The Antioxidative and Antilipidemic Effects of Different Molecular Weight Chitosans in Metabolic Syndrome Model Rats

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The effect of high and low molecular weight chitosans (HMC; 1000 kDa, LMC; 30 kDa) on oxidative stress and hypercholesterolemia was investigated using male 6-week-old Wistar Kyoto rats as a normal model (Normal-rats) and spontaneously hypertensive rat/ND mcr-cp (SHP/ND) as a metabolic syndrome model (MS-rats), respectively. In Normal-rats, the ingestion of both chitosans over a 4 week period resulted in a significant decrease in total body weight (BW), glucose (GI), triglyceride (TG), low density lipoprotein (LDL) and serum creatinine (Cre) levels. The ingestion of both chitosans also resulted in a lowered ratio of oxidized to reduced albumin and an increase in total plasma antioxidant activity. In addition to similar results in Normal-rats, the ingestion of only HMC over a 4 week period resulted in a significant decrease in total cholesterol levels in MS-rats. Further, the ingestion of LMC resulted in a significantly higher antioxidant activity than was observed for HMC in both rat models. In in vitro studies, LMC caused a significantly higher reduction in the levels of two stable radicals, compared to HMC, and the effect was both dose- and time-dependent. The findings also show that LDL showed strong binding in the case of HMC. These results suggest that LMC has a high antioxidant activity as well as antilipidemic effects, while HMC results in a significant reduction in the levels of pro-oxidants such as LDL in the gastrointestinal tract, thereby inhibiting the subsequent development of oxidative stress in the systemic circulation in metabolic model rats.

Key words metabolic syndrome; chitosan; antilipidemic effect; antioxidant activity

The metabolic syndrome is a constellation of risk factors, including atherogenic dyslipidemia, impaired fasting glucose, hypertension, and central adiposity, predisposing to higher risks of oxidative stress, type 2 diabetes and atherosclerotic cardiovascular disease.1–4 In particular, an enhancement in oxidative stress by reactive oxygen species (ROS) has been proposed as a common pathomechanism by which cardiovascular risk factors affect the vessel wall to induce and amplify vessel and organ injury. Thus, an appropriate evaluation and reduction in oxidative stress in the circulating blood is important from the viewpoint of protecting vascular endothelium and vascular smooth muscle cells against oxidative stress.5–7 Dietary modifications, which include the use of antilipidemic supplements, are one of the key elements in the management of these metabolic abnormalities. For example, chitosan has been proposed as a safe and efficacious dietary supplement that can contribute to weight loss by reducing the amount of dietary fat absorbed, thereby improving calorie balance.8,9

Chitosan, a cationic polysaccharide produced by the N-deacetylation of chitin under alkaline conditions, contains a linear sugar backbone, composed of β-1,4-linked glucosamine units. It exhibits a wide variety of biological activities except for cholesterol-lowering effects.9–11 A property of particular interest for this study is the antioxidant properties of chitosan. Santhosh et al. reported that the administration of chitosan to rats that had been treated with isoniazid or rifampicin inhibited the oxidation of hepatotoxic lipids.12 It had also been reported that chitosan, when injected, inhibited glycerol-induced renal oxidative damage in rats.13 Because of the numerous in vitro and in vivo antioxidant studies that have appeared, chitosan has attracted considerable attention from researchers. In spite of this, however, relationships between molecular weight (MW) and antioxidant activity have not been extensively investigated in in vivo studies.

In this study, we examined the effect of high and low MW chitosan supplements (HMC; 1000 kDa and LMC; 30 kDa) on oxidative stress in normal and metabolic syndrome model rats, in an attempt to better understand the potential role for HMC and LMC as an antioxidant in the systemic circulation. Oxidative stress was evaluated by monitoring oxidized serum albumin levels, a sensitive marker for protein oxidation, in the systemic circulation.14,15 We also investigated the role of HMC and LMC as a chelator, to develop a better understanding of the mechanism of the antioxidant activity of HMC and LMC in metabolic syndrome model rats.

