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Synthesis and characterization of selenium–chondroitin sulfate nanoparticles☆

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ABSTRACT

A novel selenium–chondroitin sulfate (SeCS) was synthesized by ultrasonic and dialysis method. With characterization by FTIR, XRD and TEM, the SeCS was found to form nanoparticles in distilled water through a self-aggregation progress. The SeCS nanoparticles had sizes between 30 and 200 nm with selenium entrapment efficiency of about 10.1%. The anti-toxin capacity of SeCS nanoparticles was demonstrated through MTT and apoptosis assays in vitro. Results indicated that the SeCS was less cytotoxic to chondrocytes than sodium selenite. In particular, the SeCS could obviously alleviate chondrocyte apoptosis induced by T-2 toxin compared to chondroitin sulfate. These results thus represent an advanced understanding of the properties of SeCS nanoparticles and demonstrate their exciting potential applications in therapy of Kashin–Beck disease (KBD) and osteoarthritis.

1. Introduction

Chondroitin sulfate, a sulfated glycosaminoglycan (GAG) composed of a chain of alternating sugars (N-acetylgalactosamine and glucuronic acid), is usually attached to proteins as part of a proteoglycan. Researchers distinctively identified different chondroitin sulfate with letters, including chondroitin sulfate A (chondroitin-4-sulfate), chondroitin sulfate C (chondroitin-6-sulfate), chondroitin sulfate D (chondroitin-2,6-sulfate), and chondroitin sulfate E (chondroitin-4,6-sulfate) (Levene & La Forge, 1913). A chondroitin chain usually has over 100 individual sugars, each of which could be sulfated at different positions with variable quantities. As we know, chitins and chitosans have already showed a lot of benefits for the patients when they were used in the repairing of bone and cartilage (Muzzarelli, 1993, 2009), while unlike chitins and chitosans, chondroitin sulfate is one major component of extracellular matrix, and plays an important role in maintaining the tissue structural integrity. As part of aggrecan, the chondroitin sulfate is the major component of cartilage and provides much resistance to compression (Baeurle, Kiselev, Makarova, & Nogovitsin, 2009). Loss of chondroitin sulfate will result in osteoarthritis (OA). Because the tightly packed and highly charged sulfate groups of chondroitin sulfate can generate electrostatic repulsion that provides much of the resistance of cartilage to compression, chondroitin sulfate is used for the treatment and recovery of OA and other cartilage damage diseases (Barnhill et al., 2006; Martel-Pelletier, Kwan Tat, & Pelletier, 2010; Monfort, Pelletier, Garcia-Giralt, & Martel-Pelletier, 2008).

Selenium is an essential trace element involved in several key metabolic activities, such as fertility, protection against oxidative damage, regulation of immune and thyroid function and so on (Rayman, 2000; Ryan-Harshman & Aldoori, 2005). In China, the deficiency of selenium in endemic regions is one of the causes for Kashin–Beck disease (KBD), which is a chronic endemic degenerative osteoarthritis (Ren et al., 2007; Yamamuro, 2001; Zou, Liu, Wu, & Du, 2009), similar to osteoarthritis (OA), but associated with different manifestations of cartilage damage (Duan et al., 2010; Mo, 1979; Wang, Guo, Chen, Xu, & Lammi, 2008). Sodium selenite (Na_2SeO_3), a colorless solid and water-soluble selenium compound, is commonly used in the manufacture of colorless glass (Langner, 1999) and as food supplement ingredient. Meanwhile the commercial sodium selenite products (mainly effective elements are sodium selenite and vitamin E) is used for prevention and treatment of KBD and the maximum safety dose of selenium is 400 μg per day (Wilber, 1980).

