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Antioxidant properties of high molecular weight dietary chitosan in vitro and in vivo

Makoto Anraku[a,*,1], Takeshi Fujii[a,1], Yuko Kondo[a], Eijiro Kojima[a], Toshiyuki Hata[a], Norihiko Tabuchi[a], Daiju Tsuchiya[a], Takeshi Goromaru[a], Hiroyuki Tsutsumi[a], Daisuke Kadowaki[a], Toru Maruyama[b], Masaki Otagiri[b], Hisao Tomida[a]

[a] Faculty of Pharmacy and Pharmaceutical Sciences, Fukuyama University, 1 Sanzo, Gakuen-cho, Fukuyama 729-0292, Japan
[b] Graduate School of Pharmaceutical Sciences, Kumamoto University, 5–1 Oe-honmachi, Kumamoto 862-0973, Japan

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ABSTRACT

The effect of high molecular weight chitosan supplement (HMCS), a natural polymer derived from chitin, on indices of oxidative stress was investigated in normal volunteers. The use of HMCS for 8 weeks resulted in a significant decrease in total cholesterol levels and atherogenic index, and increased levels of high density lipoprotein (HDL) cholesterol. HMCS treatment also resulted in a lowered ratio of oxidized to reduced albumin and an increase in total plasma antioxidant activity. A good correlation between the atherogenic index and oxidized albumin ratio was found. The results suggest that the ratio of oxidized to reduced albumin ratio represents a potentially useful marker of the metabolic syndrome. In in vitro studies, HMCS slightly reduced the levels of two stable radicals in a dose- and time-dependent manner. The strong binding capacity of indoxyl sulfate and low density lipoprotein (LDL) cholesterol was also observed with HMCS. These results suggest that HMCS reduces significant 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 in humans.

1. Introduction

Chitosan, a cationic polysaccharide produced by the N-deacetylation of chitin under alkaline conditions, contains a linear sugar backbone of chitosan composed of β-1,4-linked glucosamine units. It exhibits a wide variety of biological activities, including antitumor activities (Suzuki et al., 1986), immunostimulating effects (Jeon & Kim, 2001), cholesterol-lowering effects (Schipper et al., 1999), antimicrobial effects (Park, Je, Byun, Moon, & Kim, 2004), wound healing effects (Porporatto, Bianco, Riera, & Correa, 2003), antifungal activities, and free radical scavenging activities (Anraku et al., 2008; Park et al., 2004).

Property of particular interest for this study is the antioxidant properties of chitosan (Chiang, Yao, & Chen, 2000; Xue, Yu, Hirata, Terao, & Lin, 1998). Xie, Xu, and Liu (2001) reported that the scavenging of hydroxyl radicals by chitosan inhibits the lipid peroxidation of phosphatidylcholine and linoleate liposomes. Santhosh, Sini, Anandan, and Mathew (2006) reported that the administration of chitosan to rats that had been treated with isoniazid or rifampicin prevented the oxidation of hepatotoxic lipids. Similarly, chitosan, when injected, inhibited glycerol-induced renal oxidative damage in rats (Yoon et al., 2008). Owing to its many antioxidant studies in vitro and in vivo, chitosan has attracted considerable attention from researchers; however, relationships between molecular weight (MW) and antioxidant activity have not been extensively investigated.

We recently showed that the antioxidant properties of low MW chitosans are substantial, whereas high MW chitosans were much less effective in terms of antioxidant properties (Tomida et al., 2009). In recent, world-wide studies, several MW chitosans were tested as a dietary supplement (Gades & Stern, 2005; Kaats, Michalek, & Preuss, 2006). High MW chitosans would be expected to inhibit the absorption of certain lipids and bile acids. On the other hand, low MW chitosans would be predicted to absorb such substances, but would also be expected to show increased antioxidant effects. In fact, in a previous study, we showed that the administration of low MW chitosan to human volunteers strongly inhibited the oxidation of human serum albumin (HSA) in vivo (Anraku et al., 2009). Although several studies have been reported concerning the

* Corresponding author at: Department of Physical Pharmaceutics, Faculty of Pharmacy and Pharmaceutical Sciences, Fukuyama University, 1 Gakuen-chou, Sanzo, Fukuyama 729-0292, Japan. Tel.: +81 84 936 2111; fax: +81 84 936 2024.
E-mail address: anraku@fupharm.fukuyama-u.ac.jp (M. Anraku).
[1] These authors contributed equally to this work.

