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Lectin-modified solid lipid nanoparticles as carriers for oral administration of insulin

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Abstract

The aim of this study was to design and characterize lectin-modified solid lipid nanoparticles (SLNs) containing insulin and to evaluate the potential of the lectin-modified colloidal carriers for oral administration of peptide and protein drugs. SLNs were prepared by three different methods. For comparison, some insulin-loaded SLNs were modified with wheat germ agglutinin-*N*-glutaryl-phosphatidylethanolamine (WGA-*N*-glut-PE). The particle size, zeta potential and entrapment efficiency of insulin-loaded SLNs were determined. Insulin-loaded SLNs prepared by an appropriate modification of the double dispersion method yielded the highest drug entrapment efficiency, which was more than 60%. In vivo experiments were carried out using insulin-loaded SLNs and WGA-modified SLNs prepared by this method. SLNs and WGA-modified SLNs protected insulin against degradation by digestive enzymes in vitro. The stabilizing effect of WGA-modified SLNs was greater than that observed in SLNs.

After oral administration of insulin-loaded SLNs or WGA-modified SLNs to rats, the relative pharmacological bioavailabilities were 4.46% and 6.08%, and the relative bioavailabilities were 4.99% and 7.11%, respectively, in comparison to subcutaneous injection of insulin. These results demonstrated that SLNs and WGA-modified SLNs promoted the oral absorption of insulin.

Keywords: Wheat germ agglutinin; Insulin; Solid lipid nanoparticles; Oral administration

1. Introduction

Nanoparticles have been studied extensively as carriers for oral peptide delivery (Sakuma et al., 2001, 2002; Couvreur and Puisieux, 1993). Colloidal drug carriers prepared from solid lipids have been presented as promising alternatives to polymer nanoparticles since the early 1990 (MuÈller and Lucks, 1996; Yang et al., 1999; MuÈller et al., 2000). The use of solid lipids as a matrix material for drug delivery is well known from various reports on lipid pellets for oral drug delivery (MuÈller et al., 2000). An obvious advantage of SLNs is that the lipid matrix is composed of physiological lipids, which minimizes the risk of acute and chronic toxicity (Mehnert and Mader, 2001). In addition, SLNs can protect encapsulated peptides from degradation upon contact with gastrointestinal fluids. Furthermore, the carrier itself may exhibit certain absorption promoting effects (Garcia-Fuentes et al., 2002).

Optimum contact between the carrier and the target biological surface is necessary in order to increase drug absorption. Ideally, these particles should be able to form strong interactions with epithelial surfaces, preventing displacement from the site of adhesion, similar to the interactions developed by some microorganisms (Ezpeleta et al., 1999). The process of microorganism attachment to a biological surface is mediated by the physicochemical properties of the interface (Olsson et al., 1976) and by bacterial cell surface adhesins (such as fimbria, pili, or flagella) with specific carbohydrate-binding capacity. In this respect, the preparation of conjugates between colloidal carriers (i.e. liposomes and nanoparticles) and ligands for pharmaceutical purposes can offer some advantages either in targeted drug delivery applications (i.e. monoclonal antibodies) or to increase the gastrointestinal residence time of the pharmaceutical form (i.e. lectins).

Lectins comprise a structurally diverse class of proteins, found in organisms ranging from viruses and plants to humans (Barondes et al., 1994). Lectins are characterized by their ability to bind carbohydrates with considerable specificity. It has already been demonstrated that lectins play an important role

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in biological-recognition events in which some plant lectins can bind to intestinal mucosa and facilitate transport across cellular barriers (Rini, 1995; Clark et al., 2000; Bies et al., 2004).

Most cell surface proteins and many lipids in cell membranes of the GI tract are glycosylated and these glycans are binding sites for lectins. Such glycans contain carbohydrates comprising *N*-acetyl-galactosamine, *N*-acetyl-glucosamine, galactose, fucose and sialic acid. Wheat germ agglutinin (WGA, 36 kDa) binds to *N*-acetyl-D-glucosamine and sialic acid. As compared to plant lectins with different carbohydrate specificity, WGA binding has been demonstrated as somewhat more specific to intestinal cell lines of human origin, human colonocytes and prostate cancer cells (Gabor et al., 1997, 1998, 2001). Moreover, WGA not only binds to the cell membrane, but it is also taken up into the cytoplasm of enterocyte-like Caco-2 cells (Wirth et al., 1998).

