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Effects of chitosan on oxidative stress and related factors in hemodialysis patients

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ABSTRACT

In recent world-wide studies, chitosans were tested as a dietary supplement for inhibiting the absorption of certain lipids and bile acids. We previously demonstrated the antioxidative and renoprotective potential of chitosan supplementation in chronic renal failure using 5/6 nephrectomized rats. In this study, we report the effects of chitosan on oxidative stress and related factors in hemodialysis patients. The ingestion of chitosan over a 12-week period resulted in a significant decrease in serum indoxyl sulfate and phosphate levels, compared with the levels prior to the start of the study. The ingestion of chitosan also resulted in a lowered ratio of oxidized to reduced albumin and a decrease in the level of advanced oxidized protein products. In *in vitro* studies, chitosan solutions were found to bind 38.5% of the indoxyl sulfate and 17.8% of the phosphate, respectively. Further, the oxidized albumin ratio was correlated with serum indoxyl sulfate levels *in vivo*. These results suggest that the ingestion of chitosan results in a significant reduction in the levels of pro-oxidants, which include uremic toxins, in the gastrointestinal tract, thereby inhibiting the subsequent development of oxidative stress in the systemic circulation. In addition, the long-term ingestion of chitosan has the potential for use in treating hyperphosphatemia in hemodialysis patients.

1. Introduction

It is increasingly apparent that individuals with chronic kidney disease (CKD) are more likely to die of cardiovascular disease (CVD) than to develop kidney failure (Tonelli et al., 2006). A cohort study comprised of >13,000 elderly patients revealed that an increase in the incidence of cardiovascular events could partially be related to the fact that patients with kidney disease are less likely to receive preventive treatments against CVD (Shlipak et al., 2002). However, the mechanisms responsible for the enhanced susceptibility to CVD in CKD patients are not fully known. Dysfunctional kidney-specific risk factors such as endothelial dysfunction, inflammation, oxidative stress, anemia, proteinuria, hyperphosphatemia and changes in uremic toxins such as indoxyl sulfate have been proposed to play a pathophysiological role, not only in CVD, but also in the further progression of CKD (Tonelli et al., 2006; Watanabe, Miyamoto, Otagiri, & Maruyama, 2011). Among these factors, oxidative stress has attracted a great deal of interest from researchers. Oxidative stress appears to be increased in the serum of CKD patients because of increased oxidant activity as well as a reduced level of antioxidants, which is accompanied by kidney dysfunction and/or severe cardiorenal syndrome (Del Vecchio, Locatelli, & Carini, 2011; Massy, Stenvinkel, & Drueke, 2009). Therefore, the development of effective treatment methods for reducing oxidative stress associated with CKD is greatly desired.

Chitosan, a linear polymer of β(1–4) linked D-glucosamine units, has been proposed as a suitable functional material for accomplishing this, because of its biocompatibility, biodegradability, absence

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Table 1
Effects of CS treatment on serum parameters for HD patients.

	0 week	4 week	8 week	12 week
Albumin (g/dL)	3.8 ± 0.1	3.8 ± 0.1	3.8 ± 0.1	3.7 ± 0.1
BUN (mg/dL)	55.4 ± 3.5	52.7 ± 3.8	56.6 ± 5.1	53.2 ± 4.1
Creatinine (mg/dL)	10.8 ± 0.8	10.6 ± 0.7	10.5 ± 0.7	10.5 ± 0.7
ALP (g/dL)	186 ± 15.4	186 ± 19.0	174 ± 18.9	198 ± 23.2
Intact PTH (g/dL)	162 ± 48	152 ± 44	141 ± 45	101 ± 45[#]
HDL-C (mg/dL)	44.8 ± 3.1	43.7 ± 2.8	45.3 ± 3.1	45.5 ± 3.6
LDL-C (mg/dL)	90 ± 7.4	85 ± 7.4	81 ± 8.4	75 ± 7.6
TG (mg/dL)	114 ± 16	109 ± 17	101 ± 14	117 ± 19
Calcium (mg/dL)	8.8 ± 0.2	8.7 ± 0.2	8.5 ± 0.2	8.9 ± 0.3
Phosphate (mg/dL)	6.1 ± 0.2	6.4 ± 0.5	6.1 ± 0.5	5.2 ± 0.4[#]
Indoxyl sulfate (mg/dL)	357 ± 42.1	237 ± 27.5[#]	230 ± 27.3[#]	237 ± 26.8[#]

