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Effect of orally administered *N*-(2-hydroxyl) propyl-3-trimethyl ammonium chitosan on the levels of iron, zinc, copper, calcium and lead in mice

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ABSTRACT

The *N*-(2-hydroxyl) propyl-3-trimethyl ammonium chitosan chloride (HPT-chitosan) was prepared by the reaction of chitosan with glycidyl trimethylammonium chloride. The HPT-chitosan (0.75%, w/w) in diet was fed mice for 30 days. The body weights of mice in HPT-chitosan group and control group had no significant difference. The concentrations of Fe, Zn, Cu, Ca and Pb in the organs of the mice were measured by atomic absorption spectrophotometry. The concentrations of 48.24 ± 19.6 mg/kg Fe, 18.16 ± 5.11 mg/kg Zn and 39.2 ± 19.4 mg/kg Ca in mice's livers of HPT-chitosan group were significantly low compared to that of 212.6 ± 112.4 mg/kg Fe, 23.17 ± 4.29 mg/kg Zn and 261.6 ± 152.9 mg/kg Ca in the mice's livers of control group, respectively. Nevertheless, the concentrations of Cu and Pb in mice's livers of HPT-chitosan group did not decrease compared to control group. The HPT-chitosan significantly depressed the levels of Fe, Zn and Ca in the tested mice's livers.

1. Introduction

Chitosan is derived from the deacetylation of chitin, the second most abundant biopolymer isolated from insects, crustaceans such as crab and shrimp as well as from fungi (Jollès & Muzzarelli, 1999). It was composed of D-glucosamine with some degree of *N*-acetyl-D-glucosamine. It has showed favorable biological properties, low toxicity and high susceptibility to biodegradation, mucoadhesive properties (Muzzarelli, 2010; Muzzarelli & Muzzarelli, 2006). Chitosan was approved as a feed additive by FDA in 1983. Chitosan is also widely used as a pharmaceutical excipient (Kumar, Muzzarelli, Muzzarelli, Sashiwa, & Domb, 2004).

In spite of all its superior properties, plain chitosan has a major drawback: its solubility is poor above pH 6. To increase the solubility, chitosan is often be modified by quaternization or carboxylation. *N*-(2-hydroxyl) propyl-3-trimethyl ammonium chitosan chloride can be prepared by a relatively easy chemical reaction of chitosan with glycidyltrimethylammonium chloride.

Quaternized chitosan is potential to be used as a carrier for drug delivery (Mourya & Inamdar, 2009; Xu, Du, Huang, & Gao, 2003). Quaternary ammonium salt of chitosan has the flocculation and antibacterial effect (Qin, Xiao, et al., 2004), and is the potentially suitable for food processing; however, there is no report on the toxicity of quaternized chitosan in vivo. Evaluation of the safety of the modified natural products was very important for their applications in food and drug carrier. The effect of the HPT-chitosan on metal elements in vivo should be considered by researchers and users, which is the focus of the investigation in this paper.

2. Experimental

2.1. Material and chemicals

Chitosan sample was prepared in our laboratory, and the *N*-deacetylation is around 90%. Glycidyl trimethylammonium chloride was prepared in our laboratory as described elsewhere (Doughty and Klem, 1978). Nitric acid (65%, v/v) is guaranteed reagent for metal analysis. Other reagents were of analytical grade. Kunming strain mice (4 weeks old, 18–22 g) were purchased from Hubei Experimental Animal Center (China).

2.2. Preparation and characterization of quaternized chitosan

Purified chitosan (8.0 g) was dispersed in 200 mL isopropyl alcohol for 10 h, and Glycidyl trimethylammonium chloride from 40 g trimethylamine was added at 60 °C. The mixture was stirred at 60 °C for 8 h. The reaction product was ultra-filtered, concentrated, precipitated in acetone, washed with methanol, and dried under vacuum at 40 °C for 48 h to obtain the HPT-chitosan. The degree of substitution (DS) was determined by the reported method (Xiao, Fan, Du, & Huang, 2004).

FT-IR spectrum of the HPT-chitosan sample was recorded with KBr pellets on a Nicolt Impact 380 spectrophotometer. Gel perme-

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ation chromatography (GPC) of the samples was operated by our reported method (Qin, Zhou, et al., 2004).

2.3. Oral acute toxicity in mice

The mice were housed in cages in a temperature-controlled animal room (22–26 °C) for three days and were fasted overnight but given water ad libitum prior to dosage. The animals were divided into three groups of ten males and ten females at random. HPT-chitosan was dissolved in purified water and administered by oral gavage at dose of 5 g/kg body weight. The observation of general health status, toxic symptom and mortality in mice was continued for 7 days after treatment.