The objective of this work is to determine whether SLNs and WGA-modified SLNs can enhance the oral delivery of insulin.

2. Materials and methods

2.1. Materials

Phosphatidyl-ethanolamine (PE, Sigma); glutaric anhydride (Shanghai Chemical Reagent Co., Ltd., China); 1-(3-dimethylaminopropyl)-3-ethyl carbodiimide hydrochloride (EDC, Fluka); Wheat germ agglutinin (WGA, Sigma); Poloxamer 188(Sigma); Soya lecithin (Shanghai Pujiang lecithin Co., Ltd., China); stearic acid (Shanghai Chemical Reagent Co., Ltd., China); Insulin (27.7 IU/mg, purchased from Xuzhou Biochemical Pharmaceutical Co., Ltd., China); pepsin (1:3000, Shanghai Medical Chemical Reagent Co., Ltd., China); trypsin (≥2500 u/mg, Shanghai Medical Chemical Reagent Co., Ltd., China).

2.2. Preparation of WGA-N-glut-PE conjugates

The wheat germ agglutinin-*N*-glutaryl-phosphatidylethanolamine (WGA-*N*-glut-PE) conjugates were prepared by an appropriate modification of the two-stage carbodiimide method (Zhang et al., 2005). The WGA-*N*-glut-PE conjugates (WGA 0.6 mg/ml, PE 0.1 mg/ml) were dissolved in the 3.2% Poloxamer solution (1/1, v/v). This solution was then used to prepare WGA-modified SLNs.

2.3. Preparation of lectin-modified SLNs

Formulation 1. Insulin was dissolved in 1 ml of 0.1 M hydrochloric acid, then it was diluted to 15 ml with a 2% Poloxamer 188 (Sigma) solution. Five milliliters of acetone solution containing 80 mg of stearic acid (Shanghai Chemical Reagent Co., Ltd., China) and 60 mg lecithin (Shanghai Pujiang lecithin Co., Ltd., China) was added to insulin solution. The mixtures were dispersed with an ultrasonic instrument (Sonic Purger CQ250, Academy of Shanghai Shipping Electric Instrument), then the suspensions were poured into 50 ml 1.6% Poloxamer solution under continual stirring for 6 h (Kika Labortechnik) in order to form SLNs (Gasco, 1993).

Formulation 2. Insulin was dissolved in 1 ml of 0.1 M hydrochloric acid, then it was added to a 5 ml acetone solution containing 80 mg of stearic acid and 10 mg soya lecithin. The resulting mixtures were sonicated and 15 ml of a 2% Poloxamer solution was added followed by sonication. The suspensions were poured into 50 ml 1.6% Poloxamer solution under continual stirring for 6 h in order to form SLNs (Garcia-Fuentes et al., 2002).

Formulation 3. Insulin was dissolved in 1 ml of 0.1 M hydrochloric acid, then it was added to a 5 ml acetone solution containing 80 mg of stearic acid and 10 mg lecithin. The resulting mixtures were dispersed with an ultrasonic instrument, then the suspensions were poured into 50 ml 1.6% Poloxamer solution under continual stirring for 6 h in order to form SLNs (Sjöström and Bergenstahl, 1992; Siekmann and Westesen, 1996; Kawashima et al., 1998).

Fifty milliliters of conjugated WGA-*N*-glut-PE solution prepared by the method described above was used instead of 1.6% Poloxamer solution to form WGA-modified SLNs (Ahlin et al., 2002). The modified SLNs were stated as WGA-modified formulations 1–3.

2.4. Physicochemical characterization of SLNs

The morphology of particles was examined by Transmission electron microscopy (TEM) (JEM-1200EX, Japanese Electric). The average diameter, polydispersity index and Z-potential were determined by laser light scattering (Zetasizer 3000SH, Malvern Instruments Ltd.).