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antioxidant activities of low MW chitosan, relationships between high MW, low MW chitosans and their antioxidant activity have not been extensively reported in in vivo studies.

In this study, we examined the effect of high MW chitosan supplement (HMCS) on oxidative stress in human volunteers, in an attempt to better understand the potential role for HMCS as an antioxidant in the systemic circulation. Oxidative stress was evaluated by monitoring oxidized serum albumin levels in the systemic circulation, a sensitive marker for protein oxidation (Anraku et al., 2004, 2009). We also investigated the role of HMCS as a chelator, to verify the mechanism of the antioxidant activity of HMCS in human volunteers.

2. Experimental

2.1. Materials

A high molecular weight chitosan supplement (HMCS) (Chitosamin®; average molecular weight 100 kDa, degree of deacetylation 90%) was a generous gift from Nippon Kayaku Food Techno Co., Ltd (Gunma, Japan). 1,1′-Diphenyl-2-picrylhydrazyl (DPPH) and 2,2′-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) were supplied by Nacalai Tesque (Kyoto, Japan). Other chemicals were also of the highest grade commercially available, and all solutions were prepared using deionized, distilled water.

2.2. Volunteers

The volunteer subjects were 10 healthy subjects, aged 34 ± 4 (29–45) years. All participants provided informed consent according to NIH guidelines. They were not taking any antioxidants such as vitamin E or C during the 3 months before their participation in the study.

2.3. Study design

Suppliers of chitosan preparations available to the general public in Japan recommend an intake of 180 mg 3 times per day. While widely available, the supplements are normally taken by individuals who suspect that their health status is less than optimal. We reasoned that any measurable health benefits found in the healthy individuals selected for this study would be even more pronounced in subjects with a possible deficient antioxidant status. In this study, therefore, the selected healthy subjects received the recommended intake of 180 mg 3 times per day. The study consisted of a 4-week placebo baseline period, followed by an 8-week open-label treatment. After 0, 2, 4, and 8 weeks of treatment, blood samples were obtained for measurements of a range of parameters.

2.4. Blood analyses

Plasma samples obtained from each volunteer were immediately frozen and stored at −80 °C until used for analysis. Systolic blood pressure (SBP) and diastolic blood pressure (DBP) were measured by a blood pressure monitor (Omron Healthcare, Inc, Tokyo, Japan). Total cholesterol (TC), high and low density lipoproteins (HDL, LDL), atherogenic index (AI = TC − HDL/HDL) were measured by means of an enzymatic kit (Daiichi Pure Chemical, Co. Ltd., Tokyo, Japan). Blood glucose (BG) levels were determined by a testing kit (Bayer Health Care, Diabetes Care, Tokyo, Japan).

2.5. Chromatography of oxidized albumin

Plasma albumin was measured by high-performance liquid chromatography (HPLC), as described previously (Anraku et al., 2004). The frozen plasma samples obtained from each volunteer were thawed and 5 μL aliquots were analyzed on a Shodex Asahipak ES-502N column (Showa Denko Co., Ltd., Tokyo, Japan). From the HPLC profiles of plasma albumin, the ratios of oxidized to unoxidized albumin were estimated by dividing the area corresponding to the reduced form (human mercaptalbumin, HMA) by that for the oxidized form (human non-mercaptalbumin, HNMA). HNMA/HMA ratios (oxidized albumin ratio) have been previously used as an appropriate marker of oxidative stress in cases of chronic renal failure (Anraku et al., 2004; Kadowaki et al., 2007).

2.6. Total plasma antioxidant capacity assay

The evaluation of antioxidant power in plasma samples was determined by the 'TPAC' test (Cosmo Bio Co., Ltd., Tokyo, Japan). In this assay, Cu^{+} levels produced by the reduction of Cu^{2+} by the action of antioxidants present in the sample are measured. The stable complex formed from the reaction of Cu^{+} and bathocuproine was assayed at 490 nm, with a sensitivity of 22 μmol L^{-1} of reducing power. The assay was found to be linear from 1 to 2000 μmol L^{-1} of uric acid ($r = 0.99$, $p < 0.01$). Both within-run and between-run assay variability, tested by repeatedly assaying five samples, was consistently lower than 5%.

