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Effects of carboxymethyl chitosan on the blood system of rats

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ABSTRACT

Carboxymethyl chitosan (CM-chitosan), a derivative of chitosan, was extensively studied in the biomedical materials field for its beneficial biological properties of hemostasis and stimulation of healing. However, studies examining the safety of CM-chitosan in the blood system are lacking. In this study CM-chitosan was implanted into the abdominal cavity of rats to determine blood indexes at different times and to evaluate the effects of CM-chitosan on the blood system of rats. Coagulation function was reflected by thrombin time (TT), prothrombin time (PT), activated partial thromboplatin time (APTT), fibrinogen (FIB) and platelet factor 4 (PF4) indexes; anti-coagulation performance was assessed by the index of antithrombinIII (ATIII); fibrinolytic function was reflected by plasminogen (PLG) and fibrin degradation product (FDP) indexes; and blood viscosity (BV) and plasma viscosity (PV) indexes reflected hemorheology. Results showed that CM-chitosan has no significant effects on the blood system of rats, and provides experimental basis for CM-chitosan to be applied in the field of biomedical materials.

1. Introduction

Chitosan, a partially deacetylated product of chitin, is the only alkaline polysaccharide in nature [1]. Chitosan has been widely studied in the fields of biomedical materials and tissue engineering [2–4]. However, the widespread use of chitosan has been restricted due to its insolubility at neutral or high pH levels. The abundance of hydroxyl groups and highly reactive amino group or its *N*-acetyl counterpart with strong tendency for intra- and intermolecular hydrogen bonds, results in the formation of linear aggregates and rigid crystalline domains [5]. Through chemical modification, hydrophilic functional groups could be introduced into chitosan, forming a modified chitosan with better solubility and other beneficial biological functions-all of which are properties that are important for the research of chitosan. Carboxymethyl chitosan (CM-chitosan) is an important derivative of chitosan, which is carboxymethylated by chloroacetic acid. It has good water solubility and biocompatibility [6] and has been under investigation for various applications in the absorbable and degradable biomedical materials field, such as plastic surgery [7], drug delivery carriers [8], tissue engineering scaffolds [9] and hemostasis materials [10].

Biological safety is important for the long term development of absorbable biomedical materials. Our laboratory has reported the pharmacokinetics and degradation mechanism of CM-chitosan in rats. Results showed that 90% of the fluorescein isothiocyanate (FITC)-labeled CM-chitosan was absorbed by the abdominal cavity 6 h after intraperitoneal administration, less than 0.5% of the FITC-labeled CM-chitosan remained in abdominal dropsy at 3 d postinjection. It indicates CM-chitosan can be absorbed quickly and efficiently in vivo. And finally, CM-chitosan was discharged from the body through the kidney and urine with molecular weight of less than 45 kDa [11].

Many experiments have demonstrated that CM-chitosan can be applied for haemostasis and the acceleration of wound healing [12–14], however, more studies are necessary to determine whether CM-chitosan has significant effects on the coagulation and fibrinolytic functions of an organism after absorption and degradation in the blood system. The results of such studies will help to determine whether this versatile biomedical material can be applied safely to the human body as a therapeutic product.

In this study, CM-chitosan was synthesized and its molecular weight, carboxymethyl degree and the content of free amino groups were determined. A rat model was used to evaluate the safety of CM-chitosan in the blood system of rats in four aspects: coagulation function, fibrinolytic function, anticoagulation performance and hemorheology.

2. Materials and methods

2.1. Preparation of CM-chitosan

Chitosan (97% deacetylated, Qingdao Biotemed Biomaterial Co. Ltd., PR China) was added in isopropanol solution containing 40%

Abbreviations: CM-chitosan, carboxymethyl chitosan; CD, carboxylmethyl degree; TT, thrombin time; PT, prothrombin time; APTT, activated partial thromboplatin time; FIB, fibrinogen; PF4, platelet factor 4; ATIII, antithrombinIII; PLG, plasminogen; FDP, fibrin degradation product; BV, blood viscosity; BVH, blood viscosity of high shear; BVM, blood viscosity of medium shear; BVL, blood viscosity of low shear; PV, plasma viscosity.

