Butyl stearate prolongs the drug release period of isoperidone-loaded poly (lactic-co-glycolic acid) microspheres: In vitro and in vivo investigation

FENGYING SUN, CHANGHUI YU, XIMING LIU, DAN WANG, NA LIU, JIAJIN LIU, LESHENG TENG and YOUXIN LI

The Center for Pharmaceutical Research, School of Life Sciences, Jilin University, Changchun, Jilin 130012, P.R. China

Received April 11, 2018; Accepted November 28, 2018

DOI: 10.3892/mmr.2018.9797

Abstract. The present study aimed to investigate the effects of butyl stearate on t-butoxyl paliperidone derivative (isoperidone)-loaded poly(lactide-co-glycolide) (PLGA) microspheres. The mechanism of drug release rate delay by butyl stearate was examined by accelerated testing, morphological observation, thermal and fluorescence analyses. In vivo pharmacokinetic study was conducted on female beagle dogs. Spherical microspheres with smooth surfaces, small internal pores and shell structures were initially prepared. It was found that 3% (w/w) butyl stearate prolonged the in vitro drug release period from 46 to 82 days, and in vivo release period from 20 to 27 days. Furthermore, the results demonstrated that the green fluorescence imaging of isoperidone approaching the cores of microspheres with 3% butyl stearate was brighter than in microspheres without additives. In conclusion, it was shown that butyl stearate affected the microsphere structure, isoperidone microsphere distribution and isoperidone crystallinity. The results of the present study thus provide a potential method to develop sustained-release preparations.

Introduction

Biodegradable polymer-based microspheres encapsulating hydrophobic compounds have been widely studied as potential long-acting drug delivery systems (1-3). Typically, extended and zero-order release are essential requirements for the treatment of schizophrenia to reduce the dose frequency and improve patient compliance (4,5). Polylactic acid (PLA)- or polyglycolic acid (PGA)-based dosage forms can release drugs over 1-4 months (6,7). However, the remaining polymers continue to degrade for a long time following the drugs release period. Poly(lactide-co-glycolide) (PLGA) is the most commonly used polymer to encapsulate small molecule drugs as it is both biodegradable and biocompatible (8,9).

A longer period of drug release is currently achieved by regulating the molecular weight and end-capping of PLGA, adjusting the parameters of its preparation method, particle size distribution, coating, decreasing the drug loading (DL) and through additives (10,11). Acid catalysis further accelerates the degradation of PLGA. Likewise, fatty acids or esters of fatty acids increase the drug release rate from hydrophobic matrices (12). Kazuhiko et al (13) reported that ethyl and butyl esters of fatty acids enhance the release rate of aclerubicin from poly-β-hydroxybutyric acid microspheres. N atoms of these compounds may form salts with the-COO group of fatty acids. This may result in a certain percentage of fatty acids in microspheres that decrease the drug release rate (14). Fatty acids may occasionally elevate matrix hydrophobicity and slow down the isoniazid release rate from chitosan and gelatin-based microspheres (15). Angadi et al (16) reported that stearic acid-coated interpenetration polymer network blend microspheres of chitosan and gelatin reduce the burst release in the gastric stomach media. The esters of fatty acids are more hydrophobic than fatty acids as they lack the free-COOH group, which may prolong the drug release period of PLGA-based microspheres in other ways (17).

At the end of 2006, Johnson and Johnson launched paliperidone, under the trade name of Invega® for the treatment of schizophrenia (18). However, its poor absolute oral bioavailability (only 28%) and high daily dose may increase the risk of side effects and inhibit its effects. A series of paliperidone derivatives were synthesized in our laboratory. Among these derivatives, t-butoxyl paliperidone hydrochloride (isoperidone; named PD5) displays higher bioavailability and lower toxicity in animal experiments compared with Invega® (19) Isoperidone (Fig. 1) quickly hydrolyzes to paliperidone and takes effect in vivo.

