Development of brucine-loaded microsphere/thermally responsive hydrogel combination system for intra-articular administration

Zhi-peng Chen a,1, Wen Liu b,1, Dan Liu c, Yan-yu Xiao c, Hong-xuan Chen b, Jun Chen a, Weidong Li a, Hao Cai a, Wei Li b, Bao-chang Cai a,⁎, Jinhuo Pan a,⁎⁎

a Department of Pharmacy, Nanjing University of Traditional Chinese Medicine, Nanjing 210046, PR China
b Departments of Pharmacy, Henan University, Kaifeng 475001, PR China
c Pharmaceutical Research Institute, China Pharmaceutical University, Nanjing 210009, PR China

⁎ Corresponding author. Tel./fax: +86 25 86798281.
⁎⁎ Corresponding author. Tel./fax: +86 25 83811050.
E-mail addresses: czpqiu2008@hotmail.com (B. Cai), panjinhuo@163.com (J. Pan).
1 Zhi-peng Chen and Wen Liu contributed equally to the project and are considered co-first authors.

1. Introduction

Osteoarthritis and rheumatoid arthritis (OA and RA) are rheumatic diseases for which a curative treatment does not currently exist. Millions of people suffer from rheumatic diseases and lose their ability of life. At present, the principle for the treatment of RA and OA is mainly symptomatic treatment, non steroidal analgesic anti-inflammatory drugs (NSAIDs) have been used extensively for treatment of arthritic diseases owing to their quick onset of analgesic effects and mild anti-inflammatory properties despite their side effect as ulceration of the gastrointestinal tract [1].

The joint cavity constitutes a discrete anatomical compartment which allows the possibility of intra-articular injection [2]. This kind of drug delivery system was able to send the drug directly to the target area in a localized manner, avoiding the physiological barriers of transportation and changing its distribution in the human body [3], which can effectively reduce the dose of drug hence minimize the toxicity. Therefore, this delivery mode attracted more and more attention. However, most intra-articular injection was by solution, it has the disadvantage of short retention time and needs frequent injection. Therefore, the prolonged action preparations for intra-articular injection were investigated in the past few years [4–6].

It is well known that chitosan is a natural parvus polysaccharide, and possess many attractive features such as biocompatibility, biodegradability, non-toxicity, and physiological inertness, remarkable affinity to
proteins, bacterial resistance, and hemostaticity [7]. Owing to its excellent biocompatibility, chitosan has been widely used to form microsphere preparations. It has been reported that microsphere has been applied in intra-articular injection. But it had been also reported that significant initial burst release was observed and phagocytes may clear microspheres within a relatively short time period after intra-articular administration [8].

In recent years, some studies reported that chitosan can form thermosensitive hydrogel when together used with β-sodium glycerophosphate (β-GP) [9–11] or mixed with glycerol and saturated borax solution [12]. The hydrogel can respond to temperature, their initial liquid formulation can flow easily and fill any target shape. The formulation can be stored at room temperature or below before the administration. And achieves gelation when the temperature increases to 37 °C (normal body temperature). Hydrogel is filled with water which is much similar to organization. And its soft and wetting surface have good affinity with organization and reduced stimulatory to surrounding tissues, thus hydrogel has good biocompatibility. Hydrogel has porous network, has many apertures that can load microspheres. In order to retard the burst release from the microsphere, we incorporated them into a chitosan thermally responsive hydrogel. Therefore, the resulting drug delivery system can efficiently deliver entrapped drug during an extended period of time.

Strychnos nux-vomica L. has been applied clinically in traditional Chinese Medicine for a long history due to its effect of activating meridians to stop pain and subside swelling, especially for treating diseases like RA and OA [13]. It has been reported that brucine is the main active component of S. nux-vomica L. Brucine could compete with NO for its effect of suppressing cartilage cells enhancement, hence effectively boost cartilage cells regeneration and repair damage cartilage resulted by OA. Though brucine has great effect in treating arthritic diseases, unfortunately, the potential use of brucine is severely limited because of its violent toxicity. It has good liposolubility, could easily permeate the blood–brain barrier and if distributed in the brain can manifest severe toxicity to the central nerve system [14]. Until now, brucine has not been used clinically or being researched in clinical trials. Therefore, there is an urgent need to reduce its side effects.

