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Preparation and pharmacodynamics of low-molecular-weight chitosan nanoparticles containing insulin

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ABSTRACT

Low-molecular-weight chitosan (LMWC) was obtained by enzymatic degradation and ultrafiltration separation. LMWC nanoparticles with LMWC having 20 kDa weight average molecular weight (M_w) were then prepared by solvent evaporation method. The resultant nanoparticles were spherical with a narrow particle size distribution. LMWC nanoparticles loaded with insulin as a model drug were prepared. The average entrapment efficiency of insulin could reach up to 95.54%. The in vitro drug release profiles from the nanoparticles showed an initial burst of release in the first 2 h, followed by zero order release kinetics. In vivo pharmacodynamics of chitosan nanoparticles containing insulin showed that the nanoparticles showed some hypoglycemic activity. Compared with an insulin solution, a relative bioavailability of 0.737 was observed for four times the dosage of insulin in the chitosan nanoparticles after pulmonary administration.

1. Introduction

Chitosan is a polysaccharide containing glucosamine and *N*-acetylglucosamine. It is obtained by the partial deacetylation of chitin, a material found largely in shells of crustaceans. This cationic polymer has attracted a great deal of attention as a drug delivery carrier because of its unique properties, such as acceptable biocompatibility (Campos, Sanchez, & Alonso, 2001), low toxicity (Illum, Jabbal-Gill, Hinchcliffe, Fisher, & Davis, 2001) and the ability to enhance the absorption of hydrophilic molecules across the Caco-2 cell epithelium via the paracellular transport pathway (Schipper et al., 1999). Immunohistological studies have shown that chitosan could open the tight junctions between cells through an effect upon F-actin filaments (Schipper et al., 1997).

Chitosan shows important functional properties in many areas, however, the high molecular weight, high viscosity and insolubility at physiological pH values (7.2∼7.4) of chitosan restricts its uses in vivo (Tommeraas et al., 2002). In addition, the action of chitosan in vivo will be affected because the human gastrointestinal tract does not possess enzymes to directly degrade the β glucosidic linkage in chitosan and consequently the unbroken polymers may be poorly absorbed by the human intestine (Weiner, 1992). Ruth Duncan (Carreno-Gomez & Duncan, 1997) found that a variety of chitosans were cytotoxic, and the extent was dependent upon their molecular weight, degree of deacetylation and salt form. Toxicity of chitosan increased with increasing molecular weight, and chitosan hydrochloride was the most toxic form (50% growth inhibition of cell (IC_{50}) of 210 μg/ml), only fourfold less than that of the cationic reference polymer poly-L-lysine (IC_{50} of 50 μg/ml). Chitosan with much lower molecular weights showed substantially decreased toxicity, and good solubility in physiological pH environment (Azarmi, Roa, & Lobenberg, 2008).

Macromolecular drugs such as peptides, proteins and oligonucleotides are very unstable compounds that need to be protected from degradation in the biological environment. Moreover, their efficacies are strongly limited by their ability to cross biological barriers and reach the target site. The future of these molecules as therapeutic agents clearly depends on the design of an appropriate carrier for their delivery to the body. Nanoparticles can be effective carriers for macromolecular drugs protected from degradation by enzymes and transport through biological barriers. They can be administered via different routes such as parenteral, oral, intraocular, transdermal or pulmonary inhalation. Nanoparticles formed by ionic gelation of the positively charged polysaccharide chitosan with anionic biomacromolecules have been used in genes, vaccines and anticancer agent administration (Illum et al., 2001; Janes, Fresneau, Marazuela, Fabra, & Alonso, 2001). Most of nanoparticles delivery systems focused on the oral adminstration (Damge, Maincent, & Ubrich, 2007; Lin, Mi, & Chen, 2008; Liu, Gong, Fu, et al., 2008). Pulmonary administration of insulin was an alternative non-invasive approach for both local and systemic drug

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delivery. This route has lots of advantages such as a large absorptive area, extensive vasculature, easily permeable membranes and low extracellular and intracellular enzyme activity. Nanoparticle delivery system exhibits some well-defined and delicate characteristics, which have created an attractive and efficient approach for pulmonary delivery of drugs especially for proteins with higher bioavailability, controlled release properties and enzymatic tolerance (Chattopadhyay et al., 2007).