2.7. Scavenging activity of HMCS on DPPH and ABTS radicals

Radical scavenging activities of different concentrations of HMCS were tested in ethanolic solution (10 ml of ethanol, 10 ml of 50 mM 2-(N-morpholino) ethanesulfonic acid (MES) buffer (pH 5.5) and 5 ml of 0.5 mM DPPH). Radical scavenging was estimated from the decrease in the absorbance of DPPH radicals at 517 nm (Kogure et al., 1999). Stable ABTS cation radicals were generated by oxidation with potassium persulfate. The reaction mixture contained 200 μl of 70 mM potassium persulfate and 50 ml of 2 mM ABTS in distilled water. The stable $ABTS^{\bullet+}$ radical was generated on standing for 24 h and was used in the assay. The reaction of any antioxidant present with the $ABTS^{\bullet+}$ was estimated from the decrease in its absorbance at 734 nm (Leelarungrayub, Rattanapanone, Chanarat, & Gebicki, 2006).

2.8. Binding capacity on total cholesterol and LDL

HMCS (1 mg/mL) was incubated with a Multi Calibrator N solution (Wako Co., Ltd., Tokyo, Japan) for 1 h. After centrifugation at 12,000 rpm for 10 min, the supernatant was determined by the Wako L-Type LDL-C and the Wako Cholesterol E methods (Wako Co., Ltd., Tokyo, Japan).

2.9. Measurement of indoxyl sulfate (IS) binding capacity

Each chitosan sample (1 mg/mL) was incubated in IS solution (10 μM) for 1 h. After filtration on a VIVASPIN 500 (Vivascience AG, Germany) for 10 min at room temperature, IS was determined by HPLC. The HPLC system consisted of a L-6200 intelligent pump (Hitachi, Tokyo, Japan) and a F-1050 fluorescence spectrophotometer (Hitachi). A LiChrosorb RP-18 column (Cica Merck,Tokyo, Japan) was used as the stationary phase. The mobile phase consisted of acetate buffer (0.2 M, pH 4.5)/acetonitrile. The flow rate was 1.0 ml/min. IS was detected by means of a fluorescence monitor. The excitation/emission wavelengths were 300/400 nm (Tsutsumi et al., 2002).

2.10. Statistical analysis

Statistical significance was evaluated by the 2-tailed paired Student's *t*-test for comparison between 2 mean values and by ANOVA

Table 1
Effects of chitosan treatment of normal volunteers.

	0 week	2 weeks	4 weeks	8 weeks
SBP (mm Hg)	133.9 ± 12.5	132.4 ± 6.8	129.0 ± 8.2	129.8 ± 12.3
DBP (mm Hg)	80.7 ± 4.5	80.4 ± 5.3	82.5 ± 5.2	79.0 ± 3.5
BMI	24.2 ± 5.1	24.2 ± 5.1	24.4 ± 5.8	23.5 ± 6.2
BG (mg/dL)	117.0 ± 12.5	119.7 ± 13.4	114.1 ± 8.8	111.0 ± 13.5
TC (mg/dL)	172.9 ± 18.1	134.0 ± 16.6	140.3 ± 19.2	128.2 ± 8.26[a]
LDL (mg/dL)	155.8 ± 32.4	137.0 ± 28.2	143.3 ± 29.2	123.2 ± 8.59
HDL (mg/dL)	48.8 ± 2.2	51.2 ± 4.4	55.5 ± 2.7	54.6 ± 2.7[a]
AI	2.35 ± 0.3	1.69 ± 0.3	1.76 ± 0.4	1.60 ± 0.2[a]

SBP: systolic blood pressure, DBP: diastolic blood pressure, BMI: body-mass index, BG: blood glucose, TC: total cholesterol, LDL: low density lipoprotein cholesterol, HDL: high density lipoprotein cholesterol, AI: atherogenic index.
[a] $p < 0.05$ versus at 0 week ($N = 10$).

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. Results are reported as the mean ± SEM.

3. Results

3.1. Effects of HMCS on biological parameters

The results of blood parameter measurements show that the administration of HMCS for up to 8 weeks had no effect on blood pressure, body-mass index, or blood glucose levels (Table 1). Compared to the corresponding results before the treatment, there was a significant decrease in the levels of total cholesterol and atherogenic index after 8 weeks. However, the concentration of HDL continued to increase during the treatment period while the trend towards lower levels of LDL was not significant at $p < 0.05$. These results suggest that the administration of HMCS enhances resistance to the effects of oxidative stress.