In this study we prepared the microsphere/thermally responsive hydrogel combination system in order to overcome the problem of burst release and for the aim of prolonged release. Our composite hydrogel’s initial liquid formulation could flow easily under the 37 °C and after administration it could quickly turn into hydrogel form, drug releases slowly both in the form of diffusion and degradation of chitosan, it could alter the distribution of the drug, increase the concentration in the joint cavity and decrease their distribution in other tissues, reduce its central nerve toxicity, and retard the retention of the drug in target area.

It has been reported that microsphere/hydrogel composite systems were mainly used for the delivery of protein or polypeptides [15–17]. However, in clinical the treatment of OA and RA are mostly small molecular drugs, such as analgesics, NSAIDs and steroidal. Depending on their chemical structure, they are rapidly cleared from the joint, thus requiring numerous injections, which could cause infection or joint disability. There are many reports about the sustained release system for intra-articular administration. For example, it has been reported that Celecoxib was loaded in chitosan microspheres for intra-articular administration. But the significant initial burst release was observed and phagocytes may clear microspheres within a relatively short time period after intra-articular administration. Another example, Diclofenac was incorporated into albumin and poly (lactide-co-glycolide) microspheres. It also existed significant initial burst release (in vitro) and limited sustained release effect in vivo [18,19]. In order to solve the problem of burst release and obtain sustained release effect of the intra-articular drug delivery system, it is necessary to construct composite drug delivery system for a small molecular drug.

In this article we firstly loaded a small molecular drug into the system, and the feasibility was studied. And we firstly fabricated the thermosensitive composite hydrogel using chitosan as a temperature-sensitive material for intra articular administration. We also firstly adopted the technology of small animal in vivo FX imaging system for intra-articular injection from which we could visually observe the release capacity of our preparation. And the drug release, pharmacodynamics, biocompatibility and other aspects of this drug delivery system were evaluated for the feasibility of intra-articular injection which provided a reference for the application of a small molecular drug.

2. Methods and materials

2.1. Materials

Chitosan (M 600000, deacetylation degree 96.2%) was purchased from Zhejiang Aoxing Biotechnology Co. Ltd. Brucine (anhydrous, 98%) was obtained from Sigma–Aldrich. Borax, glycerol, acetic acid, glutaraldehyde, hydrochloric acid were of analytical grade.

2.2. Preparations of chitosan microspheres loaded with brucine

Chitosan microspheres have been prepared using a spray-drying method. In brief, chitosan (0.75%, w/v) was dissolved into 1% CH3COOH solution (v/v), glutaraldehyde (1% v/v) was added to a ratio of 1:20 (glutaraldehyde: chitosan = 1:20, v/v) under stirring condition. Stirring for 1 h, the microspheres were prepared by a spray drying apparatus with the condition of Q-flow at 40 m3/h, exit temperature at 120 °C, pump speed of 15 ml·min–1. The brucine-loaded microsphere (BM) was prepared by adding brucine into the chitosan solution (w/w, 1:5). The schematic illustration of the process for preparing composite hydrogel was shown as Fig. 1.

2.3. Drug loading and entrapment efficiency

The BM had been grinding sufficiently in a mortar, 10 mg of the powder was then dissolved in 100 ml 0.1 M HCl and sonicated for 3 h. The supernatant was measured at 264 nm with UV-752 spectrophotometer and equivalent concentration was determined using the calibration curve prepared using the same proportion of solvents. The percentage drug loading (DL) and percentage entrapment efficiency (EE) were calculated using the following formula.