The purposes of the current study were to prepare low-molecular-weight chitosan (LMWC) nanoparticles and evaluate the feasibility of low-molecular-weight chitosan as carriers of macromolecular drug for pulmonary administration. Insulin (INS) was chosen as a model protein drug. The characteristics of INS-loaded LMWC nanoparticles in vitro were evaluated through size distribution, zeta potential, entrapment efficiency and in vitro release. Moreover, the pharmacodynamics after pulmonary administration in vivo of chitosan nanoparticles containing insulin was reported too.

2. Experiments

2.1. Materials

Biomedical grade chitosan (M_w = 450 kDa, deacetylation degree = 95%) was obtained from Yuhuan Marine biochemistry Co., Ltd., China. Chitosanase was purchased from Chemical Industries Co., Ltd., Japan. Polysaccharide (Part Number: 2090-0100; consist of maltotriose units which form a linear macromolecular polysaccharide) with different molecular weight (M_w = 5.9, 11.8, 22.8, 47.3, 112 and 212 kDa) was purchased from Polymer Laboratories Inc. (MA, USA). Insulin was purchased from Xuzhou Wanbang biochemistry Co., Ltd., China. Span 85 was purchased from Shanghai Chemical Reagent Co., Ltd., China, all other chemicals were analytical grade.

2.2. Enzymatic degradation of chitosan and determination of molecular weight

2.2.1. Enzymatic degradation of chitosan

Enzymatic degradation of chitosan was performed as follows. Briefly, a 3% chitosan solution was prepared by dispersing 15 g of chitosan in 500 ml of distilled water, and dissolving it by stirring after adding 6.25 ml of 36.5% (w/v) hydrochloric acid. The temperature for hydrolysis of chitosan was controlled at 50 °C in a batch reactor. The reaction mixture containing 1 U/ml chitosanase was incubated for an appropriate time at this temperature. After enzymatic hydrolysis, the reaction mixture was centrifugated for 10 min at 4000 r/min. The supernatant was filtered by filter with 0.45 μm pole size, and the filtrate was separated using two different molecular weight cut off (NMWCO, 10 and 50 kDa) ultrafiltration membranes (Millipore Labscale TFF system, Millipore Co., USA). Then, the ultrafiltrate was lyophilized.

2.2.2. Determination of molecular weight

The molecular weight of the low-molecular-weight chitosan (LMWC) was determined by gel permeation chromatography (GPC) with a TSK-gel column (G3000SW, 7.5 mm ID × 30 cm) at 25 °C. The weighted lyophilized powder of LMWC was dissolved in ultrapure water and the final concentration was adjusted to 1.0 mg/ml. Then 10 μl of the sample was chromatographed using 0.1 M NaAc (pH 6.0) as the elution buffer with a flow rate of 0.8 ml/min. The LMWC were detected by monitoring the refractive index.

Master samples of polysaccharide with different molecular weight (M_w = 5.9, 11.8, 22.8, 47.3, 112 and 212 kDa) were dissolved in ultrapure water, and their final concentrations were 1 mg/ml. Calibration was performed by means of polysaccharide samples using the integral molecular weight distribution method.

