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Absorption enhancing effects of chitosan oligomers on the intestinal absorption of low molecular weight heparin in rats

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ABSTRACT

Absorption enhancing effects of chitosan oligomers with different type and varying concentration on the intestinal absorption of low molecular weight heparin (LMWH) were examined by an *in situ* closed loop method in different intestinal sections of rats. Chitosan hexamer with the optimal concentration of 0.5% (w/v) showed the highest absorption enhancing ability both in the small intestine and large intestine. The membrane toxicities of chitosan oligomers were evaluated by morphological observation and determining the biological markers including amount of protein and activity of lactate dehydrogenase (LDH) released from intestinal epithelium cells. There was no obvious change both in levels of protein and LDH and morphology in the intestinal membrane between control and various chitosan oligomers groups, suggesting that chitosan oligomers did not induce any significant membrane damage to the intestinal epithelium. In addition, zeta potentials became less negative and amount of free LMWH gradually decreased when various chitosan oligomers were added to LMWH solution, revealing that electrostatic interaction between positively charged chitosan oligomers and negative LMWH was included in the absorption enhancing mechanism of chitosan oligomers. In conclusion, chitosan oligomers, especially chitosan hexamer, are safe and efficient absorption enhancers and can be used promisingly to improve oral absorption of LMWH.

1. Introduction

Low molecular weight heparin (LMWH), an anionic polysaccharide, is preferred clinically as an improved anticoagulant compared to un-fractionated heparin for the prevention and treatment of deep vein thrombosis (DVT) and pulmonary embolism through catalyzing the inactivation of factors Xa and IIa by binding to anti-thrombin, which ultimately leads to the inhibition of the clotting cascade (Hirsh et al., 1998; Paliwal et al., 2011a; Weltermann et al., 2003). However, it has been recognized that the clinic application of LMWH is limited because of the poor oral bioavailability and consequently the requirement for daily subcutaneous injection although it provides a predictable anticoagulant response.

Drug delivery via oral route remains the most preferred administration mode due to several advantages including convenient application, patient compliance and low cost. However, the extent of oral absorption of LMWH was sometimes not enough for its therapeutic effect owing to the characteristics of LMWH such as hydrophilicity, anionic surface charge and the large molecular weight, which led to the poor membrane permeability (Jaques, 1980; Norris et al., 1998; Ross and Toth, 2005). Therefore, various strategies had been investigated to improve the oral absorption of LMWH including preparing of microparticle (Lanke et al., 2009; Meissner et al., 2007; Paliwal et al., 2011a), nanoparticle (Hoffart et al., 2006; Leung, 2012) and complexation (Lee et al., 2001; Yang et al., 2006; Grabovac and Bernkop-Schnürch, 2006), delivering by carrier system (Kast et al., 2003; Ito et al., 2006; Paliwal et al., 2011a; Schmitz et al., 2005) and using of absorption enhancers such as glycyrrhetinic acid (Motlekar et al., 2006), fatty acids and their salts (Mori et al., 2004), sodium caprate (Motlekar et al., 2005) and chitosan and its derivatives (Thanou et al., 2001a,b; Thanou et al., 2007). Unfortunately, the oral formulation of LMWH is still not available commercially up to now.

Chitosan oligomers are derivatives of chitosan and can be prepared by complete deacetylation from chitosan. Fig. 1 shows the chemical structure and physicochemical properties of chitosan oligomers. As shown in Fig. 1, different types of chitosan oligomers

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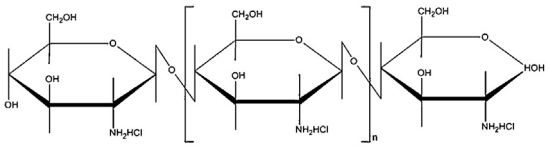

Chitosan oligomers	n	Mean M.W. (Da)	Solubility in water (g/100mL)
Chitosan dimer	0	424	190
Chitosan tetramer	2	808	158
Chitosan hexamer	4	1203	68

Fig. 1. Chemical structure and physicochemical properties of various chitosan oligomers.

including chitosan dimer, tetramer and hexamer are named according to the number of sugar rings in their chemical structures. These chitosan oligomers are of remarkable water-soluble characteristics and relative low molecular weight (Fig. 1), compared with conventional chitosan and its derivatives, which generally have poor solubility in water at physiological pH and consequently are limited in the clinic development. Previous studies reported that chitosan oligomers, as a novel type of absorption enhancer, were capable of increasing the intestinal absorption of insulin and FD4 (Gao et al., 2008) and also improving the pulmonary absorption of interferon-α in rats (Yamada et al., 2005).

