Fortunately, ceftiofur-loaded gelatin microsphere (Cef-MS) enjoys advantages of lung-targeting and can achieve an effective concentration. However, no study has been reported on this modality of drug delivery.

Objective: We investigated the properties of this delivery modality—lung targeting ceftiofur-Cef-MSs.

Methods: We prepared Cef-MS and investigated drug loading, stability and release characteristics *in vitro* and studied tissue distribution patterns and potential lung injury in mice.

Results: Results showed that the average size and span value are 21.26 μm and 1.07, respectively. Drug loading and loading efficiency were 15.31 and 76.55%, respectively. Cef-MSs were stable in light, heat and humidity, except that agglutinative phenomenon was observed in 90% humidity after 10 days. Cef-MS presented a slower *in vitro* release pattern compared to ceftiofur. Cef-MS mainly concentrates in lungs after intravenous administration. Furthermore, histopathological studies showed that Cef-MS only induces mild and reversible lung injury and is biologically safe.

Conclusion: Cef-MS is a promising alternative form with high lung-targeting properties for the treatment of respiratory infections.

Keywords: Ceftiofur, gelatin, lung-targeting, microsphere, respiratory infections

Introduction

Respiratory infections are of particular concern in livestock breeding industry since they cause considerable economic loss[1]. It has been reported that *Pasteurella multocida* (41.8%) and *Actinobacillus pleuropneumoniae* (38.8%) were the main pathogens that cause respiratory infections[2]. Ceftiofur, a third generation cephalosporin antibiotic, exerts its antibacterial activity through inhibition of mucopeptide synthesis in the bacterial wall[3]. It shows a high *in vitro* activity against the aforementioned bacteria and is used extensively to treat respiratory infections[3–5]. Moreover, recent evidence revealed that it can reduce inflammatory cytokine secretion and attenuate lipopolysaccharide-induced (the main toxin from gram-negative bacteria) lung injury[6,7]. Therefore, ceftiofur is desirable against pulmonary infections. However, ceftiofur concentration after intravenous injection is below therapeutic levels in lungs. Alternative forms like microspheres with diameter range of 12–44 μm have a notably lung-targeting action due to mechanical trapping effect of pulmonary blood vessels[8], thereby achieving an effective concentration in lungs. Moreover, gelatin, used as a coating material for microspheres, is biodegradable and biocompatible. Therefore, ceftiofur-loaded gelatin microsphere (Cef-MS) is a promising treatment form against

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respiratory infections since it can not only improve therapeutic efficacy and reduce side effects due to its targeting, but is also biodegradable and biocompatible. However, to date, no research has reported its preparation, pharmacokinetics and tissue distribution patterns, or potential lung injury. Therefore, we conducted experiments to prepare Cef-MS, to study its properties, pharmacokinetics and tissue distribution patterns, and to assess its potential lung injury in mice.

Materials and methods

Apparatus, reagent and animals

Apparatus: A chromatographic system consisted of an HPLC pump (model G1311A solvent delivery module, Ajilent Co.), a variable-wavelength UV absorbance detector (model G1314A variable-wavelength detector, Ajilent Co.) and an analytical column (TC-C18 (2), Ajilent Co., 15 cm × 4.6 mm i.d.) was used to determine drug amount. An optical microscope with camera (Olympus, Japan) and a scanning electron microscope (CamScan Series 4, Cambridge Scanning Company Limited, England) were used for physical examination of Cef-MSs. A MasterSizer 2000 (Malvern Instruments) was employed to determine the average diameter and particle size distribution of Cef-MS. A drug stability test chamber (LHH-500SDP, Shanghai Yixian Instrument Co., China) was used to obtain certain temperature, humidity and light conditions in stability tests. A radio-immunity gamma counter (SN-695, Shanghai, China) and a radioactivity meter (FT-3106, Beijing, China) were also used to determine radioactivity.

Reagents: Ceftiofur (Zhejiang Hisun Pharmaceutical Co. Ltd., China) and gelatin (Sigma Aldrich) were used to prepare microspheres (sample number with 20090304); $Na^{125}I$ (PerkinElmer Inc.), Iodogen (Sigma Aldrich) and all other reagents (liquid paraffin, isopropanol, pentanediol and acetone) were all of analytical grade (Organic Chemistry Research Institute of Chinese Academy, China).

