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RESEARCH ARTICLE

The effect of carboxymethyl-chitosan nanoparticles on proliferation of keloid fibroblast

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In this study, different molecular weight (MW) carboxymethyl chitosans (CM-chitosan) nanoparticles were prepared by ionic gelification. The particle size of nanoparticles was around 180–250 nm by dynamic light scattering (DLS) and transmission electron microscope (TEM). With the increase of CM-chitosan nanoparticles concentration from 2 to 200 μg/mL, the growth inhibition effects on the keloid fibroblast increased. At the concentration of 100 μg/mL, CM-chitosan nanoparticles with MW 6.3 kDa had a significant inhibitory effect (inhibition ratio 48.79%) of the proliferation of keloid fibroblast. Compared with CM-chitosan solution, the inhibition of CM-chitosan nanoparticles were lower in prior period and similar in later period. By analyzing the different effects of chitosan, CM-chitosan solution and CM-chitosan nanoparticles on proliferation of keloid fibroblast, we have found that the carboxylmethyl groups of CM-chitosan play an important role in inhibition of proliferation of keloid fibroblast.

Keywords carboxymethyl-chitosan, nanoparticle, keloid fibroblast, proliferation

1 Introduction

Chitosan, a-(1–4)-2-amino-2-deoxy-b-D-glucan, is a natural biodegradable and nontoxicity polymer derived from partially deacetylated of chitin. It has caused a wide concern due to its unique biologic activity, such as immune enhancing effect [1], antitumor activity [2,3] and antimicrobial activity [4–8]. Previous research showed that chitosan possesses the characteristics favorable for promoting rapid dermal regeneration and accelerating wound healing [9,10] which were closely related to the collagen secretion and keloid forming. However, chitosan is soluble only under acidic conditions, which limits its applications. The limited solubility of chitosan in water can be overcome by chemical modification. An important chemical modification method is carboxymethylation [11].

CM-chitosan is a water soluble chitosan derivative and functional biomaterial. CM-chitosan can stimulate the extracellular lysozyme activity of skin fibroblast and promote the proliferation of the normal skin fibroblast but inhibit the proliferation of keloid fibroblast. The functions of CM-chitosan on the wound healing are not fully known yet [11–13]. CM-chitosan nanoparticles can be easily formed through simple ionic gelification [14–16]. Compared with CM-chitosan solution, CM-chitosan nanoparticles also have antibacterial activity and nontoxicity. Moreover, CM-chitosan nanoparticles have lower viscosity than CM-chitosan solution so that it can be directly injected into location of a lesion as injection. Furthermore, CM-chitosan nanoparticles have the potential for drug delivery [15]. These characteristics will make it a novel delivery system for accelerating wound healing and preventing keloid formation.

Keloids are benign dermal fibroproliferative tumors only in humans with no malignant potential [17–20], and occur as a result of derangement of the normal wound healing process in susceptible individuals. They continue to grow and extend beyond the confines of the original wound, and invade the skin beyond the perimeter of the original wound with a leading edge that is often erythematous and pruritic. Some researchers believe that keloids represent an inability to halt the wound healing process [18]. Currently, except for surgical excision, glucocorticoids are often used in the treatment of keloid, but the long-term use of these steroids leads to a large number of debilitating side effects [21]. We hope to develop a novel delivery system that cannot only reduce the side effects of glucocorticoids, but also have some inhibitory effects on the keloid.

In this study, we prepared CM-chitosan nanoparticles in various MWs and examined their physicochemical characteristics with TEM and DLS. The effects of concentration, exposure time and MW of CM-chitosan nanoparticles on the proliferation of the keloid fibroblast were investigated *in vitro*. We evaluated which groups played an important role in inhibition of proliferation of keloid fibroblast by analyzing the different effects of chitosan, CM-chitosan and

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CM-chitosan nanoparticles on proliferation of keloid fibroblast.

2 Experimental

2.1 Materials

Two samples of Chitosan with different MWs (MW 160 kDa, DD 0.91; MW 5.2 kDa, DD 0.89, Biotech Co) were respectively purified by being dissolved in dilute acetic acid solution (1%; v/v), filtered, and precipitated with aqueous NaOH (10%; w/v), then washed to neutral and freeze–dried. Monochloroacetic acid, acetic acid and calcium chloride ($CaCl_2$) were purchased from Sigma (St. Louis, USA). Fresh tissue specimens (keloids) were obtained from the Affiliated Hospital of Qingdao University Medical College (Qingdao, China). They were excised by a surgeon from patients of 16–45 years old. D-Hanks (Hank's balanced salt without $CaCl_2$), Dulbecco's modification of eagle's medium (DMEM), Bovine calf serum (BCS) and 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl-tetrazolium bromide (MTT) were purchased from Sigma (St. Louis, USA).

