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Acute toxicity of high dosage carboxymethyl chitosan and its effect on the blood parameters in rats

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Abstract This experiment was aimed to study whether Carboxymethyl chitosan has acute toxicity and effects on the blood parameters of rats, which were treated with high dosage carboxymethyl chitosan (1350 mg/kg) through a laparotomy. Acute toxicity was first studied and then kinds of blood parameters were detected at different time points after the laparotomy, which contain coagulant parameters (thrombin time, prothrombin time, activated partial thromboplatin time and fibrinogen), anticoagulant parameter (antithrombin III), fibrinolytic parameters (plasminogen and fibrin degradation product) and hemorheology parameters (blood viscosity). Results showed that no acute toxicity was detected and no significant effects were found on the parameters of coagulation, anticoagulation, fibrinolysis or hemorheology of rats after the laparotomy, which indicated that carboxymethyl chitosan has no significant toxicity on the blood system of rats after being absorbed in the abdominal cavity and degraded gradually in the blood. And this study has provided experimental basis for carboxymethyl chitosan to be applied in the field of biomedical materials.

1 Introduction

Chitosan, the amino polysaccharide partly deacetylated from chitin, is the only alkaline polysaccharide in the nature [1]. Owing to its non-toxicity and antibacterial property [2], Chitosan has now been widely studied and applied in the field of medicine [3], biomedical materials [4] and tissue engineering [5]. However, chitosan can only dissolve in weak acid because of large numbers of intra- and intermolecular hydrogen bonds formed by abundant hydroxyl groups and highly reactive amino groups in chitosan itself. Obviously,the poor solubility of chitosan has greatly limited its application. Carboxymethyl chitosan (CM-Chitosan) is a novel derivative of chitosan which is carboxymethylated by chloroacetic acid under alkaline conditions. Besides basic advantages of chitosan, the CM-Chitosan has better water solubility and biological degradability [6], which enable it to be variously applied in the absorbable and degradable biomedical materials field, such as postsurgery healing [7], drug delivery carriers [8], tissue engineering scaffolds [9], anti-adhesion materials [10] and hemostasis materials [11].

Biological safety is crucial to the future study of the absorbable biomedical materials such as CM-Chitosan. Our laboratory has reported the dynamics of metabolism and degradation mechanism of CM-Chitosan in rats. The results showed that CM-Chitosan administrated intraperitoneally can be absorbed quickly, then degraded gradually in abdominal cavity, blood and liver, and finally discharged from the body through urine with molecular weight of less than 45 kDa [12]. It is necessary for the acknowledgement of CM-Chitosan's biological safety to study whether it has effects on relevant systems and organs after being absorbed and degraded gradually in vivo. However, this aspect of research has rarely been reported yet.

Previously, we have already proved that it has no significant effects on the coagulation system and fibrinolytic system of rats which were treated with CM-Chitosan at the dosage of 135 mg/Kg [13]. Since there is no use standard or regulation for CM-Chitosan in any pharmacopoeia all over the world, the dosage of 135 mg/kg was determined by referring to the daily dosage of chondroitin sulfate and glucosamine sulphate which are also polysaccharides with

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similar molecular structure to CM-Chitosan and already have use standard as dietary supplements [14]. The daily dose of chondroitin sulfate and glucosamine sulphate used in human is about 1,500 mg/70 kg [15] which is equivalent to 135 mg/kg in rats according to the dosage conversion among different animals [16]. Therefore the dosage of 135 mg/kg we chose in the previous experiment could be considered the standard dosage for dietary supplements.

But considering the wide research and potential application of CM-Chitosan in medicine and tissue engineering field [7–11], the dosage which is higher than the dosage used in the dietary supplements is the inexorable trend. Therefore in this experiment which could be considered the further experiment of the previous study, we chose the dosage of 1,350 mg/kg which is 10 times the dosage of original experiment. The dosage of 1,350 mg/kg was also determined in accordance with the limited volume of rat's abdominal cavity. Higher gradient which is also commonly adopted like 15 times(2,025 mg/kg) or 20 times(2,700 mg/kg) would definitely too excessive for the abdominal cavity of rats and that is not in accordance with Directive 2010/63/EU on the protection of animals used for scientific purposes. Moreover, no research which used the CM-Chitosan at the dosage higher than 1,350 mg/kg in similar implantation has been reported. Therefore, we chose the dosage of 1,350 mg/kg and regarded it as the high dosage in contrast to the previous standard dosage of 135 mg/kg.

