ORIGINAL ARTICLES

ORAL ASTAXANTHIN SUPPLEMENTATION PREVENTS PERITONEAL FIBROSIS IN RATS

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◆ **Background:** Preventing peritoneal damage during peritoneal dialysis is critical. Reactive oxygen species (ROS) have an important role in peritoneal damage; however, few studies have investigated this. We aimed to determine the effects of oral astaxanthin (AST) supplementation in a peritoneal fibrosis (PF) rat model.

◆ **Methods:** Thirty-seven Sprague–Dawley rats were divided into 5 groups: Control 1 (fed a normal diet without stimulation), Control 2 (fed an AST-supplemented diet without stimulation), Group 1 (fed a normal diet with 8% chlorhexidine gluconate [CG] stimulation for 3 weeks), Group 2 (fed a 0.06% AST-supplemented diet with CG stimulation), and Group 3 (fed a 0.06% AST-supplemented diet that was initiated 4 weeks before CG stimulation). Peritoneal fibrosis, vascular proliferation, and fibrosis-related factor expression were examined.

◆ **Results:** Peritoneal thickness was significantly suppressed by AST supplementation. Astaxanthin diminished the number of CD68-, 8-hydroxy-2'-deoxyguanosine (8-OHdG)-, and monocyte chemoattractant protein-1 (MCP-1)-positive cells. Type 3 collagen, tumor necrosis factor- α (TNF- α), interleukin-1 β (IL-1 β), and MCP-1 mRNA expression was significantly lower in Group 3 than in Group 1. Increased transforming growth factor- β (TGF- β) and Snail mRNA expression, vascular density, and the number of α -smooth muscle actin (α -SMA)-positive cells were also decreased in Group 3.

◆ **Conclusion:** Astaxanthin suppressed PF development through the inhibition of inflammation and oxidation in PF rats. It appears that the anti-oxidative agent AST may be useful for the prevention of peritoneal damage.

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Peritoneal dialysis (PD) is a renal replacement therapy for patients with end-stage kidney disease; however, peritoneal fibrosis (PF) can complicate PD treatment and lead to peritoneal function failure and encapsulating peritoneal sclerosis (1). Long-term exposure to a bioincompatible dialysate containing a high glucose concentration, uremic toxins, and refractory or recurrent infectious peritonitis often increases cytokine and growth factor production in the peritoneum (1). A glucose-PD solution and an icodextrin-PD solution can induce a dose-dependent increase in reactive oxygen species (ROS) generation (2). Reactive oxygen species induced by a conventional PD solution can mediate functional alterations and PF through epithelial-mesenchymal transition (EMT) (3,4).

The carotenoid family includes > 700 lipid-soluble pigments produced by phytoplankton, algae, plants, and a limited number of fungi and bacteria. Astaxanthin (AST), a xanthophyll, is one of the most prevalent carotenoids, and is abundantly present in the red pigment of crustacean shells, salmon, and asteroideans after becoming more concentrated higher up in the food chain as primary producers that are consumed as food (5). The AST molecule has a polar structure at either end and a non-polar zone in the middle, and it exhibits a strong capacity for

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quenching free radicals or other oxidants because of its 2 oxygenated groups on each ring structure (5). This polar-nonpolar-polar layout also allows the AST molecule to assume a transmembrane orientation, thereby fitting precisely into the polar-nonpolar-polar span of the cell membrane. Several previous studies have demonstrated that AST exhibits various biological activities, including an anti-oxidative effect as a ROS scavenger (6) and an anti-inflammatory effect via the inhibition of the nuclear factor kappa light chain enhancer of activated B cells (NF- κ B) pathway (7,8).

The intraperitoneal administration of anti-oxidants such as vitamin E, pyridoxamine, and N-acetylcysteine has been investigated in animal models (9–11). However, no study has assessed the effects of oral AST supplementation on peritoneal damage. In this study, we demonstrated the effects of oral AST supplementation, which is a strong lipid-soluble anti-oxidant, on chlorhexidine gluconate (CG)-induced PF in rats (12).