In the present study, it was investigated whether butyl stearate, which is rarely used in microspheres, could prolong the release period of isoperidone from PLGA microspheres (20) and affect the microsphere structures and isoperidone distribution. It was found that a number of pores surrounded by everted matrices appeared on the smooth surface of microspheres with

Correspondence to: Dr Youxin Li, The Center for Pharmaceutical Research, School of Life Sciences, Jilin University, 2699 Qianjin Street, Changchun, Jilin 130012, P.R. China
E-mail: liyouxin@jlu.edu.cn

Key words: poly (lactic-co-glycolic acid) microsphere, isoperidone, schizophrenia, butyl stearate, drug release rate
3% butyl stearate on the first day, compared with microspheres without butyl stearate. However, there were more pores in the core of the microspheres without butyl stearate. In addition, studies of the fluorescence of isoperidone in microspheres have demonstrated that the drug concentration close to the cores of the microspheres with 3% butyl stearate was higher than the microspheres without butyl stearate. Additionally, it was observed that microsphere structures and drug concentration mediated drug release. It was also demonstrated that the crystallinity in the microspheres was altered with 3% butyl stearate. The findings of the present study thus provided a novel strategy to prolong drug release by adding an appropriate amount of butyl stearate to the PLGA microspheres.

Materials and methods

**Materials.** Poly [D,L-lactide-co-glycolide (PLGA)] 7525 7E (lactide/glycolide ratio, 75/25; molecular weight, 113,000 Da; inherent viscosity, 0.74 dL/g) was obtained from Lakeshore Biomaterials, Inc. (Birmingham, AL, USA). Butyl stearate was purchased from TCI Shanghai Development Co., Ltd. (Shanghai, China). Methylene dichloride was purchased from Beijing Chemical Works (Beijing China), and polyvinyl alcohol (PVA; Mr 13,000-23,000) was supplied by Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). Isoperidone was synthesized in our laboratory. Sodium-dodecyl sulphate (SDS) was purchased from Baotaike Bio-Technology Co., Ltd, Shenyang, China.

Healthy female beagle dogs aged 1-2 years and weighing 12±2 kg were supplied by Yadong Laboratory Animal Research Center (Nanjing, China). The animals were kept in iron cages at 20±4°C, with 45-65% relative humidity and a 12 h light/dark cycle. They were fed with granulated feed and had free access to water. All animal experiments were approved by Institutional Animal Care and Use Committee of Jilin University (Changchun, China).

**Preparation of microspheres.** An O/W emulsion solvent evaporation method was used to prepare the microspheres (12). To prepare the oil phase, the isoperidone, additives and PLGA were briefly dissolved in 13.8 ml methylene dichloride (DCM). This was slowly added to 1.7 liters 0.5% (w/v) PVA aqueous solution (pre-saturated with 0.5 ml DCM) for 1.5 min and homogenized at 211 ± 2°C for another 3.5 min at 20±0.5°C. The mixture was stirred at 20±0.5°C for 4 h until the microspheres hardened. The microspheres were then screened for sizes of 25-154 µm using sieves, washed three times with 1 liter ddH2O and lyophilized with 10% (w/w) mannitol as lyoprotectant. The formulas of the isoperidone microspheres are presented in Table I.

**Morphological characterization by scanning electron microscopy (SEM).** Prior to imaging, dried microspheres were mounted onto metal stubs. Conductive double-sided adhesive tape coated it with a thin layer of gold under a vacuum was used and analyzed with an accelerating voltage of 20 kV. The microspheres were frozen in liquid nitrogen for 30 min and cut with a knife blade. For the fluorescence study, an epoxy resin embedded cutting method was used to acquire sections (epoxy resin:curating agent, 6:1 v/v) of F1 and F4. Microspheres (20 mg) were embedded in a 200 µl medium at 40°C for 2 h and cut with a knife blade. The surfaces of both the microspheres and sections were observed by scanning with an electron microscope at an original magnification of x1,000 for F1 and x750 for F4 (JXA-840; JEOL, Tokyo, Japan).

**Particle sizing.** A LS13 320 laser particle size analyzer (Beckman-Coulter, Inc., Brea, CA, USA) was used to determine the mean particle diameter and particle size distribution. The particle size was expressed as the volume-weighted mean particle diameter (µm).