2.5. The drug entrapment efficiency

The dispersions of SLNs were dissolved in methanol (pH was modified to 2.0 with H_3PO_4) under water bath at 65 °C for 10 min, then cooled to room temperature to preferentially precipitate the lipid. The drug content in the supernatant after centrifugation (4000 rpm for 15 min, 80-2, Shanghai Surgery Instruments, China) was measured by an HPLC method at 214 nm using an Inertsil ODS-3 column (4.6 mm × 25 cm, GL Sciences Inc. Japan) with a mobile phase consisting of 0.025 mol/l NaH_2PO_4–0.05 mol/l Na_2SO_4–hydrocyanic ether (36:36:28). The pH of the mobile phase was modified to 2.0 with H_3PO_4 and the flow rate was 1 ml/min (Zhang et al., 2005).Four ml of each SLN dispersion was centrifuged at 40 000 rpm for 1 h at 10 °C (L8-60M, Beckman). The precipitate was washed by distilled water to separate the free insulin. The dispersed system was then centrifuged again, and the precipitate was washed likewisely. The drug contents in each precipitate were measured by the same HPLC method previously described. The drug entrapment efficiencies were calculated from the following equation:

$$\text{drug entrapment efficiency} = \frac{\text{drug content in precipitations}}{\text{drug content in dispersions of SLNS}} \times 100 \quad (1)$$

2.6. The drug distribution characters in SLNs and the drug incorporation models

Four milliliters of each SLN dispersion was centrifuged at 40 000 rpm for 1 h at 10 °C, the precipitate was washed by distilled water. The dispersed system was then centrifuged and re-suspended in distilled water. This procedure was repeated two more times. The insulin concentration at the surface of SLNs was measured using the radioactive immunity analysis (RIA, Insulin radioactive immunity analysis box, Navy Radioactive Immunity Technology Center, China; GC-911 γ-ray radioactive immunity analysis instrument, Keda Zhongjia Technology Co., Ltd., China). The surface drug distribution ratios were calculated from Eq. (2) (Ma et al., 2001):

$$\text{surface drug distribution ratio} = \frac{\text{drug content at the surface of the SLNs}}{\text{total content of drug entrapped in SLNs}} \times 100 \quad (2)$$

2.7. Stability studies

Stability studies were carried out in pepsin solution (0.05 mg/ml, Tris–HCl buffer, pH 2) and trypsin solution (0.36 mg/ml, phosphate buffer, pH 7.4) (Wu et al., 2003). A 1.5 ml of either proteolytic enzyme solution was added into 1.5 ml of insulin-loaded SLNs, WGA-modified SLNs dispersions (formulation 2) or insulin solution (each formulation containing insulin 1 mg). The mixture was immediately incubated at 37 °C. Two hundred microliters of samples were withdrawn at selected times. The pepsin digestion reaction was stopped by adding 100 μl 0.05 mol/l NaOH, while the trypsin digestion reaction was stopped by adding 100 μl 0.1 mol/l HCl. The remaining concentrations of insulin in the samples were determined by the HPLC method described above.

2.8. The relative bioavailability studies in rats

Sprague–Dawley (SD) rats (250 ± 25 g, from the medical animal test center of Shandong University) were housed under normal conditions with free access to food and water. Twenty-four rats were separated into four groups. Rats from groups one and two were gavaged with 0.5 ml insulin-loaded SLNs or WGA-modified SLNs (formulation 2, insulin 50 IU/kg). The third group was given phosphate buffer only and was used as a control for the experiment. Insulin saline solution was injected subcutaneously to the fourth group (insulin 2 IU/kg). After the rats were anaesthetized by ether inhalation, 0.5 ml of blood was withdrawn from the subclavian vein at each time interval and was then centrifuged (8000 rpm for 15 min). Twenty microliters of serum was separated to determine the blood glucose by the glucose oxidase method (glucose determination agent box, Shanghai Rongsheng Biological Technology Co., Ltd., China) (Wu et al., 2003). The relative pharmacological bioavailability was calculated by the area over the hypoglycemic curve versus time profile after administration (Scott-Moncrieff et al., 1994;

