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Preparation and antibacterial activity of compound chitosan-compound Yizhihao-nanoparticles

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Abstract: **Objective** To prepare chitosan (CS)-compound Yizhihao-nanoparticles (NP) and to investigate its antibacterial activity. **Methods** CS NPs were formed by the incorporation of CS and Na_3PO_4. CS-compound Yizhihao NPs were prepared by ion-cross-linking. The particle sizes and surface charges of CS NPs were determined by Malvern Zetasizer 1000-HAS and atomic force microscope (AFM), respectively. The antibacterial acitivity of CS-compound Yizhihao-NPs was studied in vitro and compared with that of compound Yizhihao powder. **Results** Malvern Zetasizer 1000-HAS and AFM demonstrated that the diameter of CS-compound Yizhihao NPs was (137.00 ± 14.28) nm and CS NPs had (16.90 ± 1.32) mV positive surface charges. The minimal inhibitory concentrations (MIC) of CS-compound Yizhihao NPs on *Staphylococcus aureus*, *Pneumococcus*, β-*hemolytic streptococcus*, and *Escherichia coli* were 1∶32, 1∶32, 1∶16, and 1∶2, respectively. The minimal bactericidal concentrations (MBC) of CS-compound Yizhihao-NPs on *Staphylococcus aureus*, *Pneumococcus*, β-*hemolytic streptococcus*, and *Escherichia coli* were 1∶16, 1∶16, 1∶8, and 1∶2, respectively. The antibacterial efficacy of CS-compound Yizhihao-NPs to *Staphylococcus aureus*, *Pneumococcus*, and β-*hemolytic streptococcus* had been improved significantly ($P < 0.05$). **Conclusion** CS-compound Yizhihao-nanoparticles have obvious antibacterial activity to the *Staphylococcus aureus*, *Pneumococcus*, and β-*hemolytic streptococcus*, which lays the experimental foundation for new preparation of traditional Chinese medicine in future research.

Key words: chitosan; compound Yizhihao; nanoparticle; preparation; antibacterial activity

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Biography OU Sheng (1981-), male, master's degree, mainly engaged in the research of nanomedicine.

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复方一枝蒿-壳聚糖纳米药物的制备及其体外抗菌作用的研究

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[摘要] 目的:制备复方一枝蒿-壳聚糖纳米药物并研究其体外抗菌作用。方法:通过乳化交联法合成复方一枝蒿-壳聚糖纳米药物。分别用激光粒度分析仪(Malvern Zetasizer 1000-HAS)和原子力显微镜(AFM)检测该种纳米药物的粒径分布以及表面电位。通过体外抗菌实验检测此种纳米药物的体外抗菌能力,并与传统复方一支蒿颗粒的抗菌能力作比较。结果:该种纳米药物粒径分布均一,为(137.00±14.28) nm;表面携带正电荷,Zeta 电位值为(16.90±1.32)mV。其对金黄色葡萄球菌、肺炎球菌、乙型溶血性链球菌和大肠杆菌的最小抑菌浓度分别为 1:32,1:32,1:16 和 1:2,最小杀菌浓度分别为 1:16,1:16,1:8 和 1:2。与传统复方一支蒿颗粒相比,该纳米药物对金黄色葡萄球菌、肺炎球菌及乙型溶血性链球菌的抗菌能力均有所改进($P<0.05$)。结论:复方一枝蒿-壳聚糖纳米药物对金黄色葡萄球菌、肺炎球菌、乙型溶血性链球菌均具有明显的体外抗菌作用,为我国传统中药新剂型的开发奠定了实验学基础。

[关键词] 壳聚糖; 复方一枝蒿; 纳米粒; 制备; 抗菌作用
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Chitosan (CS) is the deacetylated derivative of chitin and largely available in the exoskeletons of shellfish or insects, which is one of the abundant, renewable, nontoxic and biodegradable carbohydrate polymers. It is singular alkaline polysaccharide in nature [1-2]. Recently, CS has attracted increasing attention as a biomaterial and pharmaceutical excipient for drug delivery because of its favorable biological properties [3-8]. Besides its low toxicity and biodegradation, CS has some properties of mucoadhesive as well as an important drug penetration enhancement capacity across mucosal barriers [9-12]. The different drug carriers of CS nanoparticles (NPs) have been explored by several researchers [13-15]. It is clear that CS NPs have such many distinctive properties as targeting, delayed releasing, and increasing drug absorption.

