Research Article

Journal of Nutritional Biology

Sacran, a High-molecular Weight Polysaccharide Inhibits Renal Injury and Oxidative Stress in Chronic Renal Failure Model Rats

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Abstract

The administration of a high-molecular polysaccharide Sacran results in a significant decrease in renal injury and oxidative stress, compared with that for the oral carbonaceous adsorbent, AST-120 (Kremezin®) or a non-treatment group in 5/6 nephrectomized rats. An oral administration of Sacran (20 mg/day) over a 4 week period resulted in a significant decrease in serum indoxyl sulfate, creatinine and urea nitrogen levels, compared with a similar treatment with AST-120 or the non-treatment group. Sacran treatment also resulted in antioxidant potential being maintained, compared with that for AST-120 or the non-treatment group. Immuno-histochemical analyses also demonstrated that CRF rats, when treated with Sacran, showed a decrease in the level of accumulated renal fibrosis and 8-OHdG compared with AST-120 or the non-treatment group. These results suggest that the ingestion of Sacran results in a significant reduction in the levels of prooxidants, such as uremic toxins, in the gastrointestinal tract, thereby inhibiting the subsequent development of oxidative stress in the systemic circulation.

Introduction

Oxidative stress is generally assumed to be closely related to the progression of Chronic Renal Failure (CRF) and the onset of various complications, including Cardiovascular Disease (CVD) [1,2]. It has recently been proposed that several factors significantly contribute to the enhancement of oxidative stress observed in CRF. Uremic toxins have been proposed as possible candidates, because they accumulate in CRF and a direct relationship exists between the redox properties of uremic toxins and their biological activity; therefore, it would be highly desirable to develop an understanding of the redox properties of uremic toxins, in terms of managing oxidative stress in CRF patients. Indoxyl sulfate, a uremic toxin, has been extensively investigated in this regard. It is derived from dietary protein, including tryptophan, and contains an indole ring. It is also present at high levels in the serum of patients with CRF, and

appears to be involved in the progression of CRF and the onset of complications associated with it. In fact, recent clinical studies have demonstrated that the serum levels of indoxyl sulfate are a powerful predictor of overall and cardiovascular mortality, and that it has an inverse relationship with renal function and a direct relationship with aortic calcification and pulse wave velocity [3,4]. In addition, several groups have reported that indoxyl sulfate has the potential to produce oxidative stress, a situation that is accompanied by the production of excess levels of reactive oxygen species (ROS) in renal tubular cells, mesangial cells, aortic smooth muscle cells and vascular endothelial cells [5,6].These actions are mediated by the intracellular uptake of indoxyl sulfate via the organic anion transporter 1 (OAT1) and/or OAT3 [7]. In contrast to the pro-oxidant properties of indoxyl sulfate in CRF, we recently demonstrated that indoxyl sulfate, at normal serum concentrations, shows radical scavenging activity, especially against superoxide anion radicals (O_2^-) that are generated from both a xanthine oxidase system and activated neutrophils [8]. The oral carbonaceous adsorbent, AST-120 (Kremezin®), is typically used in predialysis in cases of patients with uremic stage renal failure. It functions to adsorb biologically active substances, so-called uremic toxins such as indoxyl sulfate, from the circulation that accumulate during CRF, thereby prolonging the progression of CRF and the need to start dialysis treatment [9,10]. We also reported on the potential of using AST-120 as an anti oxidative agent in the systemic circulation using 5/6 nephrectomized rats [5], as an effective treatment method for reducing the oxidative stress associated with CRF. 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 procedure involves the use of activated charcoal [4,11]. Our recent studies suggest a new potential use for chitosan and nanofibers produced from it, as an alternative to AST-120 and as an antioxidant in CRF. Thus, from the perspective of antioxidant therapy, the use of nanofibers derived from chitosan would be preferable at a stage earlier than the conventional state of pre-dialysis uremia, where AST- 120 is routinely prescribed, because such chitosan-related products are widely used as a health food product and not a medicine [12-14]. It is noteworthy that another type of ampholytic polysaccharide, Sacran (Figure 1) is extracted from the Japanese indigenous Cyanobacterium Aphanothece sacrum, which is mass-aquacultured in rivers. This polysaccharide has a high ionic concentration, and contains a jelly-like Extracellular Matrix (ECM) with a high water content (97.5%) [15-17]. Sacran is a