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NaOH (w/v) at −20 °C for 24 h to be alkalized. The alkalized chitosan was collected in a triangular flask, into which chloroacetic acid and isopropanol solutions were slowly added while stirring. The reaction temperature was controlled at no higher than 50.0 °C for 8 h. The precipitant of the reaction was collected and adjusted to neutral with 0.5 M HCl solution, rinsed with 70% ethanol five times and dehydrated with absolute alcohol, respectively. The synthesized CM-chitosan sample was vacuum freeze dried and stored in desiccated condition. To ensure the success of the substitution reaction, FTIR spectra were tested before and after the reaction at 4000–400 cm^{-1} wave numbers.

2.2. Determination of the properties of CM-chitosan

Molecular weight (Mw) of CM-chitosan was determined by gel permeation chromatography (GPC) using high-performance liquid chromatograph (HPLC) on Breeze 1525 system. Two Shodex OHPak SB-806 M columns in series were used as HPLC media. The column was eluted using 0.1 M Na_2SO_4 and 0.02 M phosphate buffer (pH 8.0) at a flow rate of 1.0 mL/min under 35 °C. Dextran standards (M2000000-M180, National Institute for the Control of Pharmaceutical and Biological Products, PR China) with Mw of 2,000,000, 133,800, 41,100, 10,000, 2500, and 180 were used as molecular weight standards. Data were analyzed using the GPCW32 (version 6.11, National Institute for the Control of Pharmaceutical and Biological Products, PR China) and the molecular weight of the samples was calculated based on the standard curve.

Carboxylmethyl degree (C.D.) depends on the average number of carboxylmethyl that bind to each saccharine unit in a CM-chitosan molecule. 0.2 g CM-chitosan was accurately weighed and incinerated at 660 °C for 20 min. The burning residue was collected and added into 0.1 mol/L standard HCl solution while heating. Finally, excess HCl was titrated with 0.1 mol/L standard NaOH solution. C.D. was be calculated from the formula:

$$\text{C.D.} = 240.07 \times (C_{HCL}V_{HCl} - C_{NaOH}\ V_{NaOH})/1000W$$

where C_{HCl} and C_{NaOH} are the concentration (mol/L) of standard HCl solution and standard NaOH solution, respectively, V_{HCl} and V_{NaOH} are the volume (mL) of standard HCl solution and standard NaOH solution respectively, W denotes the weight (g) of sample, and 240.07 is the average Mw of each saccharine in a CM-chitosan molecule.

The content of free amino is defined as the average number of free amino binding to N atom of each saccharine unit in a CM-chitosan molecule. It was measured by potentiometric titration. 0.2 g CM-chitosan was added into 25.0 mL of standard HCl solution at a concentration of 0.1 mol/L and was stirred until the sample was completely dissolved. Excessive HCl was titrated with 0.1 mol/L standard NaOH solution. According to the volume of NaOH between the 2nd and 3rd pH leap, the content of amino was be calculated from the formula:

$$\text{Content of free amino} = (V_3 - V_2) \times C_{NaOH} \times 240.07/W_{sample}$$

where V_2 and V_3 are the volume (mL) of standard NaOH solution used in the 2nd and 3rd pH leap, respectively, C_{NaOH} is the concentration (mol/L) of standard NaOH solution, W_{sample} represents the weight (g) of the sample, and 240.07 is the average Mw of each saccharine in a CM-chitosan molecule.

2.3. Animal experiments

72 Wistar rats of SPF grade (Lukang Pharmaceutical, PR China), each weighing 200 ± 50 g were used in this experiment. 36 male and 36 female rats were kept separately with conditions of moderate temperature, adequate sunlight and good ventilation. Food and water were provided freely. Experiments were conducted 5 d after the rats adapted to their new environment. All rats were randomly divided into experimental and control groups, each group consisting of 18 male and 18 female rats. 2 male and 2 female rats from each group were placed into a unit at each testing time and were tested at 9 intervals: 6 h, 12 h, 1 d, 2 d, 3 d, 4 d, 7 d, 10 d and 14 d. Rats were anaesthetized with an intraperitoneal injection of 3% (w/v) pentobarbital sodium (1 mL/kg), shaved on the abdomen and fixed on their backs to the operating table. The operative area of skin was cleaned with 75% alcohol and surgical scissors and forceps were used to open the abdomen layer-by-layer longitudinally. The cut was as small as possible to reduce pain induced to the rats. CM-chitosan (135 mg/kg) was implanted into the abdominal cavities of rats in the experimental groups, but not for control groups. At the end of the operation, the abdominal cavities of all rats were closed. We confirmed that all animal experiments in this work were carried out in accordance with Directive 2010/63/EU on the protection of animals used for scientific purposes.