BUN, serum blood urea nitrogen; ALP, alkaline phosphatase; intact PTH, intact parathyroid hormone; TC, total cholesterol; LDL, low-density lipoproteins cholesterol; HDL, high-density lipoprotein cholesterol. Values are expressed as the mean ± SEM ($n = 11$ patients per group).
[#] $p \leq 0.05$ *vs.* at 0 week ($N = 11$).

of toxicity, adsorption properties and free radical scavenging activities (Anraku et al., 2009; Muzzarelli, 2010; Park, Je, Byun, Moon, & Kim, 2004). In recent world-wide studies, chitosans were tested as a dietary supplement for use in inhibiting the absorption of certain lipids and bile acids (Gades & Stern, 2005). Regarding the mechanism of lipid absorption, when ingested, chitosan develops an HCl-layer in the stomach. As capsulated particles of chitosan move into the duodenum, the HCl-layer becomes diluted and the chitosan particles form agglomerates with fatty acids and cholesterol, thus reducing the absorption of lipids in the gastrointestinal tract (Muzzarelli et al., 2006). In addition, the ingestion of chitosans would be expected to inhibit the absorption of certain lipids and bile acids, but would also be expected to show increased antioxidant effects. In fact, in a previous study, we showed that chitosan has a high antioxidant activity as well as anti-lipidemic effects in metabolic syndrome model rats (Anraku et al., 2010, 2011). We also showed that chitosan has a high antioxidant activity as well as having the ability to exert reno-protective effects in chronic renal failure (CRF) using 5/6 nephrectomized rats (Anraku et al., 2012). From these results, we hypothesize that chitosan would reduce the levels of lipids and/or uremic toxins that induce the production of reactive oxygen species (ROS) production in the intestinal tract, thereby inhibiting the subsequent development of oxidative stress in the systemic circulation in the metabolic syndrome or CRF. Thus, the indirect antioxidant activity of chitosan resulting from the inhibition of the absorption of some kidney-specific risk factors, such as uremic toxins, might lead to the development of effective new treatment methods for reducing oxidative stress associated with CKD.

In this study, we examined the effect of chitosan supplements (CS) on oxidative stress and related factors in hemodialysis (HD) patients, in an attempt to better understand the role and effectiveness of chitosan as an antioxidant in the systemic circulation.

2. Experiment

2.1. Study population and design

11 patients with chronic renal failure who were admitted to the Department of Nephrology at the Akebono Clinic of Japan, aged 52–78 years, with a dialysis period ranging between 2 and 22 years, between March 2012 and May 2013 were enrolled in this study. At enrollment, all of the HD patients were receiving regular bicarbonate hemodialysis therapy (4–5 h per session, 3 times per week) using high-flux polysulfone hollow-fiber dialyzers. None of the patients were being treated with antioxidants such as vitamin E and C in the three months before inclusion in the study. In this study, the selected 11 patients received the recommended chitosan intake of 500 mg per day. The study consisted of a 4-week placebo baseline period, followed by a 12-week open-label treatment. After 0, 4, 8, and 12 weeks of treatment, blood samples were obtained for measurements of a range of parameters. The study protocol was approved by the Institutional Review Board of Akebono Clinic, and informed consent was obtained in accordance with the Declaration of Helsinki. Blood samples were immediately centrifuged at 4 °C and plasma aliquots were stored at −80 °C until used for various analyses. Biochemical parameters were measured at a contract laboratory (SRL, Inc., Tokyo, Japan). Table 1 shows the biochemical properties of the patient groups before and after the chitosan supplements (CS) treatments.

2.2. Materials

CS (Chitosamin®; average molecular weight 100 kDa, degree of deacetylation 90%) was a generous gift from the Nippon Kayaku Food Techno Co., Ltd (Gunma, Japan), and was ingested in a biscuit-type form (Healket®) to the patients. Chitosamin® is widely used a food that promotes health. Other chemicals were also of the highest grade commercially available, and all solutions were prepared using deionized, distilled water.

2.3. Chromatography of oxidized albumin in HD patients

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 for 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 CRF (Anraku et al., 2004; Kadowaki et al., 2007).