2.2 Preparation of CM-chitosan with different MWs

CM-chitosan was prepared from chitosan [22,23]. 10 g chitosan samples with different MWs (MW 160 kDa, MW 5.2 kDa) were respectively added in 20 mL alkali solution (60%; w/w) to be swelled and alkalized at 50°C for 4 h. The temperature was maintained in a water bath (thermo-controller, Comabiotech. Co, Korea). The monochloroacetic acid (15 g) was dissolved in isopropanol (20 mL), then added into the reaction mixture dropwise within 30 min and reacted for 7 h at the same temperature, then ceased by adding 75% ethyl alcohol (200 mL). The solid was filtered and rinsed in 75% ethyl alcohol to desalt and vacuum dried at room temperature. In this way, we produced two CM-chitosan samples with different MWs of 196 kDa and 6.3 kDa.

The obtained CM-chitosan with MW 196 kDa was degraded with hydrogen peroxide to prepare CM-chitosan with different MWs. In brief, 100 mL H_2O_2 (3%; v/v) was added into 200 mL CM-chitosan solution (5%; w/v) and stirred at room temperature in dark. At predetermined time intervals (6, 9, and 12 h), Na_2SO_3 was added into the mixture dropwise to stop the oxidation. This solution was precipitated and desalted by 80% ethanol, then centrifuged for 30 min at 2000 g. The two samples of CM-chitosan with different MWs (16 kDa and 7.5 kDa) were obtained. MWs of the prepared CM-chitosan were measured by gel-permeation chromatography (GPC) [24]. Degree of substitution (DS) and degree of deacetylation (DD) values of the prepared products were determined by pH titration [25].

2.3 Preparation of CM-chitosan nanoparticles

CM-chitosan nanoparticles were prepared by a simple ionic gelation method with calcium chloride solution [15]. In brief, CM-chitosan with various MWs was dissolved in triple-distilled water at predetermined concentration. Then 2 mL of $CaCl_2$ solution was added into 5 mL of CM-chitosan solution dropwise under constant stirring for 30 min. The prepared nanoparticles were separated by centrifugation at 20,000 r/min for 1 h, dispersed in water, and then freeze-dried.

2.4 Characterizations

The morphology of the CM-chitosan nanoparticles was observed by TEM (100 CX II, Japan). The samples were placed onto copper grid and dried at room temperature, then examined without being stained.

Dynamic light scattering (DLS) (Zetasize; 3000 HS, Malvern, UK) was used to determine the mean size and size distribution of the CM-chitosan nanoparticles. All measurements were done with a wavelength of 633.0 nm at 25°C with an angle detection of 90°. Each sample was repeatedly measured three times.

2.5 Keloid fibroblast cultures

The specimens were rinsed with D-Hanks solution containing penicillin (1000 U/mL solution) and gentamicin (350 U/mL solution) three times, minced and explanted into tissue culture flasks (25 mL) with DMEM supplemented BCS (10%; v/v), incubated at 37°C, 5% CO_2, and 100% relative humidity [11]. The cells were harvested for subculture every 4 days with 0.01% EDTA–0.125% trypsin. After 3–4 generations, epithelial cells disappeared and only keloid fibroblasts were present. After 6 generations, the fibroblasts were inoculated at a 96-well tissue culture plate with (4×10^3)–(6×10^3) cells per well. The sample concentrations were 0–200 μg CM-chitosan nanoparticles per mL culture media. The cytotoxicity of cells was determined by the MTT method [26]. To compare the effects of Chitosan, CM-chitosan solution and CM-chitosan nanoparticles on the growth of keloid fibroblast, keloid fibroblast cells were seeded at 5×10^4 cells/well in 96-well plates and allowed to adhere overnight. The growth medium was replaced with DMEM that contained chitosan (MW 5.2 kDa), CM-chitosan (MW 6.3 kDa) or CM-chitosan nanoparticles (MW 6.3 kDa) at the same concentration (100 μg/mL). After specific time periods, we evaluated the cytotoxicity of the test samples by MTT method.

