—**Regular Article**—

Evaluation of Daidzein-loaded Chitosan Microspheres *In Vivo* after Intramuscular Injection in Rats

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Daidzein-loaded chitosan microspheres were prepared by emulsification/chemical-crosslinking technique. The dialysis bag method determined the release of daidzein from the microspheres. It demonstrated that the accumulative release curve *in vitro* was fit for the zero-order release equation and had good correlation with the absorptive fraction *in vivo*, suggesting the dialysis bag method evaluated the release of the microspheres well. The release of chitosan determined by the ninhydrin assay *in vitro* was very slow, less than 3 percent at 35 day. The pathological section by hematoxylin-eosin staining found the good biocompatibility of the prepared microspheres in the injective site. Combining the degradation photos by scanning electron microscopy with the plasma concentration-time data, it was speculated that the drug on the surface of the microspheres firstly released, then the major of drug near the surface and the inner of the microspheres released by diffusion through the shallow cavities and crack, lastly the drug released rapidly and completely being companied with the beginning of polymer degradation.

Key words——daidzein; chitosan microsphere; release; biocompatibility; biodegradation

INTRODUCTION

Daidzein is a natural isoflavone found in *Leguminosae* and belongs to the family of diphenolic compounds with structural similarities to natural and synthetic estrogens and anti-estrogens. It has been reported that daidzein exhibits a variety of beneficial effects on human health including chemoprevention of cardiovascular diseases and cancer as well as an alternative for estrogen replacement therapy (ERT) to prevent and treat osteoporosis (in post-menopausal women with bone loss).[1–4] Our previous studies also have demonstrated that daidzein prompts the proliferation of osteoblast cells and the mineralization of MC3T3-C1 cells.[5,6]

In the recent years, daidzein preparations and nutritional supplements containing daidzein are widely consumed for their potential health effects. However, the poor bioavailability of daidzein by oral administration is related to the low solubility and partition coefficient. Otherwise, daidzein shows the apparent permeability coefficient approach to propranolol (a transcellcular marker) across the Caco-2 cell monolayer, but the extensive metabolism in the intestine and liver.[7–12] Therefore, implantable sustained-release drug delivery system is considered to be designed for daidzein *via* the parenteral route. For the carrier, biodegradable polymer chitosan is a potential useful pharmaceutical material owing to its good biocompatibility and low toxicity. It is reported that chitosan, as a long-acting biodegradable carrier, is very suitable for controlled delivery of many drugs.[13–15]

Previously, preparation of daidzein-loaded chitosan microspheres crosslinked by glutaraldehyde has been optimized by a spherical symmetric design-response surface methodology and evaluated for the drug release behaviors *in vitro*, degradation and bioavailability *in vivo*. It shows that the absolute bioavailability greatly increases by intramuscular injection in rats.[16] In this study, the relationship between the drug release *in vitro* and the absorption fraction *in vivo* was investigated. Especially, the release of the carrier chitosan and biocompatibility study offered the further evaluation for the delivery characteristics of daidzein loaded chitosan microspheres, emphasizing on the drug and carrier releasing status *in vivo*.

MATERIALS AND METHODS

Chitosan (M_w: 100000) with 85% deacetylation degree was a present from Ocean University of China.

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The purity of synthesized daidzein (Huike Plants Exploiture Co., Shanxi, China) was 98.79%. Span 80 and ninhydrin were purchased from Fluka Chemical Co. (Switzerland) and Sigma (USA), respectively. TR-FIA kit for daidzein was purchased from Labmaster Co. (Turku, Finland). Glutaraldehyde (25%) was from Beijing Chemical Co. (China). Acetonitrile was HPLC grade (Burdick & Jackson, Honeywell International Inc., MI, USA). All other reagents and solvents were analytical grade.

Preparation of the Microspheres The daidzein loaded chitosan microspheres were prepared using the emulsification/chemical cross-linking technique according to the optimized formulation, which has been described in our previous study.[16] In brief, the aqueous phase was a chitosan solution (5%, w/v) suspended with micronized daidzein particles. The weight ratio of polymer to drug is 1 : 1.2. Firstly, aqueous phase was dropped into the oil phase consisting liquid paraffin containing 2% Span 80 (w/v) to form a W/O emulsion by stirring at 300 rpm for 15 min. Then 25% glutaraldehyde solution was added and stirred for 3 h at room temperature. The cross-linked microspheres were separated and washed by vacuum-induced filtration. The prepared microspheres were dried in a vacuum desiccator at room temperature.