2.4. Thirty days feeding study in mice

Twenty healthy Kunming strain female mice were divided into HPT-chitosan group with ten mice, and control group with ten mice. The control group was fed (5/cage) only with basic mice diet: crude fiber (4.80%), crude fat (4.25%), crude protein (19.1%), amino acid (12.13%), Ca (1.08%), 41.77 mg/kg P, 164.1 mg/kg Fe, and 54.12 mg/kg Zn. The HPT-chitosan group was fed (5/cage) with the basic mice diet containing HTP-chitosan (0.75%, w/w). Diets and water were given ad libitum for a continuous period of 30 days. All the animals were observed daily and weighted weekly to check for any signs of toxicity.

The mice were decapitated at the 31st day, and the vital organs of each mouse were excised and observed grossly. Heart, liver, kidneys, spleen, thymus and lung were weighted and the percent ratios of organ to body weight were calculated.

The livers were fixed in situ with 10% formalin in 0.1 mol/L phosphate buffer, dehydrated with alcohol and embedded in paraffin. Thin tissue sections were stained with haematoxylin and eosin, and observed under microscope.

2.5. Measurement of trace elements

Each organ (0.1–0.2 g) was digested with 5.0 mL nitric acid (65%, v/v) and $HClO_4$ (1.0 mL). The left mixture was diluted with triple-distilled water to 10.00 mL. The mineral concentrations were analyzed on a TAS 986 atomic absorption spectrometry (Beijing Purkinje General Instrument Co., Ltd., China), using standard conditions (Fe 0.2 nm, 4.0 mA and 1.6 L/min C_2H_2, Cu 0.4 nm, 3.0 mA and 1.6 L/min C_2H_2, Zn 0.4 nm, 3.0 mA and 1.6 L/min C_2H_2, Ca 0.4 nm, 3.0 mA and 1.6 L/min C_2H_2, Pb 0.4 nm, 2.0 mA and 1.6 L/min C_2H_2) and excitation lamps (Fe 248.3 nm, Zn 213.9 nm, Cu 324.8 nm, Ca 422.7 nm, Pb 283.3 nm). The element contents were expressed as milligrams of the element per kilogram of wet tissue weight (mg/kg organ). Mean values and S.D. were determined by the SPSS program, and the significance of difference was estimated by the standard Students *t*-test. A significant difference was accepted with $P < 0.05$.

3. Results and discussion

The chemical modification of chitosan with glycidyl trimethylammonium chloride resulted in *N*-substitution (Fig. 1). The GPC profiles show that the molecular weight of HPT-chitosan product only has slighter decrease (Fig. 2) than that of the chitosan, indicating that the quaternization of chitosan almost preserve the size of the molecules. The IR spectrum of HPT-chitosan is in accord with the reported spectrum (Chi, Qin, Zeng, Li, & Wang, 2007). The degree of substitution (DS) was 0.78.

Mice administered with HPT-chitosan did not develop any clinical signs of toxicity either immediately or during the post-treatment period even at the dosage of 5 g/kg body weight. The general conditions of all mice were normal. No mortality occurred either immediately or anytime during the 7-day observation period. The oral maximum tolerant dose of the sample was more than 5 g/kg body weight in mice. Thus, the median lethal dose (LD_{50}) for the HPT-chitosan is considered to be greater than 5 g/kg for the mice, indicating that the HPT-chitosan sample was no acute toxicity according to the WHO criteria of acute toxic classifications.

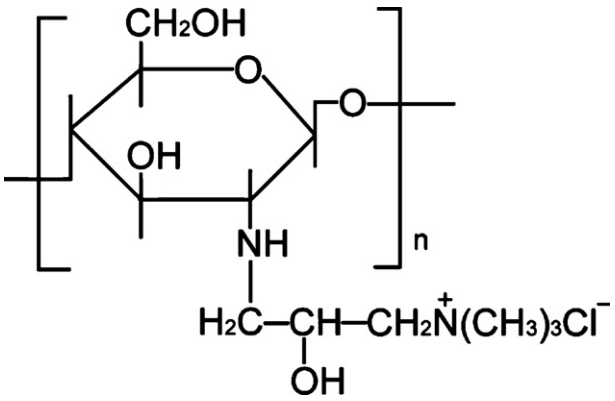

Fig. 1. Structure of HPT-chitosan.