MATERIALS AND METHODS

ANIMALS AND EXPERIMENTAL DESIGN

Thirty-seven male Sprague–Dawley rats (200 – 250 g body weight at 8 weeks; Sankyo Labo Service Corporation Inc., Tokyo, Japan) were housed in standard rodent cages under a constant temperature of 22°C and a 12-h light/dark cycle, with free access to chow and water. The control rats were fed a normal commercial diet with the following ingredients (g/100 g): moisture, 7.9; protein, 23.1; fat, 5.1; fiber, 2.8; ash, 5.8; carbohydrates, 55.3; and sufficient vitamins and minerals to maintain the health of the rats (Oriental Yeast Co., Ltd., Tokyo, Japan). The AST-supplemented chow was prepared by mixing the normal diet with 5% AST oil (Fuji Chem. Industry Co., Ltd., Toyama, Japan) to a final concentration of 0.06%. The rats were divided into 5 groups (Figure 1). Ten rats without CG administration were prepared as controls. Of those, 5 were fed a normal diet (Control 1), and the others were fed an AST-supplemented diet (Control 2). Eighteen rats were fed a normal diet for 4 weeks. Of those, 9 were simultaneously fed a normal diet and administered CG for 3 weeks (Group 1), whereas the other 9 (Group 2) were fed an AST-supplemented diet and administered CG for 3 weeks. The Group 3 rats ($n = 9$) were fed an AST-supplemented diet for 7 weeks and administered CG for the last 3 weeks of the experiment (13). All rats were sacrificed 3 weeks after CG stimulation, and peritoneum specimens were obtained. The experimental protocols were approved by the Ethics Review Committee for Animal Experimentation of Juntendo University Faculty of Medicine, Tokyo, Japan.

PERITONEAL FIBROSIS ANIMAL MODELS

The rats were administered 8% CG at an infusion rate of 2.5 μ L/h using continuous infusion pumps, as described by Komatsu *et al.* (12). Under ether anesthesia, each pump was placed in the lower abdominal cavity. The pumps were removed 21 days after placement and the peritoneum was immediately excised.

HISTOLOGICAL ANALYSIS

The maximum thickness of the submesothelial compact (SMC) zone was measured in each section, as described by Honda *et al.* (14). Five points were randomly selected for the measurement using the Kontron KS400 Imaging System (Kontron Elektronik GmbH, Eching, Germany) (15). The average was calculated for each specimen.

IMMUNOHISTOCHEMICAL ANALYSIS

Sections were deparaffinized in xylene, followed by 100% ethanol, and placed in methanol/0.3% H_2O_2 solution. Microwave antigen retrieval was performed in citrate buffer. The sections were blocked using a blocking solution, followed by overnight incubation with mouse anti-rat CD68 antibody diluted to 1:100 (Serotec Ltd, Oxford, UK), which reacts with macrophage; rabbit anti-rat monocyte chemoattractant protein-1 (MCP-1) antibody diluted to 1:500 (Abcam, Cambridge, UK); mouse anti-rat 8-hydroxy-2'-deoxyguanosine (8-OHdG) antibody diluted to 1:200 (Japan Institute for the Control of Aging, Shizuoka, Japan), which reacts with damaged DNA as a marker for oxidative stress; mouse

Figure 1 — Study design. Control 1: fed a normal diet without CG administration. Control 2: fed an AST diet without CG administration. Group 1: fed a normal diet and received CG for 3 weeks. Group 2: fed a 0.06% AST-supplemented diet and received CG. Group 3: fed a 0.06% AST-supplemented diet from 4 weeks before CG administration. CG = chlorhexidine gluconate; AST = astaxanthin.