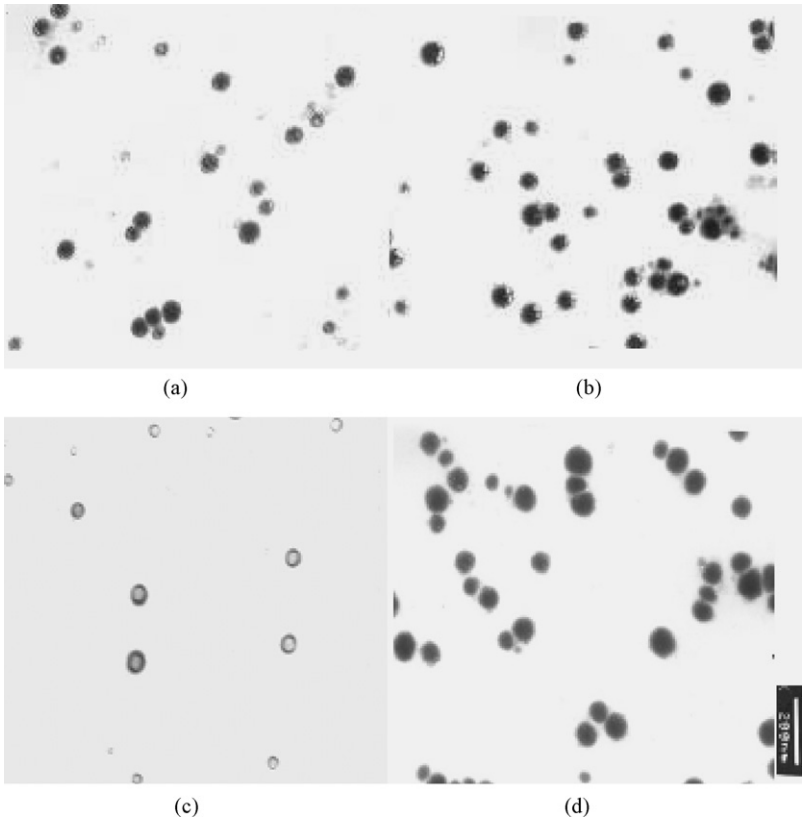

Fig. 1. Transmission electron micrographs of insulin-loaded SLNs: (a) Formulation 1, (b) Formulation 2, (c) Formulation 3 and (d) WGA-modified Formulation 2.

Table 1
Physicochemical characterization of SLNs ($n = 3$)

SLNs	Average diameter (nm)	Zeta potential (mV)
Formulation 1	58.8 ± 14.7	−41.67
Formulation 2	64.5 ± 19.56	−46.27
Formulation 3	50.2 ± 11.00	−38.07
WGA-modified Formulation 1	68.0 ± 14.65	−13.00
WGA-modified Formulation 2	75.3 ± 16.79	−13.11
WGA-modified Formulation 3	57.7 ± 10.20	−14.99

Radwan and Aboul-Enein, 2002). One hundred microliters of serum was separated to determine the insulin concentrations using the radioactive immunity analysis. The relative bioavailability was calculated by area under the curve of serum insulin concentration versus time profile. The values of the endogenous rat insulin were subtracted from the observed data.

3. Results and discussion

3.1. Preparation of lectin-modified SLNs

All insulin-loaded SLNs were of spherical or ellipsoidal shape as shown in Fig. 1. The particle sizes were slightly increased and the zeta potentials significantly increased after modification (Table 1). The drug entrapment efficiency was decreased when insulin-loaded SLNs were modified with WGA (Table 2).

WGA can be incorporated into the lecithin portion of SLNs by modifying it with a hydrophobic anchor, *N*-glut-PE. During SLN formation, increased lipophilicity will force the hydrophobic tail region to partition into the lecithin parts of SLNs and expose WGA on the SLN surface. The increases in the average diameter and the zeta potentials of insulin-loaded SLNs after the modification were evidences of the immobilized lectins on the SLNs surfaces.