MATERIALS AND METHODS

Materials LMC (MW=30 kDa) and HMC (MW=1000 kDa) were obtained from Nippon Kayaku Food Techno Co., Ltd. (Gunma, Japan). All other chemicals were of the highest grade available and were obtained from commercial sources.

Animals and Treatment Male (6 weeks old) Wistar Kyoto rats as a normal model (Normal-rats) and spontaneously hypertensive rat/ND mcr-cp (SHP/ND) as a metabolic syndrome model (MS-rats) were obtained from the Disease Model Co-operative Research Association, Japan. The experimental protocol was reviewed and approved by the Animal Care and Use Committee of Fukuyama University and the Japanese government was fully informed prior to the commencement of the study. These rats were divided into six

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groups as follows: (a) untreated normal group (n=5), (b) LMC (1 g/d) treated normal group (n=5), (c) HMC (1 g/d) treated normal group (n=5), (d) untreated MS group (n=5), (e) LMC (1 g/d) treated MS group (n=5), (f) HMC (1 g/d) treated MS group (n=5). These rats received standard rat chow.

Blood Analyses Plasma samples obtained from each rat were immediately frozen and stored at −80 °C until used for analysis. Total cholesterol (TC), high and low density lipoproteins (HDL, LDL), blood glucose (BG) and creatinine (Cre) were determined using an enzymatic kit (Wako Co., Ltd., Tokyo, Japan).

Chromatography of Oxidized Albumin High-performance liquid chromatography (HPLC) was used to analyze serum albumin, as described previously. Serum samples were immediately frozen and stored at −80 °C until used for analysis. From the HPLC profile, the content of each albumin fraction (rat mercaptalbumin, f[RMA]; rat nonmercaptalbumin, f[RNA]) was estimated as the area of the RNA fraction divided by the RMA fraction of the serum albumin peak.

Total Plasma Antioxidant Capacity Assay The evaluation of antioxidant power in plasma samples was evaluated using the 'TPA' test (Cosmo Bio Co., Ltd., Tokyo, Japan). In this assay, Cu⁺ levels produced by the reduction of Cu²⁺ by the action of antioxidants present in the sample are determined, as described previously.

Scavenging Activity of LMC and HMC on 1,10-Diphenyl-2-picrylhydrazyl (DPPH) and 2,2'-Azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) Radicals DPPH and ABTS radical scavenging activities of different concentrations of LMC and HMC were estimated as described previously.

Binding Capacity of Total Cholesterol and LDL LMC and HMC (1, 5 mg/ml) were incubated with a Multi Calibrator N solution (Wako Co., Ltd., Tokyo, Japan) for 1 h. After centrifugation at 12000 rpm for 10 min, the supernatant was analyzed by the Wako L-Type LDL-C and the Wako Cholesterol E methods (Wako Co., Ltd., Tokyo, Japan).

Statistical Analysis Statistical significance was evaluated by the 2-tailed paired Student’s t-test for comparison between 2 mean values and by ANOVA followed by the Newman–Keuls test for comparison among >2 mean values. For all analyses, values of p<0.05 were regarded as statistically significant. The results are reported as the mean±S.E.M.

RESULTS

Effects of LMC and HMC on Biological Parameters As shown in Table 1, in Normal-rats, the ingestion of both chitosans over a 4 week period resulted in a significant decrease in total body weight (BW), glucose (GI), triglyceride (TG), low density lipoprotein (LDL) and serum creatinine (Cre) levels. In MS rats, in addition to similar results to Normal-rats, the ingestion of only HMC for a 4 week period resulted in a significant decrease in total cholesterol levels. These results suggest that the difference in the MW of the chitosans had a negligible effect on the biological parameters in normal versus MS-rats.