Animals: Eighty-four 8-week-old healthy Kunming mice of both sexes (20 ± 2 g) were maintained at 20 ± 2°C with a 12-h natural light period. They were provided with commercial pellet diet (Department of Laboratory Animal Science, Fudan University, China) and tap water *ad libitum*.

Preparation of Cef-MS

Cef-MSs were prepared according to a modified method of Tabata and Ikada[9]. Briefly, ceftiofur was dispersed in 3 mL gelatin solution (0.2 g/mL) by sonication (100 W) for 5 min (2 second's work and 3 second's rest, alternatively). The formed gelatin solution of ceftiofur was added drop wise into 50 mL liquid paraffin (50°C) containing 1 mL Span80 (an emulsifying agent). The mixture was then stirred at 1800 rpm at 50°C for 15 min to yield water-in-oil emulsion. Afterwards, stirring was continued at 4°C for 30 min and then glutaraldehyde was employed to solidify gelatin particles from the formed W/O emulsion. The obtained microspheres were then separated, washed with isopropanol and acetone for three times to remove excessive liquid paraffin and glutaraldehyde, and dried directly at room temperature. Afterwards, they were passed through sieves with apertures of 15 μm.

Morphology

For morphology studies, Cef-MSs were visualized through scanning electron microscope. Specifically, samples were sprinkled on a double-sided adhesive tape attached to an aluminum stub and fixed onto a graphite surface. Excessive samples were then removed and the stub sputter was coated with gold. The coated samples were viewed under a scanning electron microscope at 25 kV to reveal the surface quality and porosity of microspheres.

Average diameter and particle size distribution

The average diameter and size distribution of Cef-MSs were measured by MasterSizer 2000 particle size analyzer based on laser light scattering. Before measurement, weighed microspheres were suspended in distilled water and vortexed. The resulting homogeneous suspension was used to determine the average diameter and particle size distribution. The average diameter was reported as a volumetric mean diameter [D (4,3)] ($\sum d^4/\sum d^3$), and the particle size distribution was evaluated by span value, defined as the following expression:

$$\text{Span Value} = \frac{D90\% - D10\%}{D50\%} \quad (1)$$

where DN% (N = 10, 50, 90) means that the volume percentage of microspheres with diameters up to DN% equals to N%. The smaller the span value, the narrower the particle size distribution is.

Drug loading and loading efficiency

Cef-MSs were dispersed in 5 mL of 0.1 M phosphate buffer (pH = 6.00) under ultrasonic action for 30 min and the resulting solution was kept on a horizontal shaker at 50 rpm for 2 h at room temperature. Afterwards, supernatant obtained by centrifuging the solution was analyzed by HPLC.

In brief, HPLC was performed by using the chromatographic system consisting of an HPLC pump (model G1311A solvent delivery module, Ajilent Co.), a variable-wavelength UV absorbance detector at 254 nm (model G1314A variable-wavelength detector, Ajilent Co.) and an analytical column (TC-C18 (2), Ajilent Co. 15 cm × 4.6 mm i.d.). The mobile phase consisted of a mixture of 0.02 M disodium hydrogen phosphate dihydrate buffer (pH = 6.0, adjusted by using ortho-phosphoric acid) and acetonitrile in 78:22 ratio. The mobile phase was pumped to the column at a flow rate of 1.0 mL/min. The chromatographic system was kept at room temperature (23 ± 1°C). The injection volume was 20 μL. Afterwards, the mobile phase was filtered through a 0.45 μm membrane filter and degassed under ultrasonic action for 30 min. Then,

quantitative analysis was performed with external standardization and measurement of peak area.

All samples were determined in triplicate. Theoretical drug loading, actual drug loading and loading efficiency were determined by using the following formulae:

$$\text{Theoretical drug loading} = \frac{drug(total)}{drug(total)+polymer} \times 100\% \quad (2)$$

$$\text{Actual drug loading} = \frac{drug(loaded)}{drug(loaded)+polymer(actual)} \times 100\% \quad (3)$$

$$\text{Loading efficiency} = \frac{\text{Actual drug loading}}{\text{Theoretical drug loading}} \times 100\% \quad (4)$$

Stabilities of the microspheres

Stability tests, including light stability, heat stability and humidity stability tests were conducted to assess its stabilities. For light stability test, Cef-MSs were stored for up to 10 days under light (4500 ± 500 lx) and sampled on 0, 5th and 10th day. For heat stability, they were preserved for up to 3 months in the dark at 4, 25 and 37°C under the humidity of 40% and sampled at the end of each month. For humidity test, they were stored for up to 10 days under the humidity of 75 and 90% in the dark at 25°C, and sampled on 0, 5th and 10th day. Since Cef-MSs were only for research but not products, we just use these conditions mentioned above. Average diameter and drug content of the samples were determined to evaluate their stabilities according to the methods described above.