hetero polysaccharide that is made up of various sugar residues, including glucose, galactose, mannose, xylose, rhamnose, fucose, galacturonic acid and glucuronic acid, as well as traces of alanine, galactosamine and muramic acid; 11% of the monosaccharides contain a sulfate group and 22% contain a carboxyl group. Sacran also has an extremely high molecular weight (approximately 20 MDa) and is comprised if surprisingly long chains of sugar units (more than 8 μm) [17]. Sacran is thought to be a safe biomaterial, because Aphanothece sacrum is a mass cultivated cyanobacterial species that has long been used as a functional food to ameliorate tendencies to develop allergeries and gastroenteritis by inhabitants of the Kyushu region in Japan. Furthermore, because of its constitution, sacran has the ability to hold large quantities of water, compared to hyaluronic acid or xantangum, and forms a nano-film. Okajima et al. recently reported that sacran can form a hydrogel through electrostatic interactions with cationic heavy metal ions and that it can be used to recover of rare metals from relatively dilute sources [18,19]. In more recent studies, Arima et al. and Motoyama et al. applied these materials as a transdermal drug carrier for use in wound healing and atopic dermatitis, etc [20,21]. However, there is currently little information available concerning the use oral preparations of sacran in the treatment of chronic diseases. In this study, we report on the effect of sacran on oxidative stress and CRF in 5/6 nephrectomized rats, compared with the effects of AST-120, in an attempt to better understand the potential role of Sacran as an antioxidant in the systemic circulation using a sensitive marker for protein oxidation [22,23].
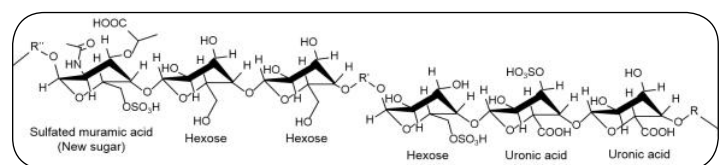
Figure 1: Structure of sacran [16].

Materials and Methods
Materials

Sacran (average molecular weight 28,000 kDa) was generously donated by Green Science Material (Kumamoto, Japan). Sodium hydroxide and acetic acid were purchased from Wako Pure Chemical Industries (Osaka, Japan) and were used as received. Other chemicals that were used were of the highest grade commercially available, and all solutions were prepared using deionized, distilled water.

Animals and treatment

Six-week-old male rats weighing 140 to 150 g that had been subjected to a 5/6 nephrectomy as a chronic renal failure model (CRF 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 Sojo University and the Japanese government was fully informed prior to the commencement of the study. The rats were divided into four groups as follows: (a) untreated nephrectomized group (N = 11). The rats received only standard rat chow. (b)Sacran (N = 11) and (c) AST-120 (N = 3) treated nephrectomized group, respectively. These rats received standard rat chow and the above samples at a daily dosage of 20 mg/day for a period of 4 weeks, respectively. Untreated CRF rats were pair-fed with the same amount of chow as was used for the samples used in the treatment of the CRF rats.

Blood analyses

After 0 and 4 weeks of the treatment, plasma samples were obtained from each of the rats and were immediately frozen and stored at -80°C until used for analysis. Biochemical parameters including serum creatinine, urea nitrogen (BUN) and indoxyl sulfate levels were determined according to previously described methods [5,24,25].

