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Preparation of Chitin-Lysozyme Anti-infective Eco-friendly Dressing and Its Effect on Wound Healing

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Abstract. This study aimed to prepare an anti-infective Eco-friendly dressing of chitin-lysozyme, which should have antibacterial activity in vitro and promote wound healing in vivo. The chitin-lysozyme was prepared using chitin (group Control) and dopamine gule (group A) methods respectively, at 2mg/ml, 4mg/ml, and 8mg /ml (Namely group B, C and D). Finally, the chitin-lysozyme was observed as lysozyme spherical particles and bedding dopamine colloid. FTIR showed that the lysozyme had been adhered and the contact angle test showed that the material was hydrophilic. In vitro antibacterial effect was significant and the role on E. coli was significantly weaker than that on S. aureus. The cytotoxicity of the chitin-lysozyme dressing was not found. On day 7, the healing rates were 35.2%, 37.9%, 56.4%, 71.9% and 75.1% in Group Control, A, B, C and D, respectively. The wound healing rate was higher in the C and D groups than these in the other three groups ($P < 0.05$).

Key words: Chitin; Lysozyme; Wound Healing; Anti-infective Dressing.

INTRODUCTION

Chitin is a natural polysaccharide made of the N-acetyl-2-amino-2-deoxy-D-glucose linked through 13-1,4 glycosidic linkages. As a natural biopolymer, chitin is widely found in the shell of lower animals, especially crustaceans, and the cell wall of lower plants, bacteria, and algae. The production of chitin is only second to cellulose in nature(Azuma et al.,2015). Chitin is widely used in the medical field, because of good biocompatibility, no toxicity, low cost, easy modification, good mechanical strength, broad-spectrum antibacterial activity and so on(Izumi et al.,2015). For example, it can be used as medical biomaterials, such as absorbent surgical sutures, hemostatic agents, immune promoters, tumor inhibitors and healing agents(Tabuchi et al.,2016)(Shao et al.,2016). However, there are regular ring structures and strong hydrogen bonds among chitin molecules, so that it has high crystallinity and poor solubility in water, dilute alkali, dilute acid and general organic solvents, which greatly limits its application. Lysozyme is a small monomer protein, containing 129 amino acid residues, with anti-inflammatory, anti-virus, bactericidal and antihistamine activities(Jiang et al.,2016)(Jing et al.,2016). It has been widely used in anti-bacterial purpose(Zheng et al.,2016). However, Gram-negative bacilli are resistant to lysozyme due to the protection from lipopolysaccharide components(Zhang et al.,2016). Besides, the lysozyme molecular structure is unstable and easy to be inactivated. Clinically, it is often used in the form of micro-balloon or in combination with other antimicrobial agents (Amara et al.,2016)(Dekina et al.,2016).

To date, the combination of lysozyme and wound dressing has become a popular research field. The combination may improve the continuous sterilization towards the gram-negative bacilli (Jiang et al.,2016)(Jing et al.,2016). Therefore, we will combine chitin and lysozyme to design a new economic and efficient antimicrobial dressing, and then assess its antibacterial activity in vitro, as well as its cytotoxicity and the effect on wound healing.

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MATERIALS AND METHODS

Dopamine Glue with Lysozyme

Tris hydrochloric acid (131.14 mg) was solved in 100 ml of deionized water, and then 200 mg of dopamine powder was added to prepare a Tris-dopamine solution (2 mg / ml, pH 8.5). The chitin was then immersed in the dopamine solution for 12 h, placed in a shaker at 37 °C (100 rpm). Lysozyme powder was dissolved in deionized water and generated as 20mg/ml lysozyme solution, and then diluted to 0, 2, 4and 8 mg/ml (ie, the A, B, C and D group). The samples were washed with deionized water carefully, immersed in different concentrations of lysozyme solution for 12 h on a 37°C shaker (100 rpm).