\[
\text{Drug Loading (DL\%)} = \frac{\text{weight of Brucine in microspheres}}{\text{weight of microspheres}} \times 100\% \quad (1)
\]

\[
\text{Theoretical loading (TL\%)} = \frac{\text{weight of Brucine added}}{\text{weight of Brucine + weight of chitosan + weight of glutaraldehyde}} \times 100\%
\]

\[
\text{EE} = 1 - \frac{\text{DL\%}}{\text{TL\%}} \times 100\%
\]

2.4. Preparation of chitosan hydrogel loaded with chitosan microspheres

The temperature-responsive chitosan hydrogel was prepared according to Chen Minyan et al. [12]. Chitosan (2%,w/v) was dissolved in 1%CH3COOH solution, then mixed with glycerol with a volume ratio of 10:1, borax solution was added to adjust the pH to 6.7. Brucine loaded microspheres were rinsed to neutrality and then dispersed in chitosan hydrogel (BMH) (30 mg/ml).
2.5. Scanning electron microscopy (SEM)

Samples of BM were observed by a scanning electron microscope (HITACHI S3000N). They were previously frozen in the refrigerator overnight and lyophilized for 24 h and then coated with platinum by ion sputter gold under vacuum. The sample surface was investigated, and photographs were taken.

2.6. Size distribution

The size distribution of BM was evaluated by a coefficient of variation (CV), which was calculated by Eq. (4):

$$CV = \left( \frac{\bar{D}}{D_{av}} \right)^2 \cdot \frac{1}{N-1} \cdot 100\%$$

Where $D_i$ is the diameter of the $i$th microsphere, $N$ is the total number of the microsphere counted, and $D_{av}$ is the arithmetic average diameter. The diameters of brucine microspheres were obtained by measuring a vision field of more than 100 microspheres.

2.7. Fourier Transform Infrared Spectral (FTIR)

FTIR spectra were used to investigate the possible chemical interactions between the drug and the polymer in the microsphere. Samples were crushed with KBr to get the pellets. The spectra of BM, the physical mixture of empty microspheres and brucine, empty microspheres, brucine and chitosan were recorded.

2.8. X-ray diffraction

Powder X-ray were obtained using a ARL XTRA X-Ray Diffractometer (Switzerland) with a Cu Kα radiation, $\theta$–$2\theta$ powder diffractometer set for an angle range of 3°–45°, The step size was 0.02°, and count times were 0.24 s per step. The analysis was carried out on the BM. As an additional experiment, X-ray determination of brucine, empty microspheres and mixture of brucine and empty microspheres were done.

2.9. In vitro drug release

The release profiles of brucine from BM, BH and BMH were determined. 20 mg of microspheres, 2.5 ml of BH and 2.5 ml of BMH (0.1% brucine) were placed respectively in a 20 ml tube, and then immersed in the matrix with 15 ml of release medium, PBS (pH 7.4, 37 °C) to maintain a sinking condition. The tubes were present in a shaker with a rate of 100 r/min, 5 ml of the medium was taken at predetermined time intervals from the release medium at preset intervals over a period of 96 h and then 5 ml fresh medium was added. Concentration of solution was analyzed by HPLC. The release percentage was calculated according to the following equation:

$$\text{Drug Release} = \frac{M_{\text{release}}}{M_{\text{total}}} \cdot 100\%$$

Where $M_{\text{release}}$ was the amount of brucine released from the formulations into release medium at scheduled intervals, $M_{\text{total}}$ was the total amount of the formulations.

The release behavior of each preparation was evaluated by first-order, Higuchi, Hixon–Crowell, Peppa’s and Double exponential bi-phasic kinetics equation [20] respectively. By comparing the $R^2$ (determination coefficient) and AIC (Akaike’s informulation criterion) the most appropriate equation of each preparation can be found.

2.10. In vivo biocompatibility studies [21]

6 male SD rats which were purchased from Sino-British SIPPR/BK Lab Animal ltd (Shanghai, China) were used to evaluate the potential biocompatibility to the synovium. The rats were housed in a room with controlled temperature and humidity and allowed free access to food and water. 0.1 ml of the BMH (0.1% brucine contained) was injected into the left knee joint, whereas 0.1 ml saline was injected into the right knee joint as control. Three days after the injection, the rats were sacrificed and the joints were isolated. They were fixed in 4% formalin and soaked in HAS decalcifying fluid for 24 h, embedded in paraffin wax. Sections were cut, stained with eosin and hematoxylin for blinded evaluation of inflammatory changes like macrophage proliferation.