2.4. Blood sample collection

At each testing time, a unit of 2 male rats and 2 female rats for each group were anesthetized with an intraperitoneal injection of 3% (w/v) pentobarbital sodium (1 mL/kg). Operative procedures were done as described above. The aorta abdominalis was separated carefully using tampons and blood was collected using 8# disposable venous infusion needles. 1 mL of the blood was placed into centrifugal tubes quiescently to fully separate the serum. The serum was used to determine PLG, FDP and PF4 indexes. 2 mL of the blood was collected into vacutainers containing heparin to determine blood viscosity indexes, which include blood viscosity of low shear (BVL), blood viscosity of medium shear (BVM), blood viscosity of high shear (BVH) and plasma viscosity (PV). Finally, 3 mL of the blood was collected into vacutainers with anticoagulant of citrate sodium to determine prothrombin time (PT), thrombin time (TT), activated partial thromboplatin time (APTT), fibrinogen (FIB) and antithrombinIII (ATIII) indexes.

2.5. Blood analysis

PLG, FDP and PF4 indexes were determined with PLG, FDP and PF4 ELISA kits (Adlitteram Diagnostic Laboratories, Inc., USA) respectively. All other indexes were determined by the affiliated hospital of medical college Qingdao University.

2.6. Statistical analysis

Data points were expressed as means ± standard deviations. Differences between means were analyzed for statistical significance by the paired Student's t-test, using SPSS 13.0 statistical analysis software. P values <0.05 were considered significant.

3. Results and discussion

3.1. Properties of CM-chitosan

The Mw of CM-chitosan prepared in this work was calculated ~107.1 kDa, with C.D. of 95.2% and 88.1% free amino group. FTIR spectra of chitosan and CM-chitosan are shown in Fig. 1. Compared to chitosan, CM-chitosan had main vibration bands at the wave numbers of 1597 cm^{-1}, 1407 cm^{-1} and 1068 cm^{-1}. The absorption peaks at 1597 cm^{-1} and 1407 cm^{-1} refer to the asymmetrical and symmetrical stretching vibration of –COO– respectively, and show that carboxyl could be introduced to chitosan. Furthermore, the new strong absorption peak of CM-Chitosan at 1068 cm^{-1} is attributed to the stretching vibration of the ether linkage, which indi-

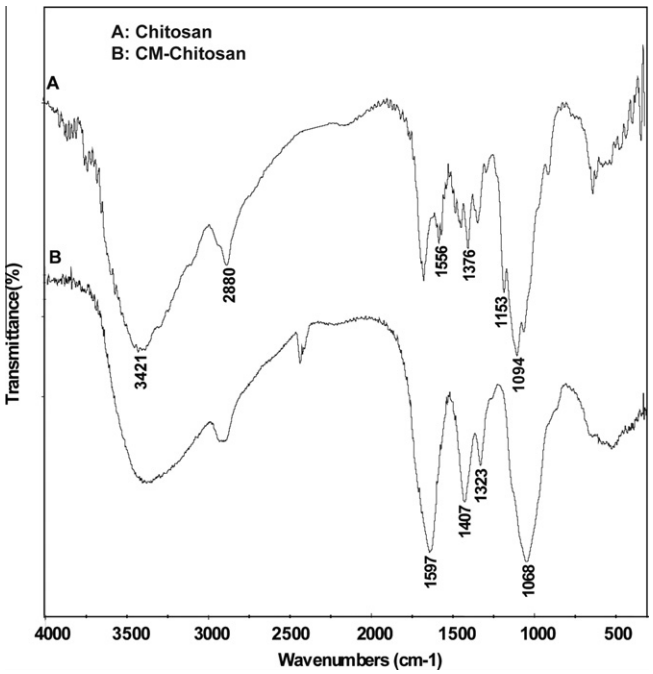

Fig. 1. The FTIR spectra of chitosan and CM-chitosan.

cates that C6–OH was the carboxymethyl substituting site [15,16]. The above results confirm the successful synthesis of CM-chitosan.