In vitro release of the drug

Ceftiofur released from Cef-MS was investigated *in vitro* by dialysis method. Drug-loaded microspheres (30 μg) were sealed into dialysis bags (Millipore dialysis tube, molecular weight cut-off of 8–14 kDa) and dialyzed against 50 mL PBS buffer (pH = 7.4) at 37 ± 0.2°C in an air-bath shaker at 50 rpm for 48 h. The system was protected from light. The released medium was collected at 0.5, 1, 2, 4, 8, 12, 24, 36 and 48 h, and the whole medium replaced with fresh PBS buffer. The released amount of ceftiofur was determined by spectrophotometer (UV-384 plus, Molecular Devices Corporation) at 254 nm. As for the control, all experimental conditions and procedures were the same with those of Cef-MS, except that ceftiofur was released from 30 mg ceftiofur powder. All experiments were run in triplicate.

Pharmacokinetics and tissue distribution patterns of Cef-MS *in vivo*

Preparation of ^{125}I-Cef-MS

Cef-MS (50 μg) were placed in test tubes coated with 100 μg Iodogen, and then 100 μL PBS buffer (0.01 mol/L, pH = 7.4) and 2 μL Na^{125}I solution (18.5×10^6 Bq) were added. After the mixture had been incubated for 10 min at room temperature, 1 mL PBS buffer (0.01 mol/L, pH 7.4) was added to stop the reaction. Then, silica gel thin layer chromatography was employed to analyze the labeled proportion. In brief, 5 μL of each sample was spotted onto silica gel thin layer chromatography plates and developed by 85% ethanol. Then, each plate was cut into 1 cm width and placed into test tubes. Radioactivity of each sample was determined by a radioactivity meter. Given that the retention fraction of ^{125}I-Cef-MS is 0.0 and that of ^{125}I is 0.85–1.00, radiochemical purity of ^{125}I-Cef-MS was defined as the ratio of radioactivity counts with retention fraction being 0.0 to radioactivity counts on the whole plate.

The labeled solution and 2 mL PBS buffer (0.01 mol/L, pH = 7.4) were placed into centrifuge tubes and centrifuged at 1500 rpm for 10 min. Then, pellets were obtained and were washed by PBS buffer for three times. Certain concentrations of ^{125}I-Cef-MS suspensions were obtained by adding appropriate amounts of Cef-MS. Thin layer chromatography and radioactivity meter were employed to determine the labeled percentage as mentioned above.

Stability test of ^{125}I-Cef-MS

^{125}I-Cef-MS was placed under 25°C and sampled on 1st, 2nd, 3rd, 4th and 5th day. Thin layer chromatography and radioactivity meter were used to determine radiochemical purity. Radiochemical purity greater than 95% after 5 days was defined as stable.

Radioactivity measurement

Ten microliters of ^{125}I-Cef-MS mentioned above was diluted to 1 mL and 10 μL out of the diluted solution was placed into plastic tubes (12 × 60 mm) and was used to determine the radioactivity by radio-immunity gamma counter. Counts per minute (CPM) was recorded for each sample and the experiments were performed in triplicate. Mean values of CPM from three samples were used for radioactive concentrations.

In vivo examinations

Prior to experiments, 60 Kunming mice were divided into 10 groups with 6 in each group (3 male and 3 female). ^{125}I-Cef-MS suspensions were injected into tail veins of each mouse with the dosage of 27.27 mg for each kilogram of the mouse. Meanwhile, for each mouse, the same amount of ^{125}I-Cef-MS suspension was used *ex vivo* as control to adjust for radioactive decay. Animals were sacrificed through cervical dislocation at 5 min, 30 min, 1, 2, 4, 8, 15, 24, 48 and 96 h. Soon after the sacrifice, blood, heart, lung, liver, kidney, spleen, stomach and small intestine were extracted and weighed by Sartorius Electronic Balance, and CPM were measured for each organ or tissue of each mouse. Then, radioactivity per gram of tissue of the whole injected radioactivity was calculated for each organ or tissue of each mouse.