2.11. In vivo FX imaging in rats [22]

In order to visually investigate the retention time of drugs in the joint cavity, we adopt the in vivo imaging technology by using Kodak FX pro imaging systems. Firstly we encapsulate infrared dye LF NIRD-15 into microspheres instead of brucine as the method mentioned above, 6 SD rats were anesthetized with urethane (1%, 30 mg/kg) divided in two groups, then respectively injected with fluorescent microspheres and fluorescence microspheres hydrogel 0.1 ml in both knees. Put them in a specimen chamber at predetermined intervals. For ex vivo imaging,
rats were anesthetized by intraperitoneal injection. Manipulate workstation to choose a suitable vision; a grayscale picture was obtained by X-ray lamp as background, 20-cm field of view, 30-s exposure time and 2.50 f-stop. Next, bioluminescence images were acquired at the same field of view, 10 s and 2.50 f-stop. Then the two images were overlapped to show the exact location and intensity of fluorochrome in rats after intra-articular injection along with time.

2.12. Pharmacokinetics in rats [23]

12 SD rats (360 g ± 18 g) were randomly divided into 3 groups, intra-articular administrated with brucine solution (BS), BM and BMH at a dose of 5 mg/kg, respectively. After administration, the rats were anesthetized with ether and a heparinized capillary was then inserted into the eyebound veins to get 0.5 ml blood into a plastic centrifuging tube at the time intervals of 0.5, 1, 1.5, 2, 4, 6, 8, 12, 24, 36 and 48 h respectively. The plasma samples were collected after centrifugation at 4000 rpm for 5 min and then stored immediately at −20 °C until analysis.

2.13. Pharmacodynamics of rabbits [24]

28 male Japanese rabbits (weighing 2.1–3.0 kg), were purchased from Nanjing Qinglongshan breeding ground. 8 rabbits were used to evaluate OA models establishing method, they were divided into two groups, normal group and model group, intra-articular injected with 0.5 ml collagenase (4 mg/ml) and saline into both knee articular of rabbits at 1st and 4th day, respectively. 4 weeks after the 4th day, 1 ml of saline was injected into the joint cavity, and then the synovial fluid was extracted and kept 20 °C before measurement of IL-1β and TNF-α. Both of the two groups were sacrificed and femoral condyles were isolated for observation. After macroscopic examination, samples were fixed with 4% neutral formalin and decalcified with HAS solution for histological evaluation.

The rest of the 20 rabbits were then divided into 5 groups, OA models were established as the method described above. Both knee joint cavities were intra-articular injected with BS, BM, BMH, Hyaluronic acid (HA), and saline 0.5 ml respectively once a week, and lasted for 4 weeks. At the 5th week 1 ml of saline was injected into the joint cavity, and then the synovial fluid was extracted and kept 20 °C before measurement of IL-1β and TNF-α. All of the groups were sacrificed and femoral condyles were isolated for observation. After macroscopic examination, samples were fixed with 4% neutral formalin and decalcified with HAS solution for histological evaluation.

The determination of TNF-α and IL-1β is according to the operation instruction of kit for quantitative determination by Enzyme Linked Immunosorbent Assay (ELISA). Firstly, the collected fluids were centrifuged 1000×g for 20 min to remove granules and polymers. Then follow the test procedure and read O.D value at 450 nm by the ELISA reader in 30 min.

2.14. Statistical analysis

All data were expressed as x ± s. Differences between groups were compared by student t-test and considered to be statistically different if p < 0.05, and considered to be statistically significant different if p < 0.01.