anti-rat α -smooth muscle actin (α -SMA) antibody diluted to 1:1000 (Abcam, Cambridge, UK) which reacts with fibroblasts; and mouse anti-rat CD31 antibody diluted to 1:100 (Thermo Scientific, Waltham, USA) for vascular density. The sections were then incubated with H_2O_2 -conjugated polyclonal goat anti-rabbit and mouse antiserum (Histofine Simple Stain MAX-PO, Nichirei Biosciences, Inc., Tokyo, Japan). The bound antibodies were visualized with 3,3'-diaminobenzidine containing 0.003% H_2O_2 . The negative control was confirmed by incubation without primary or secondary antibodies and exhibited no positive cells. All sections were counterstained with Mayer's hematoxylin solution before mounting with Glycergel mounting medium (Mount-Quick, Daido Sangyo, Saitama, Japan). The number of positive cells was counted from 10 random regions in each tissue. The average number was calculated (the number of positive cells/SMC mm²) using the KS400 Imaging System (Kontron Elektronik GmbH, Eching, Germany). Cell quantifications were performed by 2 observers blinded to the treatment groups.

DOUBLE-IMMUNOFLUORESCENCE ANALYSIS

Sections were deparaffinized in xylene, followed by 100% ethanol. Microwave antigen retrieval of the specimens was performed in citrate buffer. Sections were blocked in a blocking solution and incubated overnight with one of the following antibodies: mouse anti-8-OHdG antibody diluted to 1:200 (Japan Institute for the Control of Aging), rabbit anti-CD68 antibody (Serotec Ltd, Oxford, UK), rabbit anti-CD31 antibody diluted to 1:100 (Abcam,

Cambridge, UK), rabbit anti- α -SMA antibody (Abcam, Cambridge, UK), rabbit anti-mesothelin antibody (Santa Cruz Biotechnology, Inc., Dallas, TX, USA) which reacts with the mesothelial cell. After incubation, sections were mounted in diluted Alexa 468 or Alexa 555 (Molecular Probes, Inc., Eugene, OR, USA) as an appropriate secondary antibody. Negative controls omitted primary antibodies. In all fluorescent images, cell nuclei were stained using 4',6-diamidino-2-phenylindole (DAPI).

QUANTITATIVE REAL-TIME POLYMERASE CHAIN REACTION (PCR) ANALYSIS

Total RNA was extracted using trizol reagent (Invitrogen AG, Basel, Switzerland) and the RNeasy Mini Kit (Qiagen K.K., Tokyo, Japan). The complementary DNA (cDNA) produced using extracted RNA was used for real-time PCR as previously described (26). A 2- μ L aliquot of diluted cDNA, 1.6 μ L of forward and reverse primers, 10 μ L of SYBR Green PCR Master Mix (Applied Biosystems, Carlsbad, CA, USA), and 4.8 μ L of cDNA-free double-distilled water were then mixed. The mixture was denatured and amplified using the 7500/7500 Fast Real-Time PCR system (Applied Biosystems, Carlsbad, CA, USA). For quantification, the samples were standardized with the PCR products for glyceraldehydes-3-phosphate dehydrogenase (GAPDH). The PCR primers are listed in Table 1.

STATISTICAL ANALYSIS

All data are presented as mean $\pm$ standard deviation (SD). Differences between groups were examined for