Histopathological studies

Another 24 Kunming mice were divided into either experimental group or control group with equal gender ratio

in both groups. The experimental group received 0.1 mL Cef-MS suspension via tail vein injection while the control group received the same amount of normal saline. For each group, three mice were sacrificed immediately, 5 min, 12 or 48 h after injection. Then, lungs were dissected and washed with cold saline. The lung tissues were fixed in 10% neutral formalin, embedded in paraffin, and stained with hematoxylin and eosin. All lung samples were examined under light microscope at 500× magnification.

Statistical analysis

Results expressed as mean ± SD were analyzed by Student's *t*-test by using SPSS 16.0. A p value of less than 0.05 was considered as statistically significant.

Results and discussion

Characteristics of microspheres

As shown in Figure 1, Cef-MSs were smooth, discrete with spherical or near-spherical shape, which may be due to cross-linking of coacervates that resulted in stabilized emulsion droplets. Cef-MSs had different particle sizes, drug loadings and loading efficiencies according to different experimental conditions. In order to find an optimal condition for their preparation, the influence of initial drug to polymer ratio was examined. Results showed that the average diameter and actual drug loading increased as theoretical drug loading increased, but loading efficiency was highest when theoretical drug loading was 20% (Table 1 with "not filtered"). Then, this condition of theoretical drug loading of 20% was employed to optimize experimental conditions. However, under this condition, although it has been revealed that microspheres with diameter range of 12–44 μm have a notable lung-targeting efficacy[8], 42.60 μm was mere an average size and a relatively large portion of particles whose size were larger than 44 μm; and span value was high, which was indicative of broad particle size distribution. Thus, in an attempt to prepare high-efficacy lung-targeting and uniform particles, prepared microspheres were filtered through sieves with apertures of 15 μm. As shown in Table 1 with "filtered", the average diameter was 21.26 μm and the span value was 1.07 when theoretical drug loading was 20%, indicating that the prepared Cef-MSs were potentially lung-targeting and relatively uniform in particle sizes. Thus, these particles were used in all later experiments. However, we were curious about how it was possible to obtain 21.26 μm-averaged particles after passing through sieves with 15 μm apertures. Taken the experimental procedures into consideration, we attribute it to that gelatin in Cef-MSs swelled in water since Cef-MSs were in acetone when they were filtered and in water when we measured their particle sizes. Although gelatin swelled in water, which made their sizes different from their original sizes, we suggest that the particle sizes measured after swelling may be more accurate since Cef-MSs were also in water environment after intravenous injection.

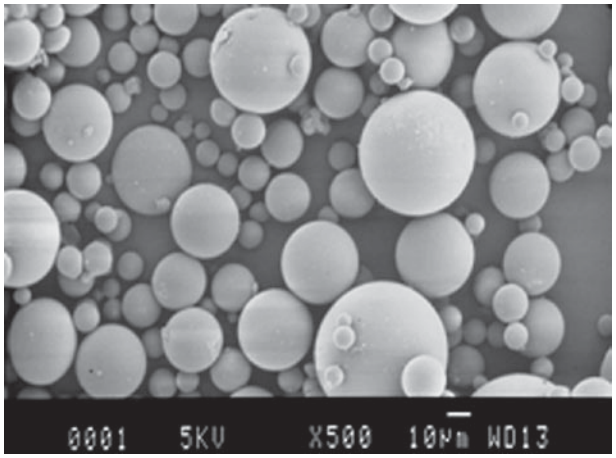

Figure 1. Scanning electron microscopy of unfiltered Cef-MSs (theoretical drug loading of 20%). Microspheres were spherical in shape with smooth surfaces but no pores.

Test for stabilities

As shown in Tables 2, 3 and 4, average diameters and drug contents did not change significantly, except for the condition of 90% humidity for 10 days. Under the humidity of 90% after 10 days, the average diameter increased significantly ($p = 5.14 \times 10^{-5} < 0.05$ and $6.37 \times 10^{-5} < 0.05$

Table 2. Light stability test of Cef-MS.

	Duration (day)		
Items	0	5	10
Average diameter (μm)	21.26 ± 0.52	21.13 ± 0.32	21.04 ± 0.22
Drug content (%)	15.31 ± 0.72	15.25 ± 0.45	15.17 ± 0.57

Table 1. Characteristics of Cef-MS with different theoretical drug loadings.

