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Effect of dietary carboxymethyl chitosans on the levels of iron, zinc and copper in mice

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ABSTRACT

Three carboxymethyl chitosan samples were prepared by the carboxymethylation of chitosans with different molecular weights (Mw). These samples (0.75%, w/w) in diet were fed mice for 30 days. Afterwards no pathological symptoms, clinical signs or deaths were observed. The body weights of mice in carboxymethyl chitosan groups and control group showed no significant difference. These carboxymethyl chitosan samples did not decrease the level of copper in the tested mice's organs. However, the level of iron in the tested mice's livers and lungs, and the level of zinc in the tested mice's livers, lungs, hearts, spleens, thymuses and kidneys were significantly decreased by oral administration of these samples.

1. Introduction

The metals Fe, Cu and Zn have been recognized as essential elements for over 60 years, which implement multiple functions in the organism (Forbes & Erdman, 1983). Changes in the levels of trace elements can damage tissue severely and cause malfunctions. Levels of the elements, Zn, Cu, and Fe, are important indicators of the condition of the tissue (Prasad, 1978).

Chitosan, derived from crustacean or fungal chitin, is a biodegradable, biocompatible and almost non-toxic polymer. It was composed of D-glucosamine with some degree of *N*-acetyl-D-glucosamine. Chitosan has a wide range of potential applications in the areas of medicine, drug delivery, water treatment, membranes, hydrogels, adhesives, biosensors, functional foods, and food packaging (Kumar, Muzzarelli, Muzzarelli, Sashiwa, & Domb, 2004). Chitosan was approved as a feed additive by FDA in 1983.

Some related applications of native chitosan are limited by its insolubility in water with pH higher than 6. To improve the solubility of chitosan, chemically modified chitosans including carboxymethyl chitosan have been prepared. The carboxymethyl chitosan has both $-NH_2$ and –COOH, which have stronger chelating ability with metal cations than the carboxymethyl cellulose and carboxymethyl starch. Both carboxymethyl cellulose and carboxymethyl starch with –COOH have been used as food additives for decades. However, there are few reports on the toxicity of carboxymethyl chitosan as a food additive (Ramesh, Viswanatha, & Tharanathan, 2004). Evaluation of the safety of the modified natural products was very important for their applications in food and feed. The effect of dietary carboxymethyl chitosans on trace 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

Crude chitosan HCS (Mw 12.2×10^4, DD 90%) was supplied by Golden-shell Biochemical Co. LTD, China. The other chitosan samples MCS (Mw 3.7×10^4), LCS (Mw 3×10^3) and chitooligomer sulfate SLCS (Mw 3×10^3) were prepared in our laboratory (Qin et al., 2004). 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 N,O-carboxymethyl chitosans

The carboxymethyl chitosans HCMCS, MCMCS and LCMCS were prepared from HCS, MCS and LCS, respectively (Muzzarelli, Lough, & Emanuelli, 1987).

50% (w/w) NaOH (80 g) was added into the mixture of chitosan (10 g) and isopropanol (100 ml) under agitation in 1 h. The alkaline slurry was stirred for an additional 30 min. Monochloroacetic acid (45 g) in isopropanol (100 ml) was added in 2 h at 60 °C, and heated at 60 °C for an additional 3 h. The mixture was filtered, and the solution was adjusted to the neutral pH by adding acetic acid.

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Table 1
Body weight changes of mice.

Group	Initial (g)	Final (g)
Control	18.62 ± 0.91	32.77 ± 2.27
HCMCS	19.57 ± 0.83	32.62 ± 2.59
MCMCS	19.98 ± 1.45	33.05 ± 2.69
LCMCS	19.51 ± 1.29	33.82 ± 3.24
SLCS	19.05 ± 0.78	34.11 ± 2.87

Ethanol (200 ml) was added into the solution, which resulted in a white precipitate. The precipitate was filtered and washed with the solution of MeOH/H_2O (50%, v/v). The precipitate was collected after drying at 60 °C in vacuum to give the carboxymethyl chitosan product (CMCS).

FT-IR spectra of the CMCS samples were recorded with KBr pellets on a Nicolt Impact 380 spectrophotometer. These CMCS samples showed characteristic absorptions at 1735 cm^{-1} due to the –COOH group, confirming a successful carboxymethylation (Nishimura, Nishi, & Tlkure, 1986).

The degree of substitution (DS) value of each CMCS sample was estimated from potentiometric titration (Chen & Park, 2003). The samples were dissolved in 0.1 mol/L hydrochloric acid (50 ml) in the presence of 0.1 mol/L sodium chloride and titrated with 0.1 mol/L sodium hydroxide. The alkalimetric curves were recorded on a pH meter. The DS of carboxymethyl chitosan HCMCS, MCMCS and LCMCS were 64.7, 63.1 and 60.5%, respectively.