***In Vitro* and *In Vivo* Release** The contents of daidzein in the microspheres were measured by HPLC instrument (Waters, 600 controller, 996 photodiode array detector, 717 plus autosampler, USA). A C_{18} column (250×4.6 mm, 5 μm, Kromasil) was selected to separate and determine the component under the wavelength of 250 nm. The mobile phase consisted of methanol - acetonitrile −1% acetic acid (50 : 10 : 40, v/v) and the flow rate was 0.8 ml/min. An aliquot of 10 μl from the taken samples was injected directly onto the HPLC. The drug release was evaluated by the dialysis bag method. 10 mg microspheres were suspend in 5 ml of PBS (pH 7.4) and placed into a cellulose dialysis bag of which the molecular weight cutoff is 12–14 kDa. The tied dialysis bag was put into 250 ml of PBS (pH 7.4) at 37±0.5℃, and the stirring speed was set at 50 rpm. The medium was replaced by the fresh medium each other 3 days. The measurement of daidzein in samples was determined by HPLC mentioned above.

Blood concentration-time curve of was measured in Wistar rats (Beijing Vital Animal Co., China). All the animal studies were performed in accordance with the experimental animal guidelines of the National institutes for Food and Drug Control, P. R. China for laboratory animal use and care. The determination of daidzein in rat plasma was carried out using the time-resolved fluoroimmunoassay (TR-FIA) kit mentioned in our previous study.[16] Briefly, after samples were treated with hydrolysis and extraction, the values of fluorescence were measured by a time-resolved fluorometry Victor^3 1420 multilabel counter (PerkinElmer, USA) according to the protocol recommended by the manufacturer. The original concentrations of drug were calculated according to the standard curve and dilution factor. Absorption percent of daidzein *in vivo* was calculated by Loo-Riegelman method, a two-compartment model according to the pharmacokinetic parameters after intravenous administration previously.

The chitosan release was measured by the paddle method. Blank chitosan microspheres were placed in the capped tube suspending on the paddle. The medium was phosphate buffer solution (PBS, pH 7.4), the rotational speed was set at 50 rpm per min and the water bath temperature was 37℃. The released samples were withdrawn at 3, 7, 14, 21, 28, 35 d. The ninhydrin assay was used to measure the chitosan release *in vitro*.[17,18] Briefly, 0.5 ml sample was added to 0.5 ml freshly prepared ninhydrin reagent (pH 5.2). Then the solution was placed under the boiling water bath for 30 min and 15 ml ethanol-water (1 : 1, v/v) was added after being cold to room temperature. The absorbent value was measured under 570 nm wavelength. The chitosan concentration in the sample was calculated by standard curve.

***In Vivo* Biocompatibility and Degradation** The *in vivo* biocompatibility study was performed for the daidzein-loaded chitosan microspheres. About 10 mg of the microspheres were suspended in 0.2 ml of CMC-Na solution and intramuscularly injected into the legs of 15 female ICR mice (8 weeks old, 5 groups), which were purchased from Beijing Vital Animal Co. (Beijing, China). The animal studies were also conducted in accordance with the experimental animal guidelines of the National institutes for Food and Drug Control, P. R. China for laboratory animal use and care. The mice were sacrificed at 3, 7, 14, 21 or 35 d to collect the muscle tissue containing microspheres. The samples were used to pathological section examination. Briefly, the muscle tissue was

placed in 4% paraformaldehyde solution under 4°C for overnight, then dehydrated with ethanol and embedded with paraffin. 4 μm thickness slice of the muscle tissue was stained by hematoxylin-eosin. Inflammatory reaction on the section was observed by microscope.

The *in vivo* degradation study of the microspheres was observed with a scanning electron microscope (SEM). Briefly, a few of the recovered microspheres *in vivo* was spread on an aluminium flat and coated with thin gold under vacuum. SEM (Model S-450, Hitachi, Tokyo, Japan) was used to record the size, shape surface characteristics and the internal structure of the microspheres. The photomicrographs were taken for the microspheres recovered at 7, 14, 21 or 35 d after intramuscular injection. The detailed method and photomicrographs have been showed in our previous study.[16] Here, the two reduced images were demonstrated repeatedly in order to show the more area of the microspheres and be convenient to assay the drug release in the following parts.