3.2. Effects of CM-chitosan on the coagulation function

TT is mainly affected by the contents of fibrinogen and fibrin in plasma and coagulation activity. The length of the TT reflects the level of common pathway of coagulation [17]. PT is an index to describe the exogenous pathway of coagulation and the length of PT reflects the level of prothrombin, fibrinogen and blood coagulation factor V, VII, X in plasma [17]. APTT is activated partial thromboplastin time, the length of which reflects the level of prothrombin, fibrinogen and blood coagulation factor V, X in plasma in endogenous pathway of coagulation [17]. FIB (also named clotting factor I) is the main protein during the process of coagulation [17]. As shown in Fig. 2D, the values of FIB at the time of 6 h for both two groups were slightly lower than those at other testing time, which suggests some fibrinogen transformed into fibrin shortly after the operation. Platelets play an important role in the process of blood coagulation. Platelet factor 4, secreted by the activated platelet, reflects the activated state of platelet [17]. These five indexes are essential to evaluate the effects of CM-chitosan on the coagulation function. Okamoto et al. studied the relationship of platelets aggregation (PA) and blood coagulation, and found that PA does not directly reflect blood coagulation, so assays about platelets were not considered in this work [18].

Results showed that there was no significant ($P > 0.05$) difference between experimental and control groups at each testing time for TT (Fig. 2A), PT (Fig. 2B), APTT (Fig. 2C), FIB (Fig. 2D) and PF4 (Fig. 2E) indexes, which indicates that CM-chitosan had no significant effects on the coagulation function of rats.