Plasma antioxidant potential (PAO)

The 'PAO' 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 that are 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. Both within-run and between-run assay variability, as tested by repeatedly assaying five samples, was consistently less than 5% [26].

Chromatography of oxidized albumin in CRF Rats

Plasma albumin was measured by High-performance liquid chromatography (HPLC), as described previously [23]. 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 (rat mercaptalbumin, RMA) by that for the oxidized form (rat nonmercaptalbumin, RNMA). RNMA/RMA ratios (oxidized albumin ratio) have been previously used as an appropriate marker of oxidative stress in cases of CRF [23,27].

Histologic examination of renal tissues

Renal abnormalities were evaluated after 4 weeks of treatment, the kidneys were removed, weighed, and fixed in 10% phosphate buffered formalin. The tissue was then dehydrated at room temperature by passage through a graded ethanol series and embedded in paraffin. The prepared tissue was then cut into 2.5 μm sections and treated with Periodic acid–Schiff (PAS) stain and Masson's trichrome for routine histology and morphometric studies. The ratio of renal tubular degeneration was measured using 20 random microscopic fields by two independent pathologists who were blind with respect to the experimental data. Once the slide had dried completely, we examined each specimen for 8-OHdG. A solution containing 50 mmol/l Tris/HCl+0.1% Tween-20 (T-TB) was used to solubilize the renal slices, followed by blocking with Block Ace (Dainippon Pharmaceutical, Osaka, Japan) at room temperature for 15 min. The primary antibody 8-OHdG (1:50) reaction was next applied overnight at 4°C. The renal slices were then washed with 50 mmol/l Tris/HCl (TB) and TTB, followed by treatment with the secondary antibody at room temperature for 90 min. The secondary antibodies for 8-OHdG were Alexa Fluor 488 goat anti-rat IgG (H + L). In each case the secondary antibody was diluted 200-fold. The slide was then examined by fluorescence microscopy using a model BZ-X700 microscope (Keyence, Osaka, Japan).

Measurement of indole binding capacity

Each sample (0.05, 0.5, 1.0, 2.5, and 5 mg/mL) was incubated in an indole solution (50 µg/mL) for 24 hours, 120 rpm at room temperature. After filtration for 1 min on a VIVASPIN 500 (Vivascience AG, Germany) at 6000 rpm at room temperature, indole concentrations were determined using a JASCO HPLC system. The indole was separated with a Hibar RT 250-4.0 Lichrosorb RP-18 (7 µm), Kanto Chemical Co., Inc. The column temperature was 40°C and the absorbance was read at 270 nm. The mobile phase used for the analysis consisted of water/acetonitrile (60/40 v/v). Analysis was complete within 15 min with a flow rate of 1.0 ml/min. The bound fraction (%) was calculated as follows: Bound fraction (%) = [1-ligand concentration in the filtered fraction / total ligand concentration (before filtration)] × 100.

Statistical analysis

Statistical significance was evaluated by the 2-tailed paired Student's t-test for comparison between 2 mean values. For all analyses, values of $p < 0.05$ were regarded as statistically significant. Results are reported as the mean ± SEM.