2.6 Statistical analysis

The assays were performed at least in triplicate on separate occasions. Statistical analysis of the differences in the measured properties of the groups was performed with one-way analysis of variance and the determination of confidence intervals, with the statistical package SigmaPlot, version 11.0 (Systat Software Inc). The data collected in this study was expressed as means and standard deviations, indicated as "mean±SD". Differences were considered to be statistically significant when the *P* values were less than 0.05.

3 Results and discussion

3.1 Preparation of CM-chitosan

The samples of CM-chitosan made by carboxymethylation had good water solubility. The MW among them was different, but DS and DD on the chitosan's backbone were almost similar. The data was shown in Table 1.

3.2 Preparation of CM-chitosan nanoparticles

CM-chitosan nanoparticles were produced by the ionic gelation between positively charged Ca^{2+} and negatively charged CM-chitosan. As the MW of CM-chitosan increased, less calcium ions were needed to form nanoparticles (Table 1). Similar tendency was also found in other researches on chitosan nanoparticles gelated by TPP [27]. This was because longer molecular chain was much easier to roll up when coordinated with calcium ions, compared to shorter chains [15]. Using dynamic light scattering (DLS) measurements, the size distributions for CM-chitosan nanoparticles with various MWs were obtained. The average diameters for CM-chitosan nanoparticles with MWs 196 kDa, 16 kDa, 7.5 kDa and 6.3 kDa were found to be 258 nm, 227 nm, 189 nm and 181 nm, respectively (Table 1). The particle size distribution for CM-chitosan nanoparticles with different MWs was shown in Fig. 1. The size of CM-chitosan nanoparticles increased with MW increasing (Table 1). This was because longer molecular chain got entangled together, giving rise to bigger nanoparticles. The typical TEM images of prepared CM-chitosan nanoparticles were shown in Fig. 2. The CM-chitosan nanoparticles with various MWs all showed a spherical morphology with diameters in the range of 150–200 nm.

3.3 The effects of CM-chitosan nanoparticles on the proliferation of keloid fibroblasts

The effects of CM-chitosan nanoparticles on the proliferation of keloid fibroblast at different MWs and concentrations by MTT method were shown in Fig. 3. The percentage cell growth inhibition was assayed as the cells surviving on the fourth day of incubation. The growth inhibition of keloid fibroblast got strengthened as MW of CM-chitosan nanoparticles decreased. Compared with MW 196 kDa and 16 kDa, CM-chitosan nanoparticles of MW 7.5 kDa had higher inhibitory effect. For either sample, the growth inhibition of keloid fibroblast got strengthened with the increase of CM-chitosan nanoparticles concentration from 2 to 200 μg/mL. Particularly, cell amount was no longer affected by the nanoparticles at concentrations above 100 μg/mL, so 100 μg/mL was utilized in the rest of the study.

The effects of CM-chitosan nanoparticles on the proliferation of keloid fibroblast upon different MWs were examined after specific periods (0 d, 1 d, 2 d, 4 d, 6 d). With the increase of the CM-chitosan MW, the growth inhibition of keloid fibroblast got strengthened in the first two days. The trend was reversed progressively in the follow up. The growth inhibition of keloid fibroblast got weakened with the increase of CM-chitosan MW as Fig. 4 showed. Less amount of calcium ions were needed for nanoparticles prepared from high MW CM-chitosan (Table 1). CM-chitosan with various MWs but similar DS had the same functional groups. CM-chitosan nanoparticles with MW 196 kDa showed higher inhibitory effects than those with MW 16 kDa and 7.5 kDa in the first two days. The reason probably was the existence of larger number of free carboxylmethyl groups in high MW CM-chitosan. As the culture time prolonged, CM-chitosan nanoparticles might gradually degrade and be inclined to resemble CM-chitosan solution in properties. Under degradation conditions, the phenomena that low MW CM-chitosan nanoparticles had higher inhibition on the proliferation of keloid fibroblast after four days, which consisted with the previous research [11]. To verify the effect of carboxylmethyl

Table 1 Reaction solvents, typical mean particle sizes and polydispersity index (PI) of CM-chitosan nanoparticles samples (n = 3)