Theoretical drug loading (%)	Actual drug loading (%)	Loading efficiency (%)	Average size (μm)	Span value	Percent yield (%)
7.69 (Not filtered)	2.51 ± 0.31	32.75 ± 4.08	29.52 ± 0.26	1.67 ± 0.03	66.91 ± 1.01
11.11 (Not filtered)	7.11 ± 0.25	63.96 ± 2.29	38.56 ± 0.14	2.43 ± 0.07	69.14 ± 0.98
20 (Not filtered)	15.14 ± 0.59	75.97 ± 3.44	42.60 ± 0.36	3.05 ± 0.14	67.77 ± 1.36
33.33 (Not filtered)	23.69 ± 0.72	71.10 ± 2.16	61.43 ± 0.28	3.95 ± 0.12	68.43 ± 1.44
7.69 (Filtered)	2.46 ± 0.27	32.46 ± 3.86	18.32 ± 0.24	0.98 ± 0.04	55.47 ± 1.82
11.11 (Filtered)	7.24 ± 0.19	61.37 ± 1.89	19.45 ± 0.31	1.03 ± 0.03	46.29 ± 0.59
20 (Filtered)	15.31 ± 0.72	76.55 ± 3.61	21.26 ± 0.52	1.07 ± 0.02	40.02 ± 1.28
33.33 (Filtered)	23.39 ± 0.68	70.97 ± 1.57	22.69 ± 0.30	1.11 ± 0.06	28.21 ± 1.51

Table 3. Heat stability test of Cef-MS.

Items	Duration (month) 0	1	2	3
4°C				
Average diameter (μm)	21.26 ± 0.52	21.19 ± 0.36	22.81 ± 0.41	23.03 ± 0.55
Drug content (%)	15.31 ± 0.72	15.26 ± 0.49	15.22 ± 0.59	15.18 ± 0.36
25°C				
Average diameter (μm)	21.26 ± 0.52	21.03 ± 0.39	22.22 ± 0.47	21.47 ± 0.31
Drug content (%)	15.31 ± 0.72	15.27 ± 0.64	15.12 ± 0.41	15.05 ± 0.69
37°C				
Average diameter (μm)	21.26 ± 0.52	22.22 ± 0.45	23.41 ± 0.37	22.73 ± 0.49
Drug content (%)	15.31 ± 0.72	15.27 ± 0.77	15.16 ± 0.59	15.11 ± 0.68

Table 4. Humidity stability test of Cef-MS.

Items	Time (days) 0	5	10
RH 75%			
Average diameter (μm)	21.26 ± 0.52	21.93 ± 0.47	21.54 ± 0.32
Drug content (%)	15.31 ± 0.72	15.36 ± 0.53	15.29 ± 0.61
RH 90%			
Average diameter (μm)	21.26 ± 0.52	22.80 ± 0.38	29.86 ± 0.59*
Drug content (%)	15.31 ± 0.72	15.15 ± 0.49	15.02 ± 0.64

*$p = 5.14 \times 10^{-5}$ and 6.37×10^{-5} for 10 d vs. 0 and 10 days vs. 5 days, respectively. RH, relative humidity.

for 10 days vs. 0 and 10 days vs. 5 days, respectively), but the drug content did not change, indicating that the high humidity can cause agglutinative phenomenon but do not affect drug content. Thus, these results suggested that Cef-MSs were stable and can be stored under these conditions, which is consistent with the study by Lu et al.[10].

In vitro release pattern

As shown in Figure 2, compared to ceftiofur powder, Cef-MS presented a significantly slower release pattern ($p = 0.001 < 0.05$, $p = 0.04 < 0.05$, $p = 3.3 \times 10^{-6} < 0.05$, and $p = 4.1 \times 10^{-5} < 0.05$ for Cef-MS vs. ceftiofur powder at 0.5, 1, 2, and 4 h, respectively). Ceftiofur released from Cef-MS was 24.90, 58.68 and 84.90% at 0.5, 4 and 48 h, respectively; whereas, ceftiofur released from ceftiofur powder was 87.69% at 4 h. Therefore, these findings indicate that Cef-MS has a well-sustained release pattern, suggesting that Cef-MS is promising to be used as a controlled release formulation. Moreover, the cumulative released amounts of ceftiofur from Cef-MS versus time were fitted to various kinetic equations to determine the release mechanism by using SigmaPlot version 11.0. As presented in Table 5, Biexponential model was an ideal fit with correlation coefficient (R^2) being 0.9905, which indicates that the mechanism of *in vitro* release is diffusion.