2.4. Total plasma antioxidant capacity assay in HD patients

The 'TPAC' test (Cosmo Bio Co., Ltd., Tokyo, Japan) was used to evaluate antioxidant activity in the plasma samples. In this assay, Cu^{+} levels produced by the reduction of Cu^{++} *via* 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} for uric acid ($r = 0.99$, $p < 0.01$). Both within-run and between-run assay variability, tested by repeatedly assaying five samples, was consistently less than 5%.

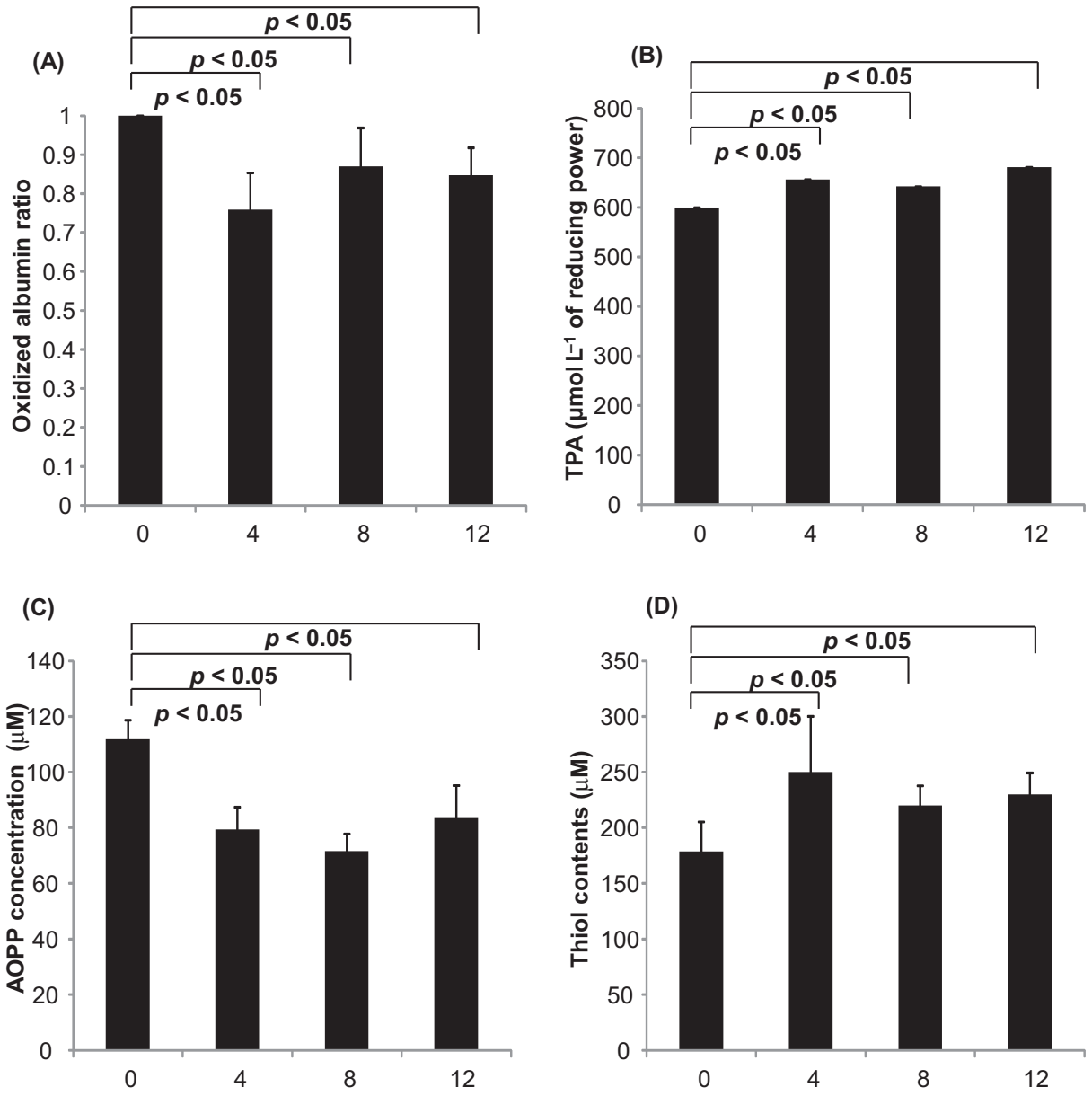

Fig. 1. The effects of CS treatment on indices of oxidative stress. Chitosan was administered daily at a dose of 500 mg per day. (A) The ratio of oxidized to reduced albumin is lowered. (B) Total plasma antioxidant capacity (TPA) is increased. (C) Levels of AOPP are lowered. (D) Thiol content is increased. Results are expressed as the mean ± SEM