In this study, high dosage CM-Chitosan (1,350 mg/Kg) was administrated intraperitoneally in rats for further evaluation of its biological safety. Acute toxicity was first studied and the effects of high dosage CM-Chitosan on the blood system of rats were studied from four aspects: coagulation function, anticoagulation function, fibrinolytic function and hemorheology.

2 Materials and methods

2.1 Materials and reagents

Sixty adult male Wistar rats (220 ± 10 g body weight) were purchased from Qingdao Laboratory Animal Center. CM-Chitosan, with an average molecular weight of 1.07×10^5 and deacetylation degree of 95.2%, was obtained from Qingdao Biotemed Biomaterial Co. Ltd, China. Fibrin degradation product (FDP) and plasminogen (PLG) ELISA kits were purchased from Adlitteram Diagnostic Laboratories, Inc., USA. All other reagents used were reagent grade.

2.2 Animal experiments

Sixty adult male Wistar rats were housed individually and kept under standard laboratory conditions with free access to food and water. Animals experiments were conducted 5 days later after the rats had adapted to new environment. Sixty rats were randomly divided into experimental group and control group, and each group consisted of 30 rats. Ten testing time points after the laparotomy, 12 h, 1d, 2d, 3d, 4d, 5d, 6d, 7, 10 and 14d, were set in this experiment. Three rats of each group were tested at each time. Rats were anaesthetized with an intraperitoneal injection of 3% (w/v) pentobarbital sodium (1 ml/kg). After shaving and disinfection of the abdomen with 75% alcohol, a middle laparotomy was made on both groups. CM-Chitosan (1,350 mg/kg) was implanted into the abdominal cavity of rats for experimental group while nothing for control group. The abdominal cavities of all rats were closed by suture after the implantation. 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.

2.3 Acute toxicity study

Rats were observed for signs of toxicity individually after the laparotomy until their testing time points. The apparent signs and behaviors of rats, including skin, fur, eyes, appetite and mental status, were recorded. The body mass of each rat was measured individually at 1d, 2d, 3d, 4d, 5d, 6d, 7d, 10d and 14d after the laparotomy. The animal that died during the observation period or at its testing time was necropsied immediately to determine whether there was pathological changes in its viscera.

2.4 Detection of blood parameters

According to the testing time set before, three rats of each group were randomly selected for detection at each testing time. The rats were anesthetized with an intraperitoneal injection of 3% (w/v) pentobarbital solium (1 ml/kg). A midline laparotomy was made on each rat after shaving and disinfection of its abdomen. The intestine was mobilized and positioned laterally, and by use of an operation microscope the abdominal aorta was carefully exposed. Then a 8# disposable intravenous infusion needle was used to collect blood from the aorta. One millilitre blood was taken into centrifugal tube to separate the serum out on standing for PLG and FDP parameters detection; 2 ml blood was taken into heparin tube for blood viscosity parameters detection, including blood viscosity of low shear (BVL) and blood viscosity of high shear (BVH); 3 ml blood was taken into sodium citrate tube for thrombin time (TT), prothrombin time (PT), activated partial thromboplatin time (APTT), fibrinogen (Fg) and antithrombin III (AT-III) parameters detection.

PLG and FDP parameters were detected with PLG and FDP ELISH kits respectively. The rest parameters were detected by the affiliated hospital of medical college QingDao university.

2.5 Statistical analysis

Data were shown as means ± SD (standard deviation). Statistical analysis of data was performed by one-way analysis of variance (ANOVA), and a value of $P < 0.05$ was considered significant (computed by SPSS version 13.0 Software).