In vivo pharmacokinetics and tissue distribution patterns of Cef-MSs

^{125}I-Cef-MSs were stable under 15°C (radiochemical purity greater than 95% after 5 days): their radiochemical purities were 98.41, 98.24, 97.81, 97.31 and 96.57% after 1, 2, 3, 4 and 5 days, respectively, which guarantees that the data observed in later experiments were

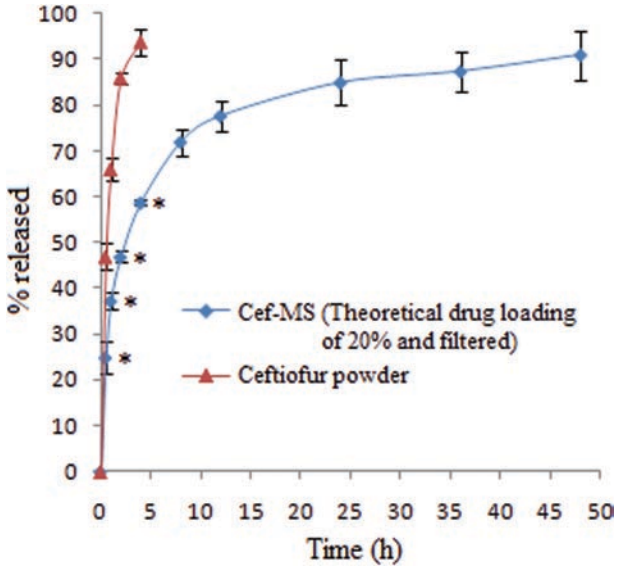

Figure 2. *In vitro* release patterns of ceftiofur from Cef-MS and ceftiofur powder were examined through dialysis methods. All the experiments were in triplicate. Cef-MS showed a significantly slower release pattern in contrast with ceftiofur. (* indicates that $p = 0.001 < 0.05$, $p = 0.04 < 0.05$, $p = 3.3 \times 10^{-6} < 0.05$, and $p = 4.1 \times 10^{-5} < 0.05$ for Cef-MS vs. ceftiofur powder at 0.5, 1, 2, and 4 h, respectively.)

free from errors induced by unstability of ^{125}I-Cef-MSs. The *in vivo* pharmacokinetics of microspheres was studied using 3P97 software offered by the Chinese Pharmacological Society and were fitted by one-compartment model, two-compartment model and three-compartment model. Goodness of fit was assessed with the Akaike Information Criterion (AIC). Based on the analysis of the models and parameters, it could be concluded that the *in vivo* pharmacokinetics of microspheres in blood could be best described by three-compartment model with intravenous injection. As are presented in Table 6, results showed that the central distribution volume (Vc) was 1.2 L/kg, indicating that Cef-MS was mainly distributed in tissues (mainly in lung as compared to other tissue, which is shown in Figure 3) rather than in blood. Moreover, we found that $T_{1/2(\alpha)}$ and $T_{1/2(\beta)}$ were 2.14 and 297.15 h, respectively. Since $T_{1/2(\alpha)}$ and $T_{1/2(\beta)}$ are distribution half-life and elimination half-life, this finding indicates that Cef-MS