3. Results and discussion

3.1. Surface morphology and size distribution analysis

The microsphere was characterized by SEM. The surface morphology of the drug loaded microsphere was shown in Fig. 2. From the

20 μl strychnine (20 μg/ml) was added into 100 μl plasma as internal standard, alkalinize the plasma with 200 μl 0.1 mol/L NaOH, then extracted by 4 ml of mixed hexane, methylene chloride, isopropyl alcohol (65:30:5, v/v) supernatant was dried with nitrogen, dissolved with 100 μl mobile phase, centrifuged at 12,000 r/min for 10 min, 20 μl of supernatant were assayed by HPLC at a wavelength of 260 nm.

2.13. Statistical analysis

All data were expressed as x ± s. Differences between groups were compared by student t-test and considered to be statistically different if p < 0.05, and considered to be statistically significant different if p < 0.01.

3. Results and discussion

3.1. Surface morphology and size distribution analysis

The microsphere was characterized by SEM. The surface morphology of the drug loaded microsphere was shown in Fig. 2. From the

Fig. 4. The X-ray diffraction spectra of (a) brucine, (b) empty microsphere, (c) mixture of brucine and chitosan, (d) brucine-loaded microsphere.

Fig. 5. Percentage cumulative in vitro release profiles of brucine from BM, BH and BMH.
micrographs, it is clearly seen that the microspheres shows good sphere structure and surface uniformity.

The size distribution of brucine microspheres was evaluated by a coefficient of variation (CV). The diameters of BM were obtained by measuring a vision field of 100 microspheres. The CV was 0.326, indicating uniform size distribution. Moreover, the size distribution was shown in Fig. 3, the size ranges from 0.5 to 4.5 μm, with an average of 2.45 μm, which meets a normal distribution. The EE of brucine microsphere was 98.60±1.1% (n=3).

3.2. FTIR analysis

FTIR spectra of BM, the physical mixture of empty microspheres and brucine, brucine, empty microspheres and chitosan were studied. In the FTIR spectrum of brucine, peaks at 2928 and 2868 cm⁻¹ are related to the C-H bond of saturated carbons. Characteristic-CO stretching at 1653 cm⁻¹, peak at 1500 cm⁻¹ related to aromatic bending of C=C. Peaks at 1287 cm⁻¹ and 1195 cm⁻¹ attributed to stretching vibration of C-O-C. Spectrum of chitosan shows broad band at 3450 cm⁻¹ which corresponds to the absorption of νNH₂ and ν-OH. Peak at 2875 cm⁻¹ related to the ν-CH₃ peak at 1422, 1380 and 1323 cm⁻¹ are bending vibrations of C-H bonds of saturated carbons. The peak at 1074 cm⁻¹ was attributed to the asymmetrical stretching vibration of C-O-C. The analysis of empty microspheres shows similar peaks as that of plain chitosan, except for the one at 1640 cm⁻¹, it corresponds to the new-C=O bond when the –NH₂ of chitosan react with glutaraldehyde. And the absorption at 1527 cm⁻¹ might attribute to the stretching vibration of C=N when –NH₂ and glutaraldehyde formed amide linkage and enol interconversion. In the spectrum, we could see the characteristic 1653 cm⁻¹ and 1500 cm⁻¹ due to the presence of brucine in the blend matrix. In the spectrum of BM the peak at

| Table 1 |
The release model parameters of BM, BH and BMH.