2.3. Preparation of LMWC nanoparticles

LMWC nanoparticles were prepared by solvent evaporation in a W/O (water in oil) emulsion system. Forty-eight milligrams LMWC and 2 mg insulin were completely dissolved in 8 ml 1% (w/v) acetic acid aqueous solution. The solution was then added into a 50 ml Span 85 ethanol solution under mechanical agitate (DC-40, Hangzhou Electrical Engineering Instruments, China) with 400 rpm at 40 °C. After the evaporation of water phase and ethanol under diminished pressure at 50 °C for about 6 h, the droplets of the water phase were transformed into LMWC nanoparticles which suspended in Span 85. The obtained nanoparticles dispersion was then centrifuged (3K30, Sigma Laborzentrifugen, Germany) at 4000 rpm for 15 min and further washed with petroleum ether three times to remove the remaining Span 85. The nanoparticles was then re-suspended in 25 ml pH 7.4 phosphate buffer solution (PBS) with probe-type sonic treatment (400 W, actively every 1 s with a 2 s duration, JY92-II ultrasonicator, Scientz Research Institute, China) in an ice-water bath to form LMWC nanoparticle suspension.

Process parameters were varied as follows. The percent of ethanol in Span 85 ethanol solution (v/v) was varied from 0% to 40%. Times of sonic treatment were 60, 90, 120, 150 and 180 s, respectively. The blank LMWC nanoparticles were prepared using the same procedures.

2.4. Physicochemical properties of LMWC nanoparticles containing insulin

The mean diameter and zeta potential of LMWC nanoparticles containing insulin were determined by a zetasizer (3000HS, Malvern Instruments Ltd., UK). The samples were diluted 20 times with the corresponding dispersed phase. The morphological observation of insulin-loaded LMWC nanoparticles was performed by transmission electron microscopy (TEM) (TECNAI 10, PHILIPS, Dutch). The samples were diluted and natively stained by phosphotungstic acid before TEM observation.

2.5. Determination of drug entrapment efficiency

To determine the drug entrapment efficiency of insulin-loaded LMWC nanoparticles, the re-suspended nanoparticles were separated by ultracentrifugation at 10,000 rpm under 10 °C for 30 min. The amount of free insulin in the supernatant was measured by HPLC using an Agilent G1310A pump (1100 Series) unit control, an Agilent G1314A Variable Wavelength Detector (1100 Series) was set at 214 nm. The mobile phase was a mixture of acetonitrile, NaH_2PO_4, and Na_2SO_4 (adjusted to pH 2.5 by adding phosphate acid) (Pan, Li, Zhao, et al., 2002). The insulin entrapment efficiencies of LMWC nanoparticles were calculated according to the following equation.

$$\text{Entrapment efficiency}(\%) = (1 - C_1/C_0) \times 100\%$$

Where C_1 and C_0 were the drug amounts in the supernatant and the initial charged drug amounts, respectively.

2.6. In vitro insulin release

2.6.1. Stability of insulin in dissolution medium

pH 7.4 PBS was used as the dissolution medium in insulin release tests. The stability of insulin in pH 7.4 PBS was investigated. Equal insulin was dissolved in pH 7.4 PBS and pH 7.4 PBS with 1.5%

glycin as stabilizer, respectively. The solution was transferred into the release tube and then shaken horizontally (Incubator Shaker HZ-88125, Hualida Laboratory Equipment Company, China) at 37 °C and 60 strokes per min. 0.5 ml solution was withdrawn from the system at each time interval. The insulin concentration of each sample was determined by the HPLC method.

2.6.2. Choice of eluting agent

Insulin was dissolved in 30 mL pH 7.4 PBS (with 1.5% glycin as stabilizer) firstly, and then blank LMWC nanoparticles were re-suspended in this PBS. The suspension was transferred into five release tubes and then shaken horizontally at 37 °C and 60 strokes per min. In the first tube, 1.0 ml suspension was withdrawn from the system after 1, 2 and 24 h, respectively. In the second to fourth tube, 0.1%, 0.3%, and 0.5% sodium dodecyl sulfate (SDS) was added after shaking the re-suspended suspension for 24 h. After another 2 h, 1.0 ml suspension was withdrawn from the system, respectively. In the fifth tube, 0.3% SDS was added after shaking the re-suspended suspension for 2 h. 1.0 ml suspension was withdrawn from the system after another 0, 0.5, 1, 2, 4, 8, 12 and 24 h, respectively.