Data Analysis Data are presented as means±S.D. Differences were considered significant at $p \leq 0.05$ using Student's *t*-test.

RESULTS

The prepared daidzein-loaded microspheres presented symmetrically orbicular shape. The mean medium particle diameter was 72.91 μm and the span was 0.553. HPLC assay showed that the daidzein loading content in the microspheres was 31.04%. The release of daidzein-loaded chitosan microspheres was evaluated by the dialysis bag method. The data were shown in Table 1. The *in vitro* accumulative release percent of daidzein in the microspheres was 6.52% and 93.13% at 3 d and 35 d, respectively. It seemed that the burst effect at the initial stage was not clear by the dialysis bag method, with the following zero-order release characteristics.

TR-FIA is a simple, specific, rapid and sensitive method for the determination of daidzein in plasma with high precision. The intravenous injection of a daidzein solution in rats was studied in our previous study.[16] The plasma concentration-time data of daidzein after intravenous injection in rats were best fitted to a two-compartment model. These data were used to calculate the absorptive fraction *in vivo* of daidzein by the Loo-Riegelman method here. Table 1 shows the result of *in vivo* data after intramuscular injection of the daidzein-loaded chitosan microspheres in rats. The linear regression was analyzed between the absorptive fraction *in vivo* (Y) and the release percent *in vitro* (X). The linear regression equation was $Y=1.096X+0.1397$ and the correlation coefficient was 0.9975. It presented a good relationship between them, suggesting that the release method by dialysis bag *in vitro* perfectly evaluated the release process *in vivo* of the daidzein-loaded chitosan microspheres.

Table 1. *In Vivo* Absorptive Fraction and *In Vitro* Accumulative Release Percent of Daidzein-loaded Chitosan Microspheres ($n=6$)

Time (h)	*in vitro* release (%)	*in vivo* absorption (%)
1	0.26±0.09	0.30±0.11
4	0.94±0.23	0.99±0.35
12	2.35±0.65	2.10±0.68
24	3.32±0.57	3.97±0.70
48	5.42±0.79	6.70±0.53
72	6.52±0.64	8.09±0.94
168	14.80±1.21	14.33±3.51
264	23.70±1.85	22.66±1.97
360	34.78±2.04	38.24±9.36
456	45.79±1.98	55.30±6.12
552	56.88±2.46	62.82±5.67
648	70.00±2.95	80.35±2.32
744	81.51±2.52	90.68±2.12
840	93.13±3.05	97.00±0.39

Chitosan release experiment was analyzed by the ninhydrin reagent. Calibration curves for the concentrations of standard chitosan solutions (Y) *versus* the absorbent value at 570 nm (X) were found to be linear over the concentration range of 0.25–1.25 mg/ml. The linear regression equation of the calibration curve was $Y=1.8612X+0.04623$ and the related coefficient was 0.999. Figure 1 shows the accumulative release curve of chitosan. The release rate was very slow and even lower than 3% at 35 d.

Figure 2 shows the photomicrographs of paraffin section. Comparing with normal muscle tissue before injection (Fig. 2(a)), wetting and infiltration of granulocyte and minor lymphocyte were observed on the site around the injected microspheres (Fig. 2(b)). This slight inflammatory reaction was clear at 3 d and then gradually disappeared (Figs. 2(c) and (d)). Macrophage and vascular proliferation was not found in the sections, presenting that the daidzein loaded chitosan microspheres had good biocompatibility.

After intramuscular injection, the morphology of the chitosan microspheres changed with the existing time *in vivo*. At 3 d and 7 d (the photos were not shown), a majority of drug crystals on the surface of the microspheres released into tissue fluid. And the inner drug gradually dissolved and diffused toward outside. At 21 d (Fig. 3(a)), the microspheres kept in spherical shape but the surface became rough with some shallow cavities and crack, indicating that degradation occurred at the out layer of the microspheres. At 35 d (Fig. 3(b)), the microspheres were degraded into a loose and porous structure and minor part changed into fragments. At this point, most of the drug was released completely through the pore structure.