Scanning Electron Microscopy (SEM)

The sample of the material wafer was carefully washed with deionized water, dried and sprayed, and the aperture structure of the film was observed under vacuum condition by scanning electron microscope and photographed.

Fourier Infrared Spectrum

Different materials characterization and chemical structure was Testing by Fourier transform infrared spectroscopy (Nicolet-460 Thermo Fisher) ,wavenumber scan range is 600-4000 cm-1.

Contact Angle Test

The film of different materials is placed on the horizontal surface, and the 1ul deionized water is dripped down from the top to the surface of the material. The contact angle size of the droplet is measured, and the average value of each sample is measured 3 times.

Drug Loading and Encapsulation Efficiency

(1) determination of lysozyme standard curve
(2) determination of drug loading and encapsulation efficiency
Drug loading = actual lysozyme concentration * volume (UG) / dissolved light wood lysozyme total mass (mg);
Encapsulation efficiency = actual lysozyme concentration / theoretical lysozyme concentration.

Tensile Property Test

The tensile properties of the films were examined by universal material testing machine. The tensile strength and elongation at break were recorded as two indexes. The operating procedure is as follows:

Drying sample: width 10mm, total length 150mm, initial distance between clamps 100mm, standard distance length 50mm. The thickness is <1mm and the thickness is measured with micrometer (5 points per sample). Test speed 50mm/min, for testing. Each sample was subjected to 3 parallel tests and statistical analysis was carried out.

Swelling sample: soak the dried sample in PBS buffer (0.05mol/L, PH7.4) and swell 1h. After removal, the surface moisture is removed by filter paper, cut into dried samples, tested for the same shape, and measured with the same parameters

Bacteria Co-Culture

S.aureus and E.coli strains were obtained from the Institute of Burns, Southwest Hospital, Third Military Medical University. The bacteria (1*104 CFU/ml) were first detected by the microplate reader (An OD value about 0.07 met the standard) and then added 200 ul into each well of the 96-well plates. Each group of materials was placed in the wells. Incubation was performed at 37 °C for 12 h and 24 h.

Assay of Cytotoxicity

Normal neonatal mice were used to isolate primary fibroblasts as routine method,(reference) and the 3rd passage cells were used to assay the cytotoxcity of the prepared chitin-lysozyme dressing. After cells attached, materials were placed into wells. Each group contained 12 wells (for day 1, 3, 5 and 7 cell proliferation assessment, triple for each time point). Each well had 150 ul medium and cells were cultured in the 37°C incubator.

Wound Healing Rate Assessment

Wound healing rate was calculated as follow. The wound area was measured by IPP6.0 software. Wound healing rate = (original wound area - residual wound area after injury) / original wound area x 100%.

RESULTS

Electron Microscopy Scanning and Tensile Tests

As shown in Figure. 1 ,the chitin retained the clear and complete ordinary structure under the magnification (control1,x 102;control2,x103;control3,x104) .The chitin-lysozyme group showed some deposition under the higher magnification (D3,2 x 104) the Paving dopamine layer and pellets lysozyme were visible.