The intrinsic viscosity [η] of each CMCS sample in 0.1 mol/L NaCl aqueous solution at 30 °C was measured by using a Ubbelodhe viscometer (Chen, Du, Wu, & Xiao, 2002), and the viscosity-average molecular weight (M_η) of the samples was calculated according to the Mark–Houwink equation $[\eta] = KM^\alpha$ with $\alpha = 1.0$ and $K = 7.92 \times 10^{-5}$ (Nishimura et al., 1986). The M_η of HCMCS, MCMCS and LCMCS were 9.3×10^4, 2.1×10^4 and 3×10^3, respectively.

2.3. Oral acute toxicity in mice

The mice were housed in cages in a temperature-controlled animal room (22–26 °C) for 3 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. CMCS was dissolved in purified water and administered by oral gavage at doses 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

Forty healthy Kunming strain female mice were divided into three carboxymethyl chitosan groups, one chitooligomer sulfate group and one control (10 $group^{-1}$, 5 $cage^{-1}$). The chitosan derivate groups were fed with diets containing the sample (0.75%, w/w), 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. 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) and excitation lamps (Fe 248.3 nm, Zn 213.9 nm, Cu 324.8 nm). The element contents were expressed as micrograms of the element per gram of wet tissue weight (μg/g organ). Mean values and S.D. were determined by the SPSS program, and the significance of difference was estimated by the standard Student's *t*-test. A significant difference was accepted with $P < 0.05$.

3. Results

3.1. Oral acute toxicity in mice

Mice administered with HCMCS, MCMCS, LCMCS and SLCS 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 these samples was more than 5 g/kg body weight in mice. Thus, the median lethal dose (LD_{50}) for the carboxymethyl chitosan is considered to be greater than 5 g/kg for the mice, indicating that these carboxymethyl chitosan samples were no acute toxicity according to the WHO criteria of acute toxic classifications.

3.2. Thirty days feeding study in mice

Throughout the 30-day dietary feeding study, no deaths were found in all 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 four samples did not cause any significant difference in body weight in comparison with the control.

As shown in Table 2, the three carboxymethyl chitosan samples had no significant effect on the weight of liver, lung, heart and kidney. The thymus/body weight ratios and spleen/body weight ratios of mice increased after 30-day dietary feeding of HCMCS and SLCS.

Table 2
Organ/body weight ratios of the mice (g/100 g body weight).

	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
HCMCS	5.74 ± 0.38	0.66 ± 0.15	0.51 ± 0.07	0.39 ± 0.09[a]	0.31 ± 0.07[a]	1.25 ± 0.16
MCMCS	5.19 ± 0.44	0.62 ± 0.13	0.46 ± 0.03	0.31 ± 0.09	0.30 ± 0.09[a]	1.19 ± 0.12
LCMCS	5.63 ± 0.37	0.68 ± 0.19	0.50 ± 0.08	0.31 ± 0.08	0.20 ± 0.06	1.28 ± 0.15
SLCS	5.63 ± 0.33	0.61 ± 0.14	0.50 ± 0.05	0.38 ± 0.13[a]	0.27 ± 0.05[a]	1.16 ± 0.12

[a] $p < 0.05$.

Table 3
The Cu level in organs of the mice (μg/g organ, $X \pm S$, $n = 10$).

	Liver	Lung	Heart	Spleen	Thymus	Kidney
Control	2.44 ± 0.39	3.78 ± 0.92	6.58 ± 1.46	7.53 ± 2.07	1.48 ± 0.59	3.28 ± 0.28
HCMCS	3.00 ± 1.34	3.24 ± 1.06	6.24 ± 1.06	6.05 ± 1.68	1.60 ± 0.34	3.44 ± 0.50
MCMCS	2.88 ± 0.59	3.50 ± 1.17	6.38 ± 0.34	7.48 ± 2.52	1.71 ± 0.58	3.61 ± 0.42
LCMCS	2.38 ± 0.48	3.47 ± 1.40	5.82 ± 0.73	6.89 ± 1.96	1.54 ± 0.38	3.00 ± 0.25
SLCS	3.11 ± 0.39[a]	3.44 ± 0.89	6.16 ± 0.64	4.81 ± 1.48	2.21 ± 0.39	3.78 ± 0.48[a]

[a] $p < 0.05$.

Table 4
The Fe level in organs of the mice (μg/g organ, $X \pm S$, $n = 10$).

	Liver	Lung	Heart	Spleen	Thymus	Kidney
Control	212.6 ± 112.4	409.4 ± 219.0	269.9 ± 156.6	813.0 ± 297.2	653.7 ± 258.6	264.1 ± 79.5
HCMCS	111.9 ± 65.4[a]	146.2 ± 41.8[a]	182.5 ± 84.1	363.1 ± 170.2[a]	686.1 ± 490.6	203.9 ± 122.2
MCMCS	110.0 ± 36.4[a]	127.1 ± 69.9[a]	179.5 ± 75.1	890.9 ± 102.8	909.7 ± 628.9	259.0 ± 127.5
LCMCS	147.9 ± 67.4[a]	220.1 ± 86.0[a]	180.9 ± 121.5	968.7 ± 423.8	1024.1 ± 492.7[a]	210.0 ± 122.1
SLCS	226.7 ± 139.1	473.1 ± 250.3	311.1 ± 156.1	528.6 ± 164.1[a]	372.3 ± 238.9[a]	131.8 ± 82.3[a]

[a] $p < 0.05$.