For long-term, the toxicity of selenium in high concentrations restricts the use of its products in the treatment of KBD and osteoarthropathy or other diseases. Meanwhile, the elemental selenium at nano size showed a potential biological effects with reduced risk of selenium toxicity. (Zhang, Wang, & Xu, 2008; Zhang,

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Gao, Zhang, & Bao, 2001). In order to gain less toxic and more bioactive nano-selenium, ultrasonic and dialysis method were used to prepare an original selenium–chondroitin sulfate (SeCS) nanoparticles. In addition, physiochemical properties of the nanoparticles were evaluated by FTIR, XRD and TEM, furthermore, the anti T-2 toxin behavior was manifested in vitro experiments.

2. Experimental

2.1. Materials

Chondroitin sulfate (CS), with molecular weight of between 3×10^4 Da and 5×10^4 Da, was obtained from Qingdao Greenextract Biology Science & Technology Co., Ltd., China. Ascorbic acid was supplied by Shanghai Shanpu Chemical Co., Ltd., China. Sodium selenite was supplied by Chongqing Yuzhou Fine Chemical Co., Ltd., China. The other chemicals were analytical reagent purchased from Shanghai Chemical Reagent Co., China.

2.2. Synthesis of SCS nanoparticles

0.2 g chondroitin sulfate and 0.1 g sodium selenite with weight ratio of 2:1 were dissolved in 1 ml pure water in a centrifuge tube (solution A) and kept in ultrasonic (40 kHz) for 1 h. 1 g ascorbic acid was dissolved in 20 ml pure water (solution B). Meanwhile, solution A was dialyzed overnight against solution B in a dialysis bag (MWCO: 5000 Da) with constant stirring. The obtained suspension in the dialysis bag was freeze dried to keep the biological activity of selenium.

2.3. Characterization

The Fourier transform infra-red (FTIR) spectra of CS and SeCS were recorded at room temperature on a Nicolet AVATR 360 spectrometer. The sample was grinded with KBr powder and pressed to be a pellet. X-ray diffraction pattern of SeCS, CS, Na_2SO_3 and CS together with Na_2SO_3 were obtained by using Philips PANalytical X'Pert powder diffraction meter with Cu Kα radiation respectively in the range of 5–60° (2θ) at 40 kV and 30 mA. Transmission electron microscopy (H-600, Hitachi, Japan, accelerating voltage 75 kV, magnification 50,000×) was used to investigate the morphology and structure the SeCS nanoparticles.

2.4. Selenium concentration in SeCS

The concentration of selenium entrapped in the SeCS nanoparticles was determined by Atomic-Fluorescence Spectrometry (AFS-2202, Beijing Kchuang Haiguang Instrument Co., Ltd., China). Standard curve method was used to measure selenium levels, according to the following measurement conditions: mobile phase with 5% HCl, resumption of diplomatic pressure at 320 V and lamp current of 60 mA, atomizer height at 10 mm, heating temperature at 200 °C, carrier gas flow rate at 500 ml/min and shielding gas flow rate to 1000 ml/min, reading time 10 s while delay 1 s. We quantified the samples with the standard curve as following: an accurately weighed sample of 11.9 mg plus 1 ml concentrated nitric acid solution, was placed in 1000 ml constant volume capacity bottle with ultrapure water to volume, mixed well, take 2 ml to 500 ml constant volume capacity bottle with ultrapure water, mixed well again, then a 0.5 ml, 0.7 ml solutions were taken into two 15 ml scaled test tubes with stopper separately, plus HCl 2.5 ml (1:1), 10% potassium ferricyanide 0.75 ml, constant volume with ultrapure water 10 ml and mix well in each tube, 30 min later, they were analyzed by AFS following the above measurement conditions.

2.5. In vitro cell toxicity

Chondrocytes from KBD patients were cultured in DMEM/F12 (HyClone, Thermo Scientific, USA), supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, 100 μg/ml streptomycin, and maintained in a humidified atmosphere at 5% CO_2 and 37 °C. Chondrocytes of passaged one were seeded into 96-well plate at 1×10^5/well. The next day, SeCS particle dispersion and Na_2SeO_3 solution with concentration of 0.5 mg/ml were diluted with the culture medium to the same concentration of selenium and used for intervene. Three days later, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Amresco, USA) was used and the absorbance of each well at 490 nm was recorded by using a 96-well plate reader (Bio-Rad Labs). Chondrocytes from three KBD patients were used, and this in vitro experiment was performed in triplets.