CM-chitosan			CM-chitosan /(mg·mL^{-1})	$CaCl_2$ /(mg·mL^{-1})	Particle size /nm	PI
MW/kDa	DS	DD				
196	0.91	0.77	0.5	1.0	258.7±5.8	0.133
16	0.88	0.81	0.5	2.5	227.1±5.3	0.166
7.5	0.92	0.79	0.5	4.0	189.3±7.2	0.049
6.3	0.94	0.78	0.5	4.5	181.1±6.4	0.163

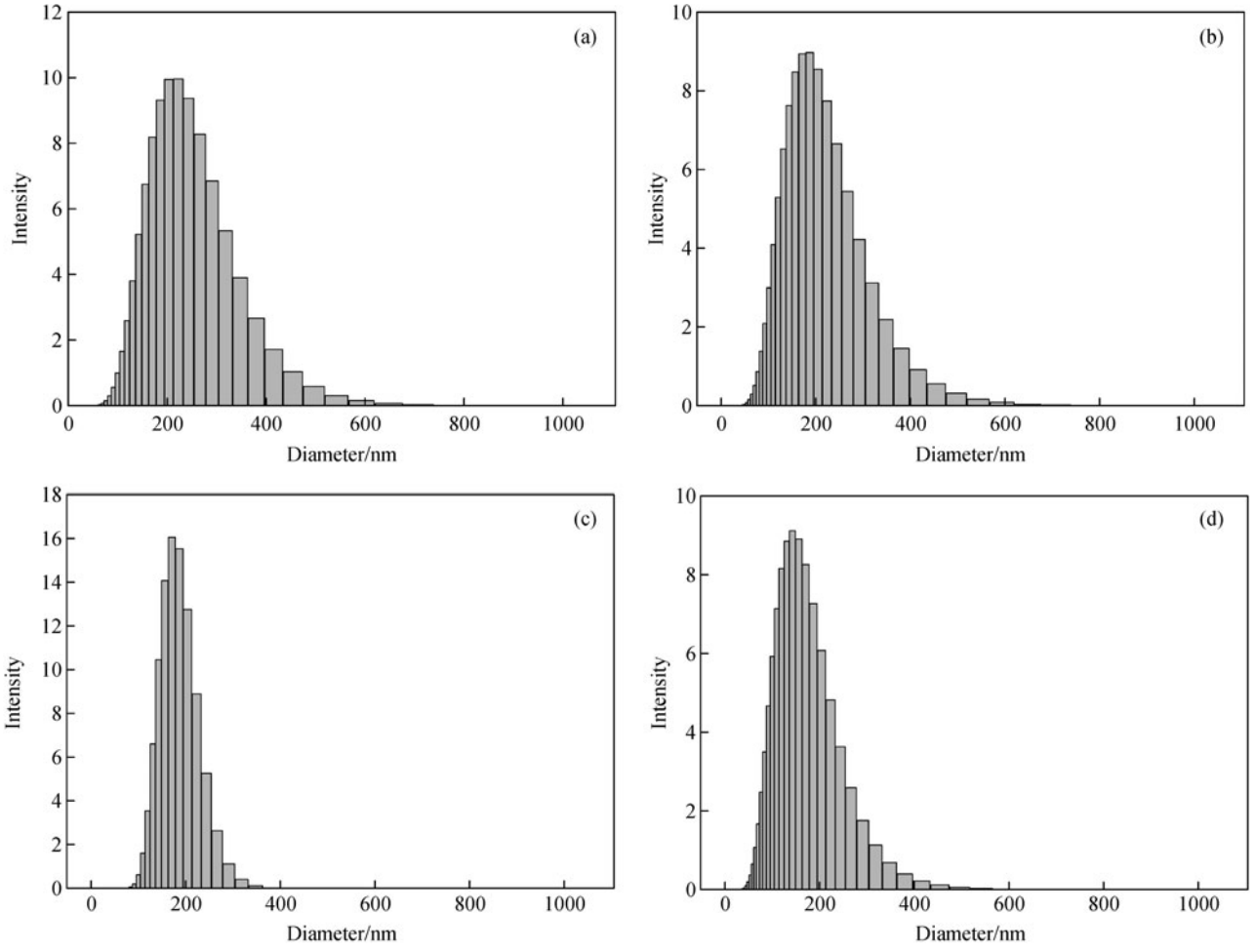

Figure 1 Particle size distribution of CM-chitosan nanoparticles with different MWs (n = 3) (a) 196 kDa; (b) 16 kDa; (c) 7.5 kDa; (d) 6.3 kDa

groups on the growth of keloid fibroblast, the efficacy of nanoparticle and solution form of CM-chitosan were compared and particularly discussed in the following section.