<table>
<thead>
<tr>
<th>Model</th>
<th>Equation</th>
<th>R²</th>
<th>SS</th>
</tr>
</thead>
<tbody>
<tr>
<td>BM First-order</td>
<td>ln(1–Q) = −0.039 t −0.831</td>
<td>0.724</td>
<td>2.618</td>
</tr>
<tr>
<td></td>
<td>Higuchi</td>
<td>0.889</td>
<td>0.429</td>
</tr>
<tr>
<td></td>
<td>Peppas</td>
<td>0.789</td>
<td>0.273</td>
</tr>
<tr>
<td></td>
<td>Double exponential</td>
<td>0.679</td>
<td>0.132</td>
</tr>
<tr>
<td></td>
<td>duplex dynamic</td>
<td>0.873</td>
<td>0.766</td>
</tr>
<tr>
<td>BMH First-order</td>
<td>ln(1–Q) = −0.021 t −0.268</td>
<td>0.932</td>
<td>5.931</td>
</tr>
<tr>
<td></td>
<td>Higuchi</td>
<td>0.902</td>
<td>0.086</td>
</tr>
<tr>
<td></td>
<td>Peppas</td>
<td>0.899</td>
<td>0.355</td>
</tr>
<tr>
<td></td>
<td>Double exponential</td>
<td>0.956</td>
<td>8.640</td>
</tr>
<tr>
<td></td>
<td>duplex dynamic</td>
<td>0.992</td>
<td>1.081</td>
</tr>
<tr>
<td>BMH First-order</td>
<td>ln(1–Q) = −0.009 t + 0.116</td>
<td>0.988</td>
<td>5.942</td>
</tr>
<tr>
<td></td>
<td>Higuchi</td>
<td>0.905</td>
<td>0.044</td>
</tr>
<tr>
<td></td>
<td>Peppas</td>
<td>0.985</td>
<td>1.338</td>
</tr>
<tr>
<td></td>
<td>Double exponential</td>
<td>0.976</td>
<td>0.384</td>
</tr>
<tr>
<td></td>
<td>duplex dynamic</td>
<td>0.983</td>
<td>13.632</td>
</tr>
</tbody>
</table>

Fig. 6. Biocompatibility of saline (a) and BMH (b).

Fig. 7. In vivo FX imaging of rats injected with fluorescence microsphere (a) composite hydrogel (b) after 6 h, 12 h, 24 h, 48 h, 4 d and 7 d, respectively.
1503 cm$^{-1}$ corresponding to the presence of brucine which means brucine is stable in the microspheres.

### 3.3. X-ray diffraction

The X-Ray diffraction of BM, the physical mixture of empty microspheres and brucine, brucine and empty microspheres were shown in Fig. 4. In the X-ray diffraction of brucine, the sharp peaks at a diffraction angle of 20 = 9.560, 11.800 and 13.540 are present which suggest that the drug is in crystalline form. There is no peak in the diffractogram of empty microsphere and three peaks in the diffraction of mixture of empty microsphere and brucine at a diffraction angle of 20 = 9.600, 11.760 and 13.600. The absence of brucine intensity of BM indicated that brucine was changed into amorphous in the molecular level when encapsulated into microspheres.

### 3.4. In vitro release studies

The profiles release of brucine from BM, BH and BMH were depicted in Fig. 5. The brucine release profile from BM were polymorphism which consisted of a burst release followed by a gradual release phase. The burst release of brucine was associated with those drug molecules, dispersing close to the microspheres surfaces or embedded in the surface layer, which diffused out in the initial incubation time. When dispersed in hydrogel, the initial burst release was obviously retarded.

The release profiles of BM, BH and BMH were assessed by first-order, Higuchi, Hixon–Crowell, Peppas and the double exponential duplex dynamic equation respectively.

According to the R$^2$ and AIC, the double exponential duplex dynamic equation was the optimal equation of BM, BH, and BMH as shown in Table 1. With diphasic release including rapid release and late slow release, from the Peppas equation of BM, $InQ = -0.753 + 0.202ln(t)$, $R^2 = 0.930$, the diffusion index (n) was 0.202, when n = 0.45, the release of drug was mainly by means of Fick's diffusion, when 0.45 < n < 0.89, diffusion and erosion were simultaneously the mechanism.

The Peppas equation of BH was $InQ = -2.045 + 0.462ln(t)$ ($R^2 = 0.956$), n = 0.462. The Peppas equation of BMH is $InQ = 0.558ln(t) + 2.689$ ($R^2 = 0.983$), n = 0.558.

The release mechanism of BM, BH, and BMH was diffusion, diffusion and erosion, diffusion and erosion, respectively.