2.5. Determination of advanced protein oxidation products (AOPP) and thiol contents in HD patients

AOPP levels were assessed by measurement of the absorbance at 340 nm and are expressed as chloramine-T equivalents after a fivefold dilution of 200 μL of plasma with 20 mmol/L of phosphate-buffered saline (PBS), pH 7.4; the addition of 80 μL of acetic acid, and reading against a blank of PBS (Witko-Sarsat et al., 1996). Serum thiol contents were determined spectrophotometrically as described previously (Ishima et al., 2007) using the thiol-specific reagent DTNB at 4, 8 and 12 weeks after the CS treatment.

2.6. Binding capacity on phosphate and indoxyl sulfate

CS (1 mg/mL) was incubated with a Multi Calibrator N solution (Wako Co., Ltd., Tokyo, Japan, Phosphate solution: 100 μM) for 1 h. After filtration on a VIVASPIN 500 (Vivascience AG, Germany), the supernatant was determined by the Wako L-Type Phosphate method (Wako Co., Ltd., Tokyo, Japan). CS (1 mg/mL) was incubated in indoxyl sulfate solution (10 μM) for 1 h. After filtration on a VIVASPIN 500 (Vivascience AG, Germany), the level of indoxyl sulfate was determined by HPLC according to previously described methods (Tsutsumi et al., 2002). The bound fraction (%) was calculated as follows: Bound fraction (%) = [1 − ligand concentration in filtered fraction/total ligand concentration (before filtration)] × 100.

2.7. Statistical analysis

Statistical significance was evaluated using the two-tailed, unpaired Student's *t*-test for comparisons between two means, or ANOVA analysis followed by Newman–Keuls method for more than two means. A value of $p < 0.05$ was regarded as being statistically significant. Results are reported as the mean ± SE.

3. Results

3.1. Effects of CS treatment on biochemical parameters in HD patients

The results of blood parameter measurements show that the CS treatment for up to 12 weeks has no effect on the levels of albumin, serum blood urea nitrogen (BUN), creatinine (Cr), alkaline phosphatase (ALP), total cholesterol, high-density lipoprotein (HDL-C) or calcium (Table 1). Compared to the corresponding results before the treatment, a significant decrease in the levels of intact parathyroid hormone (PTH) and phosphate was found after 12 weeks. In particular, compared to the corresponding results before the treatment, the CS treatment resulted in a significant decrease (33.5%) in the levels of serum indoxyl sulfate after 4 weeks ($p < 0.05$ *vs.* ratio at 0 week), which was maintained (33.4%) at 12 weeks ($p < 0.05$ *vs.* 0 week). However, the trend toward lower levels of high-density lipoprotein (LDL-C) was not significant at $p < 0.05$ (Table 1). These results suggest that the administration of CS enhances resistance to the effects of oxidative stress related factors such as phosphate and indoxyl sulfate.

3.2. Effects of CS treatment on oxidative stress in HD patients

As shown in Fig. 1A, the CS treatment resulted in a significant decrease (24.1%) in the oxidized albumin ratio after 4 weeks ($p < 0.05$ *vs.* ratio at 0 week), which was maintained (15.3%) at 12 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, it can be concluded that CS has the potential for reducing the effects of stress in HD patients. This conclusion is supported by an increase in total plasma antioxidant capacity (TPA) by the CS treatment after 4 weeks (9.6%), which was maintained (13.6%) at 12 weeks (Fig. 1B). Further, we also determined AOPP levels from HD patients during the CS treatment. As shown in Fig. 1C, compared to the corresponding results before the treatment, AOPP levels were significantly decreased at 4 weeks as the result of the treatment, and then gradually declined by 12 weeks. AOPP is a reliable marker of oxidant-mediated protein damage in uremic patients and also functions as mediators of inflammation, a condition that induces the development of cardiovascular diseases (Witko-Sarsat et al., 1998, 2003). Therefore, the significantly decreased AOPP levels are indicative of, not only a decreased risk for cardiovascular, but also a reduction in oxidative stress. As shown in Fig. 1D, the total free thiol content was significantly increased as the result of the CS treatment after 4 weeks (36.9%), which was maintained (28.7%) at 12 weeks.