**Drug loading and encapsulation efficiency.** To determine the encapsulation efficiency of isoperidone in the microspheres, 2 mg of the lyophilized microspheres were dissolved in 1 ml acetonitrile. Next, the resulting solution was diluted in methanol:ultrapure water (80:20, v/v) to 20 ml, filtered through a 0.45 µm membrane filter (Thermo Fisher Scientific, Inc., Waltham, MA, USA) and the isoperidone content was analyzed by high-pressure liquid chromatography (HPLC). The HPLC system consisted of a Waters 1525 binary pump and a Waters 2487 Dual Absorbance Detector (Waters Corporation, Milford, MA, USA) set at 280 nm. An Agilent Extend C18 column (4.6x250 mm, 5 µm: Agilent Technologies, Inc., Santa Clara, CA, USA) was used for drug separation. A mixture of methanol:water:triethylamine [80:19.5:0.5 (v/v/v)], adjusted to pH 10.22 with acetic acid, was used as the mobile phase, as previously described (21). The flow rate was set at 1 ml/min. Chromatography was performed at 25°C with an injection volume of 20 µl. The drug loading percentage and encapsulation efficiency were calculated as follows: Encapsulation efficiency (%)=(drug loading determined by HPLC/theoretical drug loading) x100 (1).

**In vitro drug release studies.** In vitro drug release was measured in PBS (pH 7.4) containing 0.02% (w/v) sodium azide and 0.1% (w/w) SDS. Approximately 1 mg microspheres were suspended in 35 ml PBS in a 50 ml centrifuge tube. The tube was shaken horizontally at 100 rpm in a shaking bath, which was maintained at 37±0.5°C. Following centrifugation at 844 x g for 5 min at 25°C, 20 ml PBS was removed from the tube at 4 h, and on days 1, 2, 4-42, 46, 50-82. The medium removed from the tubes was replaced with an equal amount of fresh PBS. The collected samples were filtered through the 0.45 µm filter and subjected to further HPLC analysis, as described above.
Table I. Formulas and characteristics of isoperidone microspheres (mean ± standard error or the mean, n=3).

<table>
<thead>
<tr>
<th>Formula code</th>
<th>PLGA</th>
<th>Theoretical DL [% (w/w)]</th>
<th>Actual DL [% (w/w)]</th>
<th>Encapsulation efficiency [% (w/w)]</th>
<th>Mean particle diameter (µm)</th>
<th>Span value</th>
<th>Additives (w/w)</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1</td>
<td>7525 7E</td>
<td>30</td>
<td>27.92±0.19</td>
<td>93.07±0.64</td>
<td>80.35±24.38</td>
<td>0.82</td>
<td>-</td>
</tr>
<tr>
<td>F2</td>
<td>7525 7E</td>
<td>30</td>
<td>26.37±0.57</td>
<td>87.88±1.89</td>
<td>85.65±25.82</td>
<td>0.86</td>
<td>1% butyl stearate</td>
</tr>
<tr>
<td>F3</td>
<td>7525 7E</td>
<td>30</td>
<td>27.11±0.03</td>
<td>90.36±0.12</td>
<td>88.35±26.53</td>
<td>0.89</td>
<td>2% butyl stearate</td>
</tr>
<tr>
<td>F4</td>
<td>7525 7E</td>
<td>30</td>
<td>26.37±0.81</td>
<td>87.91±2.70</td>
<td>85.21±25.73</td>
<td>0.86</td>
<td>3% butyl stearate</td>
</tr>
<tr>
<td>F5</td>
<td>7525 7E</td>
<td>30</td>
<td>27.04±0.61</td>
<td>90.14±2.03</td>
<td>85.14±26.06</td>
<td>0.88</td>
<td>4% butyl stearate</td>
</tr>
<tr>
<td>F6</td>
<td>7525 7E</td>
<td>30</td>
<td>26.72±1.97</td>
<td>89.07±6.57</td>
<td>86.8±27.38</td>
<td>0.84</td>
<td>5% butyl stearate</td>
</tr>
<tr>
<td>F7</td>
<td>7525 7E</td>
<td>30</td>
<td>26.31±0.43</td>
<td>87.71±1.43</td>
<td>87.18±25.53</td>
<td>0.88</td>
<td>3% stearic acid</td>
</tr>
<tr>
<td>F8</td>
<td>7525 7E</td>
<td>30</td>
<td>25.09±2.87</td>
<td>83.63±9.56</td>
<td>89.97±27.48</td>
<td>0.97</td>
<td>5% stearic acid</td>
</tr>
<tr>
<td>F9</td>
<td>7525 7E</td>
<td>40</td>
<td>38.89±1.64</td>
<td>97.23±4.11</td>
<td>85.76±25.46</td>
<td>0.89</td>
<td>-</td>
</tr>
<tr>
<td>F10</td>
<td>7525 7E</td>
<td>40</td>
<td>40.34±1.52</td>
<td>100.85±3.81</td>
<td>87.32±29.63</td>
<td>0.91</td>
<td>3% butyl stearate</td>
</tr>
<tr>
<td>F11</td>
<td>7525 7E</td>
<td>50</td>
<td>49.13±2.63</td>
<td>98.26±5.25</td>
<td>89.74±25.14</td>
<td>0.95</td>
<td>-</td>
</tr>
<tr>
<td>F12</td>
<td>7525 7E</td>
<td>50</td>
<td>47.9±2.02</td>
<td>95.8±4.03</td>
<td>85.55±22.47</td>
<td>0.87</td>
<td>3% butyl stearate</td>
</tr>
</tbody>
</table>