### 3.5. In vivo biocompatibility

The BMH was further investigating for the in vivo biocompatibility with synovium present in the joint cavity of male SD rats. And the results were shown as Fig. 6. Visual observations were made on the knee injected with physiological saline (right knee) and BMH (left knee). The gait was changed after injection for the first 2 h. However, after 2 h, return to normal. Histological examinations of rat synovium taken on the 3rd day showed the absence of inflammation (i.e. proliferation of macrophages) for saline group, and BMH group has a bit of phlogocyte imbibitions near the edge of synovium. And the BMH appeared to be generally biocompatible with synovium and hence it might be suitable for the development of treatment strategies for arthritic diseases.

### 3.6. In vivo FX imaging in rats

Fig. 7 shows a series of views of rats injected with fluorescence microsphere (a) composite hydrogel (b) after 6 h, 12 h, 24 h, 48 h, 4 d and 7 d respectively. From the frames fluorescence signals were found, we could visually observe the retention of fluorescence dye in rats' joint cavity.

From the figure we could see the intention of bioluminescence decreased as time went by in both groups as the signals attenuated and the area of fluorescence diminished. In the microsphere group there's a rapid decline of signals in first 12 h, at 7 d there's no fluorescence observed. In the hydrogel groups, the elimination was much slower than the group of microspheres, and at 7 d there's still signal in the rats' joint, the drug delivery system can approximately prolong the release of drug for over 7 days which was consistent with the results of in vitro drug release.

### 3.7. Pharmacokinetics in rats

Clearance of brucine into the systemic circulation after intra-articular administration in rats is shown in Fig. 8. From the result we could know the BS is rapidly cleared from the joint into systemic circulation because of the rapid equilibration between the synovial fluid and plasma. The clearance of BM is slower than the BS, but at 4 h and 8 h the concentration was higher as the drug entrapped led to a delay. The clearance of BMH was the slowest; the chitosan hydrogel obviously retarded the burst release of BM and minimized the exposure of the cartilage to significantly high concentration of the drug. The blood concentration peak of the three formulations occurred 1 h after intra-articular injection.

Thus, from the results obtained, we could conclude that by dispersing the microspheres into chitosan hydrogel, the clearance of the drug from the joints into systemic circulation was significantly retarded. The systemic adverse effects associated with brucine would therefore be expected to decrease. The parameters of the three groups were shown in Table 2. From the parameters we also could conclude the same conclusion.

### Table 2

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Unit</th>
<th>BS</th>
<th>BM</th>
<th>BMH</th>
</tr>
</thead>
<tbody>
<tr>
<td>$T_{1/2}$ alpha</td>
<td>min</td>
<td>114.93 ± 4.25**</td>
<td>447.04 ± 65.8</td>
<td>206.69 ± 18.69*</td>
</tr>
<tr>
<td>$t_1/2$ beta</td>
<td>min</td>
<td>694.44 ± 21.63**</td>
<td>3926.31 ± 66.85</td>
<td>2780.41 ± 98.25</td>
</tr>
<tr>
<td>$K_{10}$</td>
<td>1/min</td>
<td>0.00315 ± 0.00025*</td>
<td>0.000627 ± 0.000054</td>
<td>0.000346 ± 0.000012</td>
</tr>
<tr>
<td>AUIC$_{0.7}$</td>
<td>(Ng/ml)*/min</td>
<td>273928.78 ± 958.25*</td>
<td>484366.97 ± 584.26</td>
<td>357129.44 ± 449.83</td>
</tr>
<tr>
<td>MRT$_{0.7}$</td>
<td>min</td>
<td>344.17 ± 61.62*</td>
<td>665.34 ± 31.28</td>
<td>1165.83 ± 84.22*</td>
</tr>
<tr>
<td>CL(s)</td>
<td>µg/min/(ng/ml)</td>
<td>0.00467 ± 0.00036*</td>
<td>0.00147 ± 0.00019</td>
<td>0.00209 ± 0.00021</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SD, Compared with BM, **: p<0.05. ***: p<0.001.