3 Results

3.1 Acute toxicity of high dosage CM-Chitosan in rats

All of the rats returned to normal 12 h after the laparotomy. None of the treated animals died during the observation period and no signs of toxicity or behavioural changes were noticed in comparison with the control group. Also there was no obvious pathological change in any of the treated animals at necropsy in the end of their observation period. The body mass results showed that there was no significant difference ($P > 0.05$) between experimental group and control group at each testing time (Table 1). All above results indicated that high dosage CM-Chitosan (1,350 mg/Kg) had no acute toxicity while being absorbed in the abdominal cavity of rats.

Table 1 Body masses of control and treated with 1,350 mg CM-Chitosan per kg body mass in Wistar rats, $P > 0.05$ at each testing time

Time	Group	*N*	Body mass(g)
1d	Control group	27	250.2 ± 5.8
	Experimental group	27	248.8 ± 6.2
2d	Control group	24	254.0 ± 5.5
	Experimental group	24	252.0 ± 5.7
3d	Control group	21	258.1 ± 7.7
	Experimental group	21	258.5 ± 6.6
4d	Control group	18	261.2 ± 7.6
	Experimental group	18	262.3 ± 6.4
5d	Control group	15	268.2 ± 6.3
	Experimental group	15	267.1 ± 6.3
6d	Control group	12	275.7 ± 9.4
	Experimental group	12	275.6 ± 5.1
7d	Control group	9	279.7 ± 8.3
	Experimental group	9	281.0 ± 7.1
10d	Control group	6	293.7 ± 7.5
	Experimental group	6	294.2 ± 8.5
14d	Control group	3	314.0 ± 11.5
	Experimental group	3	319.3 ± 7.0

3.2 Effects of high dosage CM-chitosan on the coagulation parameters

Results showed that there was no significant difference ($P > 0.05$) between experimental group and control group at each testing time for TT (Fig. 1a), PT (Fig. 1b), APTT (Fig. 1c) and Fg (Fig. 1d) parameters respectively, which indicated that high dosage CM-Chitosan had no significant effects on the coagulation function of rats after being absorbed in the abdominal cavity and degraded gradually in the blood.

3.3 Effects of high dosage CM-chitosan on the anticoagulation parameters

Results showed that there was no significant difference ($P > 0.05$) between experimental group and control group at each testing time for AT-III (Fig. 2) parameters, which indicated that high dosage CM-Chitosan had no significant effects on the anticoagulation functions of rats after being absorbed in the abdominal cavity and degraded gradually in the blood.

3.4 Effects of high dosage CM-Chitosan on the fibrinolysis parameters

Results showed that there was no significant difference ($P > 0.05$) between experimental group and control group at each testing time for FDP (Fig. 3a) and PLG (Fig. 3b) parameters, which indicated that high dosage CM-Chitosan had no significant effects on the fibrinolysis functions of rats after being absorbed in the abdominal cavity and degraded gradually in the blood.

3.5 Effects of high dosage CM-Chitosan on the hemorheology parameters

Results showed that there was no significant difference ($P > 0.05$) between experimental group and control group at each testing time for BVH (Fig. 4a) and BVL (Fig. 4b) parameters, which indicated that high dosage CM-Chitosan had no significant effects on the hemorheology functions of rats after being absorbed in the abdominal cavity and degraded gradually in the blood.

4 Discussion

With the wide study and application of chitosan and its derivatives in the medical field, the safety study of them