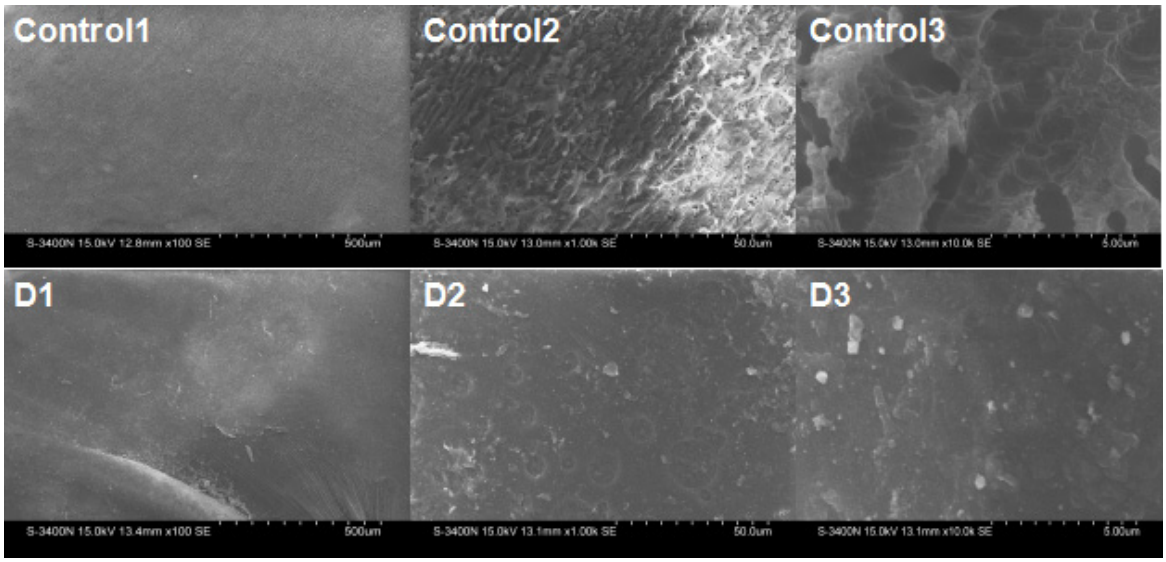

FIGURE 1. Control is the chitin, D is 8 mg/ml chitin-lysozyme

Fourier Infrared Spectrum

As shown in Figure 2, the infrared spectra of the 3 groups (Control, A, D) were approximately similar. As shown in D, lysozyme has two distinct characteristic peaks, corresponding to C=0 stretching vibrations of amide I (1634.31cm-1) and N-H, flexural vibrations and C-N stretching vibrations of amide II (1223.58cm-1).

Contact Angle Test

As shown in Figure 3, the average contact angle (θ) of Control group is 88.24± 10.76°, A is 35.1 ±6.36°,D is 6.88±1.05°.Compared with A and D, the difference was statistically significant (P<0.05).