Results

Effects of sacran treatment on biological parameters in CRF rats

The findings presented herein show that several important biological parameters can be reduced as the result of the oral administration of the sacran (Table 1). It is particularly noteworthy, that the oral administration of sacran after a 4 week period resulted in a significant decrease in the serum levels of indoxyl sulfate, creatinine, BUN and phosphorus, compared with the ingestion of AST-120 or the non-treatment groups.
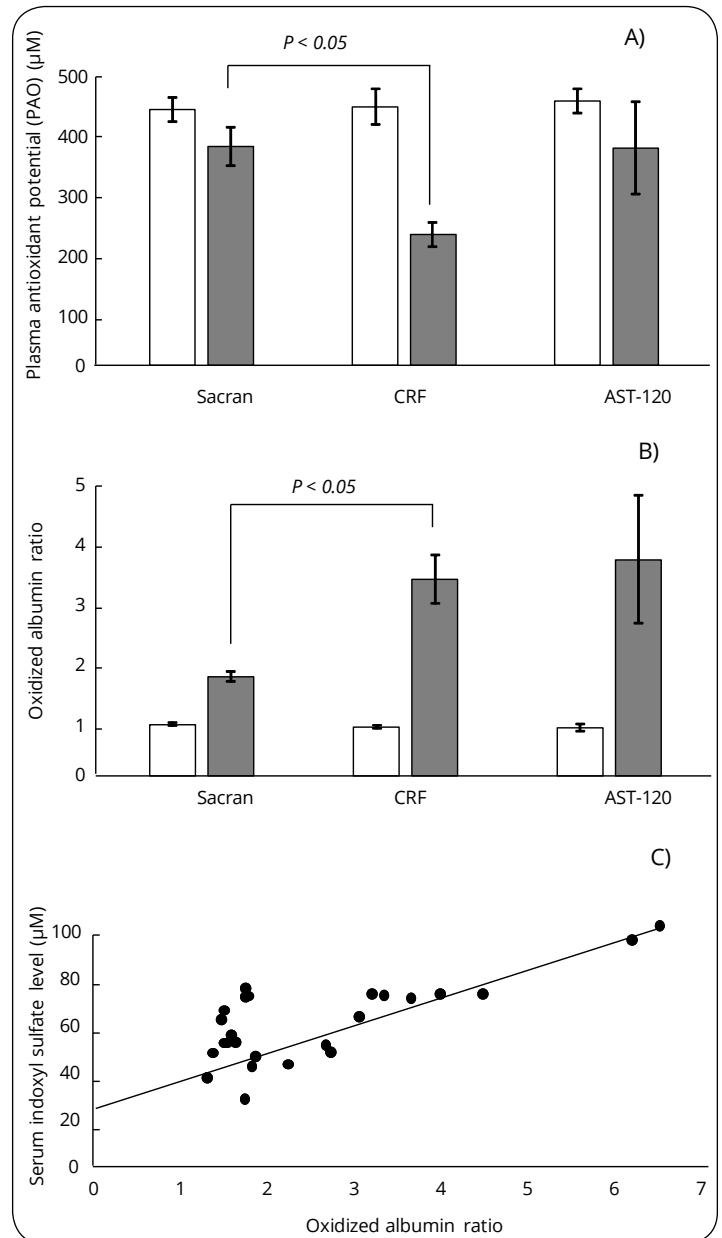
Effects of sacran treatment on oxidative stress in CRF rats

As shown in figure 2A, only the sacran treatment resulted in a significant increase in the plasma antioxidant potential (PAO) compared with the ingestion of AST-120 or nontreatment groups after 4 weeks ($p < 0.05$ vs. untreated CRF-rat). As shown in figure 2B, the sacran treatment resulted in a significant decrease in the oxidized albumin ratio, compared with the ingestion of AST-120 or the non-treatment groups after 4 weeks ($p < 0.05$ vs. untreated CRF-rat). Since the extent of oxidation of albumin, a prominent protein in serum, can be taken as an index of oxidative stress, it can be concluded that sacran has the potential for reducing the effects of stress in CRF model rats. Further, as shown in figure 2C, the relationship between the oxidized albumin ratio and serum indoxyl sulfate levels was determined. The findings indicate the existence of a good significant correlation (r = 0.752, $p < 0.05$) between the two parameters. These results also suggest that sacran reduces the levels of indole-related 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 CRF. However, no significant correlation was found between. The oxidized albumin ratio and serum phosphate levels (data not shown).

Binding capacity of indole

In an in *vitro* study, the ratio of the binding of indole to sacran was half that for the AST-120 treatment at 5

mg/mL (Figure 3). These results suggest that indole levels would be expected to be reduced in the gastrointestinal tract as the result of an administration of sacran in the CRF rats used in this study.