3.2. Effects of HMCS on oxidative stress

As shown in Fig. 1A, the HMCS treatment caused a significant decrease (12.5%) in the oxidized albumin ratio after 4 weeks ($p < 0.05$ vs. ratio at 0 week), which was maintained (11.8%) at 8 weeks ($p < 0.05$ vs. 0 week). Since the extent of oxidation of this prominent protein can be taken as an index of oxidative stress, these results demonstrate the potential of HMCS for reducing the effects of stress in vivo even in healthy subjects. This conclusion is supported by an increase in total plasma antioxidant capacity (TPA) by HMCS treatment after 4 weeks (32.6%), which was maintained (24.8%) at 8 weeks (Fig. 1B). These results suggest that HMCS itself is a powerful in vivo antioxidant. Further, the results shown in Fig. 2 show a good relationship between the oxidized albumin ratio and the atherogenic index ($r = 0.53$, $p < 0.05$). These results also suggest that oxidized albumin ratio is a reliable index of the effectiveness of HMCS treatment on the metabolic syndrome and that chitosan itself is a powerful in vivo antioxidant.

3.3. Scavenging activity of HMCS on DPPH and ABTS radicals

In order to determine whether direct radical scavenging is a general property of the chitosan used in this study, we studied its ability to scavenge radicals other than peroxyl radicals, namely the stable N-centered DPPH and $ABTS^{\bullet+}$ radicals. The DPPH radical scavenging ability of HMCS was lower than that of ascorbic acid (Fig. 3A). In the case of the $ABTS^{\bullet+}$, HMCS was a poorer scavenger of $ABTS^{\bullet+}$ (Fig. 3B). These results suggest that HMCS preparations do not generally scavenge oxygen- and nitrogen-centered radicals and suggest that its antioxidant potential in vivo shown in other systems may be due, at least in part, to this property.

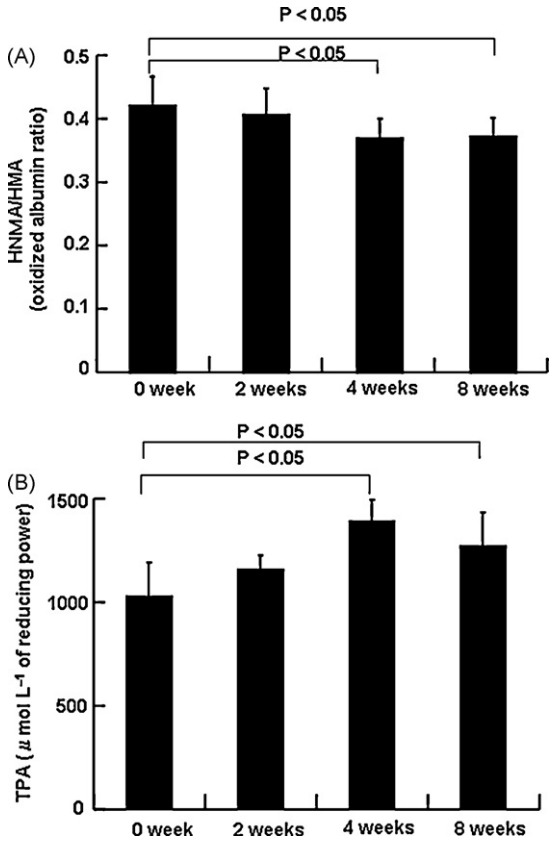

Fig. 1. The effects of HMCS treatment on indices of oxidative stress. Chitosan was administered daily at 180 mg 3 times per day. (A) Lowering of the ratio of oxidized to reduced albumin in plasma. (B) Increase in total plasma antioxidant capacity (TPA). Results are expressed as the mean ± SEM.

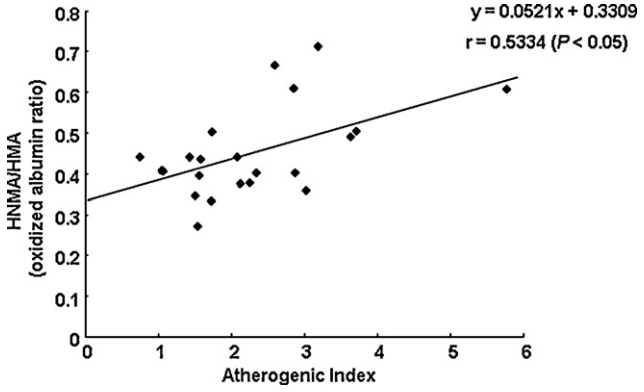

Fig. 2. Relationship between atherogenic index and oxidized albumin ratio HNMA/HMA. The line shows linear regression of the two sets of results ($N = 10$, $r = 0.53$, $p < 0.05$).