TABLE 1
Polymerase Chain Reaction Primers Design

	Forward primer	Reverse primer
GAPDH	5'-ACGTAAACGGCCACAAGTTC-3'	5'-AAGTCGTGCTGCTTCATGTG-3'
MCP-1	5'-AGGTCTCTGTACGCTTCTGGG-3'	5'-TAGCAGCAGGTGAGTGGGGCAT-3'
TNF- α	5'-TCGGTCCCAACAAGGAGGAGAAGT-3'	5'-TGATCTGAGTGTGAGGGTCTGGGC-3'
IL-1 β	5'-TGGAGAAGCTGTGGCAGCTACCTAT-3'	5'-CACAGAGGACGGGCTCTTCTTCAA-3'
TGF- β	5'-ATGGTGGACCGCAACAACGCAAT-3'	5'-CAGCTCTGCACGGGACAGCAAT-3'
VEGF	5'-ACTGGACCTGGCTTTACTGCTG-3'	5'-TTCACCACTTCATGGGCTTTCTGCT-3'
MMP-2	5'-TGCAGGGTGGTGGTCACAGCTA-3'	5'-AGCAGCCCAGCCAGTCCGATT-3'
Vimentin	5'-CCAGGCAAAGCAGGAGTCAAACG-3'	5'-TCTTCCATTTCACGCATCTGGCG-3'
Snail	5'-ACAGCGAACTGGACACACACACA-3'	5'-AGGGGAGTGAATGGAAGTGTGA-3'
Type1 collagen	5'-CTGCACGAGTCACACCGGAATT-3'	5'-TCCTGGTCTGGGGACCAATGT-3'
Type3 collagen	5'-CCACCTGAACTCAAGAGCGGAGAA-3'	5'-GGGACAGTCATGGGACTGGCATT-3'

GAPDH = glyceraldehydes-3-phosphate dehydrogenase; MCP-1 = monocyte chemoattractant protein-1; TNF- α = tumor necrosis factor- α ; IL-1 β = interleukin-1 β ; TGF- β = transforming growth factor- β ; VEGF = vascular endothelial growth factor; MMP-2 = matrix metalloproteinase-2.

statistical significance using 1-way analysis of variance. All statistical analyses were performed using Graph Pad PRISM Version 5.0 (GraphPad Software Inc., La Jolla, CA, USA). Differences with a *p* value of < 0.05 were considered statistically significant.

RESULTS

MORPHOLOGICAL CHANGES IN THE PERITONEAL INTERSTITIUM

Compared with the control groups, CG administration induced significant morphological alterations in Group 1 (Figure 2A and B). There were no differences between Control 1 and 2 in the morphological changes (Figure 2I), also in inflammatory- and fibrosis-related factors (data not shown). We did not show the results of Control 2 in order to interpret the results concisely. Remarkable interstitial thickening and monocyte infiltration were observed in Group 1 (Figure 2B and F). Astaxanthin ameliorated PF and the increase of CD68-positive cells in Groups 2 and 3 (SMC thickness; Control: $28.8 \pm 9.0 \mu\text{m}$, Group 1: $281.4 \pm 35.0 \mu\text{m}$, Group 2: $207.6 \pm 39.1 \mu\text{m}$, Group 3: $161.8 \pm 47.1 \mu\text{m}$, the number of CD68-positive cells; Control: $0.0/\text{mm}^2$, Group 1: $982.5 \pm 149.9/\text{mm}^2$, Group 2: $692.8 \pm 112.3/\text{mm}^2$, Group 3: $464.3 \pm 144.8/\text{mm}^2$) (Figure 2C, D, G–J). Increases in type 1 and 3 collagen mRNA expression were significantly attenuated in Group 3 (Figure 2K and L). However, there were no significant differences in the SMC thickness between Groups 2 and 3, and the collagen accumulation between Groups 1 and 2 and Groups 2 and 3 (Figure 2I, K, and L, respectively).

PERITONEAL 8-OHdG EXPRESSION

8-OHdG-positive cells were observed throughout the peritoneum (Figure 3A–D). 8-OHdG expression appeared in round- or spindle-shaped cells (Figure 3B). The number of 8-OHdG-positive cells was significantly lower in Groups 2 and 3 than in Group 1 (the number of 8-OHdG-positive cells; Control: $292.6 \pm 60.7/\text{mm}^2$, Group 1: $2099 \pm 857.7/\text{mm}^2$, Group 2: $681.1 \pm 157.6/\text{mm}^2$, Group 3: $545.8 \pm 250.4/\text{mm}^2$) (Figure 3E). Immunofluorescence revealed that 8-OHdG-positive cells also indicated the presence of mesothelin, CD68, CD31, and α -SMA (Figure 4A–D).