The effects of CM-chitosan nanoparticles with different MWs and CM-chitosan solution on the proliferation of keloid fibroblast were checked after four days of incubation. The keloid fibroblasts were cultured with the medium containing 100 mg sample (CM-chitosan nanoparticles or CM-chitosan solution) I (MW 196 kDa), II (MW 16 kDa) and III (MW 7.5 kDa) per mL culture media, respectively. CM-chitosan solution had stronger inhibitory effects than CM-chitosan nanoparticles with the same MW as shown in Fig. 5. That is because the ionic gelation between Ca^{2+} and negatively charged carboxymethyl groups of CM-chitosan reduced the free carboxylmethyl, which led to smaller amount of free carboxylmethyl groups in CM-chitosan nanoparticles in solution.

The effects of chitosan (MW 5.2 kDa), CM-chitosan solution (MW 6.3 kDa) and CM-chitosan nanoparticles (MW 6.3 kDa) on proliferation of keloid fibroblast after specific periods (0 d, 1 d, 2 d, 4 d, 6 d) of incubation were shown in Fig. 6. CM-chitosan with MW 6.3 kDa was produced by carboxylmethylation of chitosan with MW 5.2 kDa. Chitosan had almost no effects on proliferation of keloid fibroblast in this experiment, but CM-chitosan solution had significant inhibitory effects on proliferation of keloid fibroblast. The result indicated that carboxylmethyl groups of CM-chitosan played an important role in inhibition of proliferation of keloid fibroblast. Compared with CM-chitosan nanoparticles, the growth inhibition of CM-chitosan solution was higher than CM-chitosan nanoparticles in the first four days ($P < 0.05$), but similar on the sixth day (inhibition rate 48.79% for CM-chitosan nanoparticles and 49.69% for CM-chitosan solution, $P = 0.844$). The reason is