Figure 2: The effects of sacran treatment on indices of oxidative stress. A) total plasma antioxidant potential (PAO), B) the ratio of oxidized albumin, and C) the relationship between oxidized albumin ratio and serum indoxyl sulfate level. The line shows a linear regression of the two sets of results (r = 0.752, $p < 0.05$). Results are expressed as the mean ± SEM.
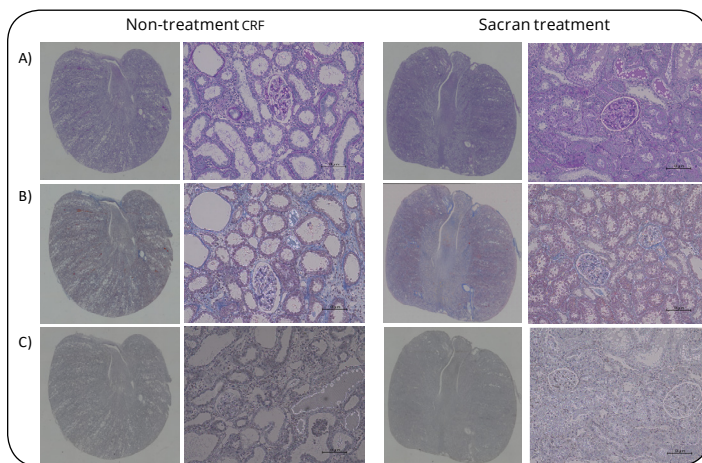
Effect of sacran on renal tissue

Figure 4 shows images of kidney tissue samples obtained from non-treatment CRF rats, and sacran treated CRF rats after staining with PAS (Figure 4A) and Masson's trichrome (Figure 4B). An examination of the

PAS-stained tissue samples showed that the sacran treated CRF rats had significantly less renal damage, especially renal tubular degeneration, compared with the CRF rats. Renal fibrosis, as detected by Masson's trichrome staining, was observed in the tubule-interstitial regions in the case of the CRF rats (Figure 4B). Figure 4C shows kidney tissue samples that were examined by immunofluorescence spectrometry for oxidative stress markers, such as 8-hydroxy-2'-deoxygenase (8-OHdG). The immunehistochemical analyses demonstrated that the accumulation of 8-OHdG in the case of the sacran treated CRF rats was decreased compared to that for the CRF group. These data suggest that the decreased oxidative stress as a result of the action of the sacran could reduce the extent of renal damage *in vivo*.
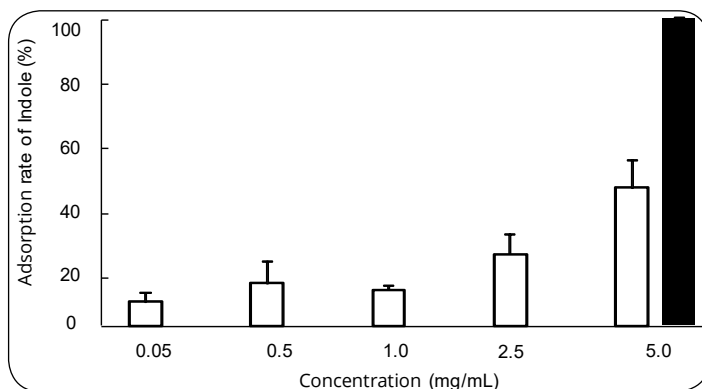
Figure 3: Binding capacity of indole for sacran and AST-120. Results are shown for sacran (□) and AST-120 (■). Results are expressed as the mean ± SEM.

Figure 4: Effect of the administration of sacran on the renal tissue of CRF model rats in kidney samples. A) PAS stain, B) Masson's trichrome and C) 8-OHdG. Non-treatment groups and sacran treatments were measured after 4 weeks, as described in Materials and Methods.