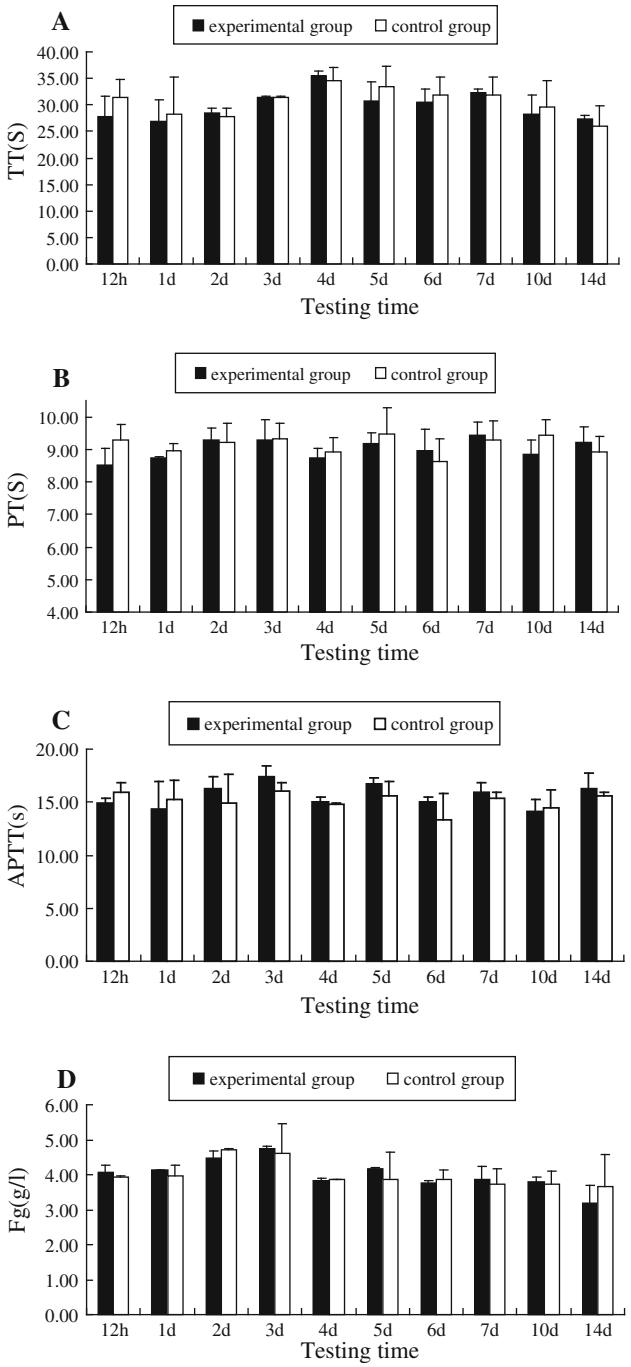

Fig. 1 Effect of high dosage CM-Chitosan on the coagulation parameters: **a** TT, **b** PT, **c** APTT and **d** Fg, $P > 0.05$ at each testing time for these four parameters

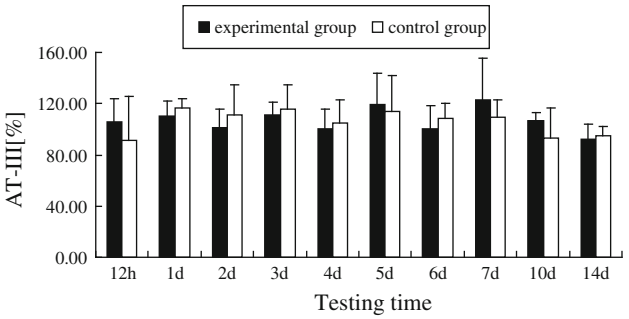

Fig. 2 Effect of high dosage CM-Chitosan on the anticoagulation parameter: AT-III, $P > 0.05$ at each testing time

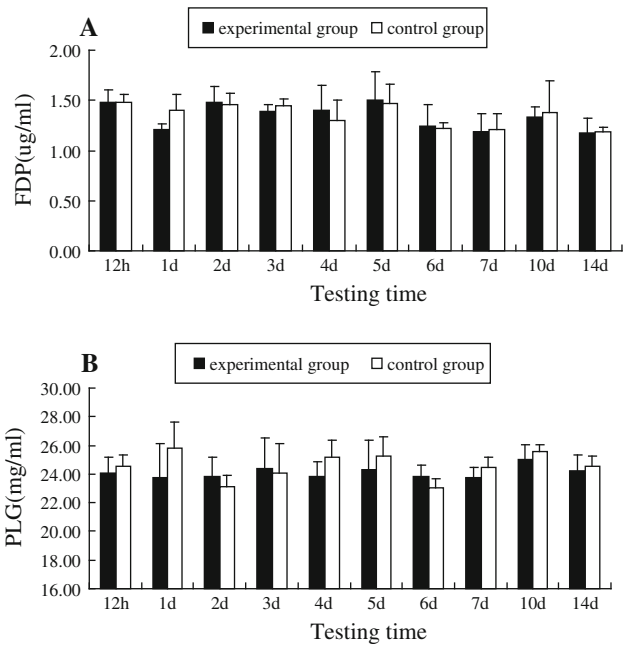

Fig. 3 Effect of high dosage CM-Chitosan on the fibrinolysis parameters: **a** FDP and **b** PLG, $P > 0.05$ at each testing time