Compound Yizhihao is a kind of Chinese herbal medicine which is often used to cure upper respiratory tract infection. In order to improve its therapeutic efficacy, CS NPs were formed during the process of incorporating CS with Na_3PO_4 and used as the drug carrier wrapped compound Yizhihao in this study. We determined the particle sizes and surface charges of the CS NPs by Malvern Zetasizer 1000-HAS and atomic force microscope (AFM), respectively, and examined the antibacterial acitivity of CS-compound Yizhihao NPs in vitro.

1 MATERIALS AND METHODS

1.1 Materials CS (deacetylation degree ≥ 90%, viscosity degree < 100 cps) was purchased from Shanghai Bo'ao Biotechnology Limited Company (China). Compound Yizhihao was made by Pharmaceutical Factory of Xinjiang Medical University (China). Na_3PO_4 was supplied by Jiangsu Lianyungang Chemical Agent Factory (China). *D*-Glucose was provided by GIBCO BRL (USA). Ultrapure water (MilliQ Plus, Millipore Iberica S.A., Spain) was used throughout.

The main instruments were Zetasizer 1000-HSA (Malvern Instruments, Southborough, MA), Asylum Research MFP-3D AFM Systems (Japan), Nanoscope IV Bioscope™ (Digital Instruments, China), Refrigeration Centrifuge (Universal 16R, German), Electronic Ana-

lytical Balance (SHIMADZU AUW120D, German), Magnetic Stirrring Apparatus (Jinfang Fuhua Apparatus Limited Company, China).

1.2 Methods

1.2.1 Preparation of CS-Na_3PO_4 NPs CS NP was prepared by polymerid dispersion method. 3 mL of CS solution (1.44 g/L in acetic acid solution, in which the mass ratio of chitosan to acetic acid was 1∶1.5) was slowly mixed together with 1.2 mL of an aqueous Na_3PO_4 solution (0.84 g/L) at room temperature under magnetic stirring, and the reaction was left overnight to assure the completion of polymerization. NPs were isolated by centrifugation at 9 000 r/min in for 40 min and the supernatant was removed; finally, the suspension was filtered by poly (ethylene terephthalate) nuclear membrane filter (diameter of pores is 0.22 μm) and CS NPs was formed.

1.2.2 Preparation of CS-compound Yizhihao NPs CS-compound Yizhihao NPs were prepared by the method of ion-cross-linking. 10 mL of compound Yizhihao solution (100 g/L in distilled water) was slowly added into 20 mL of CS NPs solution and mixed by magnetic stirrring apparatus at room temperature for 5 h, then was centrifuged at 6 000 r/min in for 30 min. The supernatant was removed and the precipitation was washed by ddH_2O before being resuspended by 150 mmol/L NaCl solution; finally, the mixture was lyophilized with steady state and CS-compound Yizhihao NPs were formed.

1.2.3 Characterization of CS NP and CS-compound Yizhihao NP The size and Zeta potentials of CS NPs prepared under the mentioned above condition was analyzed using Zetasizer 3 000 analyzer (Malvern Instruments Ltd., Southborough, MA, USA). All samples were diluted 3-fold in 0.1 mol/L sodium chloride.

The size and surface morphology of CS-compound Yizhihao NPs were analyzed by AJ-III AFM and Zetasizer 3 000 analyzer. Samples were diluted 50-fold and 3-fold in 0.1 mol/L sodium chloride respectively.