Discussion

Oxidative stress is a pathogenic element of great importance in CRF, and represents a major determinant of patient survival. Thus, the development of effective anti-oxidant therapy for the treatment of CRF would be highly desirable. One proposed mechanism for the development of oxidative stress in CRF involves the accelerated production of oxidants, such as uremic toxins and their reduced renal clearance. Thus, the removal of such substances from the systemic circulation would be expected to lead to a reduction in oxidative stress in CRF. If uremic toxins in serum could be removed or their levels reduced, then the amounts of protein-bound uremic toxins could be reduced by reducing the levels of precursors that are present in the gut. Serum concentrations of protein-bound toxins such as indoxyl sulfate can be decreased by preventing the absorption of toxins via the use of oral adsorbents. As mention above, previous studies have shown that the ingestion of AST-120 improves creatinine clearance, which then permits to be delayed [28-30]. However, AST-120 is typically administered in the form of a single, large dose (2 g), which frequently gives rise to noncompliance issues. In place of AST-120, an alternate oral adsorbent that could be administered at a lower dose is therefore needed to improve CRF. In our previous studies, were ported that chitosan powder and nanofibers derived from it showed renal protective effects as well as antioxidant activity in chronic renal failure. In particular, surfaced acetylated chitin nanofibers (SDACNFs) have highly uniform structures with diameters of 10-20 nm and, compared to chitosan and chitin, which permits them to be more readily dispersed in water due to their submicron size and high surface-to-volume ratio [31-34]. Thus, its high dispersibility in water allows it to be used as an oral adsorbent at a substantial lower dose than would be needed for chitosan powder or for AST-120 in CRF. However, chitosan itself is soluble only in acidic solvents, its taste is very bad and it tends to cause noncompliance for certain types of patients. Therefore, in place of AST-120, chitosan and nanofibers derived from it, sacran was examined as a possible alternate. In fact, sacran is thought to be a safe biomaterial, because Aphanothece sacrum is a mass cultivated cyanobacterial species that has long been used as a functional food to ameliorate allergic tendencies and gastroenteritis by inhabitants of the Kyushu region in Japan. In the present study, a sacran treatment caused a reduction in several important biological parameters (Table 1). In particular, compared to the results for AST-120 or the non-treatment group, a sacran treatment caused a significant decrease in the

Table 1: Effects of Sacran, and AST-120 treatment on serum parameters.

		Non-treatment groups	Sacran	AST-120
Body weight (g)	0 week	270.7±5.0	272.0±5.2	268.5±12.7
	4 week	371.8±7.9	340.2±7.2**	373.7±6.9
Serum BUN (mg/dL)	0 week	64.1±1.8	55.3±2.3	60.7±5.8
	4 week	78.4±5.4	52.6±1.5**	83.2±24.1
Serum creatinine (mg/dL)	0 week	0.6±0.1	0.6±0.1	0.5±0.1
	4 week	1.1±0.1	0.7±0.1**	1.1±0.3
Serum phosphorus (mg/dL)	0 week	7.7±0.1	7.3±0.2	7.3±1.1
	4 week	6.4±0.2	5.8±0.1**	6.5±0.6
Serum indoxyl sulfate (µM)	0 week	13.8 ± 3.8	14.2 ± 3.4	15.1 ± 4.1
	4 week	72.6±3.1	49.0±3.3**	67.9±17.4
Mean blood pressure (mmHg)	0 week	125.3±8.5	129.8±6.9	125.1±10.1
	4 week	156.3±10.5	139.8±6.7	149.1±8.1

BUN: serum blood urea nitrogen, Values are expressed as the mean SEM (n = 3-11 rats per group). ** $p<0.01$, compared with non-treatment groups.