*Fig. 8. Blood kinetics studies of BS, BM, BMH after intra-articular injection in rats.*
3.8. Pharmacodynamics

3.8.1. Establishment of OA models

After injected with collagenase the rabbits of model group had a change in position of rest, activity level significantly decreased and slight swelling in the joint compared to the normal group. After being sacrificed, cartilage appeared to be lackluster and its appearance displayed significant cracks and cartilage defects while normal rabbit knee articular cartilage is smooth and lustrous. The synovial fluid of the model group was light red and the volume was increased to a great extent. The synovial fluid of normal groups is a light yellow transparent fluid with viscosity. In the histological image of the normal and model group (Fig. 9), the normal group has an integrity synovial lining, in the model group numerous inflammatory cells were observed and neovessels formed. In summary, by intra-articular injected with collagenase OA model was effectively established.

3.8.2. Gross pathological observation

In the saline group, cartilage remains almost the same as was seen in OA model group, cartilage appeared to be yellow, lackluster and there were significant cracks, the synovial membrane was not integrated. In the treatment groups, these symptoms associated with OA were still seen, but the severity was milder than the saline group. Among all the treatment groups BMH was the best, no significance between BS, HA and BM in gross (figure not shown).

3.8.3. Histological evaluation

After 4 weeks treatment, the rabbits were finally sacrificed and knee joints were isolated, fixed with 4% formalin, decalcification with HAS [25] solution, embedding in solid paraffin, sliced and stained with HE. Then histological images of synovial lining was taken (Fig. 10), in the picture of saline group (a), numerous macrophage-like fibroblasts were present, and synovium epithelium was not complete, and with a bit thickening. In the picture of BS and BMH, the number of fibroblasts was significantly decreased and the integrity of synovium was improved than the saline group. In the picture of HA and BM group, the number of fibroblasts decreased but the integrity of synovium was not improved.

3.8.4. Measurement of IL-1β and TNF-α in synovial fluid

At 5 week time point, synovial fluid was collected from both of the knee before sacrifice. Fig. 11 shows the results of measurements of IL-1β and TNF-α in synovial fluid from each group. After injected with collagenase, the levels of TNF-α and IL-1β were up regulated. They were $45.234 \pm 7.286$ pg/ml and $118.213 \pm 13.507$ pg/ml after being treated with saline for 4 weeks. Differences in both IL-1β and TNF-α were significant between treatment and control groups ($p<0.01$). From the figure we could see that levels of pro-inflammatory cytokine of BS group were lower than HA ($p<0.05$), no difference in TNF-α between the determination of BS, BM and BMH group. In the determination of IL-1β, differences were observed between BS and BM ($p<0.05$), significant difference between BS and BMH ($p<0.01$) and no difference between BM and BMH.

4. Conclusion

In this study a new drug dosage form based on microspheres incorporating in hydrogel was designed to treat arthritic diseases via intra-articular administration. The thermosensitive hydrogel make it hard for drugs to flow away which increased the duration of brucine in local site, hence decreased the required injection frequencies. The composite hydrogel showed the release of brucine for more than 7 days in a controlled manner. Histological studies on the knee joints of SD rats did not show apparent aberration due to the presence of brucine-loaded composite hydrogel. The in vivo FX imaging study showed that the system could maintain in joint cavity for more than 7 days. Pharmaceutical study revealed slow clearance rate than the BS and BM. And the results of pharmacodynamics revealed that the BMH could benefit OA joint by suppressing the levels of TNF-α and IL-1β, protect the damaged joint from degradation. We successfully load brucine, a Chinese medicine active component which is toxic to the central nerve but effective for the treatment of arthritic diseases, into the microsphere/hydrogel composite system to overcome its neurotoxicity, attenuated the toxicity and reinforced the therapeutic effects. In a word, the composite hydrogel prepared in this study appears to be a promising option for the development of effective treatment methodologies of RA and OA through intra-articular administration.

Acknowledgments

The studies were supported by the National Natural Science Funds for Distinguished Young Scholars (No: 8102814) and the Major Program of Nanjing University of Chinese Medicine (No: 10XPY03).
Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.conrel.2012.07.037.

References