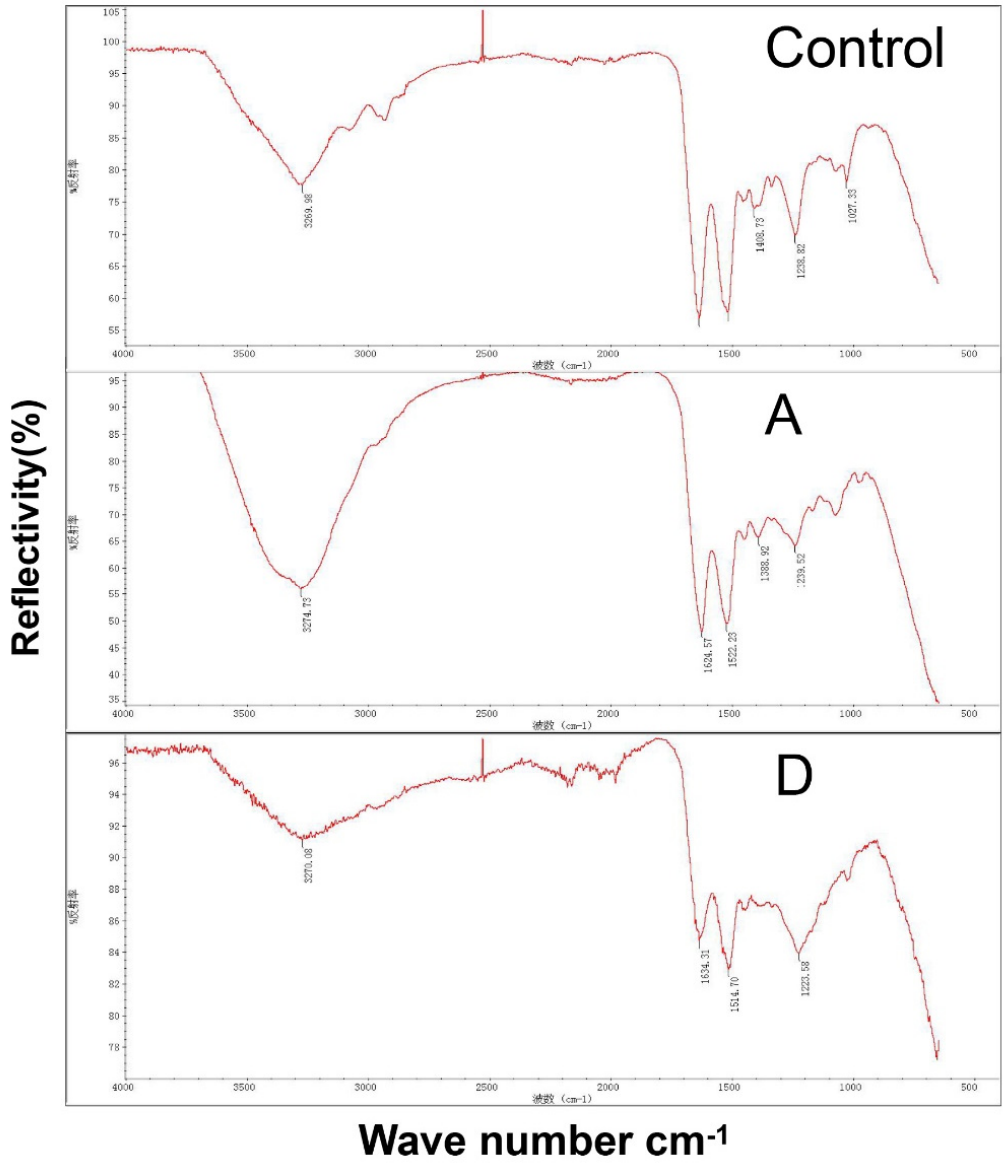

FIGURE 2. The infrared spectra of the Control,A and D groups

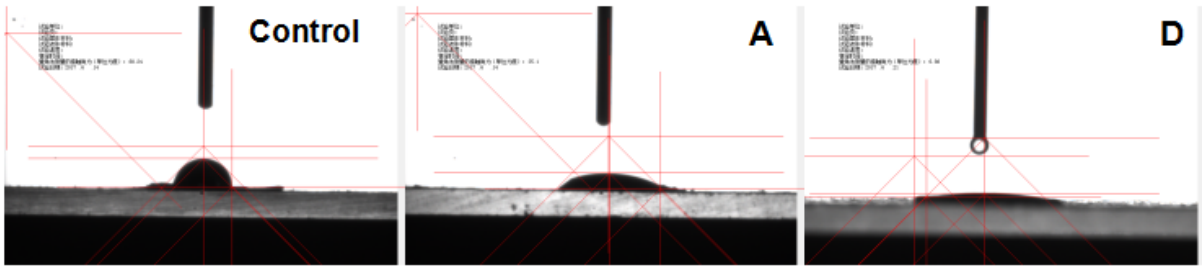

FIGURE 3. The contact angle (θ) of Control,A and D group

Enzyme Loading and Encapsulation Efficiency

As shown in Table 1,with the increasing of lysozyme concentration, the drug loading rate of Chitin-lysozyme increased gradually, while encapsulation efficiency efficiency decreased gradually ($P<0.05$). However, the encapsulation efficiency of lysozyme at different concentrations was about 60%.

TABLE 1. Drug loading and encapsulation efficiency of Chitin-lysozyme in different groups

	B	C	D
Drug loading (μg/mg)	31.8±3.6	45.7±4.0	62.7±6.2
Encapsulation efficiency (%)	77.5±6.3	67.4±5.6	57.0±4.4

Release in Vitro

As shown in Figure 4, the release rate of B, C and D groups tended to be stable and maximum at 48h, and the cumulative release percentages were 80.7%, 90.6% and 92.3% after 72h.