Table 5
The Zn level in organs of the mice (μg/g organ, $X \pm S$, $n = 10$).

	Liver	Lung	Heart	Spleen	Thymus	Kidney
Control	23.17 ± 4.29	33.87 ± 11.98	21.43 ± 4.02	45.08 ± 13.43	63.62 ± 33.49	17.88 ± 2.43
HCMCS	10.05 ± 3.53[a]	11.11 ± 3.34[a]	11.76 ± 3.99[a]	15.42 ± 6.51[a]	20.02 ± 3.74[a]	8.25 ± 2.61[a]
MCMCS	11.91 ± 3.45[a]	14.77 ± 8.11[a]	12.71 ± 2.39[a]	19.37 ± 6.67[a]	21.02 ± 9.24[a]	9.09 ± 3.57[a]
LCMCS	9.59 ± 2.54[a]	14.21 ± 6.54[a]	9.49 ± 2.45[a]	20.01 ± 6.67[a]	26.75 ± 11.7[a]	8.79 ± 2.09[a]
SLCS	22.89 ± 4.03	25.48 ± 10.18	21.91 ± 2.41	23.55 ± 7.68[a]	36.80 ± 11.90[a]	17.34 ± 3.56

[a] $p < 0.05$.

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.

3.3. Effect of dietary carboxymethyl chitosans on the levels of Cu, Fe and Zn in mice

Table 3 listed the Cu level in the organs of the mice after feeding these samples for 30 days. There was no significant difference of the Cu level in the tested six organs after administration of the three carboxymethyl chitosan samples.

Table 4 listed the Fe level in the organs of the mice after feeding these samples for 30 days. The levels of Fe in liver and lung significant decreased after administration of the three carboxymethyl chitosans.

Table 5 shows the levels of Zn in the organs of mice in this experiment. The levels of Zn significantly decreased in all tested organs for all carboxymethyl chitosan groups.

4. Discussion

The trace elements Fe, Zn and Cu as the ingested nutrient is absorbed and subsequently utilized for normal physiological functions. The small intestine is the main site of absorption of these metal cations, and the liver is the largest organ for the storage of these trace elements (Lee, Prasad, Brewer, & Owyang, 1989).

This study showed that carboxymethyl chitosans (0.75%, w/w) in diet significantly depressed the level of Fe and Zn in mice. Our previous study showed the dietary chitosans with different Mw (1.05%, w/w) did not depress the levels of Zn and Fe in mice (Zeng et al., 2008). The isolated dietary fibre preparations without phytate did not affect Zn and Fe absorption. Fe and Zn are less well absorbed trace elements, and dietary components appear to have the greatest effect on their absorption (Fairweather-Tait & Hurrell, 1996). The organic compounds forming stable complexes with Zn and Fe at intestinal pH reduce their absorption (Hurrell et al., 1992; Turnlund, King, Keyes, Gong, & Michel, 1984).

The carboxymethyl chitosan has two functional groups of $-NH_2$ and –COOH by introducing $-CH_2COOH$ into chitosan molecule, so it has stronger chelating ability with Fe^{2+} and Zn^{2+} than chitosan (Hon & Tang, 2000; Sun & Wang, 2006). These stable complexes at intestinal pH reduce their absorption.

This study showed that dietary carboxymethyl chitosan did not depress the level of Cu in mice. The level of Cu is much lower than the levels of Fe and Zn in mice. Cu is relatively well absorbed from mixed diets, and the endogenous Cu losses are lower than that of Fe and Zn (Turnlund, 1988). The content of Cu in the base diets is enough to maintain the balance of Cu in body (Turnlund et al., 1991). The dietary components appear to have not great effect on its absorption (Cherydn, 1980).

As a chelating agent, carboxymethyl chitosan may be a potential expelling agent in removing toxic metals such as Pb, Hg and Cd from the body. It is suggested that the soluble Fe and Zn salts as the supplement should be added into the diets containing carboxymethyl chitosan.

5. Conclusion

In this study, the dietary carboxymethyl chitosan samples were found to depress the levels of Zn and Fe in mice. These effects are different from that of chitosan in our previous study. It is confirmed that the introduction of –COOH increases the chelating ability of chitosan derivates with metal cations in vivo. Therefore, further studies are needed to investigate deeply the effect of the carboxylated chitosan including carboxymethyl chitosan on the absorption of trace elements in vivo, prior to their oral administration.

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