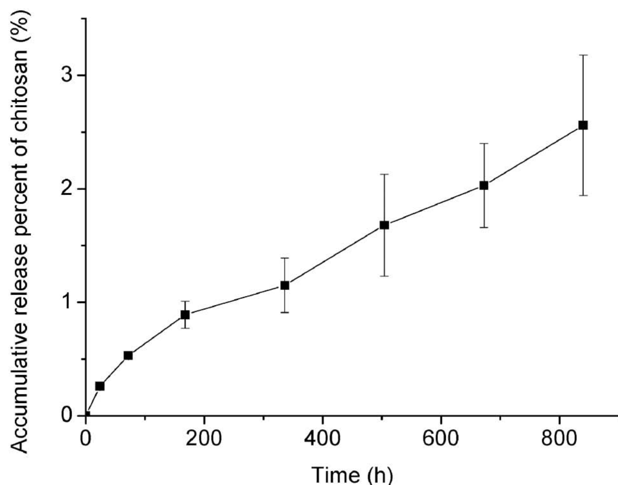

Fig. 1. *In Vitro* Release Profile of Chitosan in the Microspheres by the Ninhydrin Assay ($n=3$)

DISCUSSION

The emulsification/chemical-crosslinking method was used to prepare the daidzein-loaded chitosan microspheres. Daidzein, as a needle crystal, was micronized to decrease the particle size of the drug powder so that much more drug powder was loaded into the emulsion droplets. The result showed that the prepared microspheres showed high drug-loaded percent (up to 31.04%) and good spherical shape.

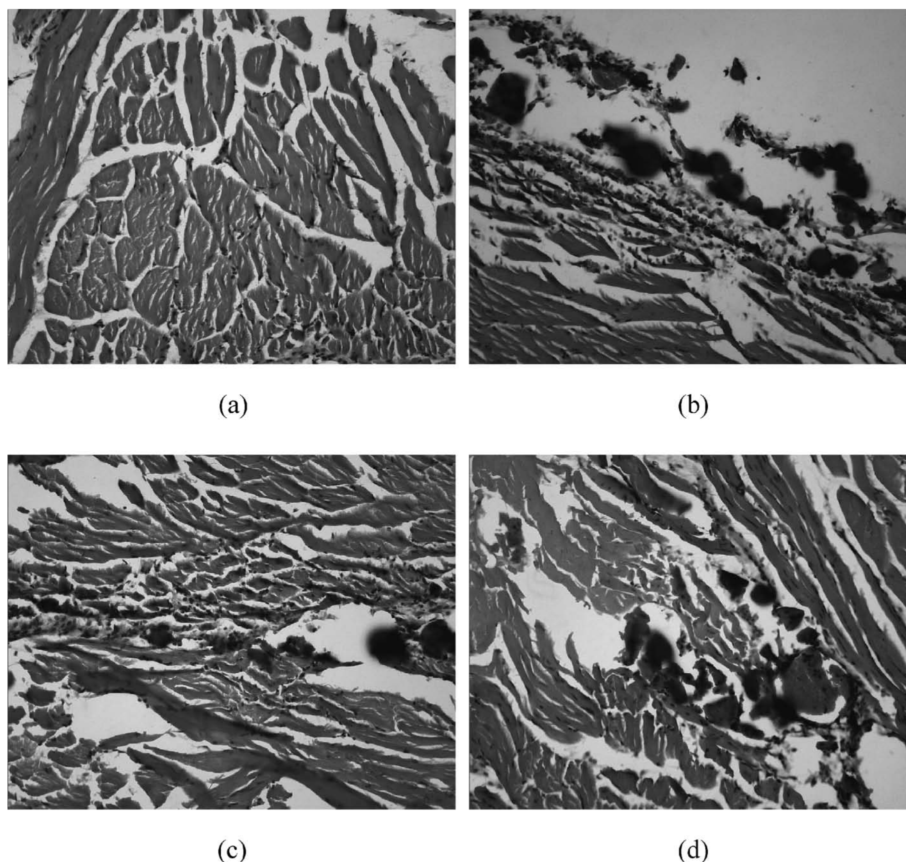

Fig. 2. Photograph of Pathological Section of Muscle before Injection (a) and after Injection with Daidzein-loaded Chitosan Microspheres Retrieved at (b) 3 d, (c) 14 d, (d) 35 d

×200.