becomes more important now. The analysis of the acute toxicity is often the basic step in the safety study of materials. The acute toxicity, which determines a lethal dose(LD_{50}), is commonly considered to be the most important in various forms of toxicity test. However, the oral LD_{50} test has been deleted by Organization for Economic Cooperation and Development (OECD) and the European Union, making the use of alternatives to the oral LD_{50} test obligatory. Therefore in this study, according to the volume of the rat's abdominal cavity, extremely high dosage CM-Chitosan (1,350 mg/Kg) was administrated intraperitoneally in rats to detect its acute toxicity by observing the apparent signs and behaviors as well as the weight changes. The results showed that high dosage CM-Chitosan had no acute toxicity in rats (Table 1), which demonstrates that LD_{50} of CM-Chitosan is greater than 1,350 mg/Kg when administrated intraperitoneally in rats. Thus CM-Chitosan appears to have low toxicity potential and relatively high safety while being absorbed in the abdominal cavity of rats.

The fluctuation of blood biochemical parameters, especially coagulation and fibrinolysis parameters, could reflect the effects that the absorbable biomedical materials have

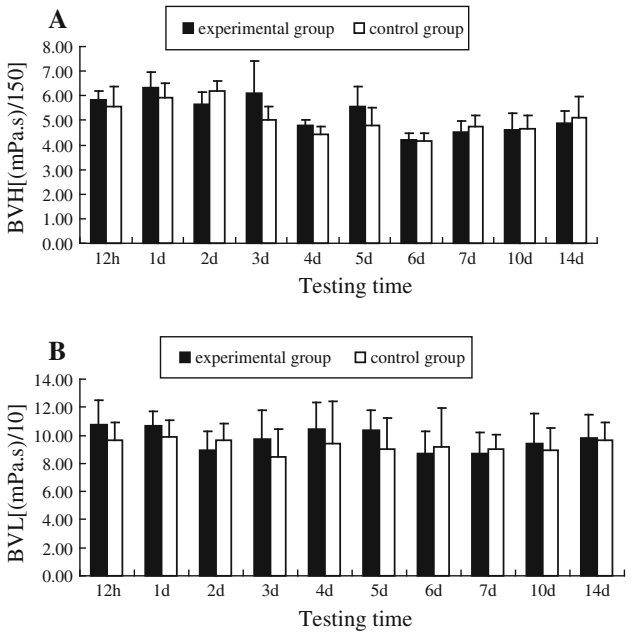

Fig. 4 Effect of high dosage CM-Chitosan on the hemorheology parameters: **a** BVH and **b** BVL, $P > 0.05$ at each testing time

produced on organism after being absorbed and degraded gradually in the blood. So it is crucial for the bio-safety study of absorbable biomedical materials to detect relevant blood parameters.

Coagulation and fibrinolysis parameters are regularly detected in clinical examination to monitor the health state. The PT is a parameter to describe the exogenous pathway of coagulation, and the length of PT reflects the level of prothrombin, fibrinogen and blood clotting factor V, VII, X in plasma; Fg, also called clotting factor I, is synthesized by liver and is the most abundant clotting factor in the plasma, and it usually piles up at prethrombotic state; TT is mainly affected by the contents of fibrinogen and fibrin in plasma, and the length of it reflects the level of common pathway of coagulation; APTT, the length of which reflects the level of prothrombin, fibrinogen and blood clotting factor V and X in plasma, stands for the status of all clotting factors [17]. FDP, the contents of which could be enhanced by primary and secondary hyperfibrinolysis, is produced by the degradation of fibrin and fibrinogen. And the amount of PLG directly reflects the fibrinolytic activity [18]. The abnormality of coagulation and fibrinolysis parameters commonly occurs in vasculopathy [19] or nephropathy [20]. In this study, we found out that high dosage CM-Chitosan had no significant effects on the coagulation and fibrinolysis parameters of rats after being absorbed in the abdominal cavity and degraded gradually in the blood (Figs. 1 and 3). However, it should be noted that the values of Fg for both two groups had an slightly upward trend within the first 3 days and returned to normal in the 4th day, which suggested the normal concentration of fibrinogen after the operation as a result of the wound healing by animals themselves (Fig. 1d).