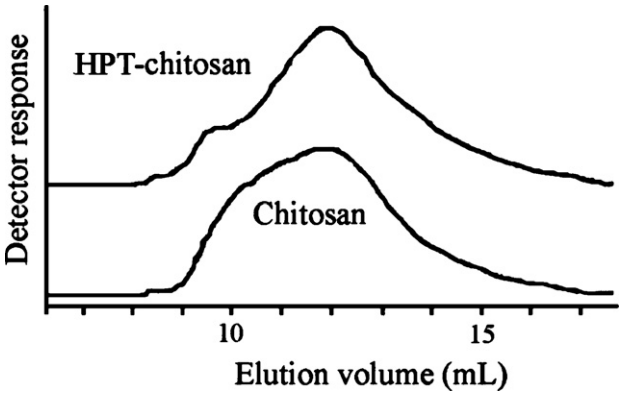

Fig. 2. GPC profiles of chitosan and HPT-chitosan.

Throughout the 30-day dietary feeding study, no deaths were found in the three groups. During the experiment period, no significant abnormality in food intake, feces, hair and behavior were observed.

The mean body weights in each group are presented in Table 1. The sample did not cause any significant difference in body weight compared to control.

As shown in Table 2, there were no marked differences in the group mean absolute or relative weights of liver, lung, heart, spleen and thymus in the HPT-chitosan-treated rats compared to control ($p > 0.05$). The kidney/body weight ratio in HPT-chitosan group was slightly higher than that of control group ($p < 0.05$).

Gross examination at necropsy did not reveal any treatment-related changes for all mice. In histopathology, gross examination did not reveal any abnormalities. Further, on microscopic examination, no treatment-related pathological lesions were evident in the tested livers.

Table 3 listed the concentrations of Fe, Zn, Cu, Ca and Pb in the organs of the mice after feeding these samples for 30 days. The

Table 1
Body weight changes of mice.

Group	Initial (g)	Final (g)
Control	18.62 ± 0.91	32.77 ± 2.27
HPT-chitosan	18.76 ± 1.17	32.84 ± 2.44

Table 2
Organ/body weight ratios of the mice.

Group	Liver	Lung	Heart	Spleen	Thymus	Kidney
Control	5.18 ± 0.42	0.62 ± 0.17	0.46 ± 0.11	0.27 ± 0.07	0.19 ± 0.08	1.17 ± 0.15
HPT-chitosan	5.07 ± 0.41	0.69 ± 0.11	0.45 ± 0.08	0.31 ± 0.10	0.19 ± 0.09	1.32 ± 0.15[a]

[a] $p < 0.05$.

Table 3
The concentrations of Fe, Zn, Cu, Ca and Pb in organs of the mice (mg/kg organ, X ± S, $n = 10$).

		Liver	Spleen	Thymus	Lung	Heart	Kidney
Fe	Control	212.6 ± 112.4	813.0 ± 297.2	653.7 ± 258.6	409.4 ± 219.0	269.9 ± 156.6	264.1 ± 79.5
	HPT-chitosan	48.24 ± 19.6[a]	497.9 ± 305.5[a]	712.3 ± 348.1	398.3 ± 41.8	335.9 ± 150.5[a]	155.8 ± 59.4[a]
Zn	Control	23.17 ± 4.29	45.08 ± 13.43	63.62 ± 33.49	33.87 ± 11.98	21.43 ± 4.02	17.88 ± 2.43
	HPT-chitosan	18.16 ± 5.11[a]	33.54 ± 18.06[a]	68.15 ± 32.37	35.79 ± 19.28	24.75 ± 3.49	24.75 ± 3.49
Cu	Control	2.44 ± 0.39	7.53 ± 2.07	1.48 ± 0.59	3.78 ± 0.92	6.58 ± 1.46	3.28 ± 0.28
	HPT-chitosan	2.49 ± 0.58	7.22 ± 2.60	3.33 ± 1.25[a]	4.92 ± 2.34	6.83 ± 0.83	3.53 ± 0.24[a]
Ca	Control	261.6 ± 152.9	1232.5 ± 616.1	1374.8 ± 551.2	440.8 ± 297.9	142.2 ± 95.7	183.6 ± 120.9
	HPT-chitosan	39.2 ± 19.4[a]	804.8 ± 458.4	1431.5 ± 855.4	464.1 ± 281.1	229.2 ± 131.8	181.7 ± 128.3
Pb	Control	0.84 ± 0.39	1.18 ± 0.33	7.44 ± 2.45	1.45 ± 0.61	0.78 ± 0.30	0.52 ± 0.05
	HPT-chitosan	1.03 ± 0.34	1.07 ± 0.18	6.38 ± 0.40	1.88 ± 0.93	1.24 ± 0.26[a]	0.47 ± 0.05

[a] $p < 0.05$.

concentrations of Fe, Zn and Ca in the livers of HPT-chitosan group significantly decreased ($p < 0.05$) while the levels of Cu and Pb in livers had no significant difference after administration of the HPT-chitosan sample.