3.3. Binding capacity on phosphate and indoxyl sulfate

In an *in vitro* study, CS was reported to bind phosphate and indoxyl sulfate in the range of 17.8–38.5% (Table 2). The binding capacity of CS for indoxyl sulfate was high, whereas these effects for phosphate were low. These results suggest that indoxyl sulfate would be expected to be reduced in the gastrointestinal tract by CS in the HD patients in this study.

Table 2
Binding capacity on phosphate and indoxyl sulfate.

	Binding capacity
Phosphate	17.8 ± 7.1
Indoxyl sulfate	38.5 ± 4.3

Results are expressed as the mean ± SEM.

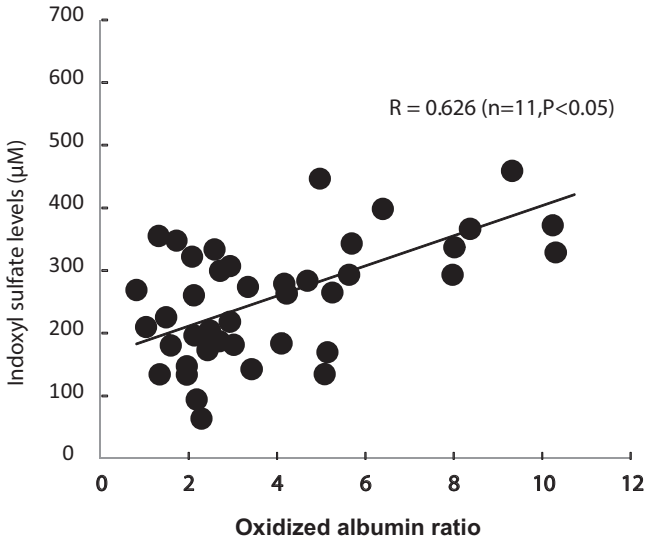

Fig. 2. Relationship between oxidized albumin ratio and serum indoxyl sulfate level. The line shows a linear regression of the two sets of results ($N = 11$, $r = 0.626$, $p < 0.05$).

3.4. Relationship between oxidized albumin ratio and serum indoxyl sulfate levels

As shown in Fig. 2, we determined the relationship between oxidized albumin ratio and serum indoxyl sulfate levels. The findings indicate the existence of a good significant correlation ($r = 0.626$, $p < 0.05$) between the two parameters. These results also suggest that CS reduces the levels of indole compounds based on indoxyl sulfate that induce the production of ROS in the intestinal tract, thereby inhibiting the subsequent development of oxidative stress in the systemic circulation in chronic renal failure. However, no significant correlation was found between the oxidized albumin ratio and serum phosphate or intact PTH levels (data not shown).

4. Discussion

Oxidative stress is a pathogenic element of great importance in HD patients, in that it has a great impact on their survival. Thus, the development of effective anti-oxidant therapy is highly desired in CKD. One proposed mechanism for oxidative stress in CKD is the accelerated production of oxidants, such as uremic toxins and their reduced renal clearance. Therefore, the removal of such substances from the systemic circulation may lead to, not only a reduction in oxidative stress, but also the prevention of CVD in CKD. This study showed that a CS treatment significantly reduced the level of oxidative stress and related substances in hemodialysis patients. To our knowledge, this is the first report to show a new role for CS treatment in reducing oxidative stress and improving dysfunctional kidney-specific risk factors related oxidative stress in HD patients.

In the present study, a reduction in several important biological parameters was found as the result of the CS treatment (Table 1). In particular, compared to the corresponding results before the treatment, a significant decrease in the levels of serum intact PTH and phosphate was found. Hyperphosphatemia is recognized as a contributor to vascular calcification in patients with CKD and HD patients and is independently associated with cardiac mortality. In fact, Ganesh et al. identified strong relationships between elevated serum phosphate, intact PTH and of death in HD patients attributable to cardiac issues, especially deaths resulting from CVD and sudden death (Ganesh, Stack, Levin, Hulbert-Shearon, & Port, 2001). From these results, our studies suggest that the binding of phosphate as the result of the CS treatment in the intestinal

tract could be a useful approach to the dietary management of serum phosphate level reduction in HD patients, as an add-on to phosphate binders at meals, thus leading to a better control of hyperphosphatemia. Further, given the fact that CS has been reported to bind phosphate in *in vitro* studies are consistent with this conclusion (Table 2). On the other hand, considering the fact that a good significant correlation between the oxidized albumin ratio and serum phosphate or intact PTH levels was not apparent, these parameters might not participate in oxidative stress directly in the systemic circulation.