MRNA EXPRESSION OF FIBROSIS-RELATED FACTORS AND VASCULAR DENSITY

Chlorhexidine gluconate stimulation induced the overexpression of tumor necrosis factor- α (TNF- α),

interleukin-1 β (IL-1 β), matrix metalloproteinase-2 (MMP-2), transforming growth factor- β (TGF- β), and vascular endothelial growth factor (VEGF; Figure 5A–E). Compared with Group 1, TNF- α and IL-1 β mRNA expression was significantly suppressed in Group 3. The levels of these factors were also suppressed in Group 2, but the differences were not statistically significant (Figure 5A and B). Furthermore, compared with Group 1, MMP-2 and TGF- β mRNA expression was significantly suppressed in Group 3 (Figure 5C and D). MMP-2 and TGF- β mRNA expression in Group 2 was minimized, but the changes were not statistically significant (Figure 5C and D). Astaxanthin did not inhibit VEGF mRNA overexpression in Groups 2 and 3 (Figure 5E). Compared with Group 1, average vascular density was significantly decreased in Groups 2 and 3 (Vascular density; Control: $46.9 \pm 10.8/\text{mm}^2$, Group 1: $261.9 \pm 25.2/\text{mm}^2$, Group 2: $170.1 \pm 53.9/\text{mm}^2$, Group 3: $143.0 \pm 28.0/\text{mm}^2$) (Figure 5F).

MRNA EXPRESSION OF MCP-1 AND MCP-1-POSITIVE CELLS IN THE PERITONEUM

In Group 1, MCP-1-positive cells were scattered throughout the peritoneum (Figure 6B). Astaxanthin attenuated the infiltration of MCP-1-positive cells (Figure 5C and D). The number of MCP-1-positive cells was significantly lower in Groups 2 and 3 than in Group 1 (Figure 5E). MCP-1 mRNA expression in Group 3 was also significantly suppressed (Figure 6F).

APPEARANCE OF α -SMA AND SNAIL

Alpha-SMA-positive cells were observed throughout the entire interstitium (Figure 7A). Compared with Group 1, the number of α -SMA-positive cells was significantly decreased in Groups 2 and 3 (α -SMA-positive cells; Control: $468.4 \pm 139.7/\text{mm}^2$, Group 1: $2104.0 \pm 506.7/\text{mm}^2$, Group 2: $1486.0 \pm 319.1/\text{mm}^2$, Group 3: $1010.0 \pm 183.1/\text{mm}^2$) (Figure 7B–E). Astaxanthin significantly suppressed Snail mRNA overexpression (Figure 7F).

DISCUSSION AND CONCLUSION

In summary, AST diminished the number of CD68-, MCP-1-, and 8-OHdG-positive cells in the CG-treated rats. Additionally, the 8-OHdG-positive cells indicated the presence of mesothelin as well as CD68, CD31, and α -SMA. Type 1 and 3 collagen, TNF- α , IL-1 β , and MCP-1 mRNA expression and vascular density were significantly lower in Group 3. Increased TGF- β and Snail mRNA expressions and the number of α -SMA-positive cells were also significantly suppressed in Group 3. To

Figure 2 — Morphological changes and macrophage infiltration in the peritoneum. A–D: The histological features on day 21 in each group are shown (Masson's trichrome staining; magnification, $\times 200$). E–H: Immunohistochemical findings of CD68 on day 21 in each group (F–H) and representative controls (E) are shown (magnification, $\times 400$). I, J: Compared with Group 1, the SMC thickness and the number of CD68-positive cells were significantly suppressed by AST supplementation in Groups 2 and 3. K, L: Increases in type 1 and 3 collagen mRNA expression were significantly suppressed in Group 3. Those in Group 2 were suppressed, but without any statistical significance. Error bars represent SD. *: significantly different ($p < 0.05$). SMC = submesothelial compact; GAPDH = glyceraldehydes-3-phosphate dehydrogenase; AST = astaxanthin; mRNA = messenger ribonucleic acid; ns = not significant.