The concentrations of the five elements in the spleens of HPT-chitosan group decreased, but only the decrease of Fe and Zn had the statistical significance ($p < 0.05$). The concentrations of the five elements in the lungs of HPT-chitosan group had no significant difference from that of control. The concentrations of the five elements in the hearts of HPT-chitosan group increased, but only the increase of Fe and Pb had the statistical significance ($p < 0.05$). The level of Cu increased in the thymuses of HPT-chitosan group, and the concentrations of Fe, Zn, Ca and Pb had no significant difference from that of the control. The levels of Fe, Ca and Pb in the kidneys of HPT-chitosan group had no significant difference from that of control group while the concentration of Fe decreased and the level of Cu slightly increased.

Essentiality of inorganic elements is not always easy to prove, but those considered to be essential for normal body functions include (i) the major elements, Na, K, Ca, Mg, P and Cl, (ii) the trace elements, Fe, Zn, Cu, Co, Mo, Se and Cr, (iii) the 'newer' trace elements, As, Pb, Li, Ni, Si, V, F and Sn (Fairweather-Tait & Hurrell, l996). The mineral absorption is mainly in the intestine, and the liver plays a major role in metabolism.

Mineral absorption in the upper part of intestine is often incomplete, and apparent absorption averages between 20 and 50%. This property is chiefly due to the presence of phytic acid in dietary fibers that reduce intestinal absorption of many metals. The negatively charged phytate ions with six phosphate groups extending from the central inositol ring can form strong insoluble complexes with cationic minerals in the gastrointestinal tract, hence possibly to alter mineral bioavailability. Previous studies showed that phytate is an efficient inhibitor of absorption of essential dietary minerals such as Ca, Zn and Fe (Hallberg, Rossander, & Skanberg, 1987; Harland & Morris, 1995; Torre, Rodriguez, & Saura-Calixto, 1991).

The intestinal microflora can express a phytase activity, which hydrolyzes the phytate (Miyazawa, Iwabuchi, & Yoshida, 1996; Wise & Gilburt, 1982; Yoshida, Shinoda, Kawaai, Iwabushi, & Mutai, 1985). The phytate-hydrolyzing activity has been demonstrated in the small intestines of rats and humans (Bitar & Reinhold, 1972; Davies & Flett, 1978), and the large intestine of rats (Delzenne, Aertssens, Verplaetse, Roccaro, & Roberfroid, 1995; Trinidad, Wolever, & Thompson, 1993). The fermentation produces short-chain fatty acids such as acetic, propionic and butyric acid and lowers cecal pH. The fermentation process produces an increase in mineral solubility, and counterbalance the inhibitory effect of phytate on mineral absorption (Lopez et al., 1998). Mineral absorption is increased by phytase supplementation (Kies, Kemme, Sebek, Diepen, & Jong, 2006).

The HPT-chitosan shows perfect solubility in water of pH 1–14. HPT-chitosan has the microbiostatic effects against the microbe, which maybe affect the production of phytase and mineral absorption. In this paper, the dietary HPT-chitosan (0.75%, w/w) significantly depressed the levels of Fe, Zn and Ca in livers of mice. Our previous study showed that the dietary chitosan (1.05%, w/w) did not depress the levels of Fe, Zn and Cu in mice (Zeng, He, Wang, Li, & Xu, 2008). The plain chitosan almost has no inhibitory effect against the microbe above pH 6.5 due to its poor solubility (Qin et al., 2006).

The essential level of Cu is much lower than the levels of Fe, Zn and Ca in mice. Cu is relatively well absorbed, and the endogenous Cu loss is low (Turnlund, 1988). The content of Cu in the base diets is enough to maintain the balance of Cu in body. The dietary phytate appears to have not negative effect on its absorption (Cherydn, 1980; Davies & Nighingale, 1975; Lee, Schroeder, & Gordon, 1988). Pb is more insoluble than Ca, Fe and Zn at physiological pH. Pb is a widespread natural and occupational environmental pollutant. The level of Pb is very low in mice, and gastrointestinal lead absorption is depressed by the dietary calcium (Varnai et al., 2004). The dietary phytate appears to have no great effect on its absorption. Thus, HPT-chitosan did not decrease the levels of Cu and Pb in livers of mice.

4. Conclusion

In this study, the orally administered HPT-chitosan was found to depress the levels of Fe, Zn and Ca in the livers of mice. These effects are different from that of chitosan in our previous study. Therefore, further studies are needed to investigate deeply the effect of the HPT-chitosan on the absorption of metal elements in vivo, prior to their oral administration.

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