Each sample was centrifuged at 10,000 rpm, 10 °C for 30 min. The amount of insulin in the supernatant was measured by HPLC. The amounts of insulin remained in the system were then calculated.

2.6.3. In vitro drug release

The release rate of insulin from nanoparticles in vitro was investigated in pH 7.4 PBS containing 0.3% SDS as eluting agent and 1.5% glycin as stabilizer. The original nanoparticles was re-suspended in 25 ml release medium into the release tube and then shaken horizontally at 37 °C and 60 strokes per min. 1.0 mL suspension was withdrawn from the system at each time interval (0, 0.5, 1, 2, 4, 8, 12, and 24 h). The released insulin was determined by ultracentrifugation as described above.

2.7. In vivo pharmacodynamics studies

Wister male rats with 200∼250 g weight and 12∼13-week-old, were made diabetic prior to the study by subcutaneous injection of 150∼200 mg/kg alloxan solution (3%, w/v) for two days. The diabetic rats were fasted for 12 h before the experiments. Formulations were administered as follows: (1) control group, insulin solution (insulin concentration at 0.5 IU/ml) by subcutaneous injection at a dose of 1 IU/kg; (2) insulin-loaded nanoparticles (insulin concentration at 1.25 IU/ml) by pulmonary administration at a dose of 4 IU/kg.

Blood samples were collected from the tail vein of the rats at different times (0, 0.5, 1, 2, 4, 8, 12, 24 h) after dosage. Blood samples for the negative control which had not been dosed with insulin were collected in the same way. Concentration of blood glucose was determined in plasma sample by glucose-oxidase method (Glucose GOD-PAD kit, Shanghai Biochemical Reagent Industry, China). Results were shown as the mean values of plasma glucose for each group.

2.8. Statistical analysis

Data were expressed as means of three separate experiments, and were compared by analysis of variance (ANOVA). A p value below 0.05 was considered statistically significant in all cases.

3. Results and discussion

3.1. Preparation of LMWC

The LMWC was obtained by enzymatic degradation of chitosan with higher molecular weight. The LMWC with relatively narrow molecular weight distribution could be obtained after the degradated LMWC was separated using 10 and 50 kDa molecular weight cut off (MWCO), ultrafiltration membranes. With the elution curves as a control, the molecular weight of LMWC was obtained by gel permeation chromatography. The M_w (weight average molecular weight), M_n (number average molecular weight) and M_w/M_n of LMWC were 20 kDa, 11 kDa and 1.8, respectively. The LMWC could easily dissolve in the aqueous phase at pH of 7.4.

3.2. Preparation of LMWC nanoparticals containing insulin

During preparation of nanoparticles containing insulin, the percent of ethanol in Span 85 ethanol solution (v/v) was fixed at 0%, 20%, and 40%, respectively. The prepared nanoparticles were re-dispersed by sonicator with 180 times sonic treatment. The volume average particle size and zeta potential of re-dispersed nanoparticles are shown in Table 1. It was found that the particle size decreased with increasing ethanol content in the oil phase. The addition of ethanol into Span 85 could reduce the viscosity of oil phase, and hence decrease the surface tension between the water phase and the oil phase, which favored the formation of W/O emulsion with smaller droplet size. Consequently, the LMWC nanoparticles with smaller size could be formed when the ethanol content in oil phase was increased. The zeta potentials of nanoparticles shown in Table 1 were above +30 mV, which implied the nanoparticle dispersion was a stable system (Rainer, Karsten, & Sven, 2000).

In addition to fix the content of ethanol in Span 85 ethanol solution (v/v) at 20%, the effect of sonication times was investigated. The volume average particle sizes and zeta potentials of obtained nanoparticles are shown in Table 2. From Table 2, it was concluded that the average particle size became smaller when the sonication time increased, while the zeta potential of nanoparticles was remained almost the same.