Table 2
Entrapment efficiencies of insulin-SLNs ($n = 3$)

SLNs	Entrapment efficiencies
Formulation 1	37.84 ± 4.64
Formulation 2	67.85 ± 5.85
Formulation 3	26.81 ± 4.73
WGA-modified Formulation 1	23.72 ± 5.06
WGA-modified Formulation 2	40.18 ± 6.32
WGA-modified Formulation 3	17.89 ± 4.41

The modifications were performed at neutral pH. Under this condition, stearic acid was negatively charged while WGA (p*I* 7.0) positively charged. The electrostatic attraction between SLNs and WGA molecules may also be a reason for the increased zeta potentials.

3.2. The drug distribution in SLNs and drug incorporation models

The drug distribution in insulin-loaded SLNs was investigated to understand the reasons for the decrease in the drug entrapment efficiency.

Based on the data, three drug incorporation models can be deduced, i.e.: (1) solid solution model; (2) core-shell model, drug-enriched shell; (3) core-shell model, drug-enriched core (Fig. 2) (MuÈller et al., 2000). By the radioactive immunity analysis, we tested the insulin amounts on the surface of SLN and calculated the surface drug distribution ratios (Table 3). The drug distribution in SLNs varied as the preparation methods changed. Ratios of surface insulin were higher than 50% for all SLNs indicating that less than 50% of the insulin was incorporated in the lipid core of SLNs. The drug incorporation model correlated well with the core-shell model. We hypothesized that insulin were dispersed in the lipid matrix when SLNs were prepared. Due to the big surface area of SLNs, some drug were adsorbed at the surface of SLNs or precipitated in the superficial lipid matrix (Hu et al., 2002). WGA molecules perhaps displaced some of the insulin molecules, which were present at the surface of SLNs, therefore decreased the drug entrapment efficiency after modification.

3.3. Stability studies

Fig. 3 shows the remaining percentage of insulin after incubation of insulin-loaded SLNs or WGA-modified SLNs in pepsin solution. The remaining ratio of insulin after incubation in

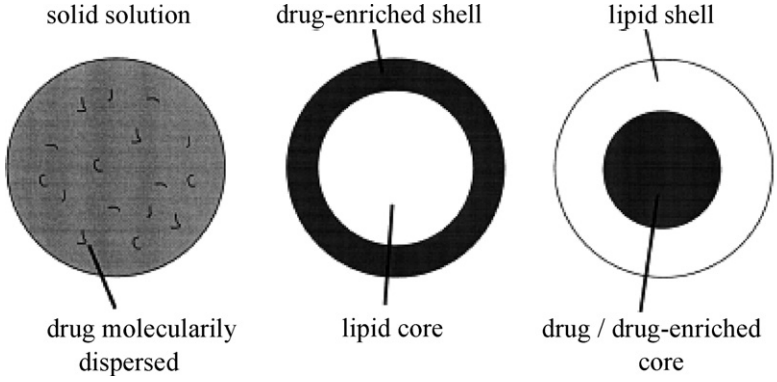

Fig. 2. Three drug incorporation models; solid solution model (left), core-shell models with drug-enriched shell (middle) and drug-enriched core (right) (MuÈller et al., 2000).

Table 3
The drug distribution characteristics in SLNs

SLNs	Insulin entrapment (μg)	Insulin on the surface (μg)	Ratio of surface insulin (%)
Formulation 1	213.2	144.0	67.54
Formulation 2	387.8	215.5	55.57
Formulation 3	151.0	131.3	86.95
WGA-modified Formulation 1	133.6	48.0	35.93
WGA-modified Formulation 2	229.6	57.7	25.13
WGA-modified Formulation 3	100.8	42.9	42.56