*Index of polydispersity. DL, drug loading.

To examine accelerated drug release, the temperature of the shaking bath was maintained at 45±0.5°C. The sampling times were 4 h, and days 1, 2, 3, 4-15. The same steps as in the 37±0.5°C in vitro drug release studies were followed. The experiment was repeated for surface observation of microspheres using SEM at an original magnification of x1,000 for F1 and x750 for F4 at 1 and 7 days.

**Isoperidone fluorescence in microspheres.** Laser-scanning confocal microscopy (LSM 710, Germany) was used to monitor the distribution of green fluorescence of isoperidone using SEM at an original magnification of x1,000 for F1 and x750 for F4 at 1 and 7 days.

**Differential scanning calorimetry (DSC) studies of microspheres.** Microspheres of 30, 40 or 50% theoretical DL with 3% (w/w) butyl stearate or without, were scanned on a DSC (Mettler-Toledo GmbH, Greifensee, Switzerland). Microspheres of 30% theoretical DL with 3% steearic acid (w/w) were also scanned as a control. Scans were carried out at 10°C/min over the temperature range of ‑50 to 200°C. The rate of nitrogen was set at 50 ml/min.

**In vivo pharmacokinetic evaluation.** Pharmacokinetic studies of F1 and F4 were performed on female beagle dogs weighing 12±2 kg (n=3). For each dog, the microspheres were suspended in a 5 ml aqueous suspension in a penicillin bottle, consisting of 0.9% (w/w) 5-carboxymethyl cellulose sodium (CMC-Na), 0.127% (w/w) sodium phosphate monobasic dihydrate, 0.1% (w/w) anhydro citric acid, 0.8% (w/w) sodium chloride and 0.086% (w/w) sodium hydroxide. The resulting suspension was drawn into a 5 ml syringe and injected slowly and deeply into the hip muscle of each dog. Blood samples were collected into heparinized tubes before (0 h), at 4 h after, and each day for 40 days after the injection. They were separated by centrifugation at 2,110 x g for 5 min at 4°C. Plasma samples were collected and stored at -80°C until analysis.

Dosage of isoperidone was calculated as: 6 mg/426.5 x (526.5/70 kg) x 0.28x1.87x30=1.66 mg/kg (2), where 6 mg is the dose of the paliperidone tablets for humans, 426.5 is the molecular weight of paliperidone, 526.5 is the molecular weight of isoperidone, 70 kg is the body weight of an adult, 0.28 is the oral bioavailability of paliperidone, 1.87 is the dosage conversion factor, and 30 is the number of days.

A liquid extraction method was used to process the plasma prior to analysis by HPLC-MS/MS (Thermo TSQ Quantum Access triple-quadrupole mass spectrometer; Thermo Fisher Scientific, Inc.), which was equipped with a positive ion electrospray ionization (ESI), and an Thermo Scientific™ Excalibur™ workstation (version 1.4 Surr1; Thermo Fisher Scientific, Inc.). A mixture of n-hexane:methylene dichloride:isopropanol (2:10:1, v/v/v) was used to extract the solvent. The internal standard was a clozapine solution (20 ng/ml). The clozapine solution (10 µl) and sodium hydroxide solution (25 µl; 0.1 mol/l) was added to 50 µl plasma. The mixture was extracted with 3 ml extracting solvent. Following vortexing for 3 min, the system was centrifuged at 492 x g for 10 min at 4°C. The supernatant was separated, dried by flowing nitrogen at 35°C, redissolved in 100 µl mobile phase [acetonitrile:water, 60:40 (v/v), containing 0.4 mmol/l ammonium acetate and 0.01% (w/w) acetic acid] and analyzed. Quantification was performed in multiple-reaction monitoring mode of the transitions m/z 427.31→207.18 for paliperidone. The nebulizer pressure was 50 psi and the flow rate was 0.2 ml/min.