2.6. In vitro anti-T-2 toxin behavior

2.6.1. Apoptosis images of chondrocyte by PI florescent staining

The chondrocytes cultured in 50 ml flasks were divided into 3 groups, group 1 as a control, group 2 were added with T-2 toxin (concentration of 20 ng/ml) alone to treat the chondrocytes, group 3 were added with SeCS (200 ng/ml) and T-2 toxin (20 ng/ml). After 5 days, all the samples in three groups are stained with Annexin-V-FITC and PI, detected by fluorescence microscope (Olympus, IX-70, Japan).

2.6.2. Early apoptosis by FCM

Chondrocytes were cultured in 50 ml cell culture flasks (BD, USA) and divided into 4 groups: (A) control, (B) CS solution and T-2 toxin, (C) SeCS solution and T-2 toxin, (D) T-2 toxin alone. Three days later, early chondrocyte apoptosis rates were determined by using Annexin V-FITC Apoptosis Detection kit according to the manufacturers' instructions (KenGEN, Nanjing, China). Cells were digested with trypsin and harvested by centrifugation, washed twice with PBS and dispersed in 400 μl binding buffer at a concentration of at least 1×10^6 cells/ml. Then a total of 4 μl Annexin V-FITC and 4 μl PI were added, the solution were subsequently incubated in the dark for 15 min before transferred into a 5 ml flow cytometry tube. Within an hour, quantitative analysis of apoptotic level was performed using a flow cytometer (Becton Dickinson, Mountain View, CA, USA). The apoptotic percentage of 10,000 cells was determined, and all experiments in this study were performed in triplicate.

3. Results and discussion

3.1. The synthesis and characterization of SeCS

FTIR spectra distinctive absorption bands of CS appeared at 3400 cm^{-1} (—OH, the presence of polysaccharide), 1620 cm^{-1} (>C=O), 1560 cm^{-1} (—NH—C=O, acetyl amino), 1200–800 cm^{-1} (belong to the sulfate base). In addition, commercial chondroitin sulfate is a mixture of chondroitin sulfate A and C whose FTIR spectra are slightly different, for example, 928 cm^{-1} and 852 cm^{-1} are characteristic adsorption peaks of chondroitin sulfate A, while 1000 cm^{-1} and 820 cm^{-1} belong to chondroitin sulfate C (Mathews, 1958). By comparing the FTIR spectra of SeCS and CS (Fig. 1), SeCS shows similar peaks location and relative intensity with that of CS. The peak from 3400 cm^{-1} to 3500 cm^{-1}(—OH) on SeCS is blue shifted, and the peaks between 3200 cm^{-1} and 2500 cm^{-1} indicating the stretching vibration of —OH intramolecular hydrogen bonding is weakened. This phenomenon suggested the intramolecular hydrogen bonding of —OH is reduced in chondroitin sulfate which might help to stabilize the introduced Se by physical adsorption, covalent or other forms of compound key. The number

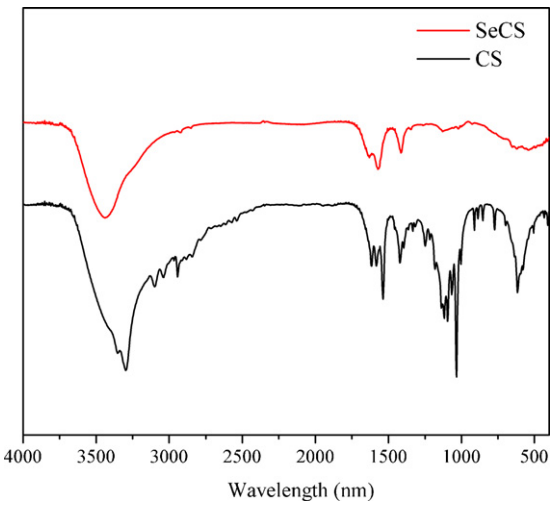

Fig. 1. FTIR spectra of selenium–chondroitin sulfate (SeCS) and chondroitin sulfate (CS).