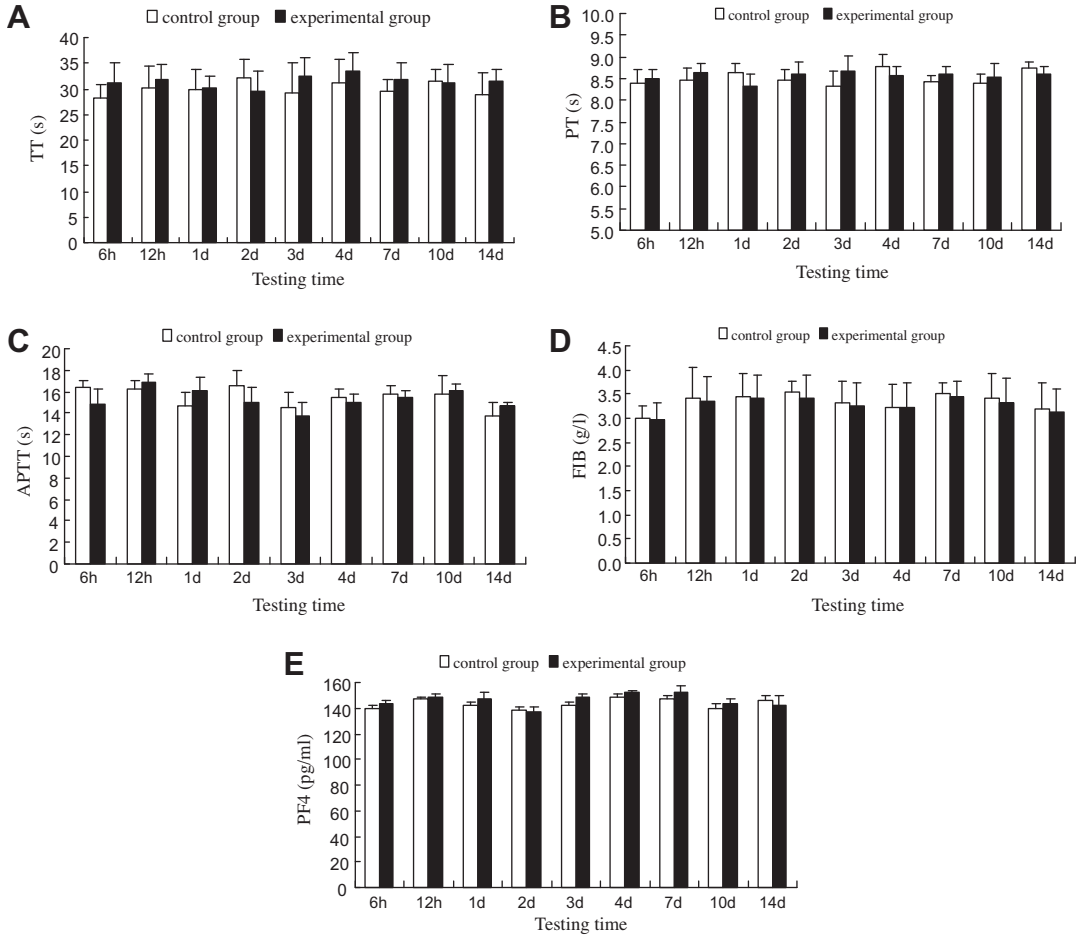

Fig. 2. Effects of CM-chitosan on indexes which reflect coagulation function of rats: (A) TT, (B) PT, (C) APTT, (D) FIB, and (E) PF4. $P > 0.05$ at each testing time for these five indexes.

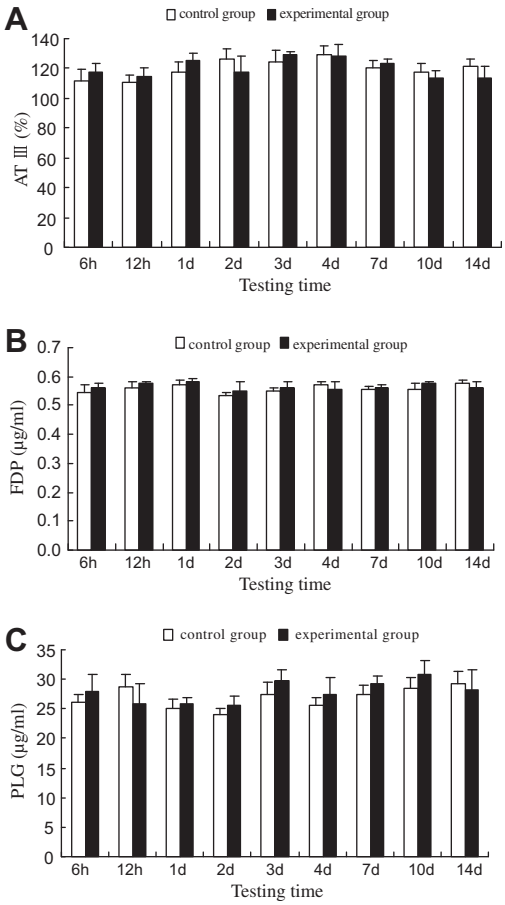

Fig. 3. Effects of CM-chitosan on (A) ATIII, (B) FDP and (C) PLG of rats, $P > 0.05$ at each testing time.

3.3. Effects of CM-chitosan on the anticoagulation performance

ATIII is an important anticoagulation substance for maintaining the balance of coagulation and anticoagulation [19]. In this study, we selected the index of ATIII to evaluate the effects of CM-chitosan on the anticoagulation performance of rats. Results showed that there was no significant difference ($P > 0.05$) for ATIII (Fig. 3A) between experimental and control groups at each testing time, which indicates that CM-chitosan, after being absorbed in vivo, had no significant effects on the anticoagulation performance of rats.

3.4. Effects of CM-chitosan on the fibrinolytic function

FDP is produced through the degradation of fibrin and fibrinogen. The contents of FDP could be enhanced by primary and secondary hyperfibrinolysis [20]. The amount of PLG has been shown to directly reflect fibrinolytic activity [21]. Results showed no significant difference ($P > 0.05$) for the indexes of FDP (Fig. 3B) and FLG (Fig. 3C) between experimental and control groups at each testing time, therefore, CM-chitosan, after it was absorbed in vivo, was considered to have no significant effects on the fibrinolytic function of rats.