Figure 3 — Immunohistochemical findings for 8-OHdG as a marker of oxidative DNA damage are shown (magnification, $\times 400$). A: The representative control. B: In Group 1, 8-OHdG-positive cells were scattered throughout the interstitium on day 21. C-E: Compared with Group 1, the number of 8-OHdG-positive cells was significantly decreased in Groups 2 and 3. Error bars represent SD. *: $p < 0.05$ vs Group 1. 8-OHdG = 8-hydroxy-2'-deoxyguanosine; DNA = deoxyribonucleic acid; SD = standard deviation.

the best of our knowledge, this is the first study to show that oral AST supplementation facilitated peritoneal repair by suppressing ROS and inflammation in rats with CG-induced PF.

Peritoneal morphological and functional alterations progress with PD duration (16). A neutral pH PD solution, which has a low glucose degradation product (GDP) concentration, ameliorated developing peritoneal damage via the suppression of peritoneal inflammation (17). We suspected that ROS facilitated GDP and advanced glycation end-product (AGE) production and PF progression through the enhancement of non-specific inflammation and EMT, as previously suggested (3,18). Several studies showed that anti-oxidative and anti-AGE substances, such as pyridoxamine and aminoguanidine, significantly improved functional and structural changes in the peritoneal membrane (10,19,20). However, there have been few studies on the effects of anti-oxidants in PF. Here, we assessed the direct and indirect roles of ROS in peritoneal damage using AST and found that AST suppressed CG-induced PF development.

Astaxanthin is a lipid-soluble agent and is located within cell membranes. The presence of hydroxyl and

keto moieties on each AST ionone ring explains the higher anti-oxidant activity and differentiates it from β -carotene and other molecules of the other carotene subclasses (6,21). Free radicals and electrons can be captured in those structures; therefore, the anti-oxidative effects of AST are stronger than those of water-soluble agents, such as N-acetylcysteine and pyridoxamine, because these agents cannot reach the cell membrane. The free-radical scavenger on cell membranes appears at AST saturation. Because it takes approximately 4 weeks to saturate the cell membrane with AST (13), we prepared 2 different AST-treatment groups. Vitamin E acts as an inhibitor of pro-inflammatory mediator production in macrophages and peritoneal mesothelial cells (22). However, no study has examined the beneficial effects of vitamin E on PF *in vivo*.

Ohgami *et al.* (23) reported that AST may decrease pro-inflammatory factor production through the suppression of the NF- κ B pathway *in vitro* and *in vivo*, particularly in a lipopolysaccharide-induced mouse uveitis model, with the effect of 100 mg/kg of AST shown to be as strong as that of 10 mg/kg of prednisolone. Our study presented that CG induced an increase of 8-OHdG in mesothelial

Figure 4 — Identification of 8-OHdG-expressing cells in the thickened peritoneum. A: double-stained with mesothelin antibody, which reacts with mesothelial cells (magnification, $\times 100$), B: double-stained with CD68, which reacts with macrophage ($\times 100$), C: double-stained with CD31, which reacts with endothelial cells ($\times 150$), D: double-stained with α -SMA antibody, which reacts with fibroblasts ($\times 100$). DAPI = 4',6-diamidino-2-phenylindole; 8-OHdG = 8-hydroxy-2'-deoxyguanosine; α -SMA = alpha-smooth muscle actin.

cells, macrophages, endothelial cells, and fibroblasts, and AST significantly suppressed it. An increase in the SMC thickness and elevated type 3 collagen mRNA expression were markedly suppressed in the AST-supplemented diet groups. Furthermore, the number of infiltrating

inflammatory cells and fibrosis-related factor mRNA levels were also ameliorated. Inflammation-induced peritoneal vascularization has been shown as an essential component of PF and associated with peritoneal solute transport (24). Vascular density in Group 3 was