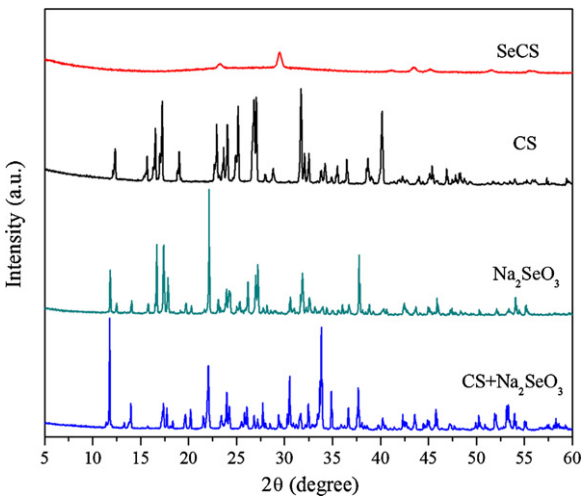

Fig. 2. XRD diffraction spectra of selenium–chondroitin sulfate (SeCS) and chondroitin sulfate (CS).

and intensity of peaks from 1500 cm^{-1} to 500 cm^{-1} (—CH) were decreased, thus exhibited the introduction of Se might affect the infrared plane deformation vibration and the out of plane bending vibration. Meanwhile, the peaks of SeCS infrared spectra near 1000 cm^{-1} was significantly reduced and weakened suggesting the possible substitution of the sulfur atoms in sulfuric acid groups by selenium due to their similar chemical properties.

Compared the XRD diffraction patterns of SeCS and CS (Fig. 2), both the peak positions and intensity of SeCS changed after modification. CS diffraction patterns had a number of strong peaks; each peak represented a crystal surface, while the diffraction pattern of SeCS became smoother as the intensity and number of peaks of the SeCS decreased after modification. We can see that Na_2SeO_3 is a crystal with a number of strong peaks as well. The XRD patterns of physical mixed Na_2SeO_3/CS showed sharp peaks which could be ascribed to Na_2SeO_3 and CS respectively. The different XRD patterns of as-prepared SeCS and the Na_2SeO_3/CS mixture indicates their different crystal structures. Simply mixing of Na_2SeO_3 and CS precursors do not change its structure and properties. This phenomenon further indicated that after modification; the crystal structure of SeCS was changed, which might be due to the introduction of Se through non-covalent or covalent bond or the removal of sulfur in sulfate functional groups. All the above characterization methods we indicated successfully modified the CS with Se.

3.2. The entrapment efficiency of selenium

The standard curve linear equations about relationship of IF (fluorescence intensity) and C (concentration of selenium) was obtained as $IF = 11.362 \times C - 17.174$, correlation coefficient $r = 0.9998$, and entrapment efficiency of selenium was 10.0986%, the sample was analyzed in triplets. If the weight ratio of chondroitin sulfate and sodium selenite at 2:1 was changed, the entrapment efficiency would have changed accordingly, while the ratio of 2:1 could have resulted in better self-assembling morphology of the nanoparticles. The reaction between sodium selenite and ascorbic acid is quite fast in water solution system, because the atomic concentration of ascorbic acid is ten times of sodium selenite, we suppose most of the sodium selenite can be restituted, and by dialysis, the SeCS can be separated from other chemicals with much smaller molecular weight.