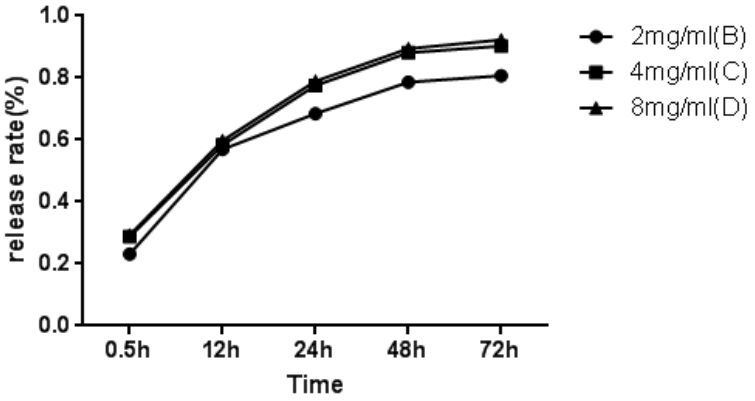

FIGURE 4. Drug release in vitro of different concentrations of wood-lysozyme

Tensile Property Test

As shown in Table 2,In dry state, the excellent degree of tensile strength and elongation at break is Control<A<B<C<D. In the swelling state, the tensile strength and elongation at break of each group are improved.

TABLE 2. The Tensile property test of chitin-lysozyme

	Dry state		Swollen state	
	Tensile strength (MPa)	Elongation at break (%)	Tensile strength (MPa)	Elongation at break (%)
Control	22.5±3.4	10.8±0.9	24.6±3.7	11.6±1.0
A	31.9±3.9	14.5±1.1	34.8±4.1	15.4±1.3
B	40.6±4.0	18.3±1.4	44.5±4.4	20.3±1.8
C	46.5±5.1	23.5±1.9	50.9±5.3	25.8±2.4
D	52.8±5.7	27.9±2.1	57.0±5.9	29.8±2.7

Assay of the Antibacterial Activity

As shown in Table 3,Table 4 and Figure. 5, the growth of S.aureus in the Control group was in accordance with the normal trend. When the concentration of lysozyme increased gradually, the inhibitory effect on the bacteria gradually increased, and the difference was statistically significant (P <0.05).

TABLE 3. OD values of co-culture of different concentrations of chitin-lysozyme dressings and S.aureus.

	Control	A	B	C	D
12h	0.635±0.109	0.592±0.097	0.473±0.092	0.379±0.089	0.355±0.066
24h	0.903±0.197	0.861±0.167	0.683±0.144	0.494±0.109	0.402±0.085

TABLE 4. OD values of co-culture of different concentrations of chitin-lysozyme dressings and E.coli.

	Control	A	B	C	D
12h	0.598±0.101	0.576±0.097	0.472±0.082	0.380±0.078	0.312±0.053
24h	0.726±0.092	0.665±0.090	0.520±0.079	0.406±0.062	0.329±0.057

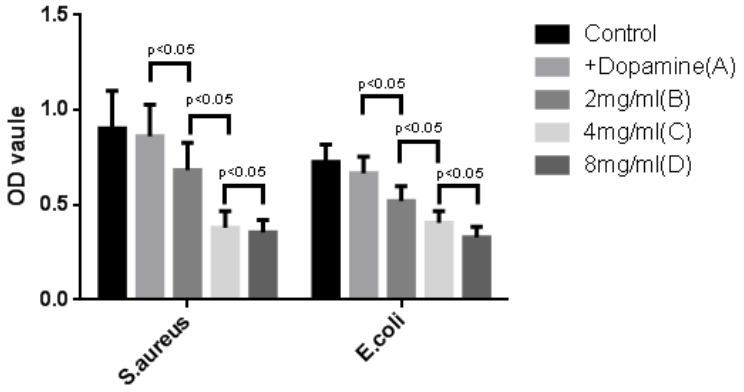

FIGURE 5. The inhibitory effects on the bacteria was gradually increased with the concentration of lysozyme (P <0.05)

Test of Cytotoxicity

As shown in Table 5 and Figure. 6, lysozyme in different concentrations was hardly inhibited the cell growth (P> 0.05).