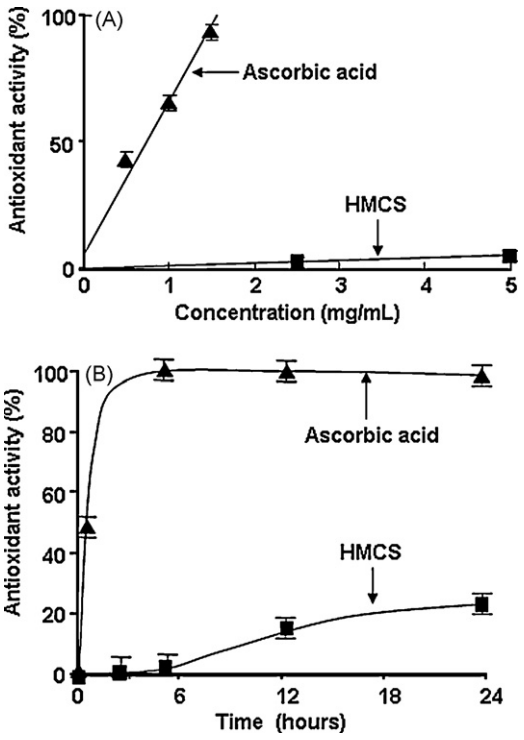

Fig. 3. Reduction of stable radicals by HMCS. (A) Decrease in 517 nm absorbance of 0.1 mM 1,1′-diphenyl-2-picrylhydrazyl (DPPH) after a 20-min exposure to HMCS. The 100% level is taken as the complete reduction of the DPPH. (B) Time course for the reduction of 2 mM 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) cation radical ($ABTS^{\bullet+}$) solution by HMCS. The decrease in absorbance was monitored at 734 nm. The concentration of HMCS and ascorbic acid was 0.5 mg/mL (▲) and 5 mg/mL (■), respectively.

3.4. Binding capacity on TC, LDL and IS

The binding capacity of HMCS for LDL, TC and IS was in the range of 11.9–38.5% (Fig. 4). The binding capacity of HMCS for LDL and IS was high, whereas the effect for TC were low. These results suggest that LDL and IS are reduced in the gastrointestinal tract by HMCS in the human volunteers in this study.

4. Discussion

The cholesterol-lowering effect of chitosan is one of its most extensively studied bioactivity. It is generally accepted that the origin of the cholesterol-lowering effect of chitosan is due to its unique ability to bind lipid and bile acids (Gallaher, Munion, Hesslink, Wise, & Gallaher, 2000; Ormrod, Holmes, & Miller, 1998; Ranaldi, Marigliano, Vespignani, Perozzi, & Sambuy, 2002). The binding results in an increased elimination of fat in the stool, reduced bile acid recycling, and the induction of hepatic synthesis of new bile acid constituents from cholesterol (Ranaldi et al., 2002; Sugano et al., 1980). In view of the different MW chitosans, since the intestinal absorption of various MW chitosans by oral administration is directly related to its MW, the amount of absorbed chitosan decreases with increasing MW (Chae, Son, Lee, Jang, & Nah, 2005). In the case of low MW chitosan, as a bioactive material, it can be absorbed from the intestinal tract and subsequently shows a number of additional bioactivities such as antitumor, cholesterol-lowering, immunostimulating, antidiabetic, 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 chitosan (Xue et al., 1998; Chiang et al., 2000). We recently showed that low MW chitosans have impressive antioxidant properties, especially antioxidant activity, whereas high MW chitosans are much less effective in terms of antioxidant properties (Tomida et al., 2009). We also showed that the administration of low MW chitosan to human volunteers prevented human serum albumin (HSA) oxidation in vivo (Anraku et al., 2009). Although several studies have been reported concerning the antioxidant activities of low MW chitosan, corresponding in vivo information on high MW chitosan, which cannot be absorbed efficiently from the intestinal tract, is not readily available. Accordingly, the objective of this study was to assess the antioxidant properties of dietary high MW chitosan in in vitro and in vivo studies.