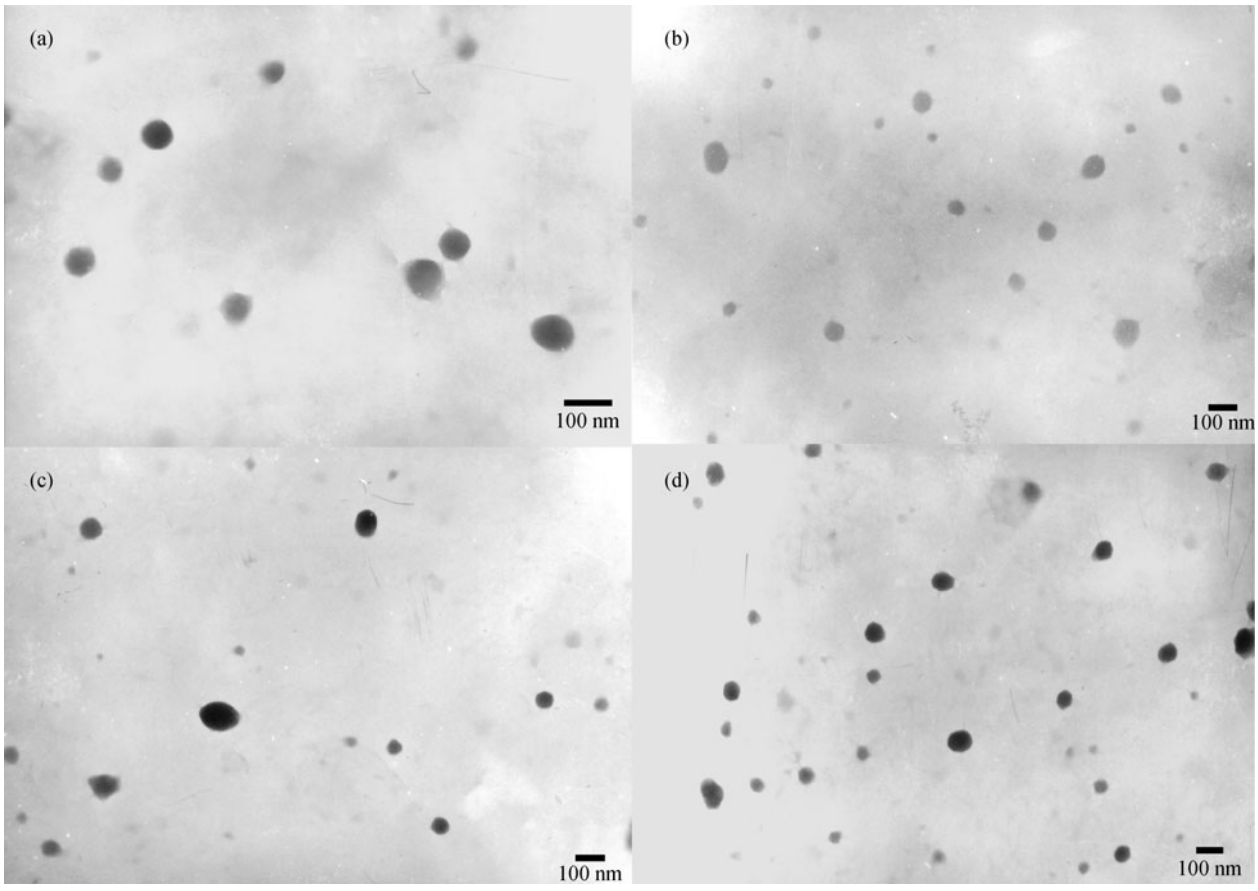

Figure 2 TEM images of CM-chitosan nanoparticles with different MWs (a) CM-chitosan nanoparticles 196 kDa; (b) CM-chitosan nanoparticles 16 kDa; (c) CM-chitosan nanoparticles 7.5 kDa; (d) CM-chitosan nanoparticles 6.3 kDa

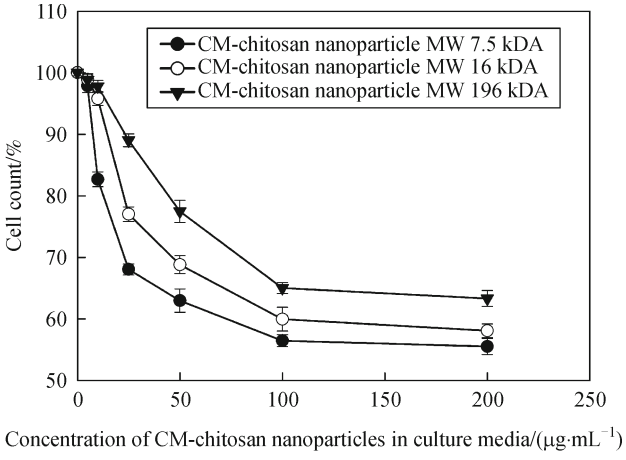

Figure 3 Effects of concentration of CM-chitosan nanoparticles with different MWs on the proliferation of the keloid fibroblast. Cell culture was in media of DMEM contained 10% BCS, 96-well tissue culture plate; initial cell number was 5×10^3 cells per well; 5% CO_2 and 37°C. Cell numbers were determined after four days of incubation by MTT method (n = 6). Control group contained 40 μg/mL $CaCl_2$. Cell count = (OD_{490nm} of test) / (OD_{490nm} of control)

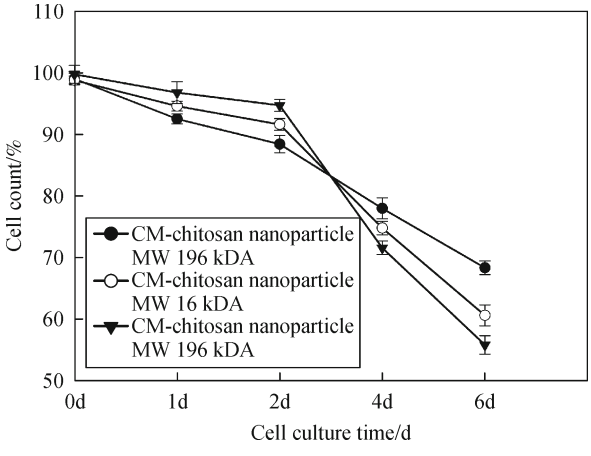

Figure 4 Effects of exposure time of CM-chitosan nanoparticles with different MWs on the proliferation of the keloid fibroblast. Cell culture was in media of DMEM contained 10% BCS, 96-well tissue culture plate; initial cell number was 4×10^3 cells per well, 5% CO_2 and 37°C. Control group contained 40 μg/mL $CaCl_2$. Cell count = (OD_{490nm} of test) / (OD_{490nm} of control). Test Groups contained 100 μg/mL CM-chitosan nanoparticles (n = 6)