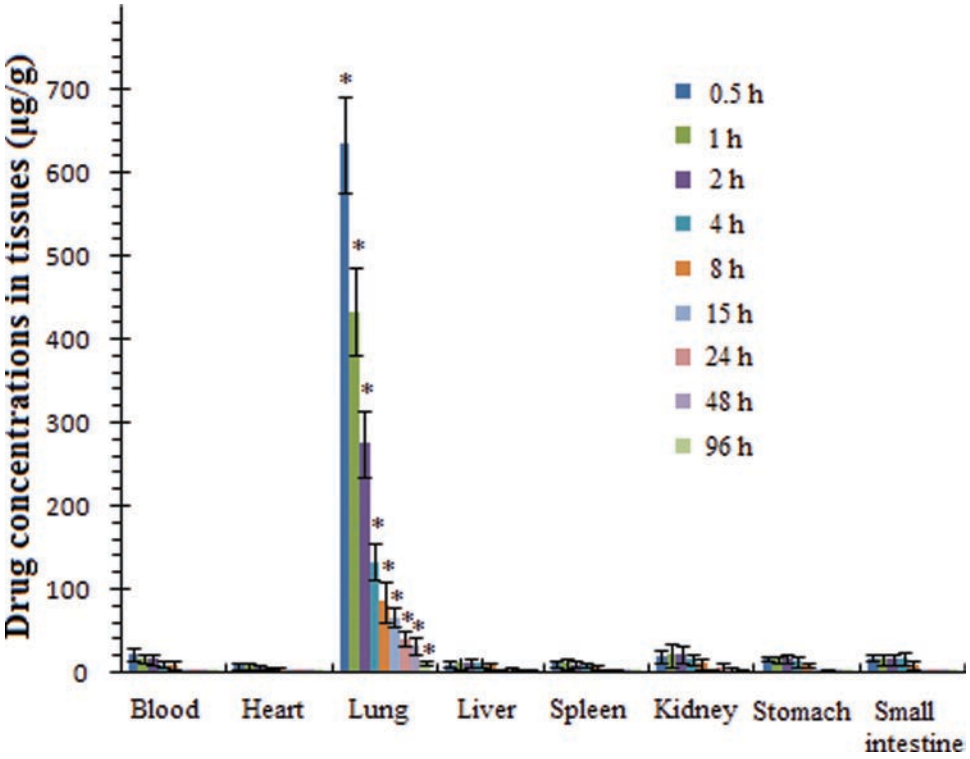

Figure 3. Drug concentrations were determined through radioactivity assay. Ceftiofur concentrations in various tissues were determined and concentrations in lung were significantly higher than other organs or tissues, e.g., concentrations of ceftiofur were significantly higher in lung than in blood (* indicates that $p = 4.22 \times 10^{-5}$, 1.91×10^{-4}, 5.12×10^{-4}, 0.001, 0.012, 6.83×10^{-4}, 9.77×10^{-5}, 0.016 and 5.61×10^{-5} for lung vs. blood at 0.5, 1, 2, 4, 8, 12, 24, 48 and 96 h, respectively.)

Table 5. Correlation coefficient for kinetic equations of mean cumulative released amount (Q) vs. time (t).

Equations	Cef-MS	R^2
Zero-order	$Q = 0.2594 \bullet t$	0.5992
Hixon & Crowell	$1-(1-Q)^{1/3} = 0.01466 \bullet t$	0.7462
Higuchi	$Q = 0.1683 \bullet t^{1/2}$	0.8243
First-order	$Q = 1-e^{-0.2312 \bullet t}$	0.8252
Barker & Lonsdale	$3/2 \bullet [1-(1-Q)^{2/3}]-Q = 0.007206 \bullet t$	0.8754
Korsmeyer & Peppas	$Q = 0.403426 \bullet t^{0.2259}$	0.9681
Biexponential	$Q = 1-(0.4093 \bullet e^{-0.0377 \bullet t} + 0.5681 \bullet e^{-0.7974 \bullet t})$	0.9905

Table 6. Pharmacokinetic parameters of Cef-MS in blood.

Parameter	Cef-MS
Vc (L/kg)	1.2
$T_{1/2(\alpha)}$	2.1354
$T_{1/2(\beta)}$	297.15
K_{12} (1/h)	2.067
K_{21} (1/h)	0.3539
K_{13} (1/h)	7.534
K_{31} (1/h)	0.0018
K_{10} (1/h)	30.6792
AUC ($(\mu g \bullet g^{-1}) \bullet h$)	724.12
CL (1/h)	0.0373

can be distributed to tissues very fast but eliminated quite slowly, which is further supported by the finding that clearance was quite slow, being 0.0373 h^{-1}. Thus, these results suggest that Cef-MSs are mainly