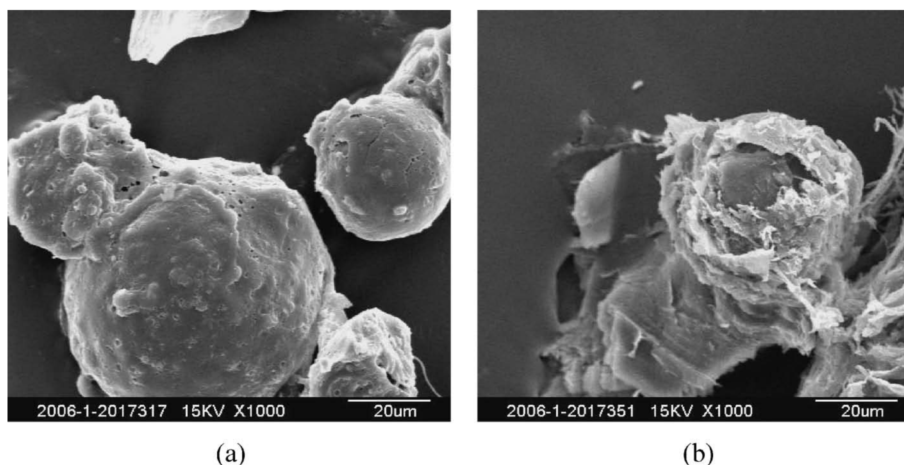

Fig. 3. Scanning Electron Microphotographs of Chitosan Microspheres Retrieved at (a) 21 d and (b) 35 d after Intramuscular Injection in Mice
×1000.

Chitosan is a carrier polymer with good biocompatibility. The degraded products by lysozyme are non-toxic, non-immunogenic and non-carcinogenic. Chitosan microspheres by intramuscular injection may product some inflammation reaction being controlled by preparing methods, crosslinking regents and air purifying condition, *etc*. In the *in vivo* experiments here, the daidzein-loaded microspheres were carried out by the aseptic technique. The pathological section by HE staining (Fig. 3) observed that only the slight inflammation happened on day 3 in the injective site and disappeared in the following days, suggesting good biocompatibility of the prepared microspheres.

Dialysis bag method is often used in the release test, especially being fit for the microspheres administered by intramuscular injection. Drugs loaded in the microspheres release into small volume of tissue fluid (alkaline solution), then diffuse into capillary through muscular tissue. So, sink condition in the gastrointestine tract does not exist here and small volume in the dialysis bag simulate the practical releasing condition in muscles. In our study, the release curve by dialysis bag method was characteristic of zero-order equation and interrelated well to the absorption fraction *in vivo* (the correlation coefficient was 0.9975). Otherwise, there was no apparent initial burst release in the microspheres. This may be attributed to the solubility of daidzein is low in pH 7.4 PBS (the solubility data is 16.22 μg/ml) and the dissolve of daidzein crystals on the surface of the microspheres controlled the release rate.[19,20]

Chitosan (the deacetylation degree is higher than 80%) is often degraded by lysozyme *in vivo*. High compactness of the matrix and crystallized area of the polymer result in the slow degrading rate of chitosan microspheres being crosslinked by glutaraldehyde.[21] SEM photos in Fig. 3 presented that the daidzein-loaded chitosan microspheres kept integrated shape with shallow crack at 21 day and minor of the microspheres partially began to degrade. The slow degrading process was content with the low accumulative release percent of chitosan *in vitro* (Fig. 1). Furthermore, the plasma concentration-time of the drug showed the three peak-valley fluctuations. The absorptive fractions *in vivo* at the three lowest points of 24 h, 72 h, 552 h and the last point of 840 h were 3.99%, 8.09%, 62.82%, 97.00%, respectively (Table 1). Combining the degradation above, these data were speculated that the drug on the surface of the microspheres first released from 0 h to 72 h, then the major of drug near the surface and the inner of the microspheres released by diffusion from 72 h to 552 h, lastly the drug released rapidly and completely after 552 h being companied with the beginning of chitosan degradation.

In conclusion, the daidzein-loaded chitosan microspheres released the drug longtime for 35 days and showed good biocompatibility by intramuscular injection. The accumulative release profile by the dialysis bag method *in vitro* had good relation to the absorptive fraction *in vivo* by the pharmacokinetic calculation. The slight degradation with the slow chitosan release supplied interpretations for the fluctuation on the plasma concentration-time of the drug *in vivo*.

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