**Statistical analysis.** SPSS 13.0 statistical software (SPSS Inc., Chicago, IL, USA) was used to perform the statistical analyses. Student’s t-test was used to compared differences between two groups. Each experiment was repeated three times. Data were presented as the mean ± standard error of the mean. P<0.05 was considered to indicate a statistically significant difference.
Results

Microsphere characterization. Table I shows the actual drug loading, encapsulation efficiency, mean particle diameter and particle size distribution of the microspheres prepared with different additives. The drug encapsulation efficiencies were between 83.63±9.56 and 100.85±3.81%, indicating that they were not significantly affected by butyl stearate and stearic acid. The mean particle size of the microspheres ranged from 80.35 to 89.97 µm and the particle size distribution was narrow.

Morphological characterization. Fig. 2 shows the spherical morphology of the microspheres. The SEM results of the accelerated testing (Fig. 2A) revealed that on day 1 a number of pores surrounded by everted matrices appeared on smooth surfaces of microspheres F4. However, fewer pores were observed in the core of microspheres with 3% butyl stearate (Fig. 2B). This phenomenon was not observed in microspheres F1. Along with the degradation of PLGA, the microspheres broke down into pieces on day 7. The pieces of F4 aggregated and became more compact, compared with F1. When the microspheres were cut after being frozen in liquid nitrogen, shell structures were
observed for both F1 and F4 (Fig. 2B). While the pores were closed in the shell for F4, they were inside for F1.

In vitro drug release rate. Fig. 3A shows the release profiles of isoperidone of F1, F2, F3, F4, F5 and F6. The profiles of all of the microspheres could all be divided into three parts, excluding F4: i) 0-8 day lag phases; ii) 8-14 day phases; and iii) 16 day quick release phases and subsequent slow release phases. Isoperidone was released from F4 with near-zero-order release kinetics. When the additional content of butyl stearate was relatively low, such as 1 or 2%, the periods of quick release phase were reduced, while the total release periods were only slightly altered. The release periods were prolonged by increasing the butyl stearate content from 1 to 3%. However, the rate of drug release increased when the butyl stearate content was above 3%. This was especially the case for F6 with 5% butyl stearate, whose quick release phase was faster than F1. The in vitro release period for the F7 and F8 formulas was greatest at 3 weeks due to the fast release period from 8 to 16 days (Fig. 3B).

In the accelerated testing, the periods of drug release decreased to 15 days (Fig. 3C). Butyl stearate still decreased the drug release rate and F5 had the longest drug release period compared with F3, F4 and F6. These results suggested that butyl stearate could increase the thermodynamic stability of the microspheres, which has been previously reported (22).

DSC studies of microspheres. Three types of peaks were observed in the thermograms: Butyl stearate melting peaks, PLGA glass-transition peaks (Tg) and isoperidone crystallization peaks (Fig. 4). The integral of the isoperidone crystallization peak was large when DL was elevated from 30 to 50%, or when 3% butyl stearate was added. The addition of 3% stearic acid did not increase the area of the isoperidone crystallization peaks of microspheres with 30% DL.

Studies on fluorescence of isoperidone in microspheres. Isoperidone emitted green fluorescence when it was excited at 488 nm due to similar structures to benzisoxazole (23). The laser-scanning confocal microscope recorded the green fluorescence intensity of each scanning layer. The green fluorescence emission intensity was relatively weak and absorbed by PLGA. This led to a darkening of the fluorescence by the scanning depth in microspheres (F1; Fig. 5A). From 12 to 44 µm scanning depth, the center of the circular scanning surface of F4 was brighter than the surrounding part, which darkened during the scanning process (Fig. 5B). Fig. 5C shows the fluorescence of sections from F1 and F4, indicating that the drug content close to the cores was higher in F4, compared with F1.