In the present study, further investigations were continuously performed to examine whether these chitosan oligomers could improve the oral bioavailability of anticoagulant LMWH based on the beneficial prevention and treatment of venous thrombo-embolism in clinic. In this regard, chitosan oligomers including chitosan dimer, tetramer and hexamer were used to investigate their absorption enhancing effects on the intestinal absorption of LMWH by an *in situ* closed loop method in rats. In addition, we also examined the intestinal membrane toxicity of chitosan oligomers by the morphological observation and determining biological markers such as LDH and protein released from the intestine epithelium. Finally, the mechanistic hypothesis that chitosan oligomers exert their absorption enhancing effects possibly through the electrostatic interactions between positively charged chitosan oligomers and negatively charged LMWH were tested by measuring zeta potential of each dosing solution of LMWH with or without chitosan oligomers and quantitative analysis of interactions between LMWH and chitosan oligomers using azure A assay method.

2. Materials and methods

2.1. Materials

Low molecular weight heparin (LMWH, Anti-factor Xa 109 U/mL, mean M.W. 5266 Da) was provided from Nanda Pharm. Co. (Nanjing, China). Chitosan oligomers were supplied by Huicheng Bio. Co. (Shanghai, China). Chromogenix Coamatic Heparin Kit was obtained from Instrumentation Lab. Co. (Bedford, MA, USA). LDH assay Kit was purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China) and protein assay Kit (BCA method) was obtained from Applygen Technologies Inc. (Beijing, China), using bovine serum albumin (BSA) as a standard. Azure A and Triton X-100 were purchased from Aladdin Industrial Inc. (Shanghai, China) and Amresco (Solon, OH, USA), respectively. All other reagents were of analytical grade.

2.2. Preparation of drug solution

Dosing solutions of LMWH were prepared in PBS (pH 7.4) to yield a final concentration of 1000 IU/mL and varying concentrations (0.1%, 0.5%, 1.0%, w/v) of each type of chitosan oligomers as absorption enhancers were added to LMWH solutions.

In certain experiment, chitosan oligomers including dimer, tetramer and hexamer with the concentration of 0.1%, 0.5%, 1.0% (w/v), were prepared respectively to evaluate the intestinal membrane toxicity of chitosan oligomers and Triton X-100 (3.0%, v/v) was used as the positive control.

2.3. Intestinal absorption studies in rats

Studies on the intestinal absorption of LMWH were performed by an *in-situ* closed loop method in rats, as reported previously (Asada et al., 1995; Fetih et al., 2005; Lin et al., 2011). The experiments were carried out in accordance with the guidelines of the Animal Ethics Committee at Xi'an Jiaotong University. Male SD rats (8–10 weeks, 250–280 g) were fasted overnight but water was freely available. Prior to the experiment, the animals were anesthetized with sodium pentobarbital (40 mg/kg body weight, i.p.) and the anesthesia was maintained with additional doses of anesthetic solution as needed throughout the experiment. The rats were placed under a heating lamp to maintain the body temperature around 37 °C. The intestine was exposed through the midline abdominal incision. After ligating the bile duct, the intestine (small intestine or large intestine) was cannulated with polyethylene tubing and then flushed by PBS (pH 7.4). The remaining buffer solution was expelled with air and the distal part of the intestine was closed by clipping a forceps. Each dosing solution of LMWH with or without chitosan oligomers, kept at 37 °C, was introduced into the lumen of the intestinal loop through an opening in the proximal cannulation of the intestine, which was then closed by clipping with another forceps. The jugular vein was exposed and blood samples were collected into heparined syringe at predetermined time intervals up to 240 min. Samples were immediately centrifuged at 12,000 rpm for 5 min to obtain the plasma fraction, which was then kept in ice until determination.

2.4. Assessment of intestinal membrane toxicity and morphological examination

PBS (pH 7.4), chitosan oligomers (0.1%, 0.5%, 1.0%, w/v) and Triton X-100 (3.0%, v/v) were injected into the intestinal loop respectively with a similar method used in the intestinal absorption experiment. And then 4 h after administration, the perfusate in the intestine was withdrawn for the evaluation of membrane toxicity by determining the release amount of protein and the activity of LDH. Finally, the intestines exposed of PBS (pH 7.4) and various chitosan oligomers were excised, fixed with 4% buffered paraformaldehyde and embedded in paraffin blocks. The sections of intestines cut from the paraffin blocks with 5 μm thick were stained with hematoxylin and eosin (H and E) to evaluate intestinal damage by light microscopy (Nikon Eclipse 80i, Japan).

2.5. Analytical methods

Absorption of LMWH was measured by determining the anti-factor Xa activity present in rat plasma with a colorimetric assay according to the protocol of Chromogenix Coamatic Heparin Kit. Samples and controls were evaluated based on the standard curve of LMWH prepared by diluting the standard LMWH with normal plasma, obtained from untreated rats and used as a negative control to account for the impact of endogenous ant-factor Xa that otherwise led to false positive results.

The area under the plasma anti-factor Xa activity versus time curve ($AUC_{0-240\,min}$) was calculated by the linear trapezoidal rule and the absorption enhancing ratio of LMWH by chitosan