levels of serum indoxyl sulfate level. We (and others) have shown that indoxyl sulfate, a widely studied uremic toxin, is one of the major risk factors for dysfunctional kidney-specific abnormalities, since it causes an increase in free radical production and induces the production of inflammatory cytokines in the kidneys and blood circulation [5,27,35]. Therefore, low indoxyl sulfate levels might lead to renal function being maintained and a lower level of oxidative stress. Furthermore, the sacran treatment caused a significant increase in PAO and a decrease in oxidized albumin ratio after 4 weeks, compared to AST-120 and the no treatment group (Figure 2). This result demonstrates the potential of sacran for reducing the effects of stress in CRF model rats, because the PAO and oxidized albumin ratio is widely used as a marker of oxidative stress in many diseases, including CRF. Further, Lim et al. reported that a reduction in the level of oxidized albumin ratio leads to a reduction in mortality in hemodialysis patients [36]. These results suggest that sacran itself functions is a powerful *in vivo* antioxidant. Further, the results shown in Figure 2C clearly point to the existence of a good relationship between the oxidized albumin ratio and serum indoxyl sulfate levels (r = 0.752, *p* < 0.05) (Figure 2C). Since neither sacran itself norAST-120 are absorbed, it is unlikely that the mechanism responsible for the antioxidant activity of sacran 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 to this material in the gastrointestinal tract, thus resulting in the suppression of their serum levels. This conclusion is supported by the fact that sacran was found to bind indole strongly, which has been reported to

be a precursor of indoxyl sulfate *in vitro* studies, provides support for this conclusion (Figure 3). Thus, the removal of indoxyl sulfate from the systemic circulation may lead to a reduction in oxidative stress in CRF rats. Moreover, the findings reported herein show that sacran-treated CRF rats had significantly decreased kidney damage and oxidative stress, compared to CRF rats (Figure 4). Based on these results, we propose that it is possible to reduce the levels of indoxyl sulfate, a pro-oxidant, in the intestinal tract by a sacran treatment and that this can be accomplished using a smaller dose or approximately the same dose as AST-120. Doi et al. recently proposed that sacran improves skin conditions, both for healthy subjects and for patients with atopic dermatitis [20,37,38]. The Stratum Corneum (SC) parameters indicated that sacran modulates the terminal differentiation of the epidermis and alleviates the inflammatory symptoms of subjects who have impaired skin barrier functions. In fact, sacran penetrates the SC to living cell layers of the epidermis, which suggests that sacran would attenuate the adverse influence in keratinocytes caused by extracellular factors such as irritants or pro-inflammatory cytokines such as interleukin 1α (IL-1α). Sacran has also been reported to markedly reduce cell damage induced by a nonionic detergent, sodium lauryl sulfate (SLS). In addition, sacran restored the elevation of intracellular (ROS) levels stimulated by SLS and by IL-1α [37]. Since intestinal flora are responsible for metabolizing amino acids to produce precursors of uremic toxins (e.g., tryptophan to indole), pre- and probiotics are considered to be potent agents for reducing the accumulation of uremic toxins. Several reports have shown that both pre- and probiotics could reduce the concentrations of uremic toxins in the

serum or in fecal and urinary excretion [39]. It is also possible that sacran functions as a prebiotic who could exhibit renal protective effects in CRF rats by modulating the environment in the gut and regulating systemic inflammation. Consequently, based on these (and other) findings, it would appear that sacran has the potential for use as a novel bio-material in the near future.

Conclusion

The findings reported here indicate that the administration of sacran has the potential to reduce the levels of uremic toxins (such as indoxyl sulfate) that induce the production of free radicals in the intestinal tract, thereby inhibiting the subsequent development of oxidative stress in the systemic circulation in CRF model rats. Thus, the removal of such substances from the systemic circulation could lead, not only to a reduction in oxidative stress, but also to the prevention of cardiovascular disease in CRF. Considering the new prophylaxis or therapy associated with the efficient use of sacran, it could be co-administered with such agents and represents a new strategy for anti oxidative treatment in cases of several types of diseases, including renal failure, because the anti oxidative effect of sacran is unique and is different from that of typical, conventional antioxidants.

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