In vivo pharmacokinetic evaluation. A very low initial burst release was observed for F1 and F4 (Fig. 6). Following the initial burst release, the drug release became very slow with the lag phase lasting for approximately 4 days. The fast release phase lasted from 4 to 22 days for F1 and from 4 to 26 days for F4. On days 16 and 20, there was F4 release was significantly higher, compared with F1 (P<0.05). The results were consistent with the in vitro drug release results, and implied that 3% butyl stearate may have produced more sustained release microspheres.

Discussion

In the DSC study, drug crystallinity increased by adding butyl stearate. The actual DL of F1 and F4 was very close, at 27.92
and 26.37%, respectively. This suggested that the drug content in the different microsphere sections was different between the two formulas. To verify this inference, the fluorescence of isoperidone at different depths of the microspheres was examined. The results suggested that butyl stearate induced isoperidone to approach the core of the microspheres.

For the O/W emulsion technique, methylene dichloride was removed from emulsions by dissolving it in the continuous phase and evaporating it to a gas phase (24-26). In the present study, a large volume of water phase (100 times more than the oil phase) was used to prepare the microspheres, which were pre-saturated with methylene dichloride. The extraction of the methylene dichloride into the continuous phase and the final hardening of the droplet surface were delayed, allowing the particles to shrink (27). For F4, the oil phase was more hydrophobic due to the addition of 3% butyl stearate and the speed of the overall

Figure 4. Differential scanning calorimetry of microspheres. (A) Alterations in isoperidone crystallinity of microspheres with different additives, as well as (B) microspheres with or without 3% stearic acid with 40% or (C) 50% theoretical DL. DL, drug loading.
solvent removal from the particles was further delayed. Polymer shells formed around the droplets for both F1 and F4. The shells of the latter kept for a longer time and the particles shrunk more slowly (26, 28). Under these conditions, the cores of the droplets may have been rich in methylene dichloride and dissolved in more isoperidone. The residual methylene dichloride in microspheres diffused across the shell, then the small part of the shells dissolved slowly. Water may have easily invaded, resulting in some porous channels adjacent to the shell. The pores could be filled by a release medium in vitro, as a result, the inside of the polymer may have degraded faster than the shells and the medium could break polymer shells from the inside. The results also implied that the porous channels accelerated the in vitro drug release rate of the quick release phase when the content of butyl stearate was 5% (F6). As the remaining methylene dichloride diffused into the phase gradually, the isoperidone close to the core of the particles may have exceeded its saturated concentration in PLGA and precipitated, leading to an increase in drug crystallinity.

While the diffusion controlled release, particle size and DL of F1 and F4 were similar, the water uptake rate of PLGA, diffusion paths, particle structure and drug state affected the drug release rate. The compact shells around the particles were the main barrier preventing the water from diffusing into the particles during the lag phase. When the PLGA matrix absorbed more water, more dissolved isoperidone passed to the particle surface through either the polymer matrix or water-filled pores. F4 showed a reduced release rate due to the longer diffusion path and through the necessity to be first dissolved from the crystalline state (29, 30). When the content of butyl stearate was >3%, the drug release rate in the quick release phase increased. This implied that the porosity of the matrix close to the shell of the microspheres could increase. A higher porosity could thus allow the release medium to penetrate the particles more easily and facilitate faster drug release by pore diffusion. This long-acting isoperidone-loaded microspheres may provide a valuable alternative for the treatment of schizophrenia.

Acknowledgements

The authors wish to thank Dr Wenyan Wang from Yantai University for her work in determining the drug plasma concentration of the samples from an in vivo pharmacokinetic evaluation.

Funding

This study was supported by the State Key Laboratory of Long-Acting and Targeting Drug Delivery System and a Jilin Province Science and Technology Development Program (grant no. 20140311072YY).
Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

CY and DW prepared and characterized the isoperidone PLGA microspheres. NL and XL performed in vitro drug release studies. FS performed in vivo pharmacokinetic evaluation, and was a major contributor in writing the manuscript. LT and JL made substantial contributions in data analysis and revised the manuscript critically for important intellectual content. YL designed the present study and critically revised the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All animal experiments were approved by Institutional Animal Care and Use Committee of Jilin University (Changchun, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References


This work is licensed under a Creative Commons Attribution-NonCommercial-NoDerivatives 4.0 International (CC BY-NC-ND 4.0) License.