Effects of LMC and HMC on Oxidative Stress As shown in Fig. 1A, the LMC and HMC treatments caused a significant decrease in the oxidized albumin ratio after 4 weeks in Normal and MS-rats (p<0.05 vs. ratio at 0 week). Furthermore, the ingestion of LMC resulted in a higher antioxidant activity than was found for HMC in both Normal and MS-rats. Since the extent of oxidation of this prominent protein serves as an index of oxidative stress, these results demonstrate the potential of LMC and HMC for reducing the effects of stress in vivo in Normal and MS-rats. This conclusion is supported by an increase in total plasma antioxidant capacity (TPA) by the ingestion of LMC and HMC after a 4 week period (Fig. 1B). These results indicate that the oxidized albumin ratio is a reliable index of the effectiveness of LMC and HMC on, not only healthy rats, but also on the metabolic syndrome and that chitosan itself is a potent in vivo antioxidant.

Scavenging Activity of LMC and HMC on DPPH and ABTS Radicals In order to determine whether direct radical scavenging is a general property of the chitosans used in

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<th>Table 1. Effects of Chitosan Ingestion on the Biochemical Properties of Normal and Metabolic Rats (n=5)</th>
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TG: triglyceride, TC: total cholesterol, LDL: low-density lipoproteins, HDL: high-density lipoprotein cholesterol, Cre: serum creatinine. *p<0.05 versus at non-treated, **p<0.05 versus at normal rats (n=5).
In this study, we evaluated its ability to scavenge radicals other than peroxyl radicals, namely the stable N-centered DPPH and ABTS·⁻ radicals. The DPPH radical scavenging ability of HMC was lower than that for LMC (Fig. 2A). In the case of the ABTS·⁻/H⁺, HMC was a poorer scavenger of ABTS·⁻ (Fig. 2B). These results suggest that the scavenging activities of LMC for DPPH and ABTS radicals are more pronounced than that of HMC.

**Binding Capacity on TC and LDL**

The binding capacity of HMC for LDL was higher than the corresponding values for LMC (Fig. 3), whereas the effects for TC were low in both chitosans (data not shown). These results suggest that LDL is reduced in the gastrointestinal tract by HMC in the MS model rats used in this study. Thus, it is possible that HMC has the ability to reduce certain levels of pro-oxidants such as LDL in the gastrointestinal tract, thereby inhibiting the subsequent development of oxidative stress in the systemic circulation, because HMC is not absorbed from the intestinal tract.

**DISCUSSION**

The cholesterol-lowering effect of chitosan has been studied extensively. It is generally accepted that the origin of this effect due to its unique ability to bind lipids and bile acids. Such binding results in an increased elimination of fat in the stool, a reduced level of bile acid recycling, and the induction of the hepatic synthesis of new bile acid constituents from cholesterol. Considering the different MW chitosans, since the intestinal absorption of various MW chitosans by oral administration is directly related to their MW, the amount of chitosan absorbed would be expected to decrease with increasing MW. In the case of low MW chitosan, as a bioactive material, it can be absorbed from the intestinal tract and would then show a variety of additional bioactivities such as antitumor, cholesterol-lowering, immunostimulating, antiabetic, antimicrobial, and antioxidant effects, etc. in both the systemic circulation and the intestinal tract. During these biological events, the property of particular interest for this study is the antioxidant activity of chi-
tosan.\textsuperscript{26,27} In fact, in a previous study, we showed (in \textit{in vitro} studies) that the antioxidant properties of low MW chitosans are substantial, whereas high MW chitosans were much less effective in terms of antioxidant properties.\textsuperscript{28} We also showed that the administration of low MW chitosan to human volunteers inhibited the \textit{in vivo} oxidation of human serum albumin (HSA).\textsuperscript{15} Although several studies concerning the antioxidant activities of low MW chitosan have appeared, relationships between MW and antioxidant activity have not been extensively reported in \textit{in vivo} studies.