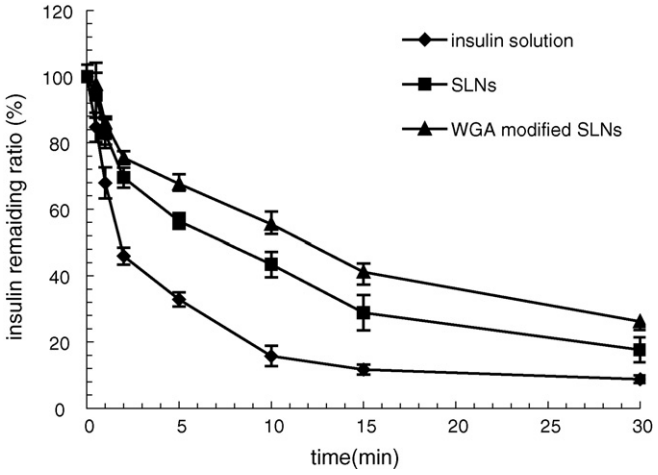

Fig. 3. Residual percentage of insulin after incubation of insulin-loaded SLNs in pepsin solution (mean ± S.D., $n = 3$).

trypsin solution is shown in Fig. 4. Protective effect was observed in both cases and the protective effect of WGA-modified SLNs was greater than that observed in SLNs.

SLNs are considered to be stable carriers for oral administration (Pinto and Muller, 1999). Poloxamer 188 was used as surfactant in the aqueous phase when preparing the insulin-loaded SLNs to increase the stability of SLNs. Lipid nanoparticles displayed an instantaneous and massive aggregation following incubation in gastric medium, whereas protective coatings such as Poloxamer 188 or PEG 2000-stearate diminished this process (Garcia-Fuentes et al., 2002). Insulin-loaded SLNs exhibited better stability than insulin solution under our experimental conditions.

For oral delivery, WGA may be a good tool due to its relatively good resistance to acidic pH and enzymatic degradation (Gabor et al., 2002). In vitro, after preincubation of WGA with abnormally high amounts of pepsin, trypsin, pancreatin, and elastase, no degradation products were observed and the cell-binding characteristics were fully retained (Gabor et al., 1997). Modified SLNs were coated by WGA and WGA is stable in the present of the enzymes, so the stability of WGA-modified SLNs was improved.

3.4. The relative bioavailability studies in rats

After oral administration of insulin-loaded SLNs and WGA-modified SLNs to rats, the blood glucose level decreased remarkably. The relative pharmacological bioavailabilities were 4.46% and 6.08%, respectively (Fig. 5). Fig. 6 shows the serum insulin concentrations after oral administration of insulin preparations to rats. The experimental data were analyzed by Pkanalyst computer program and found to best fit the one-compartment open model. The relative bioavailabilities of insulin-loaded SLNs and WGA-modified SLNs calculated by area under the curve of serum insulin concentration versus time profile were 4.99% and 7.11%, respectively, in comparison with subcutaneous injection. There was a linear relationship between blood glucose level and serum insulin concentration. These results suggested that SLNs and WGA-modified SLNs could enhance intestinal absorption of insulin.

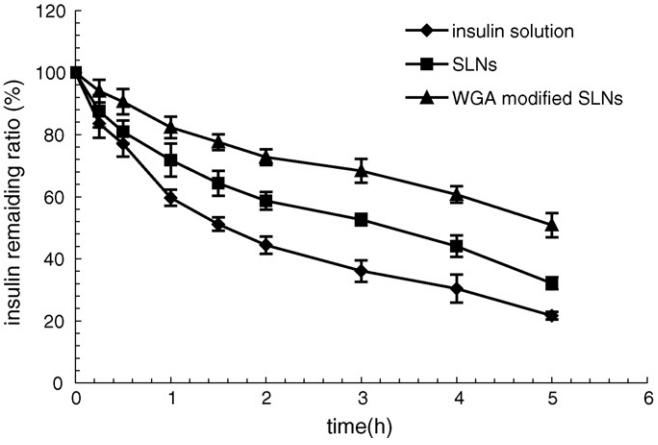

Fig. 4. Residual percentage of insulin after incubation of insulin-loaded SLNs in trypsin solution (mean ± S.D., $n = 3$).