The insulin-loaded LWMC nanoparticles was prepared using 180 times sonic treatment and 40% ethanol was used to further investigation. The TEM photograph of the insulin-loaded LWMC nanoparticles are shown in Fig. 1. It was found the LWMC nanoparticles had a spherical shape. The HPLC method was used to determine insulin content. The calibration curve of insulin obtained by

Table 1
Particle size and zeta potential of re-dispersed LMWC nanoparticles using different ethanol content in oil phase.

Ethanol content (v/v%)	Average volume particle size (nm)	PI	Zeta potential (mV)
0	1352.9 ± 128.2[a]	0.209	32.6 ± 2.6
20	442.3 ± 18.7[a]	0.198	41.7 ± 1.6
40	385.2 ± 27.6[a]	0.316	33.5 ± 1.9

PI indicates the polydispersity index of nanoparticles.
[a] $p < 0.05$.

Table 2
Effects of sonication times on average particle size and zeta potential of re-dispersed LMWC nanoparticles.

Sonication times	Average volume particle size (nm)	PI	Zeta potential (mV)
60	721.3 ± 51.3	0.345	39.2 ± 3.6[b]
90	711.6 ± 29.9[a]	0.378	42.6 ± 1.8[b]
120	613.8 ± 22.3[a]	0.318	37.9 ± 0.7[b]
150	472.2 ± 14.8[a]	0.215	40.7 ± 1.5[b]
180	442.3 ± 18.7[a]	0.186	41.7 ± 1.6[b]

PI indicates the polydispersity index of nanoparticles.
[a] $p < 0.05$.
[b] $p > 0.05$.

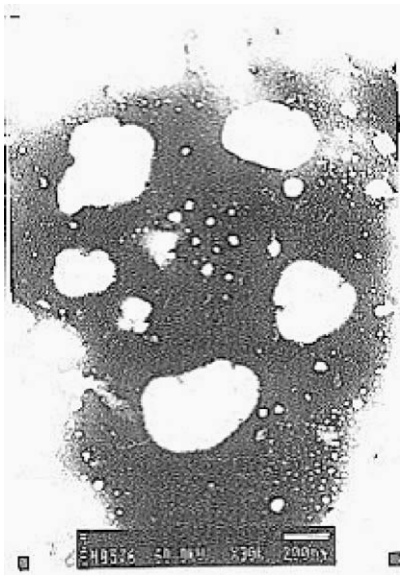

Fig. 1. TEM photograph of the insulin-loaded LWMC nanoparticles which were prepared using 180 times sonic treatment and 40% ethanol.

HPLC was $C = 8.5525 \times 10^{-5}$ A + 0.9516 ($r = 0.9996$), where A is the peak area, C is the concentration of insulin. The average drug entrapment efficiency of insulin-loaded LMWC nanoparticles was determined as 95.54 ± 1.14% ($n = 3$).

3.3. In vitro insulin release from LMWC nanoparticles

Insulin is a macromolecular protein drug. In aqueous medium, insulin is susceptible to denaturation. The stability of insulin in dissolution medium (pH 7.4 PBS) was investigated. Fig. 2 showed the variation of insulin content in pH 7.4 PBS and pH 7.4 PBS with 1.5% glycin. Glycin is a common stabilizer of insulin. From Fig. 2, it could be seen about 54.24% insulin was degraded after the insulin PBS solution was stood for 24 h under 37 °C. However, only 13.64% insulin was lost when 1.5% glycin was chosen as a stabilizer.

Table 3
Insulin contents in pH 7.4 PBS (with 1.5% glycin as stabilizer) after insulin PBS solution was incubated with blank LMWC nanoparticles for different time.