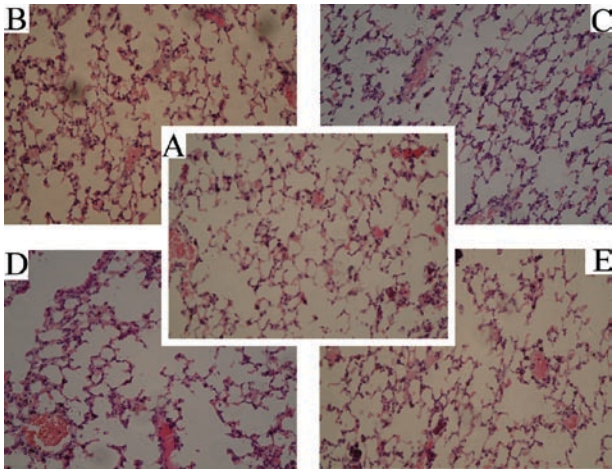

Figure 4. Both experimental group and control group were sacrificed immediately, 5 min, 12 and 48 h after intravenous injection. (A) Mice were sacrificed 12 h after injection of normal saline. No intra-alveolar hemorrhage was presented; airspaces and alveolar septa were normal (400×). (B) Mice were sacrificed immediately after injection of Cef-MS. The morphology was similar to that in (A) (400×). (C) Mice were sacrificed 5 min after injection of Cef-MS. The appearances of airspaces and alveoli were similar to those in (A) and (B) (400×), except that the alveoli were mildly distended. (D) Mice were sacrificed 12 h after injection of Cef-MS. Its overall morphology was intact, while some of the alveolar septa were damaged and several alveoli coalesced to form a large airspace. In addition, the capillaries were congested (400×). (E) Mice were sacrificed 48 h after injection of Cef-MS. Its overall morphology was normal. The alveoli and alveolar septa were normal.

Table 7. Lung-targeting efficiency of Cef-MS.

	Organs						
Parameters	Blood	Heart	Liver	Spleen	Kidney	Stomach	Small Intestine
Te	309.16	135.28	27.50	15.95	52.16	372.80	11.90

distributed in lungs and can be retained in body for long time.

Cef-MS concentrations in tissues were determined and the results showed that microspheres can deliver ceftiofur mainly to lungs after intravenous injection. Its targeting efficiency (Te) was defined as the ratio of area under curve (AUC) of between targeting tissue (lung) and non-targeting tissues. As shown in Table 7, the targeting efficiencies were over 300 for blood and stomach, and at least 12 for small intestine. Moreover, the concentrations of ceftiofur in lung were significantly higher than those in other organs (Figure 3). Thus, these findings indicate that Cef-MS, after intravenous injection, can target the lungs. As mentioned above, we found that the average diameter of the prepared Cef-MS was 21.26. The result that Cef-MS can target the lungs further supported the finding that microspheres with diameter range of 12–44 μm have a notable lung-targeting efficacy[8].

Evaluation of lung injury induced by Cef-MS

Results showed that experimental mice presented with normal alveolar architecture immediately after Cef-MS injection (Figure 4B) and 5 min after Cef-MS injection (Figure 4C), mild distention of alveoli and thinning of alveolar septa at 12 h after Cef-MS injection (Figure 4D), and gradually resolved at 48 h after injection (Figure 4E). In contrast, control group presented normal lung histology with normal sizes of airspaces and alveolar septa (Figure 4A). Except these histopathological findings, mice were normal after injection, with no other adverse effects. Thus, these findings indicate that intravenous injection of Cef-MS only induces mild and reversible lung injury and that it is relatively biologically safe.

As presented in Figure 3, the concentrations of Cef-MS in lung were highest at 0.5 h after injection of Cef-MS and gradually decreased. The highest concentration of Cef-MS and the most marked lung injury did not occur at the same time. However, since lung injury may lag behind mechanical trapping and the induced adverse effects gradually resolved with the degradation of Cef-MS after 12 h, we suggest that the induced lung injury may be due to mechanical trapping of microspheres, which is further supported by Li et al. who revealed minimal cytotoxicity of glutaraldehyde-linked microspheres[11].

Conclusion

Taken together, our study prepared ceftiofur microspheres coated by a biodegradable and biocompatible material—gelatin—for the first time. Compared to its counterpart—ceftiofur—Cef-MS enjoys many advantages in that Cef-MS released ceftiofur more slowly and stably *in vitro* and can achieve a high and effective therapeutic concentration in lung after single intravenous administration. Moreover, we found that Cef-MSs induced only mild and reversible lung injury, thus were biologically safe *in vivo*. Therefore, Cef-MS is a promising alternative form with highly lung-targeting properties for respiratory infections and we suggest that Cef-MS can be further investigated for clinical use.

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Declaration of interest

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