In the present study, we observed a reduction in several important biological parameters (Table 1) in both normal and MS rats. The results suggest that LMC and HMC have, not only a cholesterol-lowering effect, but also an enhanced resistance to the effects of oxidative stress. It should be noted that the subjects in this study were not only MS but also healthy, normal rats, leaving open the possibility that even greater benefits might be conferred by LMC and HMC in subjects who are able to resist an oxidative challenge. In general, the contributions of different plasma constituents to total antioxidant radical trapping capacity were previously estimated to be 35—65\%, due to urate, 0—24\%, ascrobate, 5—10\% and vitamin E and 10—50\% to plasma proteins.\textsuperscript{29} Proteins exert their protective effect by scavenging a wide variety of physiologically relevant oxidants and by their abundance in plasma, with albumin being the most effective extracellular antioxidant.\textsuperscript{30} As shown in Fig. 1A, the findings reported herein indicate that LMC and HMC ingestion caused a significant decrease in the oxidized albumin ratio during the 4 week period of the study. Since the extent of oxidation of this prominent protein can serve as one index of oxidative stress, these results demonstrate the potential of LMC and HMC for reducing the consequences of stress, \textit{in vivo} in not only MS but also normal rats. Further, the antioxidant activity of LMC was significantly higher than that of HMC. This conclusion is supported by the observed increase in the total plasma antioxidant capacity (TPA) as the result of the ingestion of LMC and HMC (Fig. 1B).

In order to test whether direct radical scavenging is a general property of the chitosans used in this study, we examined their ability to scavenge radicals, namely the stable N-centered DPPH and ABTS\textsuperscript{1+} radicals. The DPPH and ABTS\textsuperscript{1+} radical scavenging ability of LMC was higher than that of HMC (Figs. 2A, B). Overall, these results demonstrate that LMC has direct antioxidant activity while HMC has less antioxidant potential and suggest that its antioxidant potential shown in other systems may be due, at least in part, to this property.

In the case of cholesterol and glucose, the increase in health parameters could be due to the removal of abnormalities in carbohydrate and lipid metabolism associated with oxidative phenomena. This is supported by the use of chitosan preparations as a dietary supplement in metabolic syndromes associated with multiple risk factors such as dyslipidemia, hyperglycemia, hypertension, and abdominal obesity.\textsuperscript{31} In metabolic syndrome, LDL has, not only the potential to accelerate the progression of MS, but also the potential to produce oxidative stress in plasma and urine of subjects with MS.\textsuperscript{13} Based on these observations, we hypothesize that the levels of a pro-oxidant such as LDL can be reduced by the presence of chitosan in the intestinal tract. In fact, our results suggest that HMC strongly binds LDL-cholesterol in \textit{in vitro} studies, because HMC appears to have a compact structure, thus potentiating this stronger (Fig. 3). Therefore, HMC might reduce certain levels of pro-oxidants such as cholesterol and uremic toxins in the gastrointestinal tract, thereby inhibiting the subsequent development of oxidative stress in the systemic circulation. Furthermore, since the subjects of this study were not only MS but also healthy models and, hence, unlikely to show significant indices of oxidative damage, the administration of chitosan to patients with impaired health might have even greater beneficial effects. Thus, chitosan has the potential ability to act as an antioxidant in the metabolic syndrome to renal failure, since oxidative stress is an important pathogenic factor in uremic patients, and has a great impact on their survival. Further, we propose that, from the perspective of antioxidant therapy, the initiation of chitosan treatment such as for the metabolic syndrome is preferable at an earlier stage than the conventional late state of renal failure, because plasma levels of pro-oxidants such as protein and lipid hydroperoxides and other uremic toxins undergo a significant increase during renal failure. In conclusion, these results show that LMC has substantial antioxidant properties. In addition, HMC was much less effective in terms of antioxidant properties. On the basis of the results obtained, LMC with presumed antioxidant properties, has the potential for use as an antioxidant, as a possible food supplement or ingredient for use in the pharmaceutical industry.

REFERENCES