3.5. Effects of CM-chitosan on the hemorheology

Amongst all blood viscosity indexes, BVL shows the ability of red blood cell aggregation, where the highest value of BVL, has the most red blood cell aggregation [22]; BVH represents the deformability of red blood cells and platelets, and the ability of transforming fibrinogen into fibrin in plasma. With higher values of BVH, more fibrinogen is transformed into fibrin [22]. The value of PV mainly depends on the contents of plasma protein, such as fibrinogen, lipoprotein and globulin [22]. Our results found that there was no significant difference ($P > 0.05$) for the indexes of BVL (Fig. 4A), BVM (Fig. 4B), BVH (Fig. 4C) and PV (Fig. 4D) between experimental and control groups at each testing time, which indicates that CM-chitosan, after being absorbed in vivo, had no significant effects on the hemorheology of rats.

4. Conclusion

As the application of CM-chitosan continues to increase in the medical field, it is imperative to study its effects on the body. This study focused on the effects of CM-chitosan on the coagulation function, anticoagulation performance, fibrinolytic function and

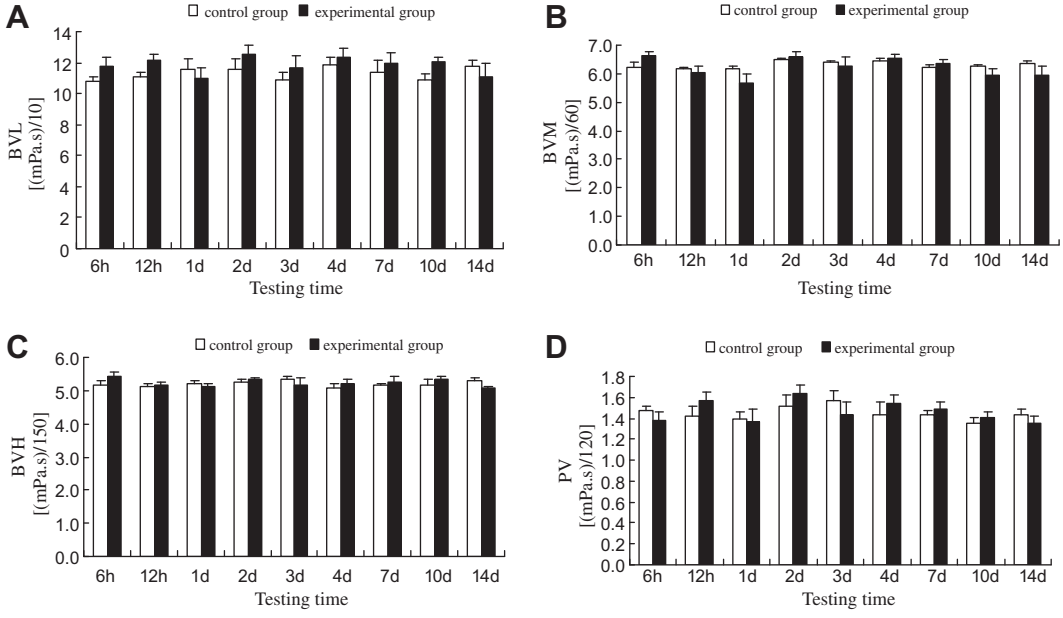

Fig. 4. Effects of CM-chitosan on the hemorheology of rats: (A) BVL, (B) BVM, (C) BVH and (D) PV. $P > 0.05$ at each testing time for all hemorheology indexes.

hemorheology of rats. The safety of CM-chitosan in the blood system was evaluated according to the physiological significance of each index and the results of statistical analysis.

Research results showed no significant difference ($P > 0.05$) for all the indexes reflecting the coagulation function, anticoagulation performance, fibrinolytic function and hemorheology of rats between experimental and control groups at each testing time, which indicates that CM-chitosan had no significant effects on the blood system of rats and can be used safely. This study has provided an important experimental basis for CM-chitosan to be studied and developed further in the medical biomaterials field.

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