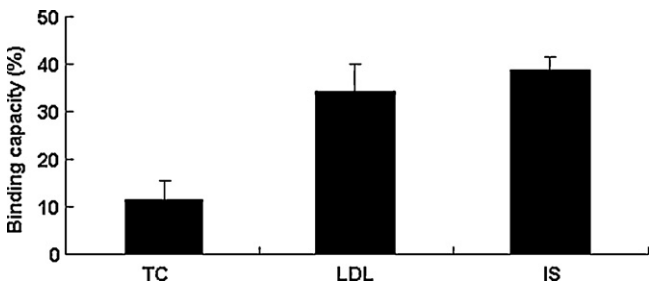

Fig. 4. Binding capacity on TC, LDL and IS. Results are expressed as the mean ± SEM.

In the present study, we observed a reduction in several important biological parameters (Table 1). The results suggest that HMCS has, not only a cholesterol-lowering effect, but also enhances resistance to the effects of oxidative stress. It should be noted that the subjects in this study were young and healthy, leaving open the possibility that even greater benefits might be conferred by HMCS in subjects who are able to resist oxidative challenge. The contributions of different plasma constituents to its total antioxidant radical trapping capacity were previously estimated as 35–65% due to urate, 0–24% to ascorbate, 5–10% to vitamin E and 10–50% to plasma proteins (Wayner, Burton, Ingold, Barclay, & Locke, 1987). Proteins exert their protective effect by scavenging a wide variety of the physiologically relevant oxidants and by their abundance in plasma, with albumin being the most effective extracellular antioxidant (Halliwell, 1988). As shown in Fig. 1A, we found that HMCS treatment caused a significant decrease in the oxidized albumin ratio during 8 week period of the study. Since the extent of oxidation of this prominent protein can be taken as one index of oxidative stress, these results demonstrate the potential of HMCS for reducing the consequences of stress, in vivo even in healthy subjects. This conclusion is supported by the observed increase in the total plasma antioxidant capacity (TPA) as the result of the HMCS treatment (Fig. 1B). Further, the results shown in Fig. 2 show a good relationship between the oxidized albumin ratio and the atherogenic index ($r = 0.53$, $p < 0.05$). The atherogenic index ratio has been reported to be associated with atherosclerosis (Schonfeld, 1979) and to be a discriminator for the presence and severity of coronary artery disease (Noma, Yokosuka, & Kitamura, 1983). Given the fact that atherosclerosis and coronary artery disease is associated with oxidative stress, the reduction in oxidative stress by the HMCS treatment might actually play an important role in protecting atherogenic lipoproteins against oxidation in vivo.

In order to test whether direct radical scavenging is a general property of the chitosan used in this study, we studied its ability to scavenge radicals, namely the stable N-centered DPPH and $ABTS^{\bullet+}$ radicals. The DPPH and $ABTS^{\bullet+}$ radical scavenging ability of HMCS was lower than that of ascorbic acid (Fig. 3). Overall, these results demonstrate that HMCS 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 dietary supplement in metabolic syndromes associated with multiple risk factors such as dyslipidemia, hyperglycemia, hypertension, and abdominal obesity (Han, Kimura, & Okuda, 1999). In chronic renal failure (CRF), IS has, not only the potential to accelerate the progression of CRF but also the potential to produce oxidative stress in renal proximal tubular cells and mesangial cells (Gelasco & Raymond, 2006; Miyazaki, Ise, Seo, & Niwa, 1997; Motojima et al., 1991). Based on these observations, we hypothesize that a pro-oxidant such as IS can be reduced by HMCS in the intestinal tract. In fact, our results suggest that HMCS strongly binds LDL-cholesterol and IS in in vitro studies (Fig. 4). Therefore, HMCS 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 healthy 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 a protein antioxidant in renal failure, since oxidative stress is an important pathogenic factor in uremic patients, and has great impact on their survival. Further, we propose that, from the perspective of antioxidant therapy, the initiation of chitosan treatment 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.

5. Conclusion

The findings reported herein serve to demonstrate the antioxidative potential of HMCS in the systemic circulation in human volunteers. From these results, we hypothesize that HMCS reduces lipid hydroperoxides and other uremic toxins that induce reactive oxygen species (ROS) production in the intestinal tract, thereby inhibiting the subsequent occurrence of oxidative stress in the systemic circulation in human volunteers. Thus, the antioxidative effect of HMCS is unique and differs from that of typical, conventional antioxidants such as antioxidant vitamins and N-acetyl cysteine. This fact suggests that HMCS can be co-administered with such agents and represents a new strategy for antioxidative treatment in cases of several diseases, including renal failure.

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