Shaking time (h)	Insulin remained (%)
0	96.8
1	95.1
2	84.7
24	17.7

The adsorption of biomacromolecules such as protein on the surface of particles through hydrophobic or static electric interaction could not be neglected in the drug delivery system, especially in the nanoparticles system with large specific surface area. Table 3 shows the variation of insulin content when insulin was incubated with blank LMWC nanoparticles in PBS (with 1.5% glycin as stabilizer) for different time. It was found that about 82.3% insulin was adsorbed on the LMWC nanoparticles after insulin was incubated with blank LMWC nanoparticles PBS suspension for 24 h under 37 °C. To prevent the adsorption of released insulin on the surface of LMWC nanoparticles, sodium dodecyl sulfate (SDS) was used as the eluting agent in this study. After insulin was incubated with blank LMWC nanoparticle PBS suspension (with 1.5% glycin as stabilizer) for 24 h, 0.1%, 0.3% and 0.5% SDS was added, respectively. The insulin contents were measured after a further 2 h incubation. The results indicated 22.0%, 84.0% and 85.4% insulin was remained in the PBS when 0.1%, 0.3% and 0.5% SDS was used as eluting agent. The addition of 0.3% and 0.5% SDS had a good eluting effect against 0.1% SDS, and no obvious difference was found between 0.3% and 0.5% SDS. Considering the SDS solution with higher concentration might increase the viscosity of samples, pH 7.4 PBS containing 0.3% SDS and 1.5% glycin was thus employed as release medium for insulin. Fig. 3 shows the variation the insulin content when insulin was incubated with blank LMWC nanoparticles in the release medium. After insulin was incubated with nanoparticles PBS suspension containing 1.5% glycin for 2 h, 0.3% SDS was added. From Fig. 3, it was found the insulin content in solution increase 12.14% after the addition of SDS for 4 h. Subsequently, the insulin content in the solution decreased 10.21% during 12–24 h. The decrease of insulin content in the later stage might contribute to the degradation of insulin. It was clear that the degradation of

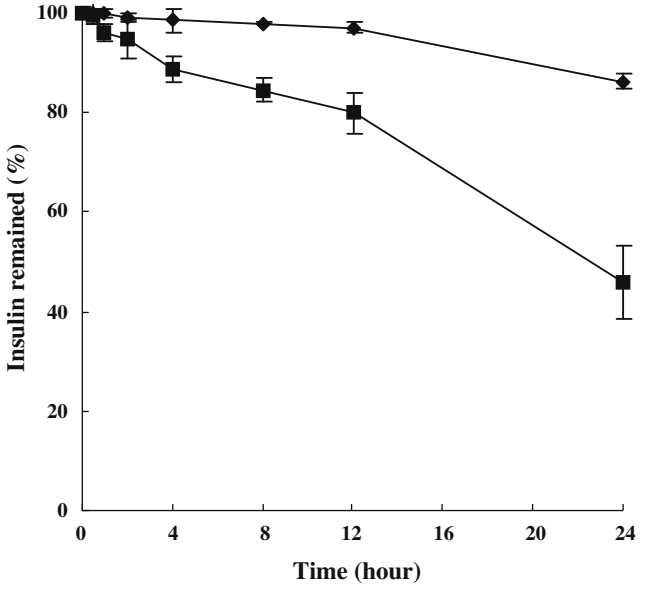

Fig. 2. Variation of insulin content in pH 7.4 PBS ($n = 3$). Key: (■) without glycin; (♦) with 1.5% glycin as stabilizer.