1.2.4 Examination of antibacterial activity of CS-compound Yizhihao NPs [16-17] MH broth and MH agar plate with 2-fold dilutions of CS-compound Yizhihao NPs solution and compound Yizhihao powder were used to invest their antibacterial activity in vitro and compare them with each other. At first, 1 mL of drug solution was diluted by MH broth or MH serum broth from the 1st tube to the 10th tube. The 11th tube was bacterial control and the 12th tube was NH broth without bacterial control. The dilutions from the 1st tube to the 10th tube were 1∶2, 1∶4 ~ 1∶224. All these tubes were aligned into 4 rows. 1st ~ 11th tubes in each row were added into 0.1 mL of different testing bacterial fluids (*Staphylococcus aureus*, *Escherichia coli*, β-*hemolytic streptococci*, *Streptococcus pneumoniae*) respectively. The 12th tube was only added into MH broth. And then, each tube was cultured at 37 ℃ for 24 h.

The 1st ~ 6th tubes were diluted to 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, respectively, each of which were added into 10 mL nutrient agar, miscing bene, and then the solution of each concentration was poured into 3 plates before being cultured at 37℃ for 24 h.

1.3 Statistical analysis SPSS12.0 software for Windows (SPSS Inc, IL, USA) was used for analysis. Continuous variables were expressed as $\bar{x} \pm s$. Statistical analyses were performed with non-parametric. A values of $P < 0.05$ was considered statistically significant.

2 Results

2.1 CS-compound Yizhihao NPs formation

The particle size and zeta potential of CS NPs were measured by Zetasizer 3 000 analyzer (Fig. 1). The size of CS NPs was (96.13 ± 11.21) nm, while Zeta potential analysis on the NP revealed a positive surface charge of (16.90 ± 1.32) mV.

AFM observation revealed that CS-compound Yizhihao NPs were well uniform and the sizes were between 80 nm and 200 nm in diameter (Fig. 2). The size of CS-compound Yizhihao NPs was (137.00 ± 14.28) nm.

2.2 Antibacterial activity of CS-compound Yizhihao-NPs

The results of this experiment were shown in Tab. 1 and Tab. 2. The minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) of CS-compound Yizhihao NPs and Compound Yizhihao powder were shown in Tab. 3.

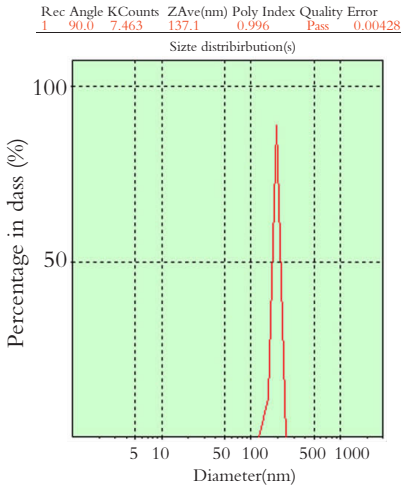

Fig. 1 Particle size distributions of the CS-compound Yizhihao NPs as measured by Zetasizer 3 000 analyzer.

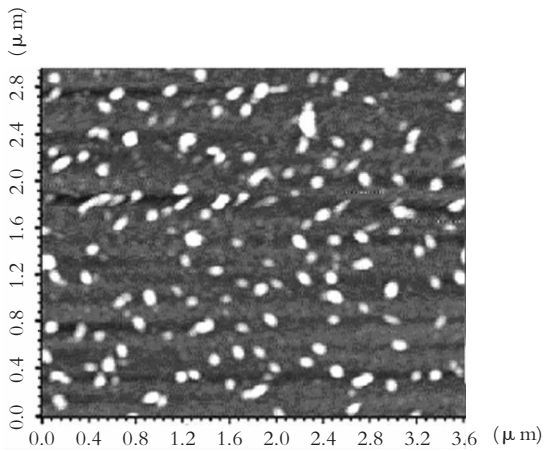

Fig. 2 AFM photograph of CS-compound Yizhihao NPs, the size of NPs ranged from 95 ~ 150 nm.