Another major dysfunctional kidney-specific risk factor, indoxyl sulfate, which is the most widely studied uremic toxin, has been shown by us and other research groups to cause an increase in free radical production and to induce the production of inflammatory cytokines in the kidneys and blood circulation (Motojima et al., 1991; Shimoishi et al., 2007). Therefore, low indoxyl sulfate levels might lead to renal function being maintained and low oxidative stress. As shown in Table 1, compared to the corresponding results before the CS treatment, a significant decrease in the levels of serum indoxyl sulfate was found. Furthermore, the CS treatment caused a significant decrease in the oxidized albumin ratio after 4 weeks, which was maintained at 12 weeks (Fig. 1A). This result demonstrates the potential of CS for reducing the effects of stress in HD patients, because the oxidized albumin ratio is widely used as an appropriate marker of oxidative stress in many diseases, including CKD. This result is also supported by an increase in total plasma antioxidant capacity (TPA), a reduction in AOPP, and an increase in thiol content as the result of the CS treatment after 4 weeks (Fig. 1B–D). These results suggest that CS itself is a powerful *in vivo* antioxidant. Further, the results shown in Fig. 2 clearly point to the existence of a good relationship between oxidized albumin ratios and serum indoxyl sulfate levels ($r = 0.626$, $p < 0.05$). Since chitosan itself is not absorbed, it is unlikely that the mechanism responsible for the antioxidant activity of chitosan involves the direct scavenging of radicals in the blood; rather, we hypothesize an indirect manifestation of activity, in which substances causing oxidative stress or their precursors are adsorbed in the gastrointestinal tract, thus suppressing their serum levels. This conclusion was supported by a correlation between the serum concentration of indoxyl sulfate, a primary substance adsorbed by chitosan, and the oxidized albumin ratio in HD patients (Fig. 2). Further, given the fact that CS was found to strongly bind indoxyl sulfate in *in vitro* studies provides support for this conclusion (Table 2). Thus, the removal of indoxyl sulfate from the systemic circulation may lead to a reduction in oxidative stress in HD patients. From these results, we propose that it is possible to reduce the levels of indoxyl sulfate, a pro-oxidant, in the intestinal tract by a CS treatment.

The oral carbonaceous adsorbent, AST-120 (Kremezin®), has been used in pre-dialysis in cases of uremic stage renal failure patients to adsorb biologically active substances, so-called uremic toxins such as indoxyl sulfate, from the circulation that accumulate during CKD, thereby prolonging the progression of CKD and the interval between the inception of dialysis (Owada et al., 1997; Sanaka, Sugino, Teraoka, & Ota, 1998). We recently reported on the anti-oxidative potential of AST-120 in the systemic circulation using 5/6 nephrectomized rats (Shimoishi et al., 2007) by the same type of CS treatment, as an effective treatment method for reducing oxidative stress associated with CKD. However, AST-120 is typically used for a very short time in cases of pre-dialysis and uremic-stage renal failure patients and non-compliance issues frequently arise, due to the fact that the dosage form contains activated charcoal (Niwa & Ise, 1994; Aoyama & Niwa, 2001). The findings reported herein suggest a new potential use for CS, namely as an alternative to AST-120 and as an antioxidant in CKD. Thus, from the perspective of antioxidant therapy, the initiation of CS administration would be preferable at a stage earlier than the conventional state of pre-dialysis uremia, that is routinely prescribed AST-120, because CS is widely used as a health food and not a medicine.

5. Conclusion

The findings reported herein serve to demonstrate the antioxidative potential of CS in the systemic circulation in HD patients. The finding reported here indicate that CS reduces the levels of uremic toxins such as indoxyl sulfate that induce the production of reactive oxygen species (ROS) in the intestinal tract, thereby inhibiting the subsequent occurrence of oxidative stress in the systemic circulation in HD patients. Thus, the removal of such substances from the systemic circulation leads, not only to a reduction in oxidative stress, but also the prevention of CVD in CKD. Considering the new prophylaxis or therapy using CS, CS could be co-administered with such agents and represents a new strategy for antioxidative treatment in cases of several diseases, including renal failure, because the antioxidative effect of CS is unique and differs from that of typical, conventional antioxidants such as antioxidant vitamins and N-acetyl cysteine.

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