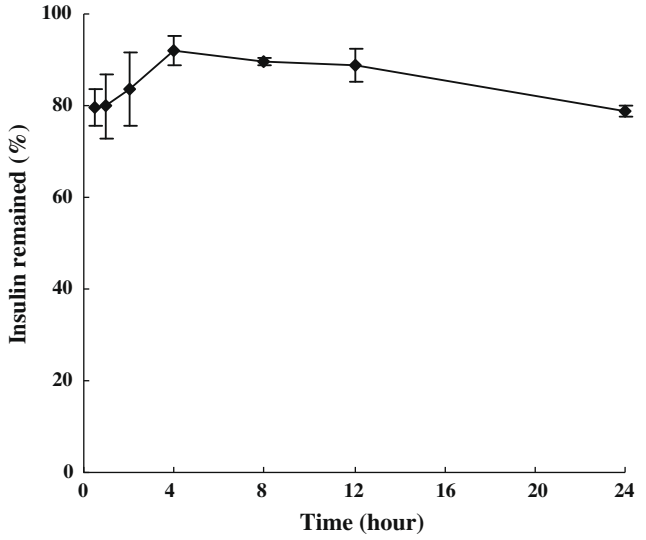

Fig. 3. Variation of insulin content when insulin was incubated with blank LMWC nanoparticles in release medium.

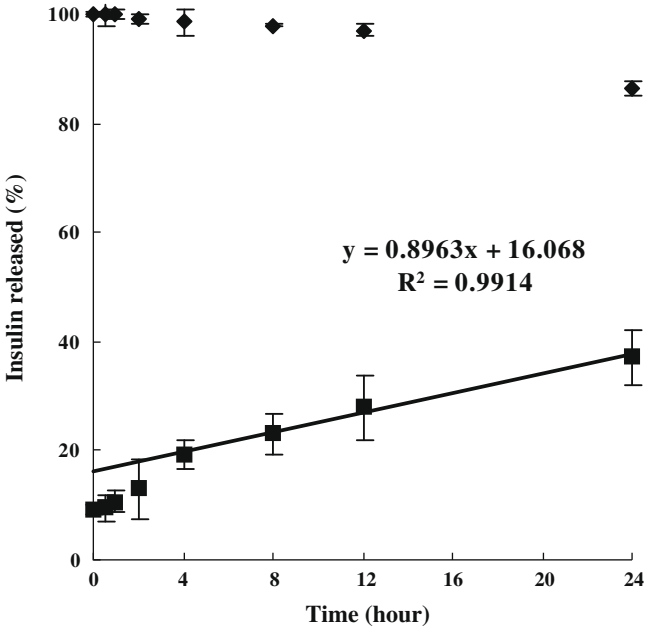

Fig. 4. In vitro insulin release profile from LMWC nanoparticles in pH 7.4 PBS containing 0.3% SDS and 1.5% glycin ($n = 3$). (■) Insulin-loaded LMWC nanoparticles; (♦) Insulin stability in release medium.

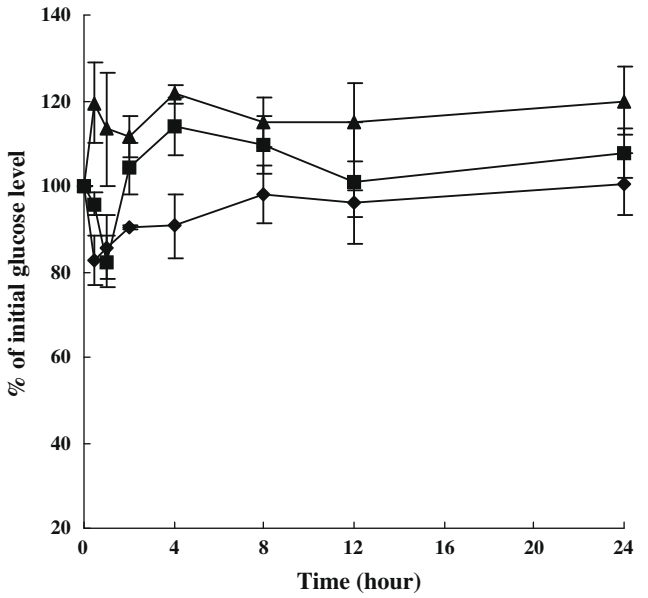

Fig. 5. Hypoglycemic effects of different formulations administered to diabetic rats. (▲) Negative control without any administration; (■) positive control insulin solution (insulin concentration at 0.5 IU/ml) by subcutaneous injection at a dose of 1 IU/kg; (♦) insulin-loaded nanoparticles (insulin concentration at 1.25 IU/ml) by pulmonary administration at a dose of 4 IU/kg.

insulin in PBS containing 1.5% glycin during 12–24 h was about 10.61%.