Tab. 1 Cultural result of bacteria in NH broth

Organism	1:2	1:4	1:8	1:16	1:32	1:64	bacterial control	non-bacterial control
Staphylococcus aureus	−	−	−	−	±	+	+	−
Pneumococcus	−	−	−	−	±	+	+	−
Streptococcus	−	−	±	+	+	+	+	−
Escherichia coli	+	+	+	+	+	+	+	−

−: Clear; ±: Little muddy; +: Muddy

Tab. 2 Cultural result of bacteria in NH agar

Dilution	Average number of bacteria			
	Staphylococcus aureus	*Pneumococcus*	β-*hemolytic streptococcus*	*E. coli*
1:8	−	−	−	−
1:16	−	−	2.2×10^{7}	2.3×10^{7}
1:32	9.0×10^{7}	1.3×10^{7}	2.8×10^{8}	2.9×10^{7}
1:64	1.2×10^{8}	2.6×10^{7}	3.6×10^{8}	3.5×10^{7}
1:128	1.6×10^{8}	1.9×10^{7}	4.5×10^{9}	4.6×10^{7}
1:256	2.6×10^{8}	4.8×10^{7}	1.5×10^{9}	1.8×10^{7}
1:512	2.5×10^{8}	2.5×10^{8}	2.5×10^{9}	2.8×10^{7}
Bacterial control	1.5×10^{9}	3.2×10^{9}	3.5×10^{9}	3.5×10^{7}

Tab. 3 MIC and MBC of CS-compound Yizhihao-nanoparticles

Organism	Compound Yizhihao powder		CS-compound Yizhihao NPs	
	MIC	MBC	MIC	MBC
Staphylococcus aureus	1:16	1:8	1:32 *	1:16 *
Pneumococcus	1:16	1:8	1:32 *	1:16 *
β-*hemolytic streptococcus*	1:8	1:4	1:16 *	1:8 *
Escherichia coli	≤1:2	≤1:2	≤1:2	≤1:2

Compared with compound Yizhihao powder, * $P<0.05$

3 Discussion

The method of emulation cross-linking was used to prepare CS-compound Yizhihao NPs in this study. The formation of CS NPs occurs spontaneously with the incorporation of Na_3PO_4 and CS [14-18]. In the present study, the negatively charged Na_3PO_4 solution and dissociative amino of CS were used to form NPs by the ionic interaction.

From these results, we found that CS-compound Yizhihao NPs can inhibit the growth of *Staphylococcus aureus*, β-*hemolytic streptococci*, *Streptococcus pneumoniae* effectively; but it did not have any therapeutic effects to *Escherichia coli*. It has been also demonstrated that the MIC and MBC values of CS-compound Yizhihao NPs were significantly decreased compared with compound Yizhihao powder ($P = 0.03$, $P = 0.02$, respectively), which indicated that the antibacterial activity of NPs has been improved effectively. The mechanism by which NP exerted its effect was thought to be a physical outcome, with the NP sterically hindering a close irreversible attachment, its good prolonged release effect enhancing the efficacy of drugs loaded by it and particle sizes in the submicron range facilitating the penetration of the NPs through the cellular membrane. *Staphylococcus aureus*, β-*hemolytic streptococci*, *Streptococcus pneumoniae* are all the pathogenic bacteria, which can cause upper respiratory tract infection. As this result, if CS-compound Yizhihao NPs have no biotoxicity, we can use it to cure upper respiratory tract infection, such as pharyngitis, laryngitis, tracheitis and so on. In order to do some further research and to promote the development of application of Chinese medicine, we should invest the biotoxicity of this kind of NPs in the future.

In this study, it is evident that CS-compound Yizhihao NPs could be prepared by the method of emulation cross-linking and the process is very easy and safe. CS NPs could be considered as a promising new drug delivery system which is able to provide high and long-lasting mucosal and humoral immune responses. Our further aim is to investigate the exact mechanism of CS-compound Yizhihao NP when it exerts the antibacterial properties.

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