TABLE 5. Cytotoxicities of different concentrations of chitin-lysozyme dressings on the mice fibroblasts.

	1Day	3Day	5Day	7Day
Control	0.279±0.038	0.415±0.049	0.658±0.059	0.768±0.075
A	0.274±0.035	0.411±0.042	0.650±0.057	0.752±0.073
B	0.271±0.037	0.402±0.044	0.641±0.052	0.749±0.072
C	0.270±0.037	0.398±0.040	0.636±0.050	0.744±0.076
D	0.265±0.036	0.395±0.041	0.633±0.051	0.740±0.069

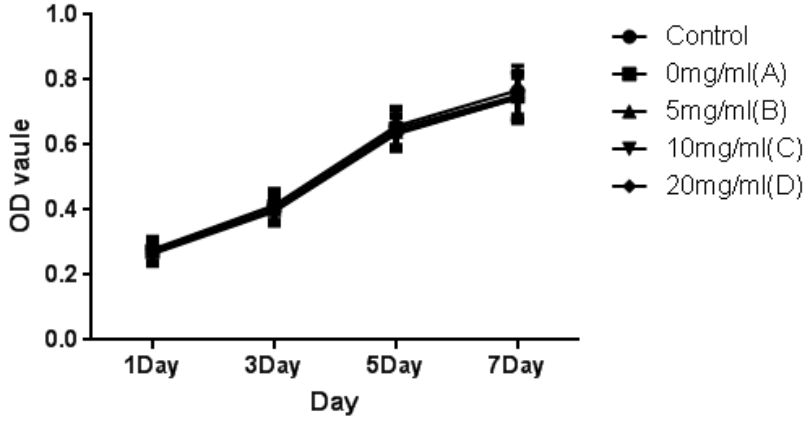

FIGURE 6. lysozyme in different concentrations was hardly inhibited the cell growth (P> 0.05)

Effect on Infectioous Wound Healing

On day 7, the healing rates were 35.2%, 37.9%, 56.4%, 71.9% and 75.1% in Group Control, A, B, C and D, respectively. The wound healing rate was higher in the C and D groups than these in the other three groups (P <0.05).. The difference between the C and D groups was not significant (P> 0.05).

DISCUSSION

For patients with severe skin lesions, there is an urgent need to quickly and effectively close the wound through various means to prevent bacterial invasion, and prevent the loss of body fluids, energy, water and electrolytes. Wound dressings can rebuild the skin barrier, accelerate wound healing, and prepare for later operation(Guo et al.,2010). Ideal wound dressings should have the following characteristics: good mechanical properties, adequate water vapor permeability and excellent biocompatibility. More importantly, the infection and inflammatory disorder may make wound healing slow down or even not heal, so that the ideal wound dressing should also provide a sterile and suitable microenvironment for the wound to grow(Chan et al.,2015). The natural materials such as chitin, chitosan, dextran, cellulose, alginate, silk protein, and so on, are considered to be suitable to make wound dressings, because of easy preparation, with good biocompatibility. Therefore, natural material loaded with a variety of antibacterial agents, growth factors or other chemicals to promote the healing of infectious wounds has become a hot spot in the field of wound repair(Dreifke et al.,2014)(Wang et al.,2015).