AT-III is single stranded glycoprotein secreted by hepatocyte and vascular endothelial cells. It can inhibit all the tivating factors in endogenous pathway of coagulation to maintain the balance of coagulation and anticoagulation [21]. The detection of AT-III as anticoagulation parameter is important in the observation of neoplasm metastasis, especially hepatic metastases [22]. In this study, we found out that high dosage CM-Chitosan had no significant effects on the anticoagulation parameters of rats after being absorbed in the abdominal cavity and degraded gradually in the blood (Fig. 2).

Hemorheology parameters are crucial basis for thrombus detecting in clinical examination [23]. BVH represents the deformability of red blood cells and platelets and also the ability of transforming fibrinogen into fibrin in plasma [24]. BVL represents the aggregation capabilities of red blood cells [25]. The abnormality of hemorheology parameters can usually be discovered in angiocardiopathy [26]. In this study, we found out that high dosage CM-Chitosan had no significant effects on the hemorheology parameters of rats after being absorbed in the abdominal cavity and degraded gradually in the blood (Fig. 4).

5 Conclusion

CM-Chitosan has been widely studied and applied in the absorbable biomedical materials field. During the initial research period, we have already proved that it has no significant effects on the coagulation system and fibrinolytic system of rats treated with standard dosage CM-Chitosan(135 mg/Kg).Research results of this experiments showed that no acute toxicity was detected and there is no significant difference ($P > 0.05$) for the parameters of coagulation, anticoagulation, fibrinolytic and hemorheology between experimental group and control group at each testing time after high dosage CM-Chitosan (1,350 mg/kg) being absorbed and degraded, which indicated that CM-Chitosan as a kind of absorbable biomedical materials has no significant toxicity on the blood system of rats after being absorbed in the abdominal cavity and degraded gradually in the blood. It provides important experimental basis for CM-Chitosan to be further studied and developed in medical biomaterials field.