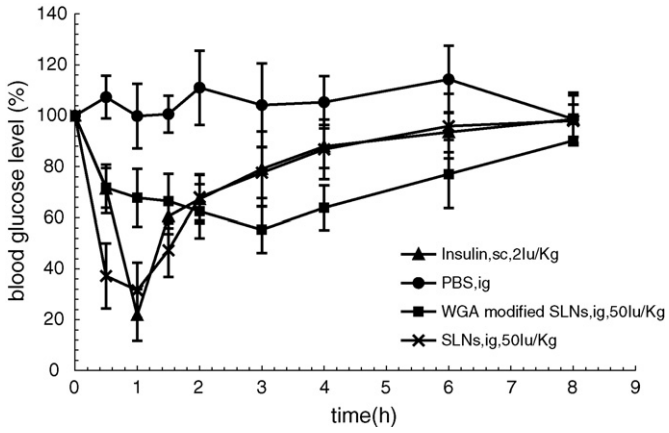

Fig. 5. Normalized serum glucose levels (%) after oral administration of insulin formulations to rats (mean ± S.D., $n = 6$).

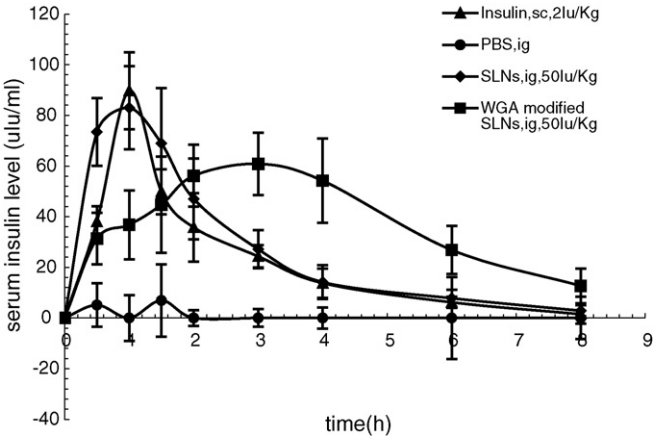

Fig. 6. Serum insulin concentration after oral administration of insulin formulations to rats (mean ± S.D., $n = 6$).

The intestinal mucous/glycocalyx layers are an insignificant impediment to diffusive *para*- and trans-cellular absorption across the intestinal membranes (Frey et al., 1996; Matthes et al., 1992; Larhed et al., 1998). It has been demonstrated that the mucous/glycocalyx layers influence insulin absorption predominantly as an enzymatic barrier not a diffusional barrier (Aoki et al., 2005). SLNs and WGA-modified SLNs attenuated insulin degradation by digestive enzymes in vitro and the stabilizing effect of WGA-modified SLNs was greater than that of SLNs. The stabilities of WGA-modified SLNs were improved so that the hypoglycemic effect after oral administration of WGA-modified SLNs was higher than that of SLNs. These results confirmed that SLNs and WGA-modified SLNs promote the oral absorption of insulin via the protection of insulin from degradation.

On the other hand, there may exit some other enhancement mechanisms, such as the interaction of WGA-modified SLNs with cells. Lectins are ligands that show affinity for receptors located in the GI cavity. They can be grafted to the surface of drug carriers and can mediate an adhesive interaction between the carrier and the biological surface (Lehr, 2004; Gabor et al., 2004). Liposomes modified with WGA were beneficial for the absorption of insulin (Zhang et al., 2005; Chen et al., 1996; Pusztai et al., 1993).

As presented here, the WGA-modified SLNs represent a powerful delivery system for transporting peptide and protein drugs. Still, it is not clear whether the transport of insulin through the intestinal mucous membrane was influenced by the increase of residence time at intestinal membrane through the specific binding of WGA-modified SLNs (Kim et al., 2005). However, it is confirmed that WGA-modified SLNs enhance the intestinal absorption of insulin sufficiently enough to drop the glucose level of blood.

The relative bioavailability calculated by area under the curve of serum insulin concentration versus time profile was 7.11% for WGA-modified SLNs in comparison to subcutaneous injection of insulin. This is still an unacceptably low oral bioavailability. Improvement of the drug entrapment efficiency and utilization of protease inhibitors simultaneously might increase the bioavailability furthermore (Ahmed et al., 2002). The characterization of lectin-modified SLNs is the first step in the successful development of nanodevices that will play an important role in a new generation of drug delivery.

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