As shown in Figure. 1 ,the chitin retained the clear and complete ordinary structure under the magnification (control1,x 102;control2,x103;control3,x104), consistent with previous studies. In the 8mg/ml chitin-lysozyme group Under the higher magnification(2× 104) dopamine layer and pellet-like lysozyme (as crystals precipitated on the surface) were visible, We think that is because the lysozyme concentration of 8mg/ml is larger than that 2-5mg/ml of Other related research (Grumezescu et al.,2017)(Lin et al.,2016) . Lysozyme is composed of 129 amino acid residues, the proportion of basic amino acid residues and the aromatic amino acids tryptophan residues is very high, with 4 of 2-disulfide bonds, which consists of two regions, which are connected by a long alpha helix, mostly alpha helical secondary structure(Zhang et al.,2016)(Amara et al.,2016). In Fig. 2, the typical two characteristic peaks of lysozyme exist(Dekina et al.,2016), but the intensity is decreased. It shows that the lysozyme molecule has been immobilized on the carrier, but the secondary structure has not changed significantly. As shown in Figure 3,the average contact angle (θ) of Control group is 88.24± 10.76°, A is 35.1 ±6.36°.Compared with Control and A , the difference was statistically significant (P<0.05), indicating that the adhesion of dopamine has changed the characteristics of the material itself. Although D group was D is 6.88±1.05°, it still has strong hydrophilicity. This may be related to the internal hydrophobicity of bacteria and the basic structure of external hydrophilic, which plays an important role in lysozyme's antibacterial function(Jing et al.,2016)(Zheng et al.,2016).As shown in Table 1 and Figure 4,with the increasing of lysozyme concentration, the drug loading rate of Chitin-lysozyme increased gradually, while encapsulation efficiency efficiency decreased gradually (P<0.05). However, the encapsulation efficiency of lysozyme

at different concentrations was about 60%.the release rate of B, C and D groups tended to be stable and maximum at 48h, and the cumulative release percentages were 80.7%, 90.6% and 92.3% after 72h.

As shown in Table 3, table 4 and Figure.5,When the concentration of lysozyme increased, the inhibitory effect on the bacteria gradually increased, and the difference was statistically significant($P < 0.05$). At present, the inhibitory effect of lysozyme on gram-positive bacteria has been known, and its mechanism is to hydrolyze the 1,4-linkage between N-acetyl- muramic acid and N-acetyl-dglucosamine of bacterial cell peptidoglycan(Yuan et al.,2013). However, for the Gram-negative bacteria, due to the protection of LPS, the sterilization effect is always limited(Zhang et al.,2016). In recent years, there have been many studies to prepare lysozyme into microcapsules, nanoparticles, pellets and other forms, and combined with different materials to improve its stability and its gram-negative bacteria bactericidal effect (Amara et al.,2016)(Dekina et al.,2016). In this study, the lysozyme was fully adhered to the pores by dopamine glue, which provided a stable releasing microenvironment towards gram-negative bacteria. On day 7(Table 5, Figure.6), lysozyme in different concentrations was hardly inhibited the cell growth ($P > 0.05$). This may be related to lysozyme, which is derived from egg white, is a natural, safe enzyme. The safety of lysozyme makes it widely used for food preserving(Tiwari et al.,2009).

The defense barrier on body surface of the burns were destroyed, and the immune capacity decreased significantly. Extensive tissue necrosis and invasion of bacteria in vivo will lead to wound infection(Fournier et al.,2015) . Wound infection is one of the major complications and key cause of death in burned patients. About 52% -70% of burns die due to wound infection(Ito et al.,2015). In this study, bacterial concentrations were 108/ml . The wound healing rate of C and D groups was higher than that of the other three groups at 3 days and 7 days after injury. There was no significant difference between the C and D groups. The results suggest that the anti-infective effect no more increases but when the concentration reaches a certain level (4-8 mg/ml).

In summary, this study prepared a natural chitin-lysozyme anti-infective dressings in different concentrations; and concentrations of 4-8 mg/ml were effective. chitin-lysozyme dressing promoted wound healing. High concentration with long-term contact might inhibit cell proliferation. This dressing provides a new means for wound healing via natural materials.

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