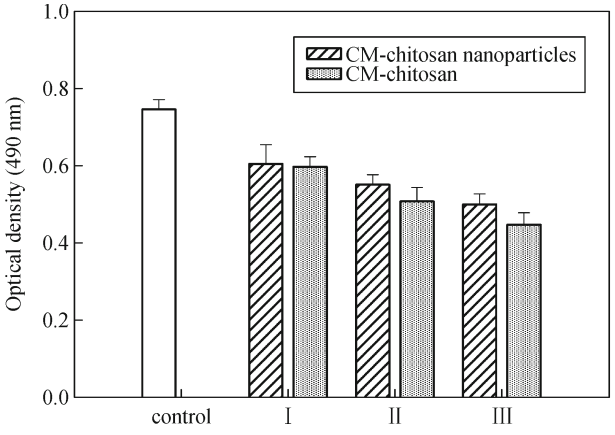

Figure 5 Effects of CM-chitosan nanoparticles and CM-chitosan solution on the proliferation of the keloid fibroblast. (I) MW 196 kDa; (II) MW 16 kDa; (III) MW 7.5 kDa. Cell numbers were determined after four days of incubation by MTT method (n = 6). Cell culture was in media of DMEM contained 10% BCS, 96-well tissue culture plate; initial cell number was 6×10^3 cells per well; 5% CO_2 and 37°C. Control group contained 40 μg/ml $CaCl_2$. Test Groups contained 100 μg/mL CM-chitosan solution or CM-chitosan nanoparticles.

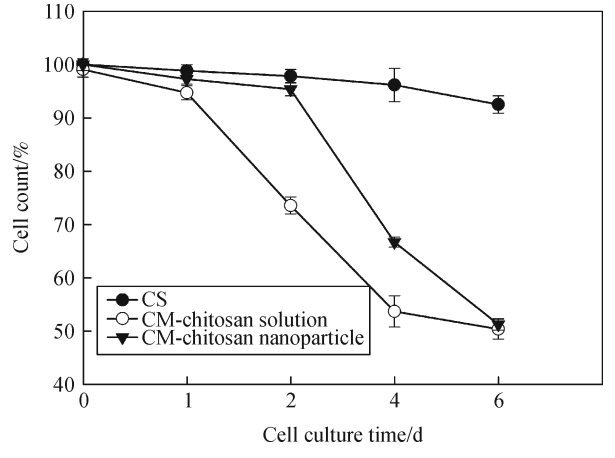

Figure 6 Effects of chitosan, CM-chitosan solution and CM-chitosan nanoparticles on the proliferation of the keloid fibroblast after specific periods (0 d, 1 d, 2 d, 4 d, 6 d) of incubation. Cell culture was in media of DMEM contained 10% BCS, 96-well tissue culture plate, initial cell number was 5×10^4 cells per well, 5% CO_2 and 37°C. Control group contained 40 μg/mL $CaCl_2$. Cell count = (OD_{490nm} of test) / (OD_{490nm} of control). Test Groups contained 100 μg/mL chitosan CM-chitosan solution or CM-chitosan nanoparticles (n = 6)

that as the culture time prolonged, CM-chitosan nanoparticles might gradually degrade and be inclined to resemble CM-chitosan solution in properties.

4 Conclusions

In this study, CM-chitosan nanoparticles were prepared by ionic gelation. The CM-chitosan nanoparticles inhibited the proliferation of keloid fibroblast and the MW 6.3 kDa CM-chitosan nanoparticles had the highest inhibition of 48.79%. Compared with CM-chitosan solution, the inhibition rate of CM-chitosan nanoparticles was lower in prior period and similar with CM-chitosan solution in later period. In contrast with the effects of chitosan, CM-chitosan solution and CM-chitosan nanoparticles on proliferation of keloid fibroblast, Carboxylmethyl groups of CM-chitosan played an important role in inhibition of keloid fibroblast proliferation. Further experimental testing of cell uptake of CM-chitosan nanoparticles and the effect of CM-chitosan nanoparticles on Cytokines growth factors of keloid fibroblast is necessary to clarify the mechanism of CM-chitosan nanoparticles in inhibition of keloid fibroblast proliferation.