3.3. The morphology of SeCS nanoparticles and proposed self-assembling mechanism

5% SeCS nanoparticle solution was prepared by dispersing SeCS powder in distilled water without ultrasonic treatment or ethanol with ultrasonic treatment for 10 min respectively. Then the samples were dripped onto copper grid, and placed under the observation of transmission electron microscope after dried. As shown in Fig. 3, SeCS nanoparticles showed spherical morphology with size between 30 nm and 200 nm both for samples that dispersed in distilled water (A) and ethanol (B). Meanwhile we can see that the outer part of the particles is quite thick when compared with the inner part which is the character of solid spheres or particles.

In the present system, there were hydrophobic long carbon chain and hydrophilic groups such as —NH—CO— and —OH, resulting

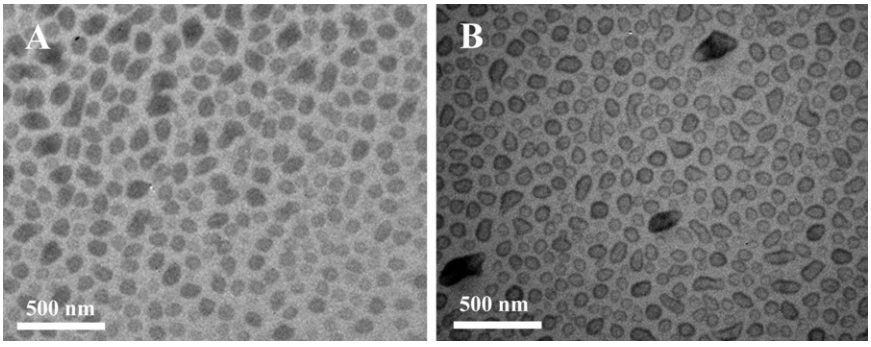

Fig. 3. TEM image of selenium–chondroitin sulfate (SeCS).

in few groups that dissociated in water. Therefore, the electrostatic interactions were not the main factor in the formation of self-aggregated SeCS. The decrease in the intermolecular H-bonding and residual sulfuric acid groups help SeCS to disperse in distilled water to form particles. However, the remaining intermolecular H bond and polysaccharide group in SeCS are not hydrophilic enough to make SeCS dispersive in water and form a homogeneous solution. Therefore, SeCS self-assembled to form nanoparticles in distilled water by the weak intermolecular H bonding between the —NH—CO— and —OH groups and hydrophobic interaction among the hydrophobic moieties in SeCS. As well as the presence of —NH—CO— can provide a site to absorb Se and stabilizing the chemical structure of SeCS, and reduce the adsorption between the selenium atoms. Therefore the elemental selenium in nanometer size is formed as SeCS without aggregation. The sulfate acid might also provide sites for substitute of Se.

3.4. The biological activity of SeCS in vitro

As shown in Fig. 4, the abscissa represents the group number of SeCS and Na_2SeO_3 with the same selenium concentration. The concentrations of selenium in SeCS and Na_2SeO_3 were 10.1% and 45.6%, respectively. The vertical axis is cell viability (%, OD ratio) of SeCS or Na_2SeO_3 compared to control; as the concentration increased, Na_2SeO_3 treated chondrocytes viability decreased, especially from group 6 to group 7, the cell viability decreased apparently; and when compared with SeCS treated groups, cell viability was higher than Na_2SeO_3 treated chondrocytes especially from group 6 to group 7 and 8, almost no decline in cell viability. This manifested