The drug release profile from insulin-loaded LMWC nanoparticles is shown in Fig. 4. There was an immediate release followed by a linear (zero order) release with an equation of $y = 0.8963x + 16.068$ ($R^2 = 0.9914$) after 2 h. After 24 h, 37.19% insulin entrapped in nanoparticles was released. From the release result, it could be presumed that LMWC nanoparticles are a matrix structure between insulin and carrier of LMWC. Most of insulin is dispersed in the LMWC skeleton, and a small portion is adsorbed on the nanoparticles surface. Most of the insulin is released through the holes in the LMWC skeleton. Due to the existed stability problem of insulin after 24 h, the release of insulin was studied during the initial 24 h.

3.4. In vivo pharmacodynamics studies

Fig. 5 exhibited the variations of blood glucose level after the diabetic rats were administered different formulations. The relative bioavailability (RB) of nanoparticles formulation was calculated by the following equation:

$$RB = AAC_{pu} \times Dose_{S.C.} \times 100/AAC_{S.C.} \times Dose_{pu}(\%)$$

where AAC_{pu} is the area above the curve of pulmonary administration in Fig. 5 and below the curve of the negative control without any administration; $AAC_{S.C.}$ is the area above the curve of subcutaneous injection in Fig. 5 and below the curve of negative control without any administration; $Dose_{S.C.}$ and $Dose_{pu}$ were the doses of the groups for subcutaneous injection and pulmonary administration, respectively. The relative bioavailability of LMWC nanoparticles containing insulin by pulmonary administration was calculated to be 73.70%. The in vivo pharmacodynamics of LMWC nanoparticles containing insulin showed that the nanoparticles had a certain hypoglycemic effect, and the effect behaved rapidly. The rapid hypoglycemic effect might contribute to the burst release of insulin adsorbed on the nanoparticle surface. The maximal hypoglycemic effect of insulin-loaded nanoparticles after pulmonary administration was similar to that of insulin solution administrated by subcutaneous injection. Compared with the insulin solution, 0.737 times relative bioavailability was observed when insulin-loaded LMWC nanoparticles was pulmonary administrated with four times dosage.

Attentively, the blood glucose level of insulin solution administrated by subcutaneous injection came back to the original level after 2 h (in 2 h, $p < 0.05$), while the glucose level of nanoparticles by pulmonary administration only came back 90% of base line even after 12 h (in 12 h, $p < 0.05$). This means that the nanoparticles can prolong the effect of pharmacodynamics in vivo. The insulin entrapped in the skeleton of nanoparticles was released slowly and thus prolonged the hypoglycemic effects.

4. Conclusions

Low-molecular-weight chitosan (LMWC) with weight average molecular weight (M_w) 20 kDa was obtained by the enzymatic degradation and ultrafiltration separation. The LMWC nanoparticles were prepared by solvent evaporation technique by controlling the ethanol content in oil phase and the time of sonication. Using the higher ethanol content, LMWC nanoparticles could be obtained, and the nanoparticles showed positive zeta potential and high insulin entrapment efficiency. The drug release profile from the nanoparticles indicated a zero-class release with burst release at the first 2 h. The in vivo pharmacodynamics of nanoparticles containing insulin showed that nanoparticles had rapid and prolonged hypoglycemic effect. Compared with the insulin solution, 0.737 times relative bioavailability was observed with the four times dosage of insulin chitosan nanoparticles after pulmonary administration.

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