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References

1. Gopalakannan, A.; Arul , V., *Aquaculture* **2006**, *255*, 179–187
2. Qin, C.; Du, Y.; Xiao, L.; Li, Z.; Gao, X., *Int. J. Biol. Macromol.* **2002**, *31*, 111–117
3. Qin, C.; Zhou, B.; Zeng, L.; Zhang, Z.; Liu, Y.; Du, M.; Xiao, L., *Food Chem.* **2004**, *84*, 107–115
4. Zheng, L. Y.; Zhu, J. F., *Carbohydr. Polym.* **2003**, *54*, 527–530
5. Lim, S. H.; Hudson, S. M., *Carbohydr. Res.* **2004**, *339*, 313–319
6. Qi, L.; Xu, Z.; Jiang, X.; Hu, C.; Zou, X., *Carbohydr. Res.* **2004**, *339*, 2693–2700
7. Jung, E. J.; Youn, D. K.; Lee, S. H.; No, H. K.; Ha, J. G.; Prinyawiwatkul, W., *Int. J. Food Sci. Technol.* **2010**, *45*, 676–682
8. Kong, M.; Chen, X.; Xue, Y.; Liu, C.; Yu, L.; Ji, Q.; Cha, D. S.; Park, H. J., *Front. Mater. Sci. China* **2008**, *2*, 214–220
9. Ueno, H.; Yamada, H.; Tanaka, I.; Kaba, N.; Matsuura, M.; Okumura, M.; Kadosawa, T.; Fujinaga, T., *Biomaterials* **1999**, *20*, 1407–1414
10. Muzzarelli, R. A. A., *Carbohydr. Polym.* **2009**, *76*, 167–182
11. Chen, X. G.; Wang, Z.; Liu, W. S.; Park, H. J., *Biomaterials* **2002**, *23*, 4609–4614
12. Hjerde, R. J. N., Vårum, K. M.; Grasdalen, H.; Tokura, S.; Smidsrød, O., *Carbohydr. Polym.* **1997**, *34*, 131–139
13. Abdull Rasad, M. S. B.; Halim, A. S.; Hashim, K.; Rashid, A. H. A.; Yusof, N.; Shamsuddin, S., *Carbohydr. Polym.* **2010**, *79*, 1094–1100
14. Dev, A.; Mohan, J. C.; Sreeja, V.; Tamura, H.; Patzke, G. R.; Hussain, F.; Weyeneth, S.; Nair, S. V.; Jayakumar, R., *Carbohydr. Polym.* **2010**, *79*, 1073–1079

15. Shi, X.; Du, Y.; Yang, J.; Zhang, B.; Sun, L., *J. Appl. Polym. Sci.* **2006**, *100*, 4689–4696
16. Anitha, A.; Divya Rani, V. V.; Krishna, R.; Sreeja, V.; Selvamurugan, N.; Nair, S. V.; Tamura, H.; Jayakumar, R., *Carbohydr. Polym.* **2009**, *78*, 672–677
17. Alaish, S. M.; Yager, D. R.; Diegelmann, R. F.; Cohen, I. K., *J. Pediatr. Surg.* **1995**, *30*, 949–952
18. Butler, P. D.; Longaker, M. T.; Yang, G. P., *J. Am. Coll. Surg.* **2008**, *206*, 731–741
19. Jagadeesan, J.; Bayat, A., *Int. J. Surg.* **2007**, *5*, 278–285
20. Tuan, T. L.; Nichter, L. S., *Mol. Med. Today* **1998**, *4*, 19–24
21. Schäcke, H.; Döcke, W. D.; Asadullah, K., *Pharmacol. Ther.* **2002**, *96*, 23–43
22. Chen, X. G.; Park, H. J., *Carbohydr. Polym.* **2003**, *53*, 355–359
23. Sun, G.; Chen, X.; Li, Y.; Zheng, B.; Gong, Z.; Sun, J.; Chen, H.; Li, J.; Lin, W., *Front. Mater. Sci. China* **2008**, *2*, 105–112
24. Shimojoh, M.; Fukushima, K.; Kurita, K., *Carbohydr. Polym.* **1998**, *35*, 223–231
25. Wan, A. C.; Khor, E.; Wong, J. M.; Hastings, G. W., *Biomaterials* **1996**, *17*, 1529–1534
26. Hansen, M. B.; Nielsen, S. E.; Berg, K., *J. Immunol. Methods* **1989**, *119*, 203–210
27. Calvo, P.; Remuñán-López, C.; Vila-Jato, J. L.; Alonso, M. J., *J. Appl. Polym. Sci.* **1997**, *63*, 125–132