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References

1. Khor E, Lim LY. Implantable application of chitin and chitosan. Biomaterials. 2003;24:2339–49.
2. Zhong ZM, Li PC, Xing RE, Liu S. Antimicrobial activity of hydroxylbenzenesulfonailides derivatives of chitosan, chitosan sulfates and carboxymethyl chitosan. Int J Biol Macromol. 2009; 45:163–8.
3. Mistra S, Gaur U, Ghosh PC, Maitra AN. Tumour targeted delivery of encapsulated dextran-doxorubicin conjugate using chitosan nanoparticles as carrier. J Control Release. 2001;74: 317–23.
4. Muzzarelli RAA, Muzzarelli C. Chitosan chemistry: relevance to the biomedical sciences. Adv Polym Sci. 2005;186:151–209.
5. Yilqor P, Tuzlakoqlu K, Reis RL. Incorporation of a sequential BMP-2/BMP-7 delivery system into chitosan-based scaffolds for bone tissue engineering. Biomaterials. 2009;30:3551–9.
6. Lu GY, Kong LJ, Sheng BY, Wang G, Gong YD, Zhang XF. Degradation of covalently cross-linked carboxymethyl chitosan and its potential application for peripheral nerve regeneration. Eur Polym J. 2007;43:3807–18.
7. Darren J, Costain BS, Renee KMD, Curtis CMD, Vivian C, McAlister MD, Timothy DGL. Prevention of postsurgical adhesions with N,O-carboxymethyl chitosan: examination of the most efficacious preparation and the effect of N,O-carboxymethyl chitosan on postsurgical healing. Surgery. 1997;121:314–9.
8. Chen SC, Wu YC, Mi FL, Lin YH, Yu LC, Sung HW. A novel pH-sensitive hydrogel composed of N,O-carboxymethyl chitosan and alginate cross-linked by genipin for protein drug delivery. J Control Release. 2004;96:285–300.
9. Shi ZL, Neoh KG, Kang ET, Poh CK, Wang W. Surface functionalization of titanium with carboxymethyl chitosan and immobilized bone morphogenetic protein-2 for enhanced osseointegration. Biomacromolecules. 2009;10:1603–11.
10. Juan Z, Clive E, Timothy DGL. Reduction in postoperative adhesion formation, re-formation after an abdominal operation with the use of N,O-carboxymethyl chitosan. Surgery. 2004;135: 307–11.
11. Yang J, Tian F, Wang Z, Wang Q, Zeng YJ, Chen SQ. Effect of chitosan molecular weight and deacetylation degree on hemostasis. J Biomed Mater Res Part B Appl Biomater. 2008;84B: 131–7.
12. Dong W, Han BQ, Feng YL, Song FL, Chang J, Jiang HP, Tang YY, Liu WS. Pharmacokinetics and biodegradation mechanisms of versatile carboxymethyl derivative of chitosan in rats: in vivo and in vitro evaluation. Biomacromolecules. 2010;11:1527–33.
13. Fu DW, Han BQ, Dong W, Yang Z, Lv Y, Liu WS. Effects of carboxymethyl chitosan on the blood system of rats. Biochem Biophys Res Commun. 2011;408:110–4.
14. The United States Pharmacopeia (32nd ed)/National Formulary (27th ed) The United States Pharmacopeial Convention. 2009; 981, 1030–1.
15. Jean YR, Rita D, Lucio CR, Richard LL, Eric L, Olivier B, Giampaolo G, Yves H, Jane ED, Christiane G. Long-term effects of glucosamine sulphate on osteoarthritis progression: a randomized, placebo-controlled clinical trial. Lancet. 2001;357: 251–6.
16. Wei W, Wu XM, Li YJ. Experimental methodology of pharmacology. 4th ed. Beijing: People's Medical Publishing House; 2010. p. 1698.
17. Wang ZY, Li JZ, Ruan CG. Thrombus and hemostasis: basic theory and clinic. 2nd ed. Shanghai: Shanghai Scientific and Technical Publishers; 1992. p. 61–90.
18. Wang HL, Bao CX, Ruan CG. Thrombus and hemostasis assay techniques. Shanghai: Shanghai Scientific and Technical Publishers; 1992. p. 96–117.
19. Christe M, Fritschi J, Lammle B, Tran TH, Marbet GA, Berger W, Duckert F. Fifteen coagulation and fibrinolysis parameters in diabetes mellitus and in patients with vasculopathy. Thromb Haemost. 1984;52:138–43.
20. Alexandre H, Eric R. Role of the coagulation/fibrinolysis system in fibrin-associated glomerular injury. J Am Soc Nephrol. 2004; 15:844–53.
21. Zhang JT. Modern experimental methods in pharmacology. Beijing: Beijing Medical University and China Concordance University Combined Press; 1997. p. 1209.
22. Cicco MD, Matovic M, Balestreri L, Angelis VD, Fracasso A, Morassut S, Coran F, Babare R, Buonadonna A, Testa V. Antithrombin III deficiency as a risk factor for catheter-related central vein thrombosis in cancer patients. Thrombosis Res. 1995;78: 127–37.
23. Wolfgang K, Edzard E. The possible role of hemorheology in atherothrombogenesis. Atherosclerosis. 1992;94:93–107.
24. Slack SM, Cui Y, Turitto VT. The effect of flow on blood coagulation and thrombosis. Thromb Haemost. 1993;70:129.
25. Zhang JF, Paul CJ, Aleksander SP. Red blood cell aggregation and dissociation in shear flows simulate by lattice Boltzmann method. J Biomech. 2008;41:47–55.
26. Zanazzi M, Fatini C, Farsetti S, Rosso G, Caroti L, Sticchi E, Liotta AA, Ricci I, Mannini L, Bertoni E, Abbate R, Salvadori M. Blood rheology and renal transplantation: an intriguing relationship for assessing cardiovascular risk. Transplant Proc. 2010;42: 1383–4.