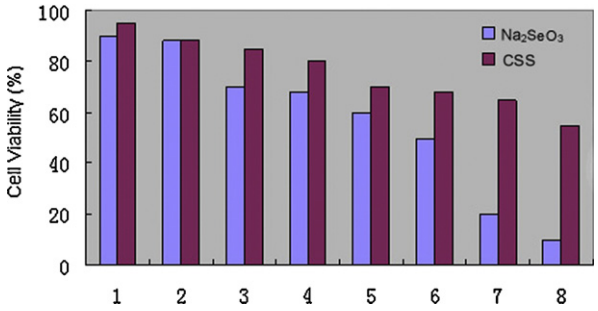

Fig. 4. MTT results of chondrocytes from Kashin–Beck disease patients (KBD) after different treatment (enumerating horizontally from left to right, the following reads as: group 1: Na_2SeO_3 1 ng/ml, SeCS 1.7 ng/ml; group 2: Na_2SeO_3 2 ng/ml, SeCS 3.4 ng/ml; group 3: Na_2SeO_3 5 ng/ml, SeCS 8.5 ng/ml; group 4: Na_2SeO_3 10 ng/ml, SeCS 17 ng/ml; group 5: Na_2SeO_3 20 ng/ml, SeCS 34 ng/ml; group 6: Na_2SeO_3 50 ng/ml, SeCS 85 ng/ml; group 7: Na_2SeO_3 100 ng/ml, SeCS 170 ng/ml; group 8: Na_2SeO_3 200 ng/ml, SeCS 340 ng/ml).

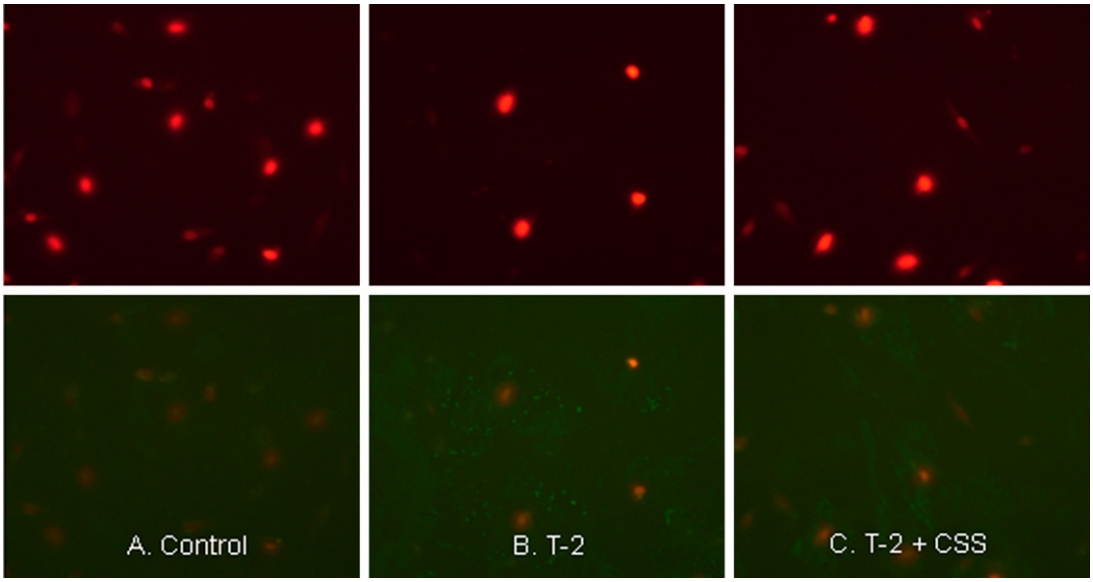

Fig. 5. Florescence images of chondrocytes from Kashin–Beck disease patients (KBD). (A) Control; (B) T-2 toxin; (C) T-2 toxin and selenium–chondroitin sulfate (SeCS).