Figure 5 — mRNA expression of fibrosis-related factors and vascular density. A, B: Compared with Group 1, TNF- α and IL-1 β mRNA expression levels were suppressed in Group 3. C–E: Compared with Group 1, MMP-2 and TGF- β mRNA expression significantly decreased in Group 3. Compared with Group 1, average vascular density at day 21 was significantly decreased in Groups 2 and 3. Error bars represent SD. *: $p < 0.05$ vs Group 1. TNF- α = tumor necrosis factor- α ; GAPDH = glyceraldehydes-3-phosphate dehydrogenase; IL-1 β = interleukin-1 β ; MMP-2 = matrix metalloproteinase-2; TGF- β = transforming growth factor- β ; VEGF = vascular endothelial growth factor; mRNA = messenger ribonucleic acid; SD = standard deviation.

significantly decreased in this study. Therefore, oral AST supplementation had beneficial preventive effects on PF development through anti-oxidative and anti-inflammatory actions.

Selgas *et al.* (25) reported that EMT was induced by several different signal pathways such as the Smads cascade, the RhoA-ROCK pathway, and the H-Ras/Raf/ERK pathway. These pathways indicate complex relationships and work in a coordinated manner. Because Snail, a transcriptional factor of E-cadherin, is ultimately promoted in any pathway when EMT is up-regulated, we also examined Snail expression as in previous reports (26,27). Snail and TGF- β mRNA expression were significantly lower in Group 3 than in Group 1; however, there was no statistically significant difference between Groups 2 and 1. Based on these results, we suspected that AST may suppress EMT through the suppression of inflammation and followed by the inhibition of TGF- β , Snail, and the α -SMA-positive fibroblasts.

From a clinical perspective, it appears that the anti-oxidative effect of AST in ameliorating peritoneal damage

may possibly be stronger than that of other anti-oxidative agents because the effect of AST was observed through oral supplementation, but not intraperitoneal administration. In a previous study, 0.02% AST was supplied as an anti-oxidative agent (28). In this study, we fed the rats a 0.06% AST-supplemented diet. There were no adverse effects of AST on liver function tests (data not shown). Because a sufficient and safe concentration of AST has not yet been defined, further studies will be required before applying the results of this study to clinical use in PD patients.

This study has several limitations. First, we did not analyze peritoneal function. Second, we did not collect urinary samples for oxidant analysis. Oxidative stress markers are excreted in urine immediately, and it is difficult to measure them using serum. Thus we had to examine the oxidative stress using urine samples. However, it has been already published that ROS are related to CG-induced PF animals (11).

In conclusion, AST suppressed PF through the inhibition of inflammation and oxidation, suggesting that AST

Figure 6 — Immunohistochemical and real-time PCR analysis for MCP-1. A–D: Immunohistochemical findings of each group are shown ($\times 400$). A: The representative control. B: MCP-1-positive cells were scattered throughout the interstitium on day 21 in Group 1. C–E: Compared with Group 1, the number of MCP-1-positive cells was significantly decreased in Groups 2 and 3. E: Compared with Group 1, MCP-1 mRNA expression was significantly suppressed in Group 3. Error bars represent SD. *: $p < 0.05$ vs Group 1. MCP-1 = monocyte chemoattractant protein-1; GAPDH = glyceraldehydes-3-phosphate dehydrogenase; PCR = polymerase chain reaction; mRNA = messenger ribonucleic acid; SD = standard deviation.

Figure 7 — Appearance of Snail and α -SMA. A–D: Immunohistochemical detection of α -SMA (A–D) in each group ($\times 400$). E: Compared with Group 1, the number of α -SMA-positive cells was significantly decreased in Groups 2 and 3. F: Compared with Group 1, Snail mRNA expression was significantly suppressed in Groups 2 and 3. Error bars represent SD. *: $p < 0.05$ vs Group 1. α -SMA = alpha-smooth muscle actin; GAPDH = glyceraldehydes-3-phosphate dehydrogenase; mRNA = messenger ribonucleic acid; SD = standard deviation.

supplementation may be useful for peritoneal damage in PD patients.

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DISCLOSURES

The authors have no conflicts of interest to declare.

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