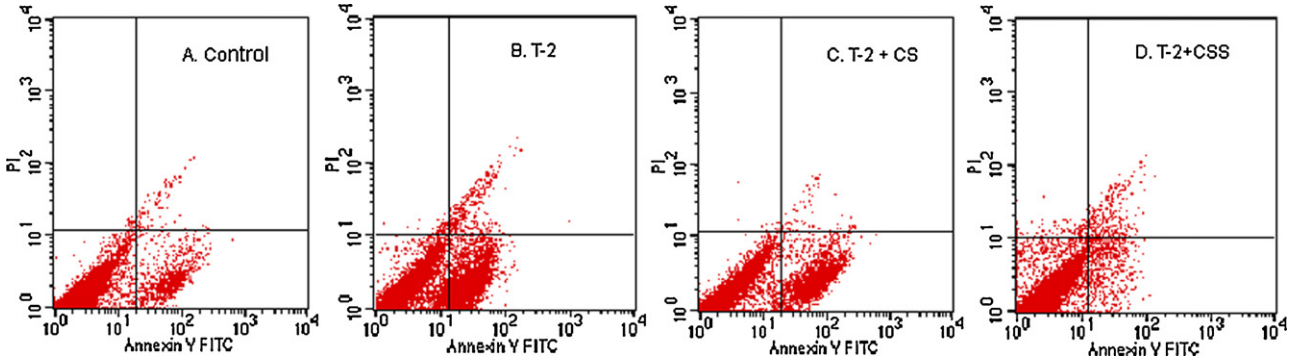

Fig. 6. Early apoptosis of chondrocytes from Kashin–Beck disease patients (KBD) by FCM. (A) Control; (B) T-2 toxin; (C) T-2 toxin and chondroitin sulfate (CS); (D) T-2 toxin and selenium–chondroitin sulfate (SeCS).

that SeCS had higher LD50 and the toxicity of SeCS was much lower than that of Na_2SeO_3.

Seen from Fig. 5 (upper and lower row of green and blue filters under the image slice, respectively), by PI staining, without any intervention, the chondrocytes in control grew favorably with morphology well kept, while after induced by T-2, we could see the obvious decrease of the cell number due to cell apoptosis. Nuclear fragments of apoptotic bodies engulfed by surrounding cells are widely found. Furthermore, when SeCS was used 30 min before T-2, apoptosis could be alleviated to some extent with more chondrocytes survival.

From previous study, 20 ng/ml of T-2 toxin had the highest early apoptosis ratio of $40.2 \pm 2.3\%$ than the others, which was chosen as T-2 toxin concentration. Therefore we divided the chondrocytes into 4 groups: A was control group, B was group with T-2 toxin (20 ng/ml). C treated with CS solution (200 ng/ml) and T-2 toxin (20 ng/ml), and D was added with SeCS nanoparticles dispersion (200 ng/ml) and T-2 toxin (20 ng/ml). Five days later, flow cytometry was used to analyze cell apoptosis. From Fig. 6, we can know that early apoptosis ratio of group B is the largest ($29.4 \pm 1.09\%$), followed by group C ($18.82 \pm 0.96\%$), D ($8.02 \pm 0.79\%$), A ($4.45 \pm 0.36\%$). Group D had relatively large apoptosis ratio than control group, but significantly lower than that of group C and A, which indicated that SeCS had lower cytotoxicity than CS which did not contain selenium. Our result showed the introducing of selenium element through substitution of CS sulfuric acid groups would greatly enhance the anti-toxicity capacity of CS, and the presence of selenium was medically active for KBD treatment.

4. Conclusions

In this study, an original biocompatible selenium–chondroitin sulfate (SeCS) was synthesized by ultrasonic and dialysis with the size ranged from 30 to 200 nm, and could be self-assembled to form nanoparticles through dialysis method. The as-prepared SeCS nanoparticles with high selenium entrapment efficiency, was less toxic to chondrocytes compared with that of sodium selenite which had been previously used for the treatment of KBD. When compared with chondroitin sulfate which has therapeutically effect toward KBD and OA, after modification, SeCS was more effective in reducing the apoptosis effect of T-2 toxin on chondrocytes for KBD patients, which is considered as one of the safety and biocompatibility concerns. These results thus indicated that SeCS nanoparticles could have potential applications in clinical treatment of KBD and osteoarthritis.

Conflict of